Targeted Expression of Plasminogen Activator Inhibitor (PAI)-1 to the Stomach Inhibits Gut-Brain Signalling by the Satiety Hormone Cholecystokinin (CCK)

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

Joanne Gamble

October 2013

For Lily, you are the sunshine in my life….

Table of Contents

Figures and Tables

ACKNOWLEDGEMENTS

This research in this thesis was completed under the excellent supervision of my primary supervisor, Professor Graham Dockray. I would like to thank him for all his help and guidance throughout, and for sharing his expert wisdom and knowledge. In addition, I offer my thanks to my secondary supervisor, Professor Andrea Varro, not only for her scientific support, but for her compassion and support during other, more personal circumstances. I am also grateful to Professor Rod Dimaline for all his PCR work, and for brightening up the early morning laboratory atmosphere with his singing. All members of green block deserve recognition, most notably Dr Galina Burdyga and Dr Susan Kenny for their assistance over the years, and also Dr Islay Steele who I called upon many times for advice. Thank you to Marie for never failing to make me laugh.

For his interesting and useful discussions, I must thank Dr Richard Morris. He supplied me with a solid background in neurobiology which has no doubt been conducive to this work. I am grateful to all the staff at the BSU, particularly Dr Lynn McLaughlin for being so accommodating. Thanks to Dr Lucy Pickavance and Dr Laiche Djourhi who allowed the use of equipment crucial to the completion of this work.

Thank you to my colleague and friend, Dr Kate Haddley, for many discussions, most often on Friday evenings over a well-earned drink, and to Dr Terry Gleave, for having me on his team of demonstrators, but mostly for lending a kind ear. I want to thank Professor David Back, Dr John Tjia, and Dr Vicky Watson for all they have done for me.

I am blessed to have made so many good friends along the journey, in addition to the support of my long-standing ones. It is hard to acknowledge you all, but I hope that you know that you all mean so much to me, a few in particular have helped alleviate the pressures towards the end of my journey and I am eternally grateful; you know who you are. Drew, Kelly and Phil have been with me from the start, I appreciate your friendship and support. Thanks to Maz for keeping me sane, my gin-twin Adrian for discussions; scientific and otherwise, Matthew for being unapologetically candid at times, and big thanks to my Green Block compadre, Suzanne.

I thank my family, particularly on my Father's side for all the words of encouragement via social media! Thanks to the Mother-in-Law for my Thursday night treats. Thank you to my parents and my brother for believing in me, and giving me the drive to achieve something worthwhile. Finally, thank you Ching, for the continual love and support throughout this process. I have achieved so much in my life simply by knowing you. I couldn't have done it without you.

Publications and Presentations

Publications

- **J. Gamble, S.H. Kenny, N. Vlatkovic, R. Dimaline, A. Varro and G. J. Dockray.** Plasminogen activator inhibitor (PAI)-1 suppresses inhibition of gastric emptying by cholecystokinin (CCK). *Regul Pept. 2013 Aug 10;185:9-13. doi: 10.1016/j.regpep.2013.06.005. Epub 2013 Jun 28.*

- **S.H. Kenny, J. Gamble, S.P.Lyons, N. Vlatkovic, R. Dimaline, A. Varro and G. J. Dockray. 2012.** Gastric expression of plasminogen activator inhibitor (PAI)-1 in mice is associated with hyperphagia and obesity. *Endocrinology: 154;718*

- Digestive Disease Week 2011, Chicago, IL. **Susan Kenny, Joanne Gamble, Suzanne Lyons, Nikolina Vlatkovic, Rod Dimaline, Andrea Varro and Graham J. Dockray.** "Gastric expression of plasminogen activator inhibitor (PAI)-1 is linked to obesity and insensitivity to the satiety effect of cholecystokinin." *Gastroenterology , vol. 140, no. 5, pp. S-33-S-33, 2011*

Presentations

- Winter Neuropeptide Conference, 2011, Liverpool. Oral and poster presentation. **Joanne Gamble**, Susan Kenny, Graham J. Dockray. "Plasminogen Activator Inhibitor (PAI)-1 inhibits the satiety effect of Cholecystokinin (CCK) Through a Mechanism Requiring the Urokinase Plasminogen Activator Receptor (uPAR)."

- Physoc/YPS 2011, Oxford. Poster presentation. **Joanne Gamble**, Susan Kenny, Graham J. Dockray. "Plasminogen Activator Inhibitor (PAI)-1 inhibits the satiety effect of Cholecystokinin (CCK) Through a Mechanism Requiring the Urokinase Plasminogen Activator Receptor (uPAR)."

- IMB symposium 2010, Glasgow. Poster presentation. **Joanne Gamble**, Susan Kenny, Graham J. Dockray. "Targeted Expression of Plasminogen Activator Inhibitor (PAI)-1 to the Stomach Inhibits Gut-brain Signalling by the Satiety Hormone Cholecystokinin (CCK)."

Abstract

Energy homeostasis is a tightly regulated system that is vital for survival involving anorectic and orexigenic signals. Obesity is a maladaptive response where the balance becomes disrupted. Obesity is one of the most concerning health problems of our time. It is no longer considered a consequence of a western lifestyle, with more developing countries now reporting an increased incidence of obesity and associated illnesses. While obesity itself can be debilitating and decrease quality of life, it is the associated comorbidities that are the main cause for concern; including type two diabetes, cancer and thrombo-occlusive diseases. One of the molecules thought to be responsible for occlusive events is plasminogen activator inhibitor (PAI)-1. This inhibitor of the plasminogen system is also reported to be up to 5 fold higher in obese subjects in plasma, and similar to leptin, is released from adipose tissue. PAI-1 is considered to play a protective role in circumstances of gastric mucosal attack, thus a transgenic mouse (PAI-1HKβ) was generated, with targeted expression of PAI-1 to the gastric parietal cells, to study this. However, an unexpected phenotype emerged, most notably hyperphagia and increased body weight, which formed the basis of these present studies.

The gut-brain axis is a major and well-studied regulator of energy homeostasis and this was the focus of this project. The PAI-1HKβ mice when compared to wild-type had decreased brain stem responses to the satiety hormone, Cholecystokinin (CCK). Brainstem responses were also attenuated in wild types pre-treated with exogenous PAI-1. Furthermore, it was shown that the urokinase plasminogen activator (uPA) receptor by which PAI-1 binds, was required to influence the observed decrease in brainstem responses.

CCK also has other physiological functions in the role of energy homeostasis, including gastric emptying. While delayed gastric emptying was observed following a protein rich liquid test meal in C57BL/6 mice, PAI-1HKβ mice had a blunted response. Blockade of the CCK_1 receptor in C57BL/6 mice also attenuated the delay in gastric emptying. Moreover, exogenous PAI-1 attenuated CCK-mediated inhibition of gastric emptying. The PAI-1HKβ mice had an attenuated inhibition of gastric emptying of a non-nutrient containing liquid test meal in response to CCK. Treatment with gastrin was shown to increase plasma PAI-1 and attenuated delayed gastric emptying in C57BL/6 mice.

Food intake is stimulated by orexigens, most notably ghrelin, working via appetite-stimulating neurons in the arcuate nucleus. While ghrelin stimulated feeding in fed *ad libitum* C57BL/6 mice, PAI-1 increased feeding in previously fasted C57BL/6 mice only. This response to ghrelin and PAI-1 was also replicated in PAI-1 $^{-/-}$ mice, suggesting PAI-1 is not required for the orexigenic effect of ghrelin. Moreover, intrapertoneal (ip.) administered ghrelin increased fos expression in arcuate neurons of both C57BL/6 and PAI-1 $^{-/-}$ mice, whereas ip. PAI-1 did not.

Weight loss in the PAI-1HKβ mice appeared to reverse the insensitivity to CCK in terms of gastric emptying. PAI-1HKβ mice were also found to be insensitive to other gut-derived satiety hormones, suggesting gastric PAI-1 is an anti-satiety factor. However, mice null for wild-type gastric PAI-1 responded normally to CCK prior to feeding, indicating that wild type is necessary for CCK insensitivity in the PAI-1HKβ mice.

The current findings demonstrate that PAI-1 plays a role in the control of food intake. PAI-1 is an example of a novel anti-satiety factor that can modulate gut-brain

signalling via the vagus nerve in order to preserve nutrient intake. This work provides a platform for future investigations into novel pathways implicated in the development and treatment of obesity.

Chapter 1

Introduction

1.1 Overview

Over a century ago Pavlov commented on the acidification of the upper intestine that resulted in pancreatic secretion (Pavlov, 1901). Wertheimer and le Page then showed that the flow of juice persisted after section of nerves to the intestine (Lepage, 1901). Bayliss and Starling, realised that the link between intestine and the pancreas was via the circulation and on 16th January 1902, showed that extracts of duodenal mucosa administered intravenously stimulated pancreatic secretion (Bayliss and Starling, 1902). The active factor was called secretin: it was the first hormone although the word 'hormone' was only introduced in 1905 to describe blood-borne messenger molecules. Shortly after, Edkins hypothesised that an analogous mechanism might regulate gastric secretion and reported the extraction of an active factor (gastric secretin, or gastrin) from antral mucosa (Edkins, 1906). The gastrins were subsequently isolated, sequenced and synthesised in 1964 by Gregory *et al.* (Gregory and Tracy, 1964). At about the same time Jorpes and Mutt isolated and sequenced secretin (Jorpes *et al*., 1962, Mutt *et al*., 1970). Other hormones discovered in the interim included insulin from pancreatic islets and in 1928, cholecystokinin, which was described as a hormone released by intestinal fat that stimulated gall bladder contraction (Ivy and Oldberg, 1928). Initially, it was suggested that secretin, gastrin and cholecystokinin (CCK) acted on the same receptor, although that was soon proved to be incorrect. Gut hormones were considered for many years to be exclusively involved in regulating digestion. However, in more recent times, it is now clear that many are involved in the control of food intake. CCK for example, is a well-established satiety hormone. Gut hormones are now thought to hold particular relevance in the development of obesity (Suzuki *et al*., 2011).

Obesity is one of the largest emerging health problems of our time. The reasons for this are the fatal consequences of co-morbidities such as, stroke, coronary heart disease, type II diabetes and also cancer. Insulin and leptin resistance are key players in obesity, where the normal mechanisms for food intake regulation are blunted (de Lartigue *et al*., 2012). Obesity is a multifactorial disease. Genetic predisposition of metabolic disorders is widely accepted (O'Rahilly and Farooqi, 2008). However, an increase in the amount of energy-rich foods available, which are high in fat and sugar, combined with an increasingly sedentary lifestyle, have proved to be important risk factors in the emergence of such a global epidemic in today's modern society (Rolls *et al*., 2007, Speakman, 2007).

The control of food intake falls into two categories of regulation: hedonic control via mesolimbic reward pathways, and homeostatic control. Homeostatic control of energy balance is primarily mediated via the feeding centres within the hypothalamus. The hypothalamus receives signals from several peripheral organs, including the liver, pancreas, stomach and adipose tissue. Hormonal signals can influence energy homeostasis by either acting directly on the central nervous system (CNS) via the circulation or by interacting with the afferent neurons of the vagus nerve (Dockray, 2009b). The main source of input is from the gastrointestinal tract via mechanoreceptors and chemoreceptors that respond to distension and nutrient composition, respectively. The brainstem is the first part of the vagal input. Many hormones activate vagal afferent neurons (VAN) via a mechanism involving second order neurons in the nucleus of the solitary tract (NTS) of the brainstem (Grill and Kaplan, 2002, Nozaki *et al*., 2002).

Multiple signals involved in the regulation of food intake are disrupted in obesity. An increase or decrease in plasma concentrations of many factors occurs in

response to the level of adiposity. One molecule that is elevated in plasma of obese individuals is plasminogen activator inhibitor-1 (PAI-1) (Shimomura *et al*., 1996, Ma *et al*., 2004). PAI-1 is expressed in adipocytes, liver and the GI tract, where the stomach holds significant importance, as levels of PAI-1 are also found to increase in the stomach with infection and inflammation.

Targeted expression of PAI-1 to the parietal cells of the stomach in mice results in hyperphagia, increased adiposity and weight gain. Previous work done by the group has revealed a desensitivity to satiety hormone CCK in behavioural studies (Kenny *et al*., 2013a). CCK is known to activate NTS neurons when administered to rodents, via a vagally-mediated pathway. Investigating gut-brain signalling by CCK in these mice will be an important aspect of this thesis. This current research focuses on the general hypothesis that PAI-1 expressed in the stomach acts as a novel signalling molecule involved in the disruption of energy homeostasis.

1.2 The Gastrointestinal Tract and Digestive Function

The gastrointestinal tract, or alimentary canal is essentially a long cavity which is designed for the digestion and absorption of nutrients. Food is initially broken down during chewing by amylase contained within the saliva. Swallowed food forms a bolus which is propelled to the stomach via peristaltic waves. The stomach has two openings, located at the oesophageal and duodenal junctions. It contains four regions known as the cardia, pylorus, fundus and the corpus. Each region performs specific functions with regards to digestion. The stomach

liquifies its contents and controls the rate at which food enters the duodenum. There are different types of cell in the stomach that are responsible for different roles in digestion. Parietal cells are responsible for the secretion of hydrochloric acid, chief cells release pepsinogen that on activation digests protein, and the enteroendocrine cells release hormones. Mucous neck cells cover the whole luminal surface and secrete bicarbonate mucus which enables lubrication and also protects the epithelium from chemical insults. Once the food is liquified into chyme, and the liquefied contents are passed to the duodenum, whose function is to receive partly digested food and continue the digestive process. Here, it mixes with digestive enzymes from the pancreas and bile from the gall bladder, before moving further along the small intestine. The small intestine is divided into 3 sections; the duodenum, the jejunum and the ileum. The jejunum and ileum are about 4-6 metres long in humans and maximise water and nutrient absorption due to their large surface area. Surface area is further increased by the structure of the villi and the microvilli. Digestive products absorbed here are transported to the liver via the blood stream, known as the hepatic portal system. Once all the nutrients have been absorbed, digestive products enter the large intestine, or the colon. Here, water is removed from the remainder, resulting in the formation of fecal waste products. Faeces pass to the rectum, where they are expelled from the body via the anus. The cells and hormones that govern this system are highly regulated involving multiple mechanisms and cell types.

1.2.1 Distribution, Structure and Biology of Enteroendocrine (EEC) Cells

The enteroendocrine system within the gut is a one of the largest endocrine systems in the body terms of cell number. There are at least 15 subtypes of enteroendocrine cells that secrete various peptide hormones and biogenic amines (histamine, 5-hydroxytryptophan [5-HT]) involved in the regulation of postpradial secretion and motility (Moran *et al*., 2008). It is now widely accepted that some EECs are capable of secreting more than one hormone.

1.2.2 Luminal Sensing

It is commonly accepted that the major function of the majority of EECs is to sense the luminal contents. EECs described as 'open' are able to function as transepithelial signal transducers by having contact with the lumen, leading to the release of biological mediators. This may be via a 'classic' endocrine mechanism or via a paracrine, or local, effects on nearby cells; ie vagal afferent fibres. Expression of taste receptors such as G-protein coupled receptor (GPCR) families T1 and T2 have been identified in EECs, and are considered key components that trigger gut peptide release (Moran-Ramos *et al*., 2012). More specifically, there are GPCRs involved in sensing sweet, bitter, fatty acids and amino acids. In addition, there are other mechanisms of luminal sensing involved, including ATP-sensitive K^+ channels and sodium-glucose linked transporter 1 (SGLT1). All these mechanisms ultimately increase intracellular calcium which triggers exocytosis.

1.3 Energy Homeostasis

Energy homeostasis is a key survival mechanism that allows the storage and metabolism of ingested food. Energy expenditure and meal size, composition and frequency vary from day to day but generally, body weight remains stable for most people. The drive for hunger is mediated by orexigenic systems used by ghrelin during the interdigestive period, whereas signals to terminate a meal, like CCK, are released in response to nutrients and gastric distension (Tack *et al*., 2006, Raybould *et al*., 1994). These processes are controlled by both peripheral and central mechanisms. The CNS receives signals from the gut that aid the surveillance of energy status (Figure 1.1).

The brain stem receives signals via the vagus nerve (Williams 2001) following food intake. The NTS is where these signals terminate before projecting to second order neurons in the hypothalamus. It was discovered by Grill and Smith in 1988, that the brainstem is important in regulating meal size in short term feeding, although the hypothalamus is required for more long-term control of food intake (Grill and Smith, 1988). The hypothalamus is made up of several nuclei each with their own functions. The projections between these nuclei and other brain areas facilitate the integration of signals from the periphery, the gut and the brain in order to regulate energy homeostasis (Neary *et al*., 2004). The arcuate nucleus is located at the base of the hypothalamus containing cell bodies that express receptors for hormones and neuropeptides that regulate food intake. Hormones reach the arcuate via the circulation through permeable capillaries in the median eminence. The arcuate contains specialised NPY/AgRP (neuropeptide

Y/ agouti-related protein) neurons (Goldstone *et al*., 2002). These neurons are inhibited by insulin and leptin and activated by ghrelin (Kalra *et al*., 1999, Nakazato *et al*., 2001). In fasted states, circulating levels of peptide YY (PYY) 3-36, insulin and leptin are low and levels of ghrelin are high. This increases NPY/AgRP neuronal activity and thus appetite is increased. In contrast, leptin stimulates POMC (pro-opiomelanocortin)-expressing neurons (Cowley *et al*., 2001). Furthermore, administration of PYY 3-36 increases POMC expression and inhibits NPY expression (Batterham *et al*., 2002). The melanocortin receptors 3 (MC3) and 4 (MC4) are activated and inhibited by neuropeptides, POMC and NPY/AgRP, respectively. This results in efferent signals that control food intake and energy expenditure (Cone, 1999). Another hypothalamic region important in energy homeostasis is the paraventricular nucleus (PVN). Located adjacent to the third ventricle and protected by the blood brain barrier, this region receives neuroendocrine signals via the arcuate nucleus as both POMC and NPY/AgRP neurons have dense projections into the PVN. Here, anorexigenic hormones such as thyrotropic-releasing hormone (TRH), corticotropin-releasing hormone (CRH) and oxytocin are released. When adiposity signals reach the PVN, a catabolic mechanism is activated, which switches to an anabolic mechanism when adiposity signals fall, informing the brain that stores are low and require replenishment (Vettor *et al*., 2002). The gut hormones activate vagal and sympathetic pathways and work with central mechanisms to regulate energy intake and expenditure (Figure 1.1).

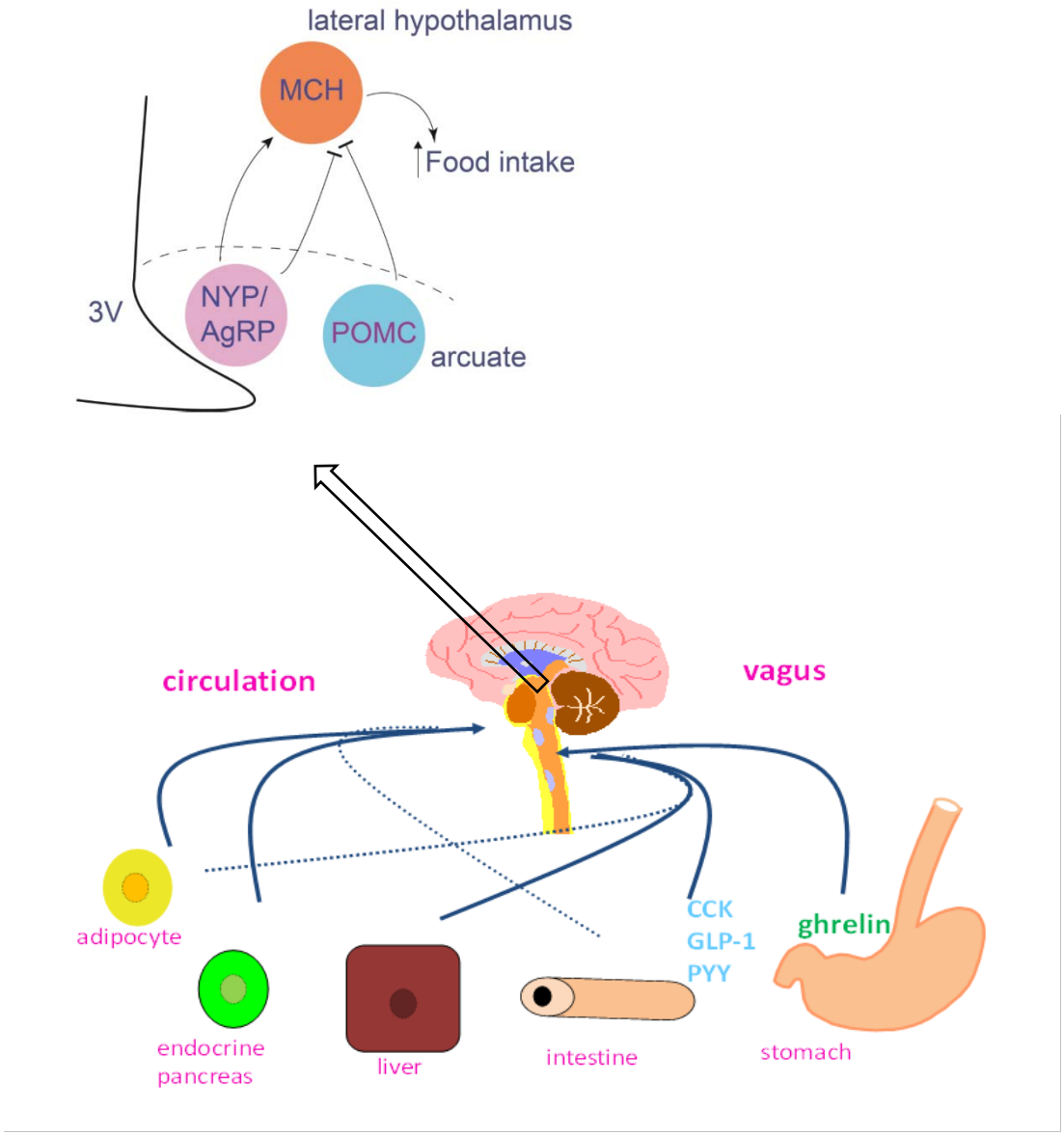

Figure 1.1

Energy homeostasis (Adapted from Dockray *et al., Regul Pept* 2009;155:6-10*)*

Energy homeostasis is controlled via both peripheral and central signal. Many of which act via the vagus (Dockray, 2009a) and trigger ascending pathways from neurons in the brainstem to specific neurons in the hypothalamus. (Melanin Concentrating Hormone, MCH; Pro-opiomelanocortin, POMC; Neuropeptide Y, NPY; third ventricle, 3V).

1.3.1 Gut Hormones

The gut hormones together maintain homeostasis via various mechanisms and interactions with other molecules, including neuropeptides, cytokines and adipokines. Gut hormones exert their effects via exocrine glands, smooth muscle, other endocrine cells and the peripheral nervous system (Murphy and Bloom, 2006). The majority of the gut hormones are released post-prandially (Adrian *et al*., 1985, Le Quellec *et al*., 1992), but some, for example, motilin and ghrelin are released in the interdigestive phase (Tack *et al*., 2006, Hellemans *et al*., 1976). Those that are released following the ingestion of nutrients including CCK, PYY and glucagon-like peptide (GLP)-1, regulate food intake by increasing satiety, but ghrelin released in the interdigestive phase stimulates food intake (Tack *et al*., 2006). Furthermore, satiety hormones such as CCK, are able to mediate inhibitory feedback mechanisms, thus delaying gastric emptying (Raybould *et al*., 1994).

1.3.1.1 The Gastrin Family

In mammals, gastrin and CCK are the only gut peptides that belong to this particular family. They are structurally and functionally related. Both are important in gastric and pancreatic secretion and smooth muscle contraction. As with many regulatory peptides, they are produced as larger precursors that are cleaved enzymatically to generate their active forms.

1.3.1.1.1 Gastrin

Gregory and Tracy chemically characterised the gastrins are peptides of 34 and 17 amino acid residues, both of which exist as sulphated and unsulphated peptides. They proved that amidation at the C-terminal was important for the stimulation of gastric acid secretion (Gregory and Tracy, 1964).

The main forms of gastrin are known as 'classic gastrins' (Dockray *et al*., 2005, Pauwels *et al*., 1986) and include G-34 (big gastrin), G-17 (little gastrin), and G-14 (mini gastrin). In humans, gastrin is expressed in both the G-cells of the pyloric antrum and the duodenum. The presence of food in the stomach is a strong stimulator of gastrin expression. There are several meal-associated mechanisms that do this, including gastric distension, vagal stimulation, and dietary protein. Following a meal, gastrin levels increase two- to three-fold (Dockray, 2004). The release of gastrin is inhibited by gastric acid in a negative feedback mechanism involving somatostatin.

Gastrin originates from a 101 amino acid precursor that undergoes a proteolytic cleavage by endopeptidases, resulting in the 80 amino acid, progastrin (Bishop *et al*., 1998). This precursor is subsequently cleaved before secretion. (Dockray *et*

al., 2005). Gastrins act at CCK-2 receptors which belong to the GPCR superfamily (Dockray *et al*., 2012). Once bound to these receptors, gastrin stimulates the biosynthesis and release of histamine (Blackmore *et al*., 2001) from enterochromaffin-like cells that in turn stimulates parietal cells to release hydrogen ions into the stomach. In addition to the stimulation of acid release, gastrin has many biological functions including stimulating gastric epithelial cell proliferation and secretion of pepsinogen from chief cells.

Increased gastrin levels are associated with *Helicobacter pylori* infection (Steele *et al*., 2007). *Helicobacter pylori* was first identified by Marshall and Warren as a bacteria that was present in patients with gastric ulcers and chronic gastritis. Conditions in which acid secretion is reduced or lost (thereby removing the negative feedback control of gastrin release) especially following infection with *Helicobacter pylori,* is thought to play a role in gastric pathology. Other examples that disrupt gastrin release are pernicious anaemia and proton-pump inhibitors (PPI) that inhibit the proton pump in parietal cells (Burman *et al*., 1989). Gastritis and hypergastrinaemia have been associated with the development of gastric cancer (Fox *et al*., 2003, Przemeck *et al*., 2008).

1.3.1.1.2 Cholecystokinin (CCK)

CCK is a primary regulator of small intestinal fat and protein digestion: it determines the capacity for digestion by regulating the secretion of pancreatic enzyme and delivery of bile salt, and it controls substrate delivery to the small intestine by inhibition of food intake and gastric emptying. CCK is produced in the I-cells (Figure 1.2) of the duodenum and the jejunum, but is also widely

expressed in neurons (Morino *et al*., 1994), including the afferent neurons of the vagus. It was first identified as a 33 amino acid protein (Mutt and Jorpes, 1971). Although there are several forms of circulating CCK across various species, CCK-58, CCK-39, CCK-33, CCK-8 have been identified in humans (Eberlein *et al*., 1988). The half-life of CCK-33 is relatively short, being rapidly degraded within 2.5 minutes (Thompson *et al*., 1975). Post-prandial release of CCK is stimulated by proteins and free fatty acids (FFA) (Liddle *et al*., 1986a). Interestingly, there is a sharp cut-off point in fatty acid chain length that is required for the functional release of CCK. Saturated fatty acids with a chain length of 12 or more carbon atoms are able to stimulate CCK release, while those with shorter chains are not (McLaughlin *et al*., 1999). One mechanism that has been proposed is that fatty acids act at GPCR GPR40, which is highly expressed in I-cells to trigger CCK release (Liou *et al*., 2011). However, binding at GPCR GPR120 is another possibility (Tanaka *et al*., 2008).

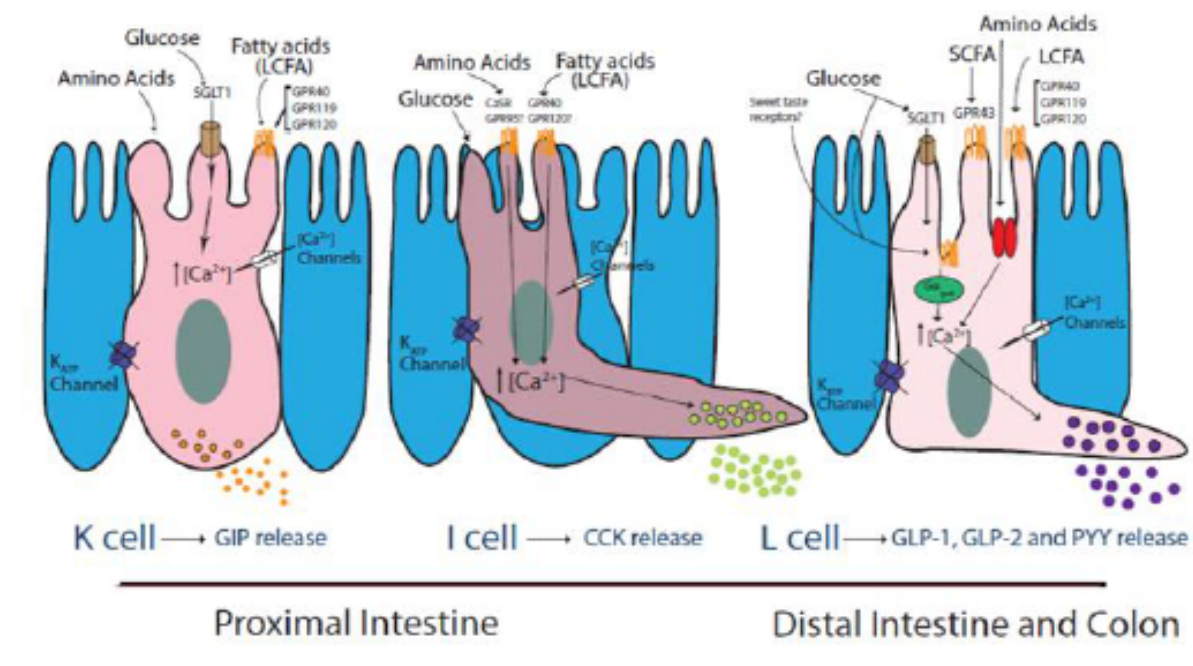

Figure 1.2

Nutrient Sensing in the Gut (adapted from Moran-Ramos *et al.Adv. Nutr 2012;3:8-20*

K and I cells are located in the proximal intestine, L cells are located in the distal intestine and colon. Macromolecules from ingested food stimulate release mechanisms within the enteroendocrine cells. Dietary fat potently induces release of CCK. Carbohydrates induce the release of GLP-1. Fats and proteins are potent secretagogues of PYY (CaSR, calcium sensing receptor; GPR, G protein-coupled receptor; LCFA, long-chain fatty acids; SGLT1, sodium-dependent glucose transporter 1) (Moran-Ramos et al., 2012).

1.3.1.1.3 CCK Receptors and the Vagus

The receptors (CCK-1 and CCK-2) at which CCK exerts its effects are both GPCRs, that when bound result in the activation of phospholipase C followed by an intracellular increase in Ca^{2+} (Wank, 1995). It has been proposed that CCK release from the gut acts directly via the CCK-1R (Wank, 1995). The CCK-1Rs are expressed in the pancreas, gall bladder, stomach kidney, lung and in parts of the CNS. In addition, they are also expressed by vagal afferent neurons (VANs) that serve the stomach and upper small intestine (Schwartz et al., 1993c, Moran et al., 1990, Moriarty et al., 1997). CCK-induced satiety occurs predominantly via interactions with CCK-1R. It has been proposed that CCK acts via a peripheral mechanism, as shown by Passaro *et al.* where CCK could not cross the blood brain barrier (BBB) (Passaro *et al.*, 1982), but may, however, pass through where the BBB is leaky such as the area postrema. Vagal afferent fibres originating in the gut respond to administration of CCK-8. Okano-Matsumoto *et al.* described how the excitation of intestinal vagal afferent terminals by CCK released post-prandially from nearby intestinal endocrine cells primarily drives inhibition of vagal excitatory motor drive to the hindstomach. Whereas the effects of CCK on gastric emptying may occur only later, as CCK reaches the circulation in amounts sufficient to influence the activity of gastric afferent fibres (Okano-Matsumoto *et al.*, 2011). Furthermore, vagal deafferentation abolishes the anorectic effect of peripheral administration of CCK (Smith *et al.*, 1981).

CCK is capable of influencing gene expression within VANs and inducing subsequent satiety. On the one hand, CCK suppresses the expression of orexigenic signals including cannabinoid receptor, CB1, melanin-concentrating hormone (MCH) and its receptor, MHCR (Burdyga *et al.*, 2004, Burdyga *et al.*,

2006), and on the other, it increases the expression of anorectic factors including cocaine amphetamine regulated transcript (CART) and neuropeptide tyrosine 2 (Y2) receptor (de Lartigue et al., 2007b, Burdyga et al., 2008).

In electrophysiological recordings of vagal afferent fibres, it was demonstrated that these fibres are sensitive to 5-HT (Iggo, 1955, Paintal, 1973) and later, CCK could be shown to stimulate brainstem neurons including neurons stimulated by gastric distension (Raybould *et al*., 1988). Moreover, capsaicin-treatment to lesion small diameter vagal afferents abolishes the satiety and gastric-emptying effects of CCK (Raybould and Tache, 1988, South and Ritter, 1988, Forster *et al*., 1990). Vagally-mediated satiety effects of CCK work via a mechanism involving second-order neurons in the NTS of the caudal brainstem (Patterson *et al*., 2002, Reidelberger *et al*., 2004, Smith *et al*., 1985, Smith and Gibbs, 1985). Furthermore, it has long been established as was first reported by Luckman *et al.*, that peripheral administration of CCK induces fos protein abundance in brainstem neurons (Luckman, 1992). Fos belongs to a family of intermediate early genes, the products of which, form dimer complexes with either itself or Jun, a protein encoded by *c-jun* (Stein *et al*., 1992) which is another intermediate early gene (Angel and Karin, 1991). This complex becomes the transcription factor, AP-1, which is important for the activation of other genes. Fos is detectable in neurons at around 20-30 minutes after depolarisation.

1.3.2 PP-fold Family

1.3.2.1 Peptide Tyrosine Tyrosine (PYY)

Tatemoto and Mutt discovered PYY during their studies on C-terminally amidated peptides in extracts of porcine upper intestinal tissue (Tatemoto and Mutt, 1980).

Later Adrian *et al.* described it as a putative gut hormone involved in post-prandial satiety (Adrian *et al*., 1985). PYY shares structural features with pancreatic polypeptide (PP) and neuropeptide Y (NPY). All are 36 amino acids long with a characteristic hairpin PP fold (Berglund *et al*., 2003). PYY is secreted as the 36-residue form (PYY_{1-36}) which is converted to PYY_{3-36} (Grandt *et al*., 1994) by DPP-IV after secretion (Medeiros and Turner, 1994). PYY is found in L-type enteroendocrine cells (Figure 1.2) that are most abundant in the ileum and colon (Adrian *et al*., 1985). PYY is released in proportion to the caloric content of meals. Concentrations rise within 30 minutes of feeding and remain elevated for up to 6 hours (Batterham *et al*., 2003).

PYY and other PP-fold peptides act on several different G-protein coupled receptors: Y1, Y2, Y4 Y5 and Y6. Each receptor differs in tissue distribution, function and selectivity for NPY, PP and PYY (Berglund *et al*., 2003). PYY_{3-36} selectively binds to Y2 receptors (Y2R) which are known to be involved in energy homeostasis (Grandt *et al*., 1994, Browning and Travagli, 2009). Y2R is widely expressed in the CNS and exerts its effects via inhibition of cAMP production. Deficiency of Y2R abolishes the satiety effects of PYY_{3-36} - the effects of which are also attenuated by Y2R inhibitors (Browning and Travagli, 2009). PYY knockout mice are hyperphagic with increased body weight and adiposity. This phenotype is reversed by treatment with exogenous PYY_{3-36} (Batterham *et al*., 2002). Some studies have demonstrated that administration of PYY to rodents and humans inhibits food intake. However, in direct contrast, other studies using PYY have failed to reproduce the inhibitory effect on food intake (Tschop *et al*., 2004). In addition, others have demonstrated that administration of PYY can inhibit food intake in the morning but not in the evening (Koegler *et al*., 2005).

PYY_{3-36} has also been implicated in increased energy expenditure and thermogenesis (Guo *et al*., 2006, Sloth *et al*., 2007).

The mechanisms by which PYY_{3-36} induces satiety remain unclear. The effects appear to be mediated centrally via the arcuate nucleus (ARC) (Batterham *et al*., 2002). While some groups have described exogenous PYY_{3-36} acting by a decrease in expression and release of NPY combined with activation of POMC neurons, others have reported an inhibitory effect of PYY_{3-36} on POMC neurons via post-synaptic Y2R (Ghamari-Langroudi *et al*., 2005). Furthermore, POMC knockout mice retain their responses to exogenous PYY_{3-36} , indicating that POMC is not critical in the satiety effects of $PYY_{3-36,}$ but instead works via NPY family of receptors (Larhammar, 1996). A vagally-mediated pathway has also been implicated. Y2Rs have been located on the vagus nerve (Koda *et al*., 2005, Burdyga *et al*., 2008) and vagotomy abolishes all activation of ARC feeding neurons following exogenous administration of PYY_{3-36} (Abbott *et al*., 2005). PYY_{3-36} has also been reported to influence gastric motility, pancreatic secretions and some effects of PYY_{3-36} may be mediated by gastric distension following a delay in gastric emptying (Savage *et al*., 1987).

Levels of PYY_{3-36} are elevated in individuals diagnosed with anorexia nervosa, but lower levels are reported in obese subjects (Pfluger *et al*., 2007). Furthermore, obese individuals have a blunted response to satiety (Ashby and Bloom, 2007), although following bariatric surgery, PYY_{3-36} levels are elevated and are consistent with the incidence of a reduced appetite in these patients (Korner *et al*., 2006).

1.3.2.2 Pancreatic polypeptide (PP)

PP was first described in chickens by Kimmel (Kimmel *et al*., 1968) and later isolated from other mammalian species by Chance *et al.* (Lin and Chance, 1974). Later, it was discovered by Larsson to be released by cells in pancreatic islets, exocrine pancreas and the gut (Larsson *et al*., 1975). Further work by Adrian *et al.* reported that it was released post-prandially by pancreatic PP cells that are stimulated by vagal efferent fibres (Adrian *et al*., 1976, Taylor *et al*., 1978). There are many similarities between PYY and PP, including secretion in proportion to caloric intake and fluctuations in plasma concentrations depending on feeding status (Adrian *et al*., 1976). PP release is also stimulated by a several gut peptides including CCK, ghrelin and secretin during exercise and states of hypoglycaemia (Kojima *et al*., 2007). Post-prandial levels are also elevated to a greater extent following a meal ingested in the evening than one eaten earlier in the day (Johns *et al*., 2006). PP binds to all Y receptors but has the highest affinity for the Y4 receptor (Y4R) (Michel *et al*., 1998), acting via this specifically to reduce food intake. The effects of PP are mediated through the ARC and the AP in the brainstem. As these neurons are located near semi-permeable blood-brain barriers, it is possible that the actions of PP are exerted directly on CNS neurons delivered via the circulation. However, the vagus is thought to play a significant role in the anorectic effect of PP, as vagotomy abolishes satiety in rats (Asakawa *et al*., 2003). Thus, it has been proposed that PP mediates its actions via a combination of both brain regions and vagal afferents.

PP has various biological actions including the regulation of gallbladder motility, inhibition of pancreatic secretions and delayed gastric emptying (Adrian *et al*., 1979, Adrian *et al*., 1976, Greenberg *et al*., 1979, Kojima *et al*., 2007). In addition, PP has been shown to augment basal glucagon and insulin release in rats via a direct paracrine mechanism (Szecowka *et al*., 1983). Obese individuals have an attenuated PP satiety response, whereas anorexic patients show an exaggerated response (Lassmann *et al*., 1980, Uhe *et al*., 1992).

Peripheral administration of PP decreases food intake in both humans and mice (Asakawa *et al*., 1999). Furthermore, PP influences energy expenditure by increasing oxygen consumption in PP-treated mice (Asakawa *et al*., 2003). PP has a short half-life of approximately 7 minutes due to enzymatic degradation by dipeptidyl peptidase (DPP)-IV and neprilysin.

1.3.2.3 Neuropeptide Y (NPY)

NPY was first discovered by Tatemoto and Mutt and was later shown by Lundberg *et al.* to be localised to neurons in the brain and enteric nervous system (Tatemoto *et al*., 1985) (Lundberg *et al*., 1982). Unlike PYY and PP, it is expressed throughout the gut-brain axis, and is abundant in the sympathetic neurons to the vasculature (Lomax and Vanner, 2010). The major receptors for NPY are Y1 and Y2. NPY is a potent orexigen that increases appetite and food intake and decreases energy expenditure (Holzer *et al*., 2012). Y2R antagonists have been investigated as potential therapeutic agents in the treatment of obesity (Brothers and Wahlestedt, 2010).

1.3.3 Secretin/Glucagon Superfamily

The glucagon superfamily is the largest and most diverse family of gut hormones. It includes secretin, glucagon, glucagon-like peptides 1 and 2 (GLP-1/GLP-2), gastric inhibitory peptide (GIP), vasoactive inhibitory polypeptide (VIP), and peptide histidine isoleucine (PIH). As well as being found in the gut, they are also found in the central and peripheral nervous systems. Some of them act as insulinotropic agents and many have been investigated as potential therapeutic targets for obesity and type II diabetes.

1.3.3.1 Secretin

Secretin is a 27 amino acid gastrointestinal hormone, released from S cells in the duodenum following food intake resulting in increased secretion of bicarbonate into the lumen (Pelletier *et al*., 1978, Kim *et al*., 1979, Draviam *et al*., 1991). Secretin exerts its effects via its receptor, SctR. This type of receptor, along with receptors for other peptides of the glucagon superfamily, belong to the B1 subclass of GPCRs. The SctR is expressed in both pancreatic acinar cells and ductal epithelial cells in high levels, with much lower levels found in pancreatic islets (Ulrich *et al*., 1998). They are also found in the epithelial cells of the bile duct, in the stomach and on vagal afferent fibres that innervate the stomach (Steiner *et al*., 1993, Gespach *et al*., 1981, Bawab *et al*., 1988, Li *et al*., 1998). The receptors are also found in many regions of the brain, as well as in the brainstem (Yung *et al*., 2001, Fremeau *et al*., 1983, Nozaki *et al*., 2002).

Although the main physiological actions of secretin involve the exocrine pancreas, and thus, increasing the secretion of bicarbonate, it can also stimulate the release of bile, increase pepsinogen release and inhibit gastric acid in the stomach (Babu and Vijayan, 1983). It has other potential physiological functions including in the CNS where it has recently been considered a neuropeptide that can influence food intake (Cheng *et al*., 2011).

1.3.3.2 Glucagon

Glucagon was first discovered by Kimball and Murlin in 1923 following extraction of insulin from the pancreas (Kimball CP, 1923). They found additional substances that were hyperglycaemic. Glucagon was subsequently sequenced in 1957 by Bromer *et al.*, as a 29 amino acid peptide secreted from α-cells in the islets of Langerhans (Bromer *et al*., 1957). Glucagon is released into the portal vein during periods of fasting and in response to physical activity, and acts on the liver to promote glycogenesis (Stevenson *et al*., 1987, Striffler *et al*., 1981, Wasserman *et al*., 1989). These effects are mediated via the glucagon receptor which is a GPCR. It is expressed in the gut, adrenal glands, heart, spleen, pancreas, brain and adipocytes. However, it is predominantly expressed in the liver and the kidney (Svoboda *et al*., 1994).

Glucagon has been shown to increase energy expenditure in both rats and humans, particularly during insulin deficiency (Nair, 1987). Administration to rats has also shown to significantly reduce food intake. Further studies in diet-induced obese mice have demonstrated weight loss, making glucagon an attractive target for obesity therapy (Geary *et al*., 1993, Day *et al*., 2009).

1.3.3.3 Products of Preproglucagon Cleavage

Preproglucagon is a 160 amino acid pro-hormone containing a signal sequence of 20 amino acids at its N-terminal (Kieffer and Habener, 1999). It is expressed in the α-cells of the islets of Langerhans, L-cells of the intestine, and within the CNS. The manner in which this pro-hormone is cleaved gives rise to tissue-specific production of biologically active products including GLP-1, -2 and oxyntomodulin, as well as glucagon itself.

1.3.3.3.1 Glucagon-like peptide-1 (GLP-1)

Lund and Goodman were the first to make the discovery that proglucagon mRNA encoded not just glucagon but also two other glucagon-related peptides (Lund *et al*., 1980, Lund *et al*., 1981), later to be named GLP-1 and -2. GLP-1 exists in two biologically active forms - GLP-$1_{7\text{-}37}$ and GLP-$1_{7\text{-}36}$ (Dhanvantari *et al*., 1996). It was reported that the latter was found at a higher circulating concentration (Orskov *et al*., 1994).

Partial post-translational processing in L-cells gives rise to GLP-1, GLP-2 and oxyntomodulin, but not glucagon; the former are co-localised in L-cells with PYY (Figure 1.2), and are released into the circulation in response to food intake (Herrmann *et al*., 1995), in particular, dietary carbohydrates and lipids (Reimann, 2010). Upon release, GLP-1 is readily degraded by DPP-IV. GLP-1 exerts its effects in pancreatic islets and centrally in the ARC, PVN and the supraoptic nucleus (SON) via its receptor, GLP-1R (Shughrue *et al*., 1996) (Holst, 2007, Yamato *et al*., 1997).

GLP-1 stimulates glucose-dependent insulin release (MacDonald *et al*., 2002b, MacDonald *et al*., 2002a), inhibits glucagon secretion and glucose production (Murphy *et al*., 1996). It has also been implicated in delayed gastric emptying and promoting satiation (Schirra *et al*., 2006, Punjabi *et al*., 2011). Thus both peripheral and central administration to rats inhibits food intake (Tang-Christensen *et al*., 2001, Turton *et al*., 1996). Furthermore, chronic administration significantly reduces body weight (Meeran *et al*., 1999). In obese subjects, GLP-1 has been shown to reduce food intake in a dose-dependent manner (Verdich *et al*., 2001a, Verdich *et al*., 2001b) and also to delay gastric emptying (Naslund *et al*., 1999).

Since peripheral administration of GLP-1 activates neurons within the brainstem (Imeryuz *et al*., 1997), it is believed to be acting via a vagally-mediated mechanism. Moreover, vagotomy completely abolishes the satiety effects of GLP-1 (Abbott *et al*., 2005, Imeryuz *et al*., 1997).

Due to its rapid degradation, GLP-1 has not been suitable for therapeutic use. GLP-1 analogues, on the other hand, have been developed in recent years to combat type II diabetes (Joy *et al*., 2005). Exendin-4, which was originally isolated from the venomous saliva of the Gila monster (Eng, 1992), has led to the development of a long-acting competitive GLP-1 receptor antagonist, exenetide (Drucker and Nauck, 2006). Following additional work on GLP-1 mimetics, reports of reduced appetite and body weight became apparent (Buse *et al*., 2004, DeFronzo *et al*., 2005, Kendall *et al*., 2005). The prevention of degradation of GLP-1 by means of DPP-IV inhibitors has made this approach an attractive and effective therapy (Astrup *et al*., 2012).

1.3.3.3.2 Glucagon-like peptide-2 (GLP-2)

GLP-2 is a 33 amino acid peptide secreted by L-cells (Figure 1.2), along with GLP-1 following the ingestion of food (Damholt *et al*., 1999) (Xiao *et al*., 1999). GLP-2 is also rapidly degraded by DPP-IV. GLP-2 interacts with a specific GPCR, increasing intracellular cAMP (Munroe *et al*., 1999). Receptors are located throughout the CNS and periphery, primarily the stomach, large and small intestines, brainstem and lung (Yusta *et al*., 2000). GLP-2 is capable of stimulating intestinal growth and is potentially responsible for intestinal adaptation, where the presence of nutrients in the gut stimulate mucosal growth. It is thought that this occurs via a mechanism involving enteric neurons (Bjerknes and Cheng, 2001). GLP-2 inhibits food intake in ICV-injected rats, although peripheral injections in both rats and humans has not demonstrated the same inhibition (Scott *et al*., 1998, Schmidt *et al*., 2003).

1.3.3.3.3 Oxyntomodulin (OXM)

Another preproglucagon product, OXM, is a 37 amino acid peptide corresponding to glucagon extended at its C-terminus and was first described by Bataille *et al* (Bataille *et al*., 1981) following its isolation from pig intestine. It is released from L-cells following food intake (Le Quellec *et al*., 1992). Although OXM is thought to act at the GLP-1 receptor (Dakin *et al*., 2004), OXM also acts via a GLP-1 independent pathway and operates as a glucagon/GLP-1 receptor co-agonist (Dakin *et al*., 2001, Sowden *et al*., 2007). Studies employing magnetic resonance imaging (MRI) have shown that administration of OXM reduces neuronal activity in the ARC, PVN and SON, indicating a separate mechanism to that of GLP-1 (Chaudhri *et al*., 2006). OXM delays gastric emptying, reduces acid

secretion (Schjoldager *et al*., 1989) and induces satiety in rodents (Dakin *et al*., 2001, Dakin *et al*., 2004, Wynne *et al*., 2005). Furthermore, it can inhibit food intake in humans, reducing body weight in obese subjects (Wynne *et al*., 2005). GLP-1 co-agonists such as OXM have proved to be extremely effective in producing weight loss in rodent models of obesity making them an attractive future obesity therapy (Liu *et al*., 2010, Wynne *et al*., 2010).

1.3.3.4 Gastric Inhibitory Peptide GIP

GIP was first described by Brown *et al* in 1971as a peptide capable of inhibiting gastric secretion (Brown, 1971). Later, it was shown by Dupre *et al* that GIP was responsible for enhancing glucose-induced insulin secretion (Dupre *et al*., 1973). It is a 42 amino-acid derived from a 133-residue precursor, preproGIP. GIP is released by K cells (Figure 1.2), a type of enteroendocrine cell that is open to the gut lumen, and mainly located in the duodenal mucosa (Theodorakis *et al*., 2006). The release of GIP is dependent on food intake and circulating levels closely mirror that of insulin (Elliott *et al*., 1993, Cataland *et al*., 1974).

Plasma levels in fasted states are low and rapidly increase following food intake (Cho and Kieffer, 2010). Dietary fat is the most potent stimulator, followed by carbohydrates, with proteins being the weakest stimulus (Elliott *et al*., 1993, Drucker, 2007). The metabolism of GIP occurs much in the same way as with GLP-1; it is inactivated by DPP-IV. GIP is further degraded by the peripheral tissues (Deacon, 2004).

The receptor at which GIP exerts its actions is a GPCR. It is widely expressed in peripheral tissues, including pancreatic islets, adipose tissue, the stomach, small intestine, heart lung, bone, brain, kidneys, thyroid and various CNS sites (Usdin *et al*., 1993, Baggio and Drucker, 2007, Kim and Egan, 2008). The highest numbers of GIP receptors (GIPRs) are found in the pancreatic β-cells, which is consistent with its main physiological role as an incretin. However, the level of expression in other tissues suggests it has functions outside of the pancreas. GIP has been shown to play an important role in lipid metabolism and reformation of bone (Tsukiyama *et al*., 2006, Irwin and Flatt, 2009). A role for GIP has also been suggested in obesity. The ob/ob mouse when knocked out for GIPR is protected from obesity (Miyawaki *et al*., 2002). Furthermore, GIPR antagonism has been shown to reverse obesity in rats fed a high fat diet (HFD) (McClean *et al*., 2007).

1.3.3.5 Vasoactive intestinal polypeptide (VIP)

VIP was first discovered by Said and Mutt following its isolation from porcine ileum (Said and Mutt, 1970a). It was named after its property of exerting long-lasting vasodilatory effects (Said and Mutt, 1970b). VIP is a 28 amino acid polypeptide (Mutt and Said, 1974), and was initially thought to be a gut hormone, but is now considered an important neurotransmitter.

VIP is produced in various tissues, including the gut, pancreas and the hypothalamus. VIP can act via any one of three GPCRs; PAC_1, $VPAC_1$, $VPAC_2$ (Shivers *et al*., 1991, Laburthe *et al*., 1996, Laburthe *et al*., 2002). $VPAC_1$ is widely expressed through the CNS and in peripheral tissues of the liver and intestines (Ishihara *et al*., 1992, Usdin *et al*., 1994). $VPAC_2$ receptors are also

found in the peripheral tissues of the GI tract (Usdin *et al*., 1994), with PAC_1 receptors predominantly distributed in the CNS (Hashimoto *et al*., 1996, Shioda *et al*., 1997)

VIP's actions on autonomic peripheral and central nerves control motility, blood flow and secretion within the gastrointestinal (GI) tract (Fahrenkrug, 1993). VIP stimulates the secretion of water and bicarbonate by the in pancreas. It also inhibits gastric acid secretion (Schubert, 2002). PAC_1 and $VPAC_2$ receptors are found on neurons also possessing anorectic POMC mRNA, indicating a role for VIP in the regulation of food intake (Mounien *et al*., 2006).

1.3.4 Gut Hormone Interactions

1.3.4.1 Synergistic Interactions - CCK & Leptin

Leptin is a 16kDa adipokine secreted from white adipose tissue in concentrations proportional to the level of adiposity (Loftus, 1999). It has long been associated with the regulation of energy homoestasis in rodents and humans. The existence of leptin was originally postulated following studies on the obese mutant *ob/ob* mouse at the Jackson laboratories in 1950 (Ingalls *et al*., 1950). Later, Friedman identified the gene encoding for leptin by positional cloning of the mouse *ob* gene (Zhang *et al*., 1994). They showed that due to a genetic mutation, the *ob/ob* mouse produces no leptin (Zhang *et al*., 1994). Further to that, administration of leptin reversed the obese phenotype of the *ob/ob* mouse (Halaas *et al*., 1995, Pelleymounter *et al*., 1995, Rentsch *et al*., 1995). These discoveries have added

weight to the hypothesis that central mechanisms interact with peripheral signals in order to regulate long-term energy balance (Loftus, 1999).

Leptin acts at the Ob-R receptor; there are several splice variants of the single gene exist encoding the 'long form' of the receptor (Mercer *et al*., 1996). Upon ligand-receptor interactions, activation of the JAK-STAT signalling pathway occurs. Administration of recombinant leptin causes activation of STAT-3 in the hypothalamus of both wild type and *ob/ob* mice (Vaisse *et al*., 1996). Circulating leptin is transported in its intact form to the brain where it influences energy homeostasis. It works via interactions with the long form, which are biologically functional in terms of energy homeostasis, of the leptin receptor (Tartaglia *et al*., 1995). Long form leptin receptors are predominantly expressed in the arcuate, lateral, ventromedial and dorsomedial nuclei of the hypothalamus, whereas the short forms are found in various tissue sites throughout the body (Schwartz *et al*., 1996, Mercer *et al*., 1996).

Leptin is also expressed, along with several isoforms of its receptor, in gastric epithelial cells, indicating a role in gastric epithelial function (Mix *et al*., 2000). More recently, it was proposed by Cammisotto *et al.* that gastric leptin participates in a physiological axis to rapidly control food intake and nutrient absorption and that the combined secretion of adipose and gastric leptins ensures proper management of food processing and energy storage (Cammisotto *et al*., 2010).

Initially, leptin was considered to act directly as an adiposity signal in the brain. Work by Wang *et al.* indicated a peripheral mechanism was involved (Wang *et al*., 1997). Using electrophysiological recordings, they demonstrated that leptin

may also increase vagal afferent activity and that CCK modulates the sensitivity of gastric afferents to leptin. It is now known that the VANs that express the CCK-1 receptor, also express receptors for leptin (Burdyga *et al*., 2002, Buyse *et al*., 2001). Overall, these discoveries describe the synergy between an adipokine and a short-term satiety hormone in the regulation of homeostasis.

CCK is a relatively short-acting and acute modulator of food intake, whereas leptin is slow in onset (Barrachina *et al*., 1997a). It is now well established that leptin and CCK work synergistically in the regulation of food intake (Wang *et al*., 2000, Matson *et al*., 1997). Furthermore, co-administration in mice of intracerebroventricular (icv.) injections of leptin and ip. CCK has the ability to regulate body weight in rats (Matson and Ritter, 1999, Matson *et al*., 2000). The reduction in body weight was not only attributable to the decrease in food intake; the enhancement of thermogenesis and changes in metabolic rate has also been implicated (Matson *et al*., 2000, Matson and Ritter, 1999). The modulation of CCK in energy homeostasis does not appear to be specific to leptin. Other hormones are now known to potentiate the effects of CCK, including insulin (Riedy *et al*., 1995) and glucagon (Le Sauter and Geary, 1990).

1.3.4.2 Orexigenic & Anorexigenic Interactions

1.3.4.2.1 Ghrelin

Ghrelin is an acylated 28 amino acid peptide which is released from the endocrine cells of the stomach during the interdigestive period. It was first identified by Kojima *et al.* as an endogenous ligand for the growth hormone secretagogue

receptor, GHS-R (Kojima *et al*., 1999). It was found shortly afterwards, that the acylation of ghrelin is vital for its biological activity. The enzyme responsible for acylation is ghrelin O-acyl transferase (GOAT). Gutierrez *et al.* first described how knockout of GOAT in mice leads to a loss of activity of ghrelin (Gutierrez *et al*., 2008). The mice also displayed dramatic decreases in plasma glucose during fasting, indicating that ghrelin acts to preserve glucose status in cases of starvation (Li *et al*., 2012). Ghrelin is also a unique and powerful orexigen important in hunger and meal initiation, as demonstrated by pre-meal plasma surges (Cummings *et al*., 2002). Secretion of ghrelin prior to food intake is considered to stimulated by sympathetic mechanisms (Mundinger *et al*., 2006). In contrast to satiety gut hormones, plasma levels of ghrelin are decreased post-prandially (Ariyasu *et al*., 2001, Tschop *et al*., 2001). Furthermore, ghrelin increases GI motility and insulin secretions (Masuda *et al*., 2000, Date *et al*., 2002b). The ingestion of carbohydrates is thought to have the most pronounced effect on decreasing levels of ghrelin. However, attenuation of ghrelin is considered to be independent of nutrient sensing in the stomach and the duodenum, as the main mechanisms of action occurs via neural pathways (Cummings *et al*., 2005).

Ghrelin inhibits growth hormone secretion and stimulates NPY and growth hormone releasing hormone (GHRH)-producing neurons in rats (Date *et al*., 2002a). Ghrelin mediates its actions via receptors expressed in NPY/AgRP neurons in the arcuate nucleus (Hewson and Dickson, 2000) and can act on these neurons following peripheral administration. Damage to the arcuate in rats inhibits the response to peripheral administration of ghrelin (Tamura *et al*., 2002). Central administration of ghrelin increases neuronal responses in other hypothalamic nuclei including the paraventricular (PVN), dorsomedial, and lateral

nuclei. It also stimulates neurons in the NTS (Lawrence *et al*., 2002). NPY and AgRP mRNA levels are significantly increased following central administration of ghrelin (Kamegai *et al*., 2001).

Ghrelin has also been considered a key player in the development of obesity, by inducing hyperphagia. However, high fat diets have been shown to inhibit the orexigenic effects of ghrelin (Gardiner *et al*., 2010, Perez-Tilve *et al*., 2011). High fat also results in ghrelin resistance in NPY/AgRP neurons (Briggs *et al*., 2010).

In addition to role in energy homeostasis, ghrelin has also been implicated in the hedonic responses to food. Malik *et al.* demonstrated that exposure to food pictures during administration of ghrelin activated brain regions associated with reward, including the amygdala, orbitofrontal cortex, anterior insula and the stratium (Malik *et al*., 2008).

In addition to the central effects of ghrelin, the interactions between CCK and ghrelin are thought to take place with the neurons and cell bodies of the vagus. Electrophysiological studies have shown ghrelin is responsible for the inhibition of basal discharge of VANs (Asakawa *et al*., 2001, Date *et al*., 2002a). In cultured VANs, ghrelin blocks the effect of CCK in stimulating the expression of CART. One way it is thought to do this is by excluding phosphoCREB from the nucleus (de Lartigue et al., 2007b).

1.3.4.2.2 Orexin & CCK

Another orexigenic peptide, orexin, is also thought to modulate the actions of CCK on VANs. Orexin has been identified in both the hypothalamus and in the endocrine cells of the gut (Kirchgessner and Liu, 1999). Orexin receptors are found throughout the CNS and also in VANs. Orexin-A has been found to inhibit the effect of CCK on vagal afferent nerve discharge (Burdyga *et al*., 2003). Pre-treatment with orexin-A in rats inhibits the responses in nerves to CCK (Burdyga *et al*., 2003); clearly demonstrating that a peripheral mechanism is at work.

1.4 The Cannabinoid System

1.4.1 Lipid Amides

There is a regulatory system involving the lipid amides that are capable of operating alongside the gut hormones released from the EECs in the regulation of energy homeostasis.

1.4.1.2 Oleoylethanolamide (OEA)

OEA is formed from dietary oleic acid and phosphatidylethanolamine in the small intestine, where levels decrease in fasted states and increase following food intake, specifically containing oleate (de Fonseca *et al*., 2001). OEA has a short half-life, being rapidly degraded by fatty acid amide hydrolase (FAAH) or N-

acylethanolamine-hydrolyzing acid amidase (ASAH)-like protein (Sun *et al*., 2005). OEA is also involved in lipolysis, via a peroxisome proliferator-activated receptor (PPAR)-α mediated pathway (Fu *et al*., 2003, Lambert and Muccioli, 2007). Additionally, OEA is an agonist at transient receptor potential cation channel subfamily V member (TRPV)-1 receptors that are expressed in pre-adipocytes (Matias *et al*., 2007) but also in peripheral vagal sensory nerves involved in the control of food intake (Ahern, 2003). GPCR GRP119 is now also considered to be an OEA receptor (Lauffer *et al*., 2009). Intestinal OEA is considered to induce satiety signals via a peripheral mechanism (Petersen *et al*., 2006). Ip. injections of OEA produce a dose-dependent inhibition of food intake in rats, along with a significant reduction in body weight (Yang *et al*., 2007). Moreover, icv. administration of OEA does not induce the same response (de Fonseca *et al*., 2001). In terms of OEA's interactions with other gut-derived hormones, it has been found to decrease plasma ghrelin, but has no effects on satiety hormones including CCK, PYY and GLP-1 (Proulx *et al*., 2005, Cani *et al*., 2004).

1.4.1.3 Anandamide (AEA)

AEA has been the subject of great interest since it was discovered to be an endogenous ligand for the cannabinoid (CB)-1 receptor which upon activation, stimulates appetite (Devane *et al*., 1992). Both OEA and AEA are structurally similar, although in direct contrast to OEA, AEA levels increase dramatically on the withdrawal of food, most notably within the limbic forebrain and small intestine. AEA activates CB1 receptors within the mesolimbic pathway, resulting

in hyperphagic behaviour, in particular of more palatable foods (Kirkham *et al*., 2002). Hypothalamic mechanisms are also activated upon food withdrawal, indicating a dual mechanism of action (Jamshidi and Taylor, 2001, Kirkham *et al*., 2002). Just as leptin controls the levels of orexigenic factors in the hypothalamus, it also decreases the levels of anandamide (Di Marzo *et al*., 2001). Furthermore, increased endocannabinoid tone is considered to result in augmented levels of plasma ghrelin, the orexigenic effects of which can be blocked by CB1 antagonist, rimonabant (Tucci *et al*., 2004). The system by which anandamide works is also thought to involve vagally-mediated signals. Rats deprived of food have increased anandamide levels within the duodenum. Peripheral injection of rimonabant decreases food intake in these rats. Furthemore, destruction of capsaicin-sensitive nerves that are also involved in CCK-induced satiety, reverses the orexigenic effect of anadamide (Gomez *et al*., 2002). Withdrawal of food also enhances CB1 expression in CCK1 receptor-expressing neurons in the nodose ganglion. Re-feeding or exogenous CCK returns the neurons to low levels of CB1 receptor expression (Burdyga *et al*., 2004).

1.5 Obesity

1.5.1 Background

Obesity can be defined as an abnormal or excessive fat accumulation that has significant implications on an individual's health. Obesity can be classified using a simple index of weight to height ratio known as BMI (body mass index). An individual with a BMI of over 30 is considered to be obese. In the UK alone, it is

estimated that 65% of men and 58% of women, and 3 in 10 children (between ages 2-15) are classed as overweight or obese as of 2010 (Statistics on Obesity, Physical Activity and Diet: England, 2013 https://catalogue.ic.nhs.uk/publications/public-health/obesity/obes-phys-acti-diet-eng-2013/obes-phys-acti-diet-eng-2013-rep.pdf. Last accessed on 4th April 2014 (Health and Social Care Information Centre, 2013)).

1.5.2 Comorbidities

There are many common health complications associated with obesity. Vascular occlusive disease, which results in heart attacks and strokes, kills over 17 million people worldwide per year. Cases of adult onset diabetes have also been on the increase, with the World Health Organisation (WHO) predicting that death from diabetes will increase by 50% within the next ten years. Osteoarthritis can occur as well as many forms of cancer.

1.5.3 Obesity - A Multifactorial Condition

Obesity was originally considered to arise solely from an imbalance between the amount of calories consumed and energy expended. A combination of an increase in the amount of energy-rich foods available, which are high in fat and sugar, plus an increasingly sedentary lifestyle, are indeed factors responsible. However, evidence has emerged that other various factors, including genetics, are also partly responsible. Genes are thought to have a role in the risk of developing obesity; leptin deficiency and FTO polymorphisms are just a few of the genetic conditions

that have been implicated in the development of obesity (Meyre *et al*., 2010). Epigenetics are now considered to have more of an influence. Epigenetic factors, such as DNA methylation or chromatin remodelling, increase an individual's susceptibility to go on to develop obesity and obesity-related illnesses (Symonds *et al*., 2011, Slomko *et al*., 2012). Hales *et al.* discovered that low birth weight was a strong predictor for the development of type II diabetes in later life (Hales *et al*., 1991).

1.5.4 Gut Microbiota

The microbiota that reside in the gut are increasingly recognised as having multiple biological functions, including intestinal defence and immunity, assistance in the development of microvilli and degradation of undigested poly saccharides (Delzenne *et al*., 2011, Delzenne and Cani, 2011). It has also been implicated in the development of fat mass and influencing energy homeostasis. The microbiome was first associated with obesity when Backhed *et al.* reported that germ-free mice had 40% less body fat than normal wild type mice (Backhed *et al*., 2004). Furthermore, they discovered that the introduction of microbiota to the germ-free mice resulted in a 60% increase in body fat and insulin resistance. Further work indicated that germ-free mice were resistant to diet-induced obesity (Backhed *et al*., 2007). It has also been reported that gut microbiota can increase the energy harvested from food, is capable of influencing the body's signalling pathways, thus leading to disruption of energy homeostasis (Backhed *et al*., 2004, Turnbaugh *et al*., 2006, Hildebrandt *et al*., 2009). Furthermore, obese subjects have been shown to have an increased capacity for harvesting energy from food

due to the growth of microvilli (Turnbaugh *et al*., 2006). As will be discussed later in this chapter, low grade inflammation has become a common feature of obesity and related metabolic disorders. Lipopolysaccharide (LPS) has been proposed as an important modulator in the development of metabolic conditions (Cani et al. (2007), (Shen et al., 2013) via metabolic endotoxemia.

The two major phyla of gut microbiota are *Firmicutes* and *Bacteroidetes* (Turnbaugh *et al*., 2006). A link was first discovered by Backhed *et al.* between high fat diets and altered gut microbiota in mice (Backhed *et al*., 2007). Other groups have since reported an increase in Firmicutes and a decrease in Bacteroidetes in mouse models of obesity (Hildebrandt *et al*., 2009, Geurts *et al*., 2011, Turnbaugh *et al*., 2008, Murphy *et al*., 2010). The Firmicutes/Bacteroidetes ratio in obese individuals has become a candidate biomarker for the development of insulin resistance and type II diabetes. Both *Bifidobacterium* and *Faecalibacterium* levels are lower in obese patients suffering with type II diabetes, than in healthy, lean subjects (Furet *et al*., 2010). Further investigations carried out by Vrieze *et al.* demonstrated that insulin resistance could be temporarily reversed when intestinal microbiota from lean subjects were given to metabolic syndrome patients (Vrieze *et al*., 2012).

The use of prebiotic foodstuffs is another area that has stimulated considerable interest in the manipulation of gut microbiota in obesity. Evidence has shown that prebiotic drinks can decrease appetite, improve glucose response via fermentation by the gut microbiota (Cani *et al*., 2009). Furthermore, a sustained ingestion of such prebiotic drinks caused a significant rise in GLP-1 and PYY plasma levels (Cani *et al*., 2009). Thus, gut microbiota may be a key factor in the development of obesity, suggesting the use of probiotics may be useful in individuals with a

predisposition to obesity (Suzuki *et al*., 2011). Raybould *et al.* have reported that shifts in the gut microbiota can lead to intestinal inflammation, leading to changes in tight junction proteins and subsequent intestinal permeability (Raybould, 2012). This allows the transfer of LPS from the lumen into the gut interstitial tissues, causing the activation of toll-like receptors (TLR-4) located on vagal afferent nerve terminals. It is thought that this inhibits leptin action at the site of vagal afferents (Raybould, 2012).

1.5.5 Obesity - An Inflammatory Disease?

Obesity is now considered an inflammatory condition involving multiple cellular and molecular mechanisms. The nature of the condition involves multiple organs and can lead on to many other disease states. There is a dose-response relationship between nutrient excess and the disruption of cellular and molecular mediators of the immune and inflammatory systems (Lumeng and Saltiel, 2011). Obesity has been described as a low-grade chronic inflammation; referred to as 'meta-inflammation' (Hotamisligil, 2006). Metainflammation is a tonic, low-grade activation of the innate immune system that can affect metabolic homeostasis (Hotamisligil, 2006). Immune activation can also occur following periods of fasting or the ingestion of high fat foods (Kosteli *et al*., 2010, Alipour *et al*., 2007). Expression of pro-inflammatory cytokines is elevated in the adipose tissue of obese patients, providing more evidence of links between the immune and metabolic systems (Emilsson *et al*., 2008). There are multiple inflammatory inputs that can affect metabolism, including circulating cytokines and decreases in protective adipokines (Hotamisligil *et al*., 1995, Hotta *et al*., 2000). Furthermore, macrophages and T-cells have been shown to play pivotal roles in the

development of insulin resistance (Varma *et al*., 2009, Huang *et al*., 2010). Some adaptive immune responses are beneficial to maintain metabolic homeostasis. Adipose tissue macrophages (ATMs) are recruited when chemokine release or lipolysis is triggered. This could promote lipid storage by inhibiting lipolysis (Lumeng and Saltiel, 2011, Kosteli *et al*., 2010).

It is also known that both LPS and IFN-γ encourage macrophages to assume a pro-inflammatory activation state known as M1 that generates Th1 responses observed in obesity (Lumeng and Saltiel, 2011). Conversely, Th2 cytokines such as IL-4 and IL-3 encourage an M2 activation state that promotes fibrosis via inhibition of NF-κB pathways (Zeyda *et al*., 2010). Increased adiposity results in a switch from one state (M2) to a pro-inflammatory response (M1) (Lumeng *et al*., 2007).

There is thought to be over 20 million macrophages within each kilogram of excess fat in humans (O'Rourke *et al*., 2009). Endoplasmic reticulum (ER) stress, adipose tissue hypoxia and adipocyte death are all hallmarks of the inflammatory response in obesity (Liu *et al*., 2009). Insulin resistance can also influence the expression of genes and macrophages. Natural killer cells and mast cells are also considered to be contributors to metainflammation (Ohmura *et al*., 2010). Although metainflammation in obesity occurs within the adipose tissue, there are other locations that undergo pro-inflammatory changes. Inflammation in pancreatic islets decreases insulin secretion and promotes beta cell apoptosis, leading to the development of diabetes (Ehses *et al*., 2008, Donath *et al*., 2010). Macrophages are also known to aggregate within the islets in diet-induced obesity (Ehses *et al*., 2007).

Changes within the liver also occur. Non-alcoholic fatty liver disease (NAFLD) is a well-known risk factor for insulin resistance, steatohepatitis and dyslipidaemia (Fabbrini *et al*., 2009). In this condition, M1/Th1 cytokine levels are increased, similar to observations in adipose tissue (Li *et al*., 2005, Kremer *et al*., 2006). Myocytes within muscle are also found to respond to inflammation via pattern recognition receptors such as toll-like receptor (TLR)-4 (Frisard *et al*., 2010). Interestingly, inflammation within the hypothalamus occurs following high fat diet and lipid infusion (Thaler and Schwartz, 2010). Here, diaglycerols and ceramides accumulate and promote increased feeding and nutrient storage via leptin and insulin resistance. It is thought to be partly mediated by saturated fatty acids that stimulate JNK and NF-κB signalling in neurons (Zhang *et al*., 2008). Inflammation in the hypothalamus can impair insulin release and its peripheral actions (Calegari *et al*., 2011, Purkayastha *et al*., 2011). Hypothalamic inflammation can also lead to oxidative stress in the renin-angiotensin system and potentiate the hypertensive response (Kang *et al*., 2009).

1.5.6 Treating Obesity

Along with significant health problems, obesity decreases quality of life and life expectancy dramatically. As little as 5% reduction in body weight has been shown to be effective in reducing the risks associated with obesity (Tsigos *et al*., 2008). Lifestyle modifications are first employed to reduce caloric intake, increase physical activity and modulate behaviour through cognitive behavioural therapy (Tsigos *et al*., 2008, WHO, 2000). Whilst these approaches are considered essential, long-term effects on weight loss are difficult to achieve. Thus, other

pharmacological and surgical approaches have been introduced in weight loss management programmes.

1.5.6.1 Drugs and Targets

Pharmacological interventions have included many drug types working on various targets either in the CNS or peripheral tissues of the muscle, fat, and the GI tract (Schwartz *et al*., 2000). Drugs generally fall into one of three categories. The first category of drug aims to reduce appetite and or induce satiety by working via specific receptors in the brain, such as CB1 antagonists and neurotransmitter reuptake inhibitors. The second type aims to increase physical activity and lipid oxidation via catecholaminergic systems within the CNS and periphery. The final type aims to reduce the absorption of lipids from the diet via lipase inhibition (Hainer and Hainerova, 2012). Orlistat, a derivative of lipostatin, falls into the latter drug category. It inhibits the lipases found in the stomach and pancreas that are responsible for the hydrolysis of ingested dietary fat (Hanif and Kumar, 2002). It is currently the only drug on the market specifically aimed at reducing weight loss in obese patients. However, despite what seems to be promising results, effective weight loss is only obtained with a hypocaloric diet. Rimonabant, an antagonist of CB1 receptors in both the periphery and the CNS was shown to be an efficient anti-obesity drug in trials. It is capable of influencing both homeostatic and hedonistic mechanisms (Cota *et al*., 2006, Cota, 2008). However, in 2009, it was withdrawn from the market due to serious adverse events associated with depression and suicidal ideation. Other drugs of this class, including taranabant and otenabant (Aronne *et al*., 2010) have also proved to

influence mood and were subsequently pulled from late phase trials. Another drug, ecopipam, a dopamine (D1/D5) receptor also had effects on mood and was also withdrawn from trials (Astrup *et al*., 2007). There are other drugs that have shown promise in effective weight loss management. GLP-1 analogues, although used primarily for the treatment of diabetes, have been effective in reducing body weight during treatment (Bunck *et al*., 2009).

1.5.6.2 Surgery

Bariatric surgery has long been established as an effective approach to sustained weight loss in obese patients (Brolin, 2002, Cummings *et al*., 2004). Surgery for obesity is either malabsorptive or restrictive. The former involves bypass of the proximal jejunum, which allows nutrients to pass directly to more distal regions of the intestine (Brolin *et al*., 2002). Restrictive surgery involves the use of a gastric band to restrict the size of the stomach, and thus limit meal size. Although weight loss results following this procedure is not as dramatic as a bypass, it is considered the safer option (Favretti *et al*., 2009). A procedure known as roux-en-Y gastric bypass combines both malabsorptive and restrictive methods, but may put patients at increased risk of complications (Tadross and le Roux, 2009). An interesting outcome of bariatric surgery is the modulation of gut hormone release, which in turn has been implicated in the long-term maintenance of weight. Ghrelin levels decrease whilst levels of PYY and GLP-1 rise (Cummings *et al*., 2002, Korner *et al*., 2009, le Roux *et al*., 2007). Gastric bypass has also shown to have dramatic, positive effects on diabetic patients; most notably by improving

their overall glycaemic profile within 48 hours of surgery (Laville and Disse, 2009).

1.5.7 Animal Models of Obesity

One of the first genetic mutations associated with obesity was observed in the Agouti mouse, which was described over a century ago (Little, 1913). A further particularly well known example is the *Ob/ob* mouse (Ingalls *et al*., 1950) described in section 1.3.4.1 (Zhang *et al*., 1994). There have been many more single-gene loss of function defects discovered, but only a small number of models have shown an obese phenotype that have mutations within these genes (Speakman *et al*., 2007). The diabetic *db/db* model has an Ob-R mutation, therefore administration of leptin shows no effect on obesity, but has been used as a leptin-resistance model. The *tubby* and *fat* models (Coleman, 1982) have been shown to develop late-onset obesity along with insulin resistance, however the mechanism is unknown. Deafness and retinal degeneration have also been implicated with such mutations. Several other mutant transgenic organisms have become useful in further research, making the mutant mouse a powerful tool in the investigations of defects that lead to obesity (Table 1.1).

Gene	Product	Mutation/Deficiency	Function and Phenotype
Aʸ	Agouti signalling peptide	*Agouti yellow*	Antagonist of melanocortin-4 receptor/ yellow fur and obese
Lep	Leptin	*ob (Ob/ob)*	Satiety hormone in fat cells/ deficiency causes massive obese phenotype
Lepr	Leptin receptor	*db (Db/db)*	Lack of receptor produces a diabetic model
Cpe	Carboxypeptidase E	*Fat*	Production of active forms of proinsulin and proopiomelanocortin, obese model
Tub	Insulin signalling protein	*tub (tubby)*	Insulin signalling, obese

Table 1.1: Mouse models of obesity

1.6 The Urokinase Plasminogen Activator System

Fibrinolysis was first discovered by McFarlane *et al.* (Macfarlane and Pilling, 1947). Subsequent investigations found that a substance, urokinase (uPA), within the urine could stimulate plasmin activity from plasminogen (Williams, 1951). uPA is a 53 kDa extracellular matrix enzyme which hydrolyses a specific Arg^{560}-Val^{561} bond resulting in the activation of plasminogen (Lesuk *et al.*, 1965). uPA is a multi-domain protease with a large C-terminal region. Its N-terminal has a growth factor (GF)-like domain, a kringle domain and a connecting linker region. The kringle domain is a folded protein stabilised by 3 disulphide bridges, and holds significant importance in blood clotting proteins.

1.6.1 Urokinase and the uPA Receptor

The uPAR is glycosylphosphatidyl inositol (GPI)-anchored receptor that binds uPA via the GF-like domain. Once bound, uPA catalyses plasminogen activation via a positive feedback loop (Figure 1.3). uPAR is able to mediate other intracellular signalling processes involved in cell proliferation, migration, invasion and survival (Collen and Lijnen, 2004). One of the unique aspects of uPAR is that is has neither cytoplasmic nor transmembrane domains but is involved in ligand-dependant signalling transduction (Ploug *et al.*, 1991). It is now an accepted that uPAR interacts with other transmembrane receptors including integrins, epidermal growth factor (EGF)- and platelet-derived growth factor (PDGF)- receptors, and GPCRs. uPAR also interacts with the extracellular matrix (ECM) protein, vitronectin. The association with vitronectin activates a signalling cascade leading to rearrangements of the cytoskeleton and stimulation of cell migration (Kjoller and Hall, 2001). Interestingly, uPAR can bind both

vitronectin and uPA, due to two distinct binding sites (Wei *et al*., 1994, Huai *et al*., 2008). Furthermore, binding of uPA to uPAR enhances vitronectin binding by uPAR (Sidenius *et al*., 2002).

Various cell types express uPAR including epithelial and endothelial cells (Limongi *et al*., 1995, Pepper *et al*., 1993). Under normal physiological conditions, uPAR has low tissue expression (Solberg *et al*., 2001). However, certain conditions have been identified in mice that induce the expression of uPAR, including tissue remodelling and wound healing (Solberg *et al*., 2001, Floridon *et al*., 1999, Romer *et al*., 1994). uPAR is also expressed in response to stress, tissue injury and inflammation (Beschorner *et al*., 2000, Plesner *et al*., 1994, Nykjaer *et al*., 1994).

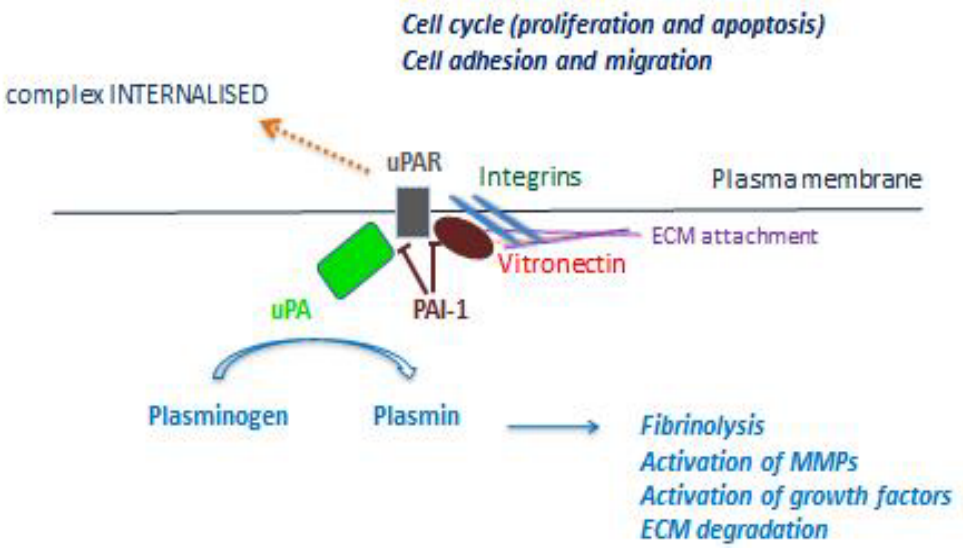

Figure 1.3:

The uPA system

Illustration showing the interactions between uPA and its receptor, uPAR. uPAR interacts with integrins and vitronectin when bound to uPA.

1.6.1.1 Plasminogen Activator Inhibitor (PAI)-1

PAI-1 belongs to the family of serine protease inhibitors (SERPINs). It was first discovered in 1981 by Loskutoff *et al.* in the endothelial cells of the rabbit (Loskutoff and Edgington, 1981). PAI-1 is an inhibitor of plasminogen activation by both tissue-type (t-) and urokinase-type (u-) plasminogen activator (PA) (Carmeliet *et al*., 1993a, Carmeliet *et al*., 1993b). It is a single chain glycoprotein with a molecular weight of 50 kDa (Lawrence *et al*., 1989). PAI-1 has a reactive centre loop, located at the carboxy terminus (Figure 1.4) and is a pseudosubstrate for serine proteases (Loskutoff *et al*., 1987). PAI-1 is known to exist in various conformational states: active, inactive and latent states have all been identified (Nar *et al*., 2000, Levin and Santell, 1987). The active form spontaneously converts to its latent form with a half-life of approximately one hour (Hekman and Loskutoff, 1988). Latent forms of PAI-1 are capable of being converted into the active forms via mechanisms including treatment with denaturants, negatively-charged phospholipids, and also vitronectin (Lambers *et al*., 1987).

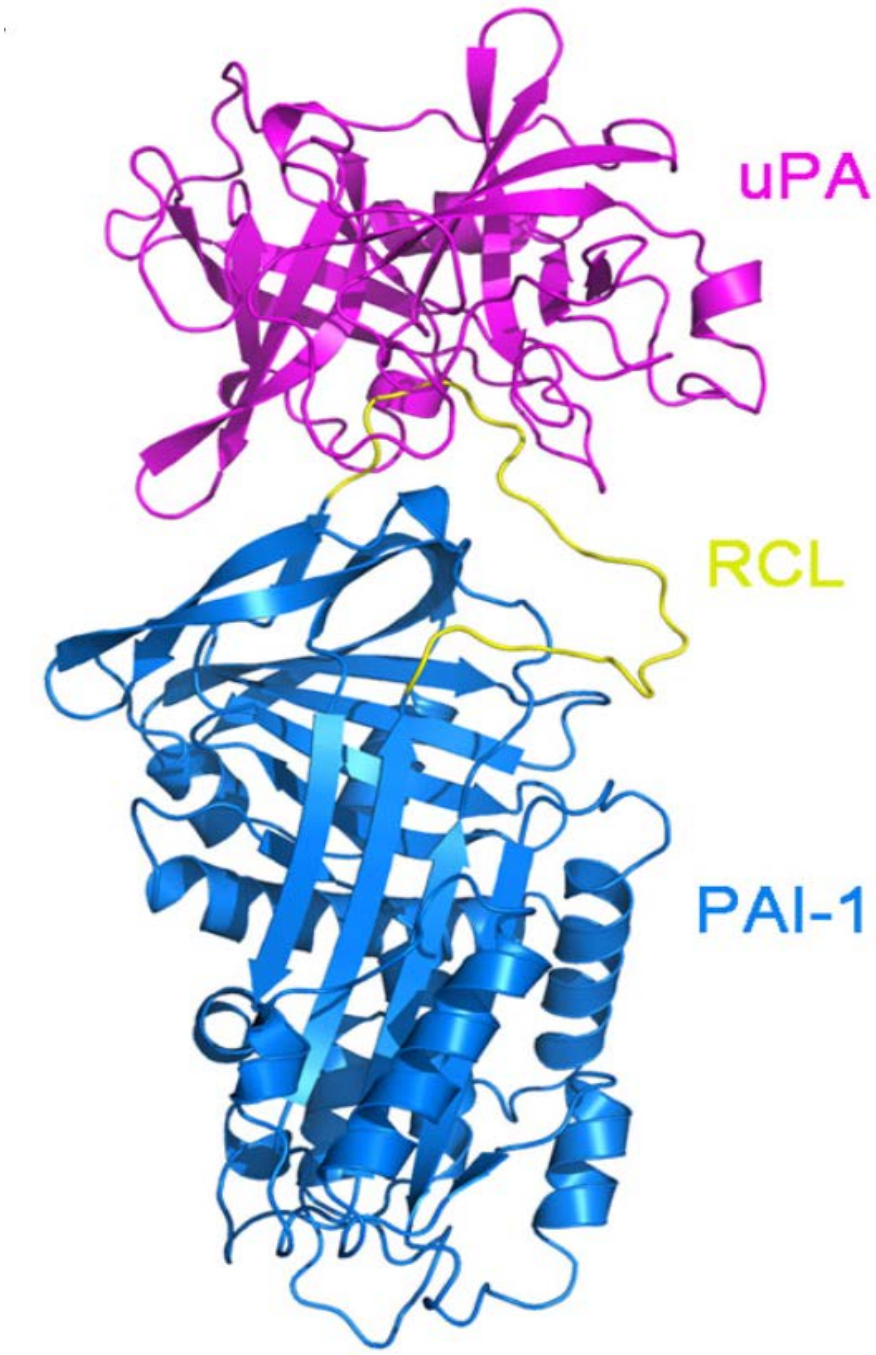

Figure 1.4:

Structure of uPA and PAI-1

This figure represents PAI-1 interacting with free uPA via its reactive centre loop. Figure was originally published in the Journal of Biological Chemistry (Lin *et al.*, 2011)

1.6.1.1.1 PAI-1 in disease

PAI-1 is an inhibitor of fibrinolysis, and therefore, deficiency was originally considered to cause problems with blood clotting. However, individuals and mice lacking PAI-1 have minor issues with bleeding and hyperfibrinolytic states, they also have normal development, and display no other major health concerns (Fay *et al*., 1997, Carmeliet *et al*., 1993a, Carmeliet *et al*., 1993b).

Elevated levels of plasma PAI-1 have been associated with an increased risk of developing cardiovascular diseases, myocardial infarction and inducing vascular occlusive episodes such as coronary sclerosis and deep vein thrombosis (Thogersen *et al*., 1998, Kohler and Grant, 2000). Moreover, increased expression of PAI-1 has been identified in atherosclerotic plaques which are indicative of the role of PAI-1 in diseases of vessel walls (Lupu *et al*., 1993, Schneiderman *et al*., 1992). PAI-1 deficient mice appear to protected against venous thrombi induced by endotoxin

Increased PAI-1 expression has been linked to fibrosis in the lung and liver. It is also increased in cases of glomernulonephritis and diabetic nephropathy (Yamamoto and Loskutoff, 1996, Loskutoff and Quigley, 2000, Tang *et al*., 2000). PAI-1 knock-out mice demonstrate an attenuated fibrinogenic phenotype when compared to wild-type or PAI-1 over-expressing mice following a fibrotic challenge (Eitzman *et al*., 1996a, Eitzman *et al*., 1996b).

PAI-1 has also been implicated in the development of tumours by influencing processes including tumour cell invasion, metastasis and angiogenesis (Jung *et al*., 2011). Elevated levels of PAI-1 are associated with poor prognosis in many different tumour types (Foekens *et al*., 2000).

1.6.1.1.2 PAI-1 and Obesity

PAI-1 is known to be elevated in individuals with obesity (Landin *et al*., 1990, Loskutoff and Samad, 1998). Adipose tissue is a source of PAI-1 and is considered to directly contribute to the observed elevation of PAI-1 in obesity. The adipose tissue of mice has high expression of PAI-1 mRNA (Sawdey and Loskutoff, 1991). In humans, weight loss from calorie controlled diets or surgical intervention reduces plasma PAI-1 levels (Calles-Escandon *et al*., 1996, Juhan-Vague and Alessi, 1997, Nielsen and Jensen, 1997). The increase in size of adipocytes is a characteristic of obesity, which in turn leads to a surge in PAI-1, compared to other tissue sites. Plasma PAI-1 is also increased up to five-fold in obese mice and humans (Samad and Loskutoff, 1996, Alessi *et al*., 1997). Omental (visceral) fat produces more PAI-1 than subcutaneous adipose tissue. In addition, triglycerides and fatty acid levels stimulate the expression of PAI-1 in 3T3-L1 adipocytes (Loskutoff and Samad, 1998).

1.6.1.1.3 Gastric expression of PAI-1

Increased expression of gastric PAI-1 has been documented following chemical insult, mucosal irritation and *Helicobacter* infection. It is thought that PAI-1 plays a protective role in mucosal gastric defence mechanisms (Kenny *et al*., 2013b, Kenny *et al*., 2008); see Figure 1.5. The generation of a mouse model by Kenny *et al.* with targeted expression of PAI-1 to gastric parietal cells (Figure 1.6) resulted in the serendipitous observation of an obese phenotype. This has encouraged further investigations into the underlying mechanisms of obesity relevant to PAI-1 (Kenny *et al*., 2013a).

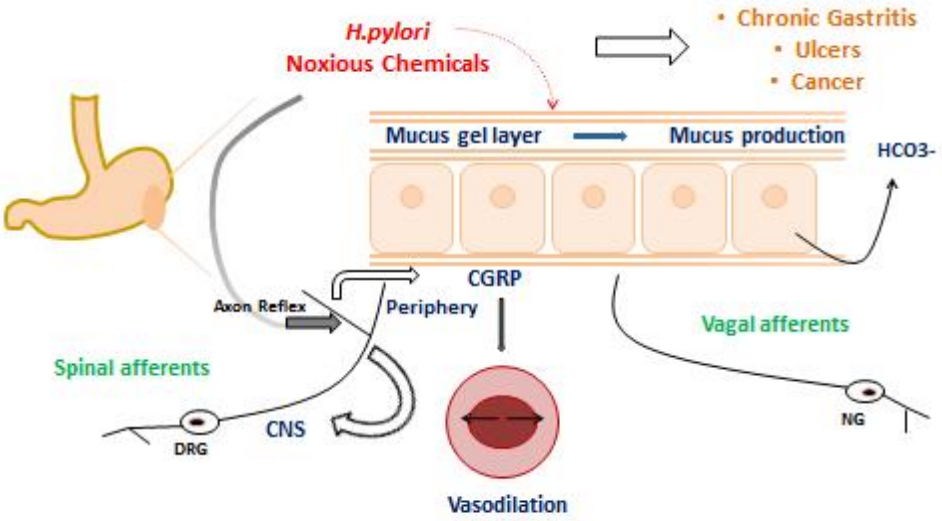

Figure 1.5: Attack and Defence within the Gastric Mucosa

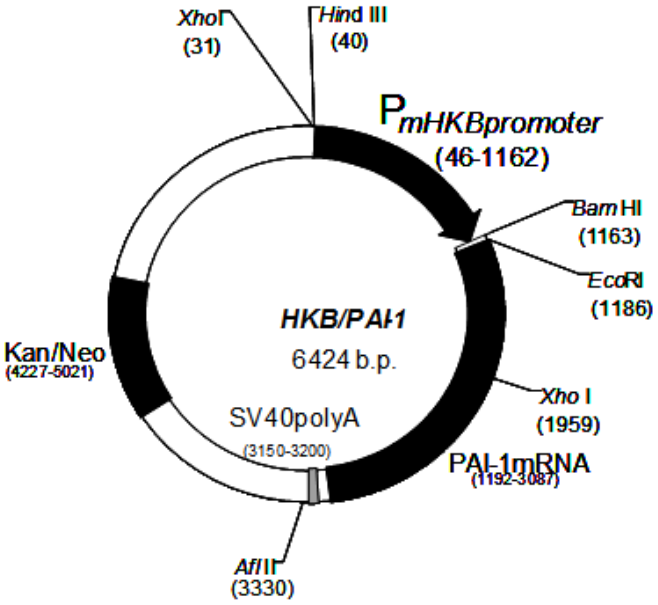

Figure 1.6: Vector for gastric expression of PAI-1

The promoter region corresponds to the beta subunit of gastric parietal cells, which drives the expression of mouse PAI-1 mRNA, producing a transgenic mouse with targeted expression of PAI-1 to the gastric parietal cells (Kenny *et al*., 2013a)

1.7 Aims and objectives

The PAI-1HKβ mice have a moderately obese phenotype, displaying hyperphagia and increased body weight and adiposity (Kenny *et al*., 2013a). Despite what is known about PAI-1, for example PAI-1 plasma levels being increased in obesity; its specific role in metabolic function is yet to be explored. This project aimed to investigate the gut-brain signalling in the PAI-1HKβ mice, with particular emphasis on the satiety hormone CCK. CCK is a well-studied gut peptide which has several biological actions, including stimulation of brainstem neurons and delayed gastric emptying. The attenuation of satiety signals has been implicated in the development of obesity, and thus, experiments analysing these process in the PAI-1HKβ mice would provide mechanistic insights into the role of PAI-1 in appetite regulation. The specific objectives of this project are listed below.

1. To investigate the effect of ip. CCK and feeding on gut-brain signalling in PAI-1-HKβ compared to C57BL/6 mice by observing neuronal fos expression in the NTS.
2. To identify the role of the uPAR receptor in PAI-1 signalling using uPAR null mice.
3. To develop a model for studies of CCK-regulated gastric emptying in mice and investigate the hypothesis that PAI-1 modulates CCK-regulated gastric emptying.
4. To test the hypothesis that PAI-1 stimulates food intake in both C57BL/6 and PAI-$1^{-/-}$ and to determine whether this is influenced by nutritional status (fasted or fed *ad libitum*).

5. To determine whether the effect of ghrelin on food intake is preserved in PAI-$1^{-/-}$ mice.
6. To elucidate the neuronal responses to PAI-1 in CNS regions influenced by orexigenic stimulation.
7. To compare the effects on food intake in PAI-1-HKβ mice of a variety of satiety factors.
8. To determine whether CCK resistance in PAI-1-HKβ mice is a consequence or cause of obesity by studies of NTS fos labelling and gastric emptying after calorie-restriction. In addition, investigate whether the obese phenotype in PAI-1-HKβ mice is solely attributable to the transgene

Chapter 2

Materials and Methods

2.1 Chemical reagents

ABC Vectarstain Elite	Vector Labs (Peterborough, UK)
Acetic Acid	Sigma (Poole, UK)
Anandamide	Sigma
Anti-c-Fos (Ab-5) (4-17) Rabbit pAb	Calbiochem (Middlesex, UK)
Camostat Mesilate (FOY-305)	Tocris (Bristol, UK)
CCK8-s	Tocris
DAB Peroxidase Substrate kit, 3, 3'-diaminobenzidine	Vector labs
Distilled Water	
DMSO	Sigma
Exendin-4	Tocris
Gastrin (G17ns)	Tocris
Ghrelin	Tocris
Hydrogen Peroxide	Sigma
Lorglumide	Rotta Research Laboratories, (Milan, Italy)
Methanol	Sigma
Methyl Cellulose	Sigma
Normal Donkey Serum	Stratech (Suffolk, UK)
Normal Goat Serum	Stratech
Oleoylethanolamide	Sigma
Orexin-A	Tocris
Oxyntomodulin	Tocris
PAI-1	Calbiochem
PAI-1 ELISA	Molecular innovation, MI, USA

Paraformaldehyde	Sigma
Pentoject (Pentobarbitone)	Animal Care Ltd (York, UK)
Peptone	Sigma
Phenol Red	Sigma
Phosphate-buffered Saline	Life technologies (paisley, UK)
Polyclonal Goat Anti-Rabbit Immunoglobulins/Biotinylated	Dako (Glostrup, Denmark)
Saline	Baxter (Berkshire, UK)
Sodium Azide	BDH (Poole, UK)
Sodium Chloride	BDH
Sodium Hydroxide	BDH
Sucrose	BDH
Triton-X100	Sigma
Trisodium citrate	Sigma

2.2 Equipment

Microscope (Axioplan 2 with AxioCam HRm)	Zeiss (Oberkochen, Germany)
Spectrophotometer	WPA Biowave II (Biochrom, Cambridge, UK)
Nanodrop (2000C)	Thermo Scientific (Leicestershire, UK)
Freezing microtome (SM2000R)	Leica (Newcastle-Upon-Tyne, UK)

2.3 Animals

C57BL/6 mice were purchased from Charles River, (Wilmington MA, USA). Mice null for PAI-1 (PAI-$1^{-/-}$) or for uPAR and PAI-1-HKβ mice were maintained on a C57BL/6 background. Transgenic PAI-1-HKβ mice exhibiting overexpression of PAI-1 targeted to the gastric partial cells were generated using 1.1 kb of the proximal promote of H^+/K^+ATPase β-subunit coupled to the coding sequence of mouse PAI-1 (PAI-1-HKβ mice) have previously been described (Kenny *et al*., 2008). The transgenic mice null for wild type PAI-1 and uPAR were purchased from The Jackson Laboratory (Bar Harbour ME, USA) and a breeding colony established in house. Mice expressing only the transgene (tg) for PAI-1 were generated by crossing PAI-$1^{-/-}$ with PAI-1-HKβ mice over three generations. Genotyping of PAI-$1^{-/-}$, $tg^{+/+}$ and PAI-$1^{-/-}$, $tg^{+/-}$ populations were carried out and confirmed by Prof. Rod Dimaline using PCR (http://jaxmice.jax.org/protocolsdb/f?p=116:2:1668577038540339::NO:2:P2_MASTER_PROTOCOL_ID,P2_JRS_CODE:6284,002507. Last accessed 4th April 2014) where the expected amplicon for the mutant was 290 bp. All mice were housed in a controlled environment in a light to dark cycle of 12 hours. Animals were fed on a commercial pellet diet and water was available *ad libitum*. Where fasting took place, animals were subjected to removal of food for no more than 24 hours and full access to water was given, unless otherwise stated. All experiments were approved by the University of Liverpool Animal Welfare Committee and were conducted in compliance with the UK Laboratory Animals (Experimental Use) Act, 1986 and performed under the Home Office personal licence of Joanne Gamble (PIL number 40/9281).

2.4 Animal treatments

Animals were fasted for 24 hours unless otherwise stated, prior to procedures. For experiments involving expression of c-fos in the NTS of the brainstem, mice received an ip injection of either CCK8s (2.5nmol/kg, Tocris Biosciences, Bristol, UK) in a 100µl of saline, or reintroduction of food for a period of 90 minutes before perfusion. C57BL/6 mice received an interperitoneal injection of stabilized human recombinant PAI-1 (2.5nmol/kg; Calbiochem, Hertfordshire, UK) prior to refeeding. In some experiments, C57BL/6 and PAI-$1^{-/-}$ were allowed free access to food prior to an interperitoneal injection of either PAI-1 (2.5nmol/kg), ghrelin (3nmol/kg, Tocris Biosciences, Bristol, UK) both in 100µl saline. Food was removed for 90 minutes before the animals were perfused. In gastric emptying assays, animals were fasted overnight and water was removed 1 hour prior to procedures. ip injections (100µl; CCK8s or PAI-1 at 2.5nmol/kg, and CCK-1 receptor antagonist, lorglumide [Rotta Research Laboratories, Milan, Italy] at 4ng/kg in saline or saline alone for controls) prior to gastric emptying experiments. In additional experiments, mice received ip. injections of human gastrin (G17ns; Tocris Biosciences, Bristol, UK; 20nmol/kg) 6 h prior to gastric emptying studies. Where mice received two *ip.* injections, a period of five minutes was timed in between injections, with the exception of gastrin.

2.4.1 Whole animal fixation via transcardial perfusion

Animals received a terminal ip injection of pentobarbitone (pentoject; Animalcare York, UK) at a volume range of 300-400µl or 40-50 mg/kg. Paw reflexes were checked prior to making an abdominal incision to expose the thoracic and peritoneal membrane. This was cut to gain access to the ribcage and diaphragm. The ribcage was cut at each lateral aspect along with the diaphragm and removed to gain access to the heart. The vena cava was cut before a perfusion syringe (29 Gauge) was injected into the left ventricle of the heart. A physiological saline solution (0.9%) was delivered (approximately 100ml) prior to switching the flow to 4% ice-cold paraformaldehyde, both at a rate of approximately 12mls per minute. Once the whole animal had become stiff, the head was removed for dissection.

2.4.2 Murine *Helicobacter felis* infection

Helicobacter felis (*H. felis* ATCC 49179) was cultured by Ms Debbie Sales (Medical Microbiology, The University of Liverpool). Animals aged 6-8 weeks were infected by oral gavage with 0.5ml (10^{10} colony forming units/mL) of a *H. felis* suspension in TBS broth, three times over a 1-2 week period (Kenny *et al*., 2013a). In order to ensure quality control of the bacteria, the optical density of suspension was above 1.5 and *H. felis* had been passaged no more than 5 times. Positive *H. felis* infection was confirmed by rapid antral urease test (Prontodry, Medical Instruments Corporation, Solothrum, Switzerland) or by antral histology performed by Dr. Suzanne Lyons and Dr. Susan Kenny.

2.5 Immunohistochemistry for the detection of fos in the brainstem and hypothalamus

2.5.1 Brainstem

Brains were dissected from the skull with the brainstems attached and post-fixed for four hours prior to storing in 30% sucrose. Brainstems were detached from the cerebellum, trimmed and cut on a freezing microtome at 40µm and stored as free floating sections in 0.1M phosphate buffered saline (PBS). Sections from approximately -7.76 mm to -7.32mm from Bregma were then processed for immunological localisation of fos in the nucleus of the solitary tract (NTS). Firstly, sections were incubated in a peroxidase deactivating solution (20% methanol, 0.2% Triton X100, and 1.5% hydrogen peroxide) for 30 minutes. Next, they were incubated for an hour in blocking agent of 10% donkey serum. The sections were then incubated in a primary rabbit anti-c-fos antibody (1:2000) for 48 hours at 4°C.

Sections were washed three times in 0.1M PBS prior to incubation with biotinylated secondary anti rabbit immunoglobulins (Ig) antibody (1:200) for 2 hours. A further three washes in 0.1M PBS was conducted before the addition of ABC (Vector) kit to the sections and incubated for 1 hour. Three more PBS washes were performed prior to the 3,3'-Diaminobenzidine reaction (DAB kit, Vector labs). Sections were watched closely for colour change from white to brown before a rapid wash in 0.1M PBS. Sections were then transferred to distilled water and mounted onto slides with vectarshield mountant.

2.5.2 Quantification of fos

Sections from the level of the area postrema were analysed using the bright field on the Zeiss axioplan-2 microscope at x10 magnification. All neurons within the areas corresponding to NTS nuclei with black/brown staining within the nucleus were counted as fos-positive. On average, 4-6 sections per brainstem were quantified, with the mean number of fos-positive neurons. Sample datasets were independently scored by an observer blinded to the experimental groups.

2.5.3 Hypothalamus

Brains were dissected from the cerebellum to the top of the olfactory bulb and sectioned on a freezing microtome at 40µm as previously described at the level of the third ventricle and median emenence.

2.6 Gastric Emptying Assays

Mice were fasted overnight and water was removed Mice received a liquid test meal with either methyl cellulose (1.5% w/v in distilled water) or peptone (4.5% w/v in methyl cellulose) with both containing 50mg/L phenol red, at a volume of 600µl via a steel gavage needle. All test meals were previously stored at 4°C and were pre-warmed for the mice to 37°C. Mice were culled after ten minutes with rising CO_2 followed by cervical dislocation. An abdominal incision was made, the pylorus and oesophageal junctions were clamped then ligated and the intact stomach was securely removed with the gastric contents collected into Eppendorf tubes. The contents were centrifuged at 15294 RCF for five minutes; the

supernatant was collected and the volume measured. Samples were alkalinised with a 1:5 dilution with 0.1M NaOH and the absorbance was determined using a spectrophotometer at 550nm. Samples of methyl cellulose and peptone test meals were used as controls. The equation, taken from Debas *et al.*, was used to calculate the rate of gastric emptying for each mouse (Debas *et al*., 1975).

2.7 Feeding studies

2.7.1 Anorexigenic peptides

Wild type C57BL/6 and PAI-1-H/Kβ mice were fasted overnight prior to procedures. Mice received ip. injections (100µl; CCK8s at 2.5nmol/kg, Exendin-4 at 0.75nmol/kg, oxyntomodulin at 3nmol/kg in saline and Oleoylethanolamide at 5mg/kg in 4% DMSO, with saline and 4% DMSO as controls) prior to access to a pre-weighed measurement of food. The food was weighed after 30 and 60 minutes and this amount was subtracted from the intial weight to give the amount of food eaten.

2.7.2 Orexigenic peptides

Wild type and PAI-$1^{-/-}$ mice were either fasted overnight with some allowed free access to food, with all mice allowed free access to water. Mice received ip. injections (100µl; PAI-1 at 2.5nmol/kg, ghrelin at 3nmol/kg, Orexin-A at 3nmol/kg in saline, anandamide at 14nmol/kg in 4% DMSO, with saline or 4% DMSO as controls) prior to returning pre-weighed food to all mice. The food was weighed at time points at 30 and 60 minutes, subtracted from the initial weight to calculate

amount of food eaten. All animals were given 2-3 days in between experiments to recover from injections and fasting.

2.7.3 Short term fasting studies

In some studies, C57BL/6 and PAI-1 $^{-/-}$ male mice were fasted for 6 hours prior to a period of re-feeding. Food intake was measured at time points of 30 and 60 minutes.

2.8 Enzyme-linked immunosorbent assay (ELISA) studies

Blood was obtained from C57BL/6 mice via cardiac puncture, collected in 0.1M trisodium citrate, centrifuged and concentrations of plasma PAI-1 were determined by ELISA (Molecular Innovations, MI, USA) according to manufacturer's instructions.

2.9 Statistical analyses

Data was analysed with GraphPad® Prism software (version 5) using unpaired, two-tailed t-tests. All data was expressed as mean ± standard error of the mean (SEM) unless otherwise stated. In other analyses, one-way or two-way analyses of variance (ANOVA) were performed where the data warranted such tests. Where the experimental design indicated a three-way ANOVA test was required, but effect of genotype or time had little/or no impact on experimental outcomes, a two-way ANOVA was performed. Bonferroni post-hoc tests were performed, unless otherwise stated.

Chapter 3

PAI-1 inhibits gut-brain signalling by CCK via the uPA receptor

3.1 Introduction

CCK is one of the most studied of all the satiety hormones. Since its discovery in 1928, it has been shown to be a short-term satiety signal released post-prandially. Peripherally administered CCK stimulates neurons within the brainstem and the effects of CCK on these neurons are mediated via vagal afferent neurons (Raybould *et al*., 1988). Increased fos expression in the neurons located in the nucleus tractus solitarii (NTS) can be observed following either exogenous administration CCK ip. or postprandial release of CCK after feeding (Chen *et al*., 1993, Luckman, 1992, Fraser *et al*., 1995, Wang *et al*., 1999, Zittel *et al*., 1999a). The NTS is located in the dorsal medial medulla, ventral to the area postrema (AP) and 4th ventricle and dorsal to the efferent system of the dorsal motor nucleus of the vagus nerve (DMV).

There are different NTS subnuclei arrayed rostrocaudally that process respiratory, gustatory and gut afferent signals (Grill and Hayes, 2009, Grill and Hayes, 2012). Vagal afferents originating from the stomach and small intestine project into neurons within the medial (Sol M), gelatinous (Sol G), dorsolateral (Sol DL) and commissural (Sol C) NTS subnuclei and amongst others are activated by satiation signals. Neurons expressing fos following ingestion of nutrients, or gastric distension, reach a peak at around -7.5mm from Bregma (figure 3.1), at the level of the AP (Fig 3.1). From around -8.12mm to -7.92mm from Bregma, the point where the AP is disappears and the DMV is smaller than it is more caudally, the appearance of additional NTS subnuclei becomes apparent. These disparate NTS neurons within the ventrolateral and interstitial NTS subnuclei receive vagal afferent inputs originating from receptors in the trachea, larynx and the bronchi.

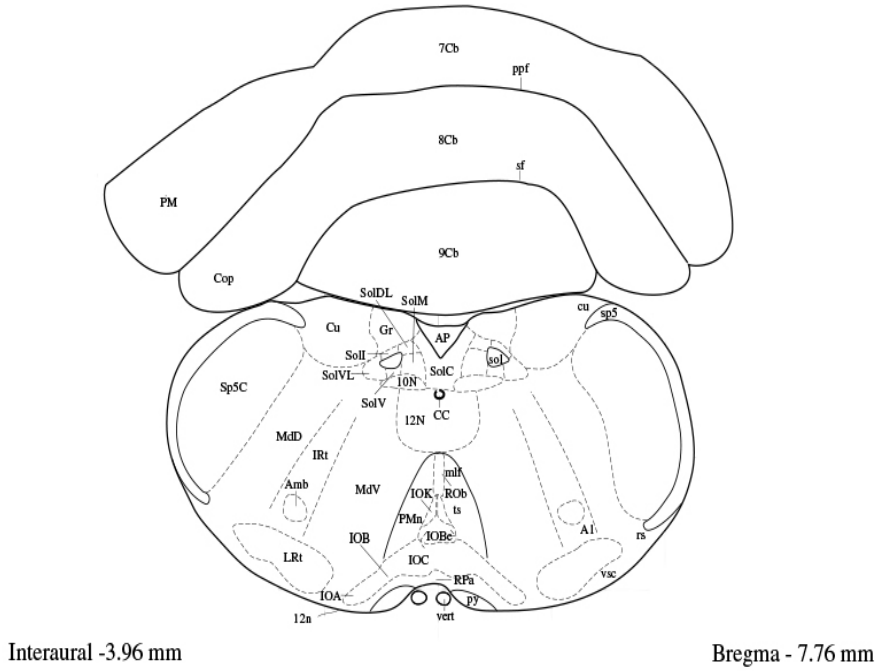

Figure 3.1

Mouse brainstem section.

Example of an annotated brainstem section at the level of the area postrema (AP) including the subnuclei regions of the NTS; adapted from Paxinos and Franklin. Permission was granted for this figure by Elsevier. Copyright Elsevier (Paxinos & Franklin, 2001) .

Previous work in the group resulted in the generation of a transgenic mouse model over-expressing PAI-1 in the stomach. These mice exhibit moderate obesity (Kenny *et al*., 2013a) and hyperphagia. The role of PAI-1 in gut-brain signalling remains to be explored. The experiments described in this chapter sought to address this.

3.1.1 Aims

The aim of the work in this chapter was to determine the role of PAI-1 in gut-brain signalling. The specific objectives were:

1. To investigate the effect of CCK on gut-brain signalling in PAI-1-HKβ compared to C57BL/6 mice by observing neuronal fos expression in key areas in the brainstem.
2. Using fos expression, to compare gut-brain signalling in PAI-1-H^+/K^+β and C57BL/6 mice following feeding.
3. To determine the effect of exogenous PAI-1 administration on brain stem fos in C57BL/6 mice.
4. To identify the role of the uPAR receptor in PAI-1 signalling using uPAR null mice.
5. To determine the effect of chronic *H.felis* infection on brainstem fos in responses to CCK8s in wild type an PAI-1 null mice.

3.2 Methods

3.2.1 Animals (C57BL/6, PAI-1-HKβ , uPAR$^{-/-}$ and PAI-1 $^{-/-}$)

Wild type (C57BL/6) and PAI-1-$H^+/K^+\beta$ mice, previously described in chapter 2, were fasted for no longer than 24 h prior to treatments. In some experiments, C57BL/6 and PAI-$1^{-/-}$ mice were infected with *H.felis* as described in chapter 2.

3.2.2 Brainstem fos labelling

Mice were culled 90 min after either reintroduction of food or administration of CCK8s. Mice were anaesthetised and perfused transcardially. Dissected brainstems were post-fixed and stored in 30% w/v sucrose. Free-floating sections (40µm) approximately -7.76 to -7.32 from Bregma were processed for immunohistochemistry using a peroxidase labelling technique described in Chapter 2. Neurons in the NTS with nuclear black brown staining at the plane of view were counted as positive. On average, 4-6 sections per brainstem were quantified and the mean number of fos-positive neurons was determined.

3.2.3 *H.felis* infection

C57BL/6 and PAI-$1^{-/-}$ mice were infected with *H.felis* were verified by an antral urease test or by antral histology as described in Chapter 2. Following 6 months of infection, mice were used in experiments to investigate fos labelling in the NTS after ip. CCK8s (2.5nmol/kg) administration.

3.3 Results

3.3.1 Mouse brainstem

In C57BL6 mice, neurons expressing fos were identified in several subnuclei of the NTS following *ip* injections of CCK (2.5nmol/kg; figure 3.2) or refeeding. Thus, in both cases, positive neurons were observed within the medial (Sol M), gelatinous (Sol G), dorsolateral (Sol DL) and commissural (Sol C). In subsequent experiments quantification of fos in PAI-1-H/Kβ mice, C57Bl/6 or PAI-1 null mice was carried in these subnuclei and the data aggregated.

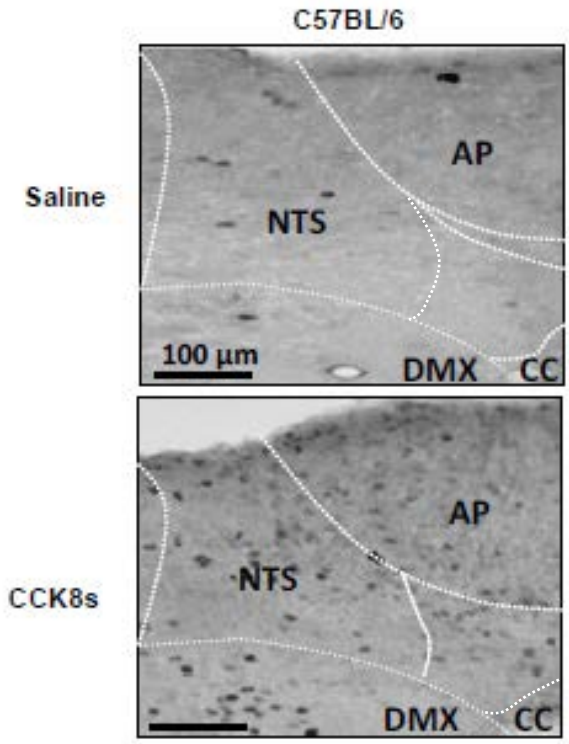

Figure 3.2

Expression of c-fos in NTS neurons in C57BL/6 mice in response to ip. CCK8s.

Neurons with stained nuclei (in focus in a single focal plane) in the NTS were counted for quantitative studies following ip injections with either saline or CCK8s (2.5nmol/kg). **NTS**, nucleus tractus solitarius; **AP**, area postrema; **DMX**, dorsal motor nucleus of the vagus; **CC**, central canal. Scale bar, 100µm.

3.3.2 PAI-1 suppresses neuronal activation by CCK

The physiological effects induced by the release of CCK are mediated via vagal afferent neurons, which increases expression of c-fos in the neurons located in the NTS. Fasted C57BL/6 mice had minimal expression of brainstem fos compared to mice that were refed following an overnight fast for 90 min (fasted C57BL/6 mean of 2.0 ± 0.2 neurons per mouse, compared to a mean of 18.0 ±1.4 neurons per mouse in previously fasted refed mice; Figure 3.3). These neurons were observed in populations located in SolG, Sol M and Sol DL subnuclei of the NTS. Expression in fasted and refed PAI-1-HKβ mice showed minimal brainstem fos expression, which was significantly lower than that of C57BL/6 fasted and refed mice (fasted PAI-1-HKβ mice; a mean of 1.0 ± 0.4 neurons per mouse and 5.0 ± 0.6 neurons in the fasted and refed mice; P = 0.0001, n=8).

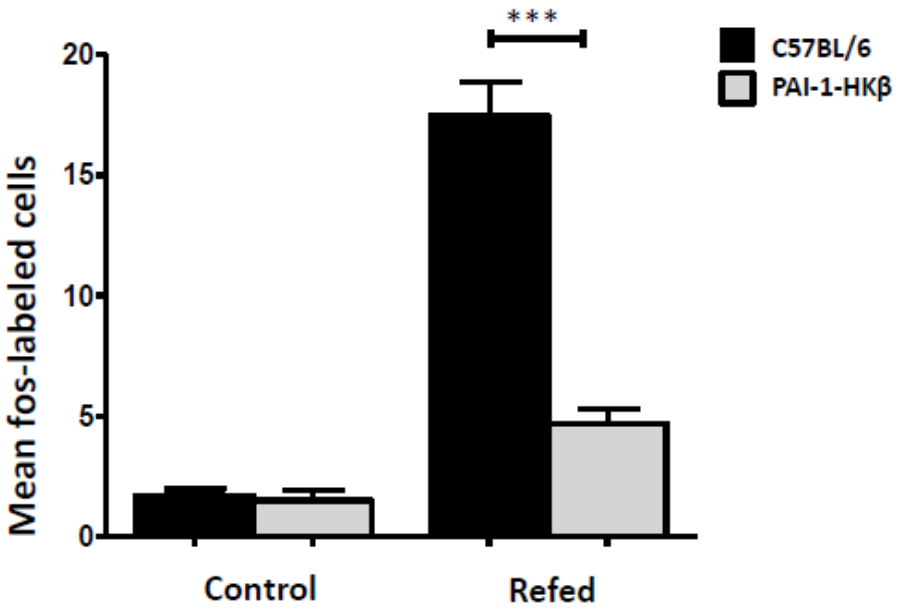

Figure 3.3.

PAI-1-H/Kβ mice exhibit decreased brain stem fos labelling following refeeding. Quantification of NTS c-fos labelled neurons in response to feeding after a 24 hour fast in PAI-1-H/Kβ and C57BL/6 mice. C57BL/6 mice showed an increase of fos-positive neurons when refed after a fast. In comparison, PAI-1-H/Kβ showed a reduced number of fos-positive neurons;***P = 0.001 C57BL/6 compared to PAI-1-H/Kβ, one-way ANOVA, Bonferroni post-test.

3.3.3 PAI-1-H/Kβ have decreased brainstem fos associated with neuronal activation by exogenous CCK8s

Just as postprandial release of CCK stimulates fos expression in the NTS, exogenous administration of CCK8s also increases fos expression in NTS neurons (Luckman, 1992). Thus both C57BL/6 and PAI-1-HKβ mice were treated with either saline or CCK (2.5nmol/kg) to investigate expression of fos in NTS subnuclei associated with gastrointestinal signals (figure 3.4. In PAI-1-HKβ mice, fos expression in the NTS was significantly less than in C57BL/6 after ip CCK8s (2.5nmol/kg; Figure 3.5; C57Bl/6: 10.0 ±1.2 neurons vs PAI-1-HKβ mice: 3.0 ±0.7 neurons; P=0.0014, n=8).

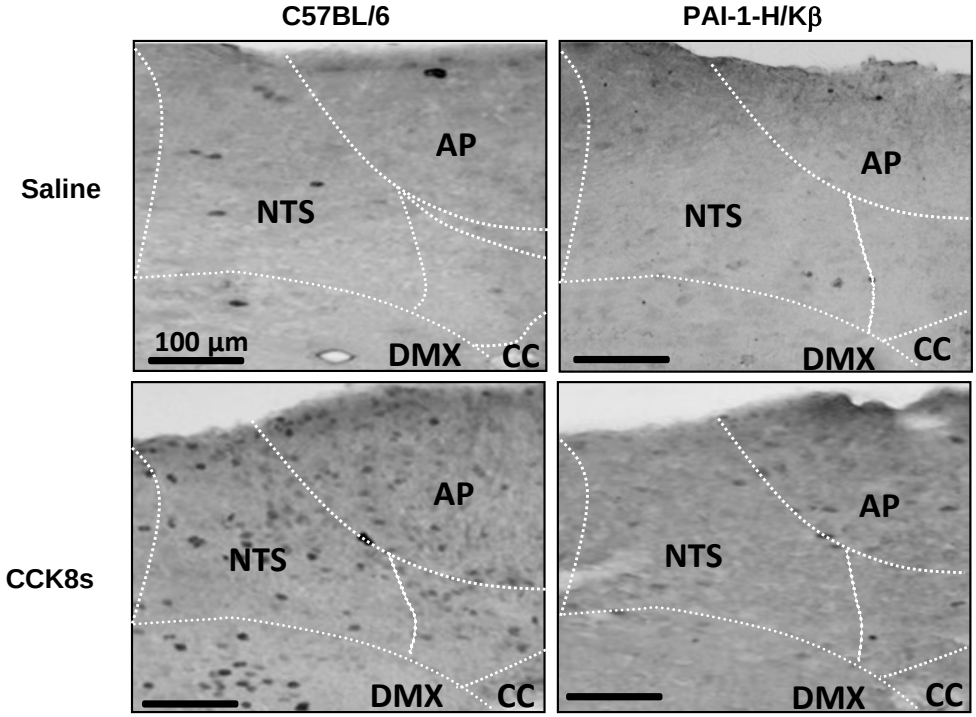

Figure 3.4
Suppressed stimulation of c-fos in NTS neurons in PAI-1-H/Kβ mice in response to *ip* CCK8s.

Neurons with stained nuclei in the NTS were counted for quantitative studies following ip injections with either saline or CCK8s (2.5nmol/kg). C57BL/6 mice displayed fos expression in the NTS compared to a decrease in fos in PAI-1-HKβ mice. Scale bar, 100µm.

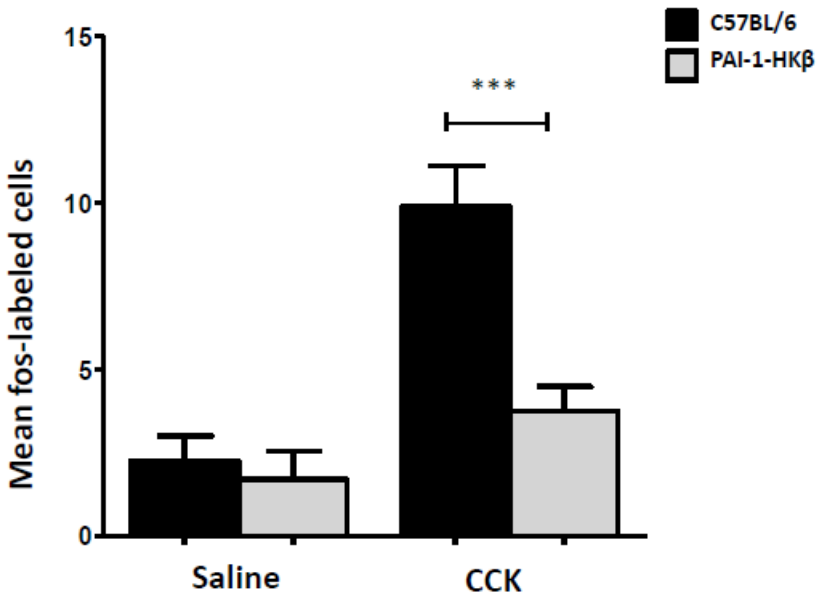

Figure 3.5

PAI-1-H/Kβ mice are resistant to CCK8.

Quantification of NTS fos labelled neurons in response to CCK8s (ip 2.5 nmol/kg) in PAI-1-H/Kβ and C57BL/6 (n=8) mice. C57BL/6 mice showed an increase in the number of fos-positive neurons following treatment with CCK. In comparison, PAI-1-H/Kβ treated with CCK had a reduced number of fos-positive neurons; ***P = 0.001 C57BL/6 compared to PAI-1-H/Kβ, one-way ANOVA, Bonferroni post-test.

3.3.4 Exogenous PAI-1 suppresses neuronal activation following feeding

The results demonstrate a marked difference between brainstem fos expression in PAI-1-HKβ and C57BL/6 mice. The data suggest that gastric expression of PAI-1 in the transgenic mice attenuates brainstem neuronal responses to feeding. To further examine this idea, recombinant PAI-1 (2.5 nmol/kg, ip) was administered to C57BL/6 mice immediately prior to re-feeding after a 24 h fast. The data indicated a significant decrease in fos-positive neurons in the NTS (Figure 3.6)

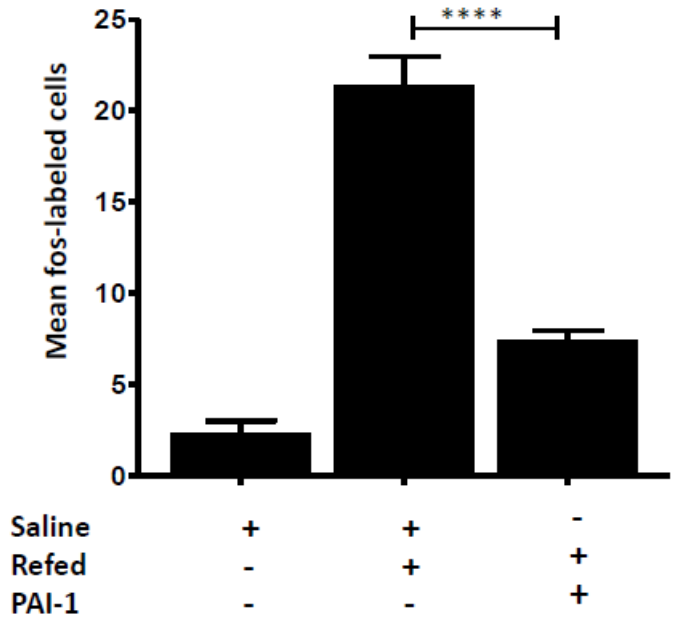

Figure 3.6

Administration of exogenous PAI-1 (2.5 nmol/kg) inhibits brain stem c-fos responses to refeeding.

C57BL/6 mice were fasted for 24 hours prior to ip injections of saline, PAI-1 and a period of refeeding. There was a decrease in fos-positive neurons in C57BL/6 mice that were refed and injected with recombinant PAI-1 (****P= 0.0001 where fasted and refed saline-treated mice were compared to fasted and refed mice receiving an injection of PAI-1, one-way ANOVA, Bonferroni post-test; n=8).

3.3.5 uPAR is required for the action of PAI-1

Some actions of PAI-1 are mediated by interaction with the urokinase plasminogen activator receptor (uPAR) (Binder *et al*., 2002). To identify if the uPA receptor was implicated in the effects on brainstem fos brought about by exogenous PAI-1, PAI-1 (or saline) was administered to refed C57BL/6 and $uPAR^{-/-}$ mice (Figure 3.7). While PAI-1 suppressed fos labelling in C57Bl/6 mice as already noted, The expression of c-fos in the NTS in $uPAR^{-/-}$ mice following re-feeding was not significantly different after PAI-1 compared with saline; the data are therefore consistent with the idea that the action of PAI-1 requires uPAR.

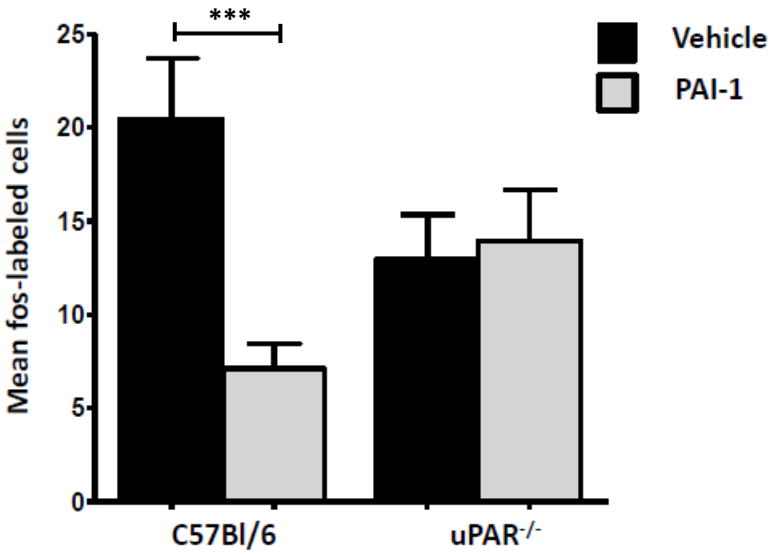

Figure 3.7

uPAR mediates the action of PAI-1 on brain stem fos in response to feeding.

In uPAR$^{-/-}$ mice, refeeding stimulated brainstem fos and this was not blocked by exogenous PAI-1 (2.5 nmol/kg). Re-fed C57BL/6 mice that were given an ip injection of recombinant PAI-1, showed a significant decrease in fos-positive neurons in the NTS, compared to controls. uPAR$^{-/-}$ mice showed no significant difference (*** P = 0.0001 n= 6-8).

3.3.6 *H.felis* infection does not have a significant effect on brainstem fos expression

Helicobacter infection increases gastric PAI-1(Kenny *et al*., 2008) and could decrease expression of fos as a potential consequence of inhibition of gut-brain signalling. C57BL/6 and PAI-$1^{-/-}$ mice were infected with *H.felis* and infection was verified by antral urease and histology (Kenny *et al*., 2013a). After CCK8s administration there was stimulation of fos in both control and infected wild type mice. PAI-$1^{-/-}$ mice responded with less fos expression following treatment with CCK8s; ip 2.5nmol/kg in both infected and non-infected groups. Interestingly, infected C57BL/6 mice had a slight decrease in the number of fos labelled cells compared to the non-infected, however this was shown to be not significant (Figure 3.8; n=5-8). Helicobacter-driven effects were observed, however, in feeding studies carried out by Kenny *et al* (Kenny *et al*., 2013a).

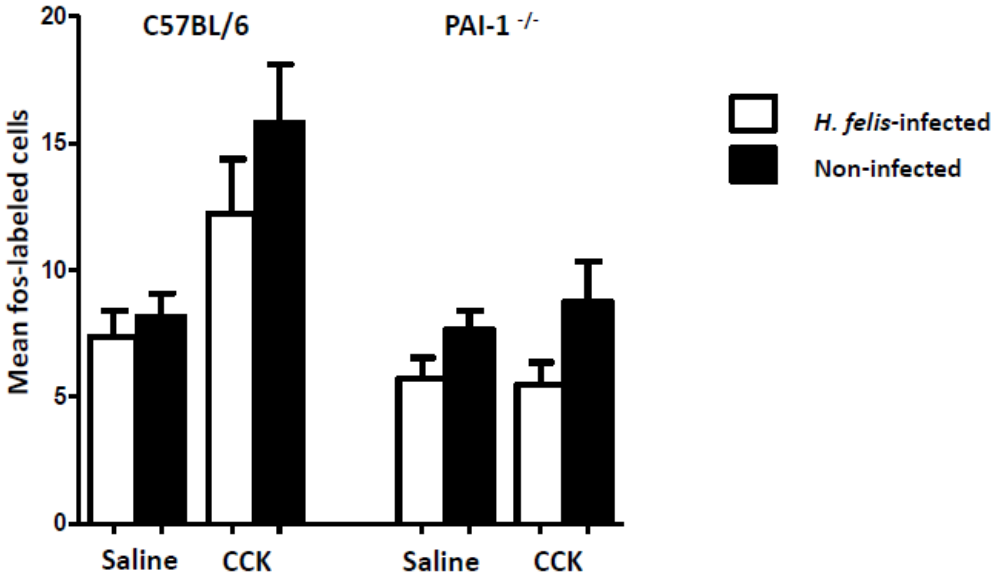

Figure 3.8

***H. felis* infection shows no significant effect on brainstem fos following ip CCK8s (2.5nmol/kg)**

Chronic infection with *H.felis* marginally decreased the number of fos labelled cells in the brainstem in response to CCK8s in C57BL/6 mice, although there was no significant difference between infected and non-infected groups for both C57BL/6 and PAI-1 $^{-/-}$ mice (n=5-8).

3.4 Discussion

The results described in this chapter suggest that PAI-1, which is increased in the plasma of obese individuals (Shimomura *et al*., 1996) and in the stomach of *H.pylori*-infected patients (Kenny *et al*., 2008), can play a role in suppressing brainstem stimulation by the satiety hormone CCK. The current data imply that PAI-1 is a locally-acting gastric factor that is capable of suppressing gut-brain signalling by CCK.

The PAI-1-HKβ mouse was generated to investigate the gastric mucosal biology of PAI-1. The entire coding region of the mouse PAI-1 gene and ~1.1kb of the promoter region of the mouse H^+/K^+ATPase β-subunit gene, was inserted into the pEGFP vector to generate the transgene. The H^+/K^+ATPase β-subunit promoter has been used to direct gene expression specifically to parietal cells in a number of previous transgenic studies (Shimomura *et al*., 1996, Landin *et al*., 1990) and specificity of transgene expression was confirmed by using PCR. The phenotype of transgenic PAI-1-HKβ mice has been described by Kenny *et al.* (Kenny *et al*., 2013a). Overexpression targeted to the gastric parietal cells was verified and results showed that there was a significant increase in plasma PAI-1 and gastric PAI-1 mRNA abundance in the transgenic PAI-1-HKβ compared to C57BL/6 mice. Overexpression was associated with a moderately obese phenotype. These mice also had hyperphagic traits; eating approximately 25% more than C57BL/6 mice in a 24 h period. Furthermore, exogenous CCK8s did not inhibit food intake in these mice until higher doses of 10nmol/kg were administered. Kenny *et al.* also described the effect of a high fat diet on C57BL6 and PAI-1-HKβ mice. Both transgenic and C57BL/6 mice alike were shown to be susceptible to diet-induced

obesity. Interestingly, mice null for PAI-1 (PAI-1-/-) were not susceptible to diet-induced obesity. Similarly, PAI-1$^{-/-}$ mice maintained sensitivity to CCK following chronic infection with *H.felis*, although C57BL/6 mice did not, compared to non-infected C57BL/6 controls. On a molecular level, PAI-1 was also shown to inhibit nodose ganglion neuron responses to CCK with regard to nuclear translocation of the immediate early gene early growth response (EGR)-1 and stimulation of Y2 receptor expression. Furthermore, uPAR was shown to be expressed by nodose neurones, consistent with the findings described in this chapter that uPAR is required for PAI-1 to elicit its apparent attenuation of fos-responses to CCK.

Compatible with the findings from Kenny *et al.*, the current study has found that fos expression in the brainstem, either after re-feeding or exogenous administration of CCK8s, was reduced in PAI-1-HKβ mice and also in C57BL6 mice treated with exogenous recombinant PAI-1. The data imply that normal gut-brain signalling is attenuated by PAI-1; these effects could, of course, account for the hyperphagia and weight gain observed in PAI-1-HKβ mice (Kenny *et al.*, 2013a). The mechanism by which gastric PAI-1 influences gut-brain signalling remains unclear, but the results from experiments in mice lacking uPAR indicates that the effects of PAI-depend on interactions with uPAR .

The characteristic obese phenotype found in PAI-1-HKβ mice has not been reported in other transgenic mice in which PAI-1 is overexpressed. Physiologically, PAI-1 is found in many different tissue types, including adipose tissue (Shimomura *et al*., 1996, Landin *et al*., 1990), the liver and endothelial

cells. Other groups have generated transgenic mice with increased expression of PAI-1; Elevated levels of PAI-1 have been shown to exacerbate fibrosis (Eitzman *et al*., 1996b) following injury when PAI-1 transgenic mice are generated under the CMV promoter, which is expressed almost ubiquitously. Another issue which has been described is tail auto-amputation (Erickson *et al*., 1990), where there is a subcutaneous haemorrhage in the tail and it eventually becomes necrotic and is lost. Due to the mechanisms in which PAI-1 is involved, it plays a significant role in venous occlusions and coronary thrombosis (Nordstrom *et al*., 2007).

It has long been established that satiety signals originating from the upper small intestine act locally on vagal afferent fibres to delay gastric emptying and decrease food intake (Gibbs *et al*., 1973, Dockray, 2009b). More specifically, CCK acts via its receptor, CCK1, on vagal afferent neurons that terminate in the NTS. The method of fos labelling to identify neuronal responses to vagal afferent stimulation was first reported by Luckman *et al.* (Luckman, 1992) who showed that 90 min following an *ip* injection of CCK in rats, fos protein was expressed in the NTS in sections at the level of the area postrema. The stimulated neurons were also found to be catecholaminergic following further work involving double labelling for tyrosine hydroxylase. This method provides useful information on which populations of neurons can be excited following CCK administration. Many other studies have adapted this method in investigations of various mechanisms in several species including gastric distension, feeding behaviour and gastric nutrient content (Rinaman *et al*., 1998, Zittel *et al*., 1999b, Boswell and Li, 1998, Blumberg *et al*., 2007). However, there are limitations; the method does not show inhibition of neurons, or indeed the intensity or strength of the signal being

produced. There are additional points to consider when using fos as a marker. Fos protein forms dimers with Jun to bind to AP-1 sites of which there are a number of variants. AP-1 sites are carried by many gene promoters and it is difficult to identify specifically the downstream targets. In addition, fos can increase rapidly in some neurons and more slowly in others, so it is important that appropriate time points are considered. It is widely accepted that prolonged stimulation of neurons increases the expression of fos. However this is not always the case; some neurones located in the dorsal root ganglion (DRG), for example, stain more readily for Jun following nerve damage (Kenney and Kocsis, 1997, De Leon *et al*., 1995).

Alternative approaches to investigate the possible inhibitory role of PAI-1 on these neurons may be able to answer specific questions regarding the potential attenuation of CCK in the presence of PAI-1. One alternative technique could involve electrophysiological techniques. Daly *et al.*, investigated vagal afferent neurons in diet-induced obesity in mice by recording vagal afferent discharge. Their results showed a marked reduction in afferent sensitivity to satiety related stimuli following exposure to CCK and 5-HT after a chronic high fat diet with reduced excitability of the neuronal cell membrane (Daly *et al*., 2011). Potentially, PAI-1 may be influencing this effect. Another electrophysiological technique, used by Raybould *et al.*, recorded responses in brain stem neurons following peripheral CCK-8 administration. They concluded that CCK acts directly on vagal afferents within the gastric wall (Raybould *et al*., 1988). Some neurons that express fos upon stimulation with CCK are known to release trigger the release of oxytocin from oxytocin-releasing neuron populations in the paraventricular nucleus via an ascending pathway (Verbalis *et al*., 1991).

Functional magnetic resonance imaging (fMRI) could be used to identify neuronal activity and this would be potentially informative in both animal and human studies (Miller *et al.*, 2007). Finally, as the specific manner by which PAI-1 attenuates CCK-induced fos expression is not yet fully understood, one way to identify if PAI-1 is producing an effect at peripheral vagal afferents or direct effects on the CNS, would be to use a transgenic mouse, null for uPAR expression in vagal afferent neurons. Vianna *et al.* investigated the involvement of CB_1 receptor on vagal afferents in energy homeostasis. They generated a transgenic mouse null for the receptor specifically on the afferent and efferent branches of the vagus (Vianna *et al.*, 2012). A similar method could be employed for these studies although the specific knockdown of CCK_1 receptors would provide important insights into the role of PAI-1.

In the current studies, wild-type mice that are re-fed after a period of fasting have shown there is a substantial expression of fos-positive neurons in the NTS. However, in comparison, the PAI-1-HKβ mice had significantly less expression of fos in neurons of the NTS. Upon further investigation, experiments described in this chapter have shown this also to be true when exogenous CCK was administered peripherally in place of feeding. The dose of CCK used is considered to mimic the physiological post-prandial release of CCK. Following dose-response studies, 2.5nmol/kg was effective in the inhibition of food intake after a 24 h fast in C57BL/6 mice (Kenny *et al.*, 2013a).

As previously reported (Kenny *et al.*, 2008), individuals infected with Helicobacter are known to have an increased expression of gastric PAI-1. Results

from experiments in wild-type mice and mice null for PAI-1 infected with *H.felis* showed that wild-type mice did not appear to develop a significant resistance to CCK8s with regards to a decrease in NTS fos expression. Interestingly, they did develop a resistance to CCK8s in feeding studies, showing that feeding was maintained following treatment (Kenny *et al*., 2013a). The relationship between CCK and PAI-1 may not be as clear cut in cases of infection, as the balance between orexigenic and anorexic signals may be affected. Following infection with *Helicobacter*, gastric expression of leptin has been found to be elevated (Azuma *et al*., 2001). It has also been reported that pro-inflammatory cytokines, such as IL1β can increase leptin in response to infection (Bornstein *et al*., 1998, Faggioni *et al*., 1998, Sarraf *et al*., 1997) as well as lower ghrelin concentrations (Osawa, 2008). Therefore, PAI-1 is potentially capable of counterbalancing the anorexic effects of stomach infection and maintain food intake (Kenny *et al*., 2013a).

PAI-1 has been associated with many of the co-morbidities that present in obese patients. This could explain why there is a higher risk of heart attacks and strokes in obese subjects. The present study suggests that PAI-1 can influence the certain aspects of the homeostatic control of food intake which potentially could indicate a therapeutic target for an ever increasing obese population.

CCK was the first gut hormone implicated in the control of food intake (Maclagan, 1937). In recent years, additional research has discovered the neural mechanisms that mediate the satiety effect of CCK (Rogers and Hermann, 2008). CCK is regarded as a short-term satiety factor, whereas another regulator of appetite homeostasis and adipokine, leptin, acts to control energy balance in a more long-term manner (Villanueva and Myers, 2008). Leptin monitors the

peripheral energy stores and relays this information centrally to suppress food intake but also increase energy expenditure. Not only is leptin capable of acting directly on hypothalamic neurons, leptin can also act via vagal afferent pathways, by synergistic interactions with CCK in vagal nodose ganglia; potentiating CCK's short-term satiety effect (Barrachina *et al*., 1997b, Heldsinger *et al*., 2011). Furthermore, diet-induced obesity has been found to decrease sensitivity to leptin in vagal afferent neurones (de Lartigue *et al*., 2007a). From the results obtained in this study, it appears that PAI-1 can elicit effects that are in direct contrast to the actions of leptin.

Vagal afferent neurons also express receptors that can stimulate food intake including the GHS-1 receptor for ghrelin. Ghrelin has been shown to negatively modulate satiety by CCK by inhibiting vagal discharge (de Lartigue et al., 2007a). Interestingly, CCK is capable of abolishing the orexigenic drive of ghrelin presumably because both ghrelin and CCK_1 receptors are expressed in the nodose ganglia (Date et al., 2005, de Lartigue et al., 2007a).

The results obtained from this work are compatible with evidence that resistance to CCK involves changes to vagally-mediated gut-brain signalling mechanisms (Covasa, 2010, Daly *et al*., 2011). The evidence presented in this chapter also suggests that PAI-1 is an adipokine but unlike leptin, it is producing opposite effects on food intake by suppressing vagal afferent responses to CCK (Kenny *et al*., 2013a).

This study supports a role for PAI-1 and has wider implications for obesity. We have investigated one line of evidence in this chapter; however, CCK is involved in various other mechanisms that are associated with energy homeostasis. The

expression of fos upon stimulation with CCK has been used here as a marker for neuronal activation in key brainstem regions involved in satiety. However, stimulation of these neurons could reflect other mechanisms, including CCK-induced pancreatic secretion, gastric inflammation and also the control of gastric emptying. CCK is known to play a role in the delay of gastric emptying in response to ingested protein and fat via a vagally-mediated pathway (Raybould and Tache, 1988). Studies into this area will no doubt provide further insights into the effect of PAI-1 on the satiety hormone, CCK.

3.5 Conclusions

1. PAI-1-HKβ mice exhibit decreased brain stem neuronal responses to CCK8s and refeeding that could contribute to an obese phenotype.
2. PAI-1 administration decreases brain stem neuronal responses to refeeding in C57BL/6 mice.
3. The uPA receptor, with which PAI-1 interacts, is required for the action of PAI-1 in influencing brain stem responses to CCK.
4. Infection with *Helicobacter felis* did not have a significant effect on brainstem fos responses to CCK8s.

Chapter 4

Plasminogen activator inhibitor (PAI)-1 suppresses inhibition of gastric emptying by cholecystokinin (CCK)

4.1 Introduction

Nutrient contents of gastric loads determine the rate of which they are delivered to the duodenum for absorption. Dietary fat and protein in particular can delay gastric emptying. Gastric emptying is under the control of several neuro-endocrine mechanisms that influence the pressure differences across the pylorus. CCK is considered to act as a mediator of gastric emptying in response to the ingestion of fat and protein (Debas *et al*., 1975, Raybould and Tache, 1988, Forster *et al*., 1990, Fried *et al*., 1991). The inhibition of gastric emptying by CCK is also thought to be mediated through the stimulation of vagal afferent neurons that express CCK-1 receptors (Dockray, 2009b). The vago-vagal reflex has been shown to cause a relaxation within the corpus of the stomach and thus, decreases the pressure difference across the pylorus (Raybould and Tache, 1988, Forster *et al*., 1990). In addition, cells containing CCK are stimulated by trypsin-sensitive releasing factors. Local trypsin inactivates CCK-releasing factors, although nutrients or chemicals such as FOY-305 compete with trypsin and upregulate CCK release (Iwai *et al*., 1987).

The presence of PAI-1 in the stomach released from gastric parietal cells is considered to be increased in response to infection, such as *H. pylori* (Kenny *et al*., 2008) and high gastrin concentrations (Norsett et al., 2011); (Kenny et al., 2013a). The evidence described in chapter 3 suggests that PAI-1 attenuates gut-brain signalling by CCK. Since this pathway has also been implicated in the inhibition of gastric emptying, the following experiments were designed to investigate the potential effect of PAI-1.

4.1.1 Aims

1. To develop a model for studies of CCK-regulated gastric emptying in mice.
2. To investigate the hypothesis that PAI-1 modulates CCK-regulated gastric emptying.

4.2 Methods

4.2.1 Animals

Male mice of 10-12 weeks (C57BL/6, PAI-1-HKβ and PAI-$1^{-/-}$) were used for all studies.

4.2.2 Gastric emptying assay

Mice were fasted overnight (>12 h) and access to water was removed 1 h prior to procedures. Mice received ip. injections (100µl; CCK8s or PAI-1 at 2.5nmol/kg in saline, or saline alone) 5 minutes prior to gastric emptying assays. Some experiments included ip. administration of gastrin (20nmol/kg) 6 h prior to gastric emptying assays. Mice received liquid test meals of either methyl cellulose (1.5% w/v) or peptone (4.5% w/v) at a volume of 600µl by oral gavage that were prewarmed to 37°C. In studies where FOY-305 was used, animals were pretreated by oral gavage 5 minutes before the test meal was administered (100µl, 100mg/kg). Mice were culled 5 minutes after gavage and stomach contents were collected for analysis. Samples of methyl cellulose and peptone were used as

controls and the rate of gastric emptying was calculated as described by Debas (Debas *et al*., 1975)

4.2.3 PAI-1 ELISA

Blood was obtained from C57BL/6 mice via cardiac puncture, collected into 0.1M trisodium citrate, centrifuged and concentrations of total plasma PAI-1 were determined by ELISA according to the manufacturer's instructions.

4.2.4 Immunohistochemistry of gastric corpus tissue

Stomachs were fixed and rings were cut on a cryostat in 7-10µm sections and prepared for immunohistochemistry as described in Chapter 2 using primary antibodies to substance P, Calcitonin gene-related peptide (CGRP) and Met-encaphalin (MetEnk).

4.3 Results

4.3.1 Peptone delays gastric emptying in C57BL/6 mice via a CCK-mediated pathway

A method to study CCK-regulated gastric emptying in mice was adapted from that previously used in rats (Forster *et al*., 1990). In particular, peptone which is known to release CCK was used as a physiological test meal. Initial studies suggested almost complete emptying of control solutions of saline in 5 min and it was considered advantagous to use test meals of increased viscosity by employing

1.5% (w/v) methyl cellulose. Even so, in C57BL/6 mice over 85% of a liquid test meal of methyl cellulose emptied within 5 minutes of oral gavage. However, a peptone-containing liquid test meal prepared in methyl cellulose significantly delayed gastric emptying compared with methyl cellulose alone (Figure 4.1) The action of peptone was reversed following administration of the CCK-1 receptor agonist, lorglumide (Figure 4.2) consistent with a role for endogenous CCK mediating the action of peptone on gastric emptying in C57BL/6 mice. The data obtained from this study was considered to indicate this was a useful model for investigative studies of gastric emptying in PAI-1HKβ mice.

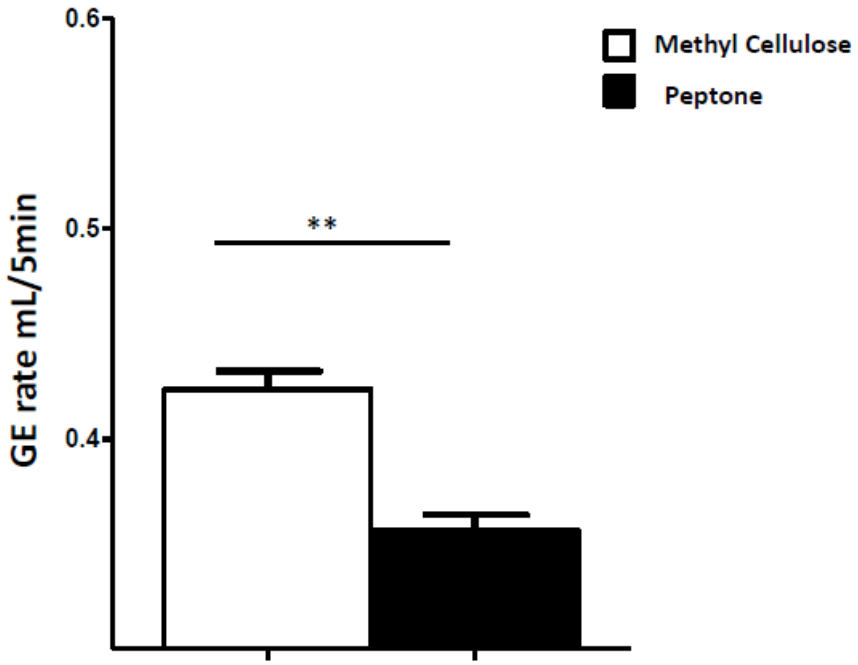

Figure 4.1

Peptone delays the rate of gastric emptying compared to a control liquid test meal of methyl cellulose in C57BL/6 mice.

C57BL/6 male mice were fasted overnight (>12hours) and water removed 1 hour before procedure. A liquid test meal of either methyl cellulose or peptone in methyl cellulose was administered orally. Peptone significantly decreased the rate of gastric emptying (GE). **P= 0.016 when comparing methyl cellulose-treated and peptone treated C57BL/6 mice, unpaired t-test (n=14-17, pooled controls).

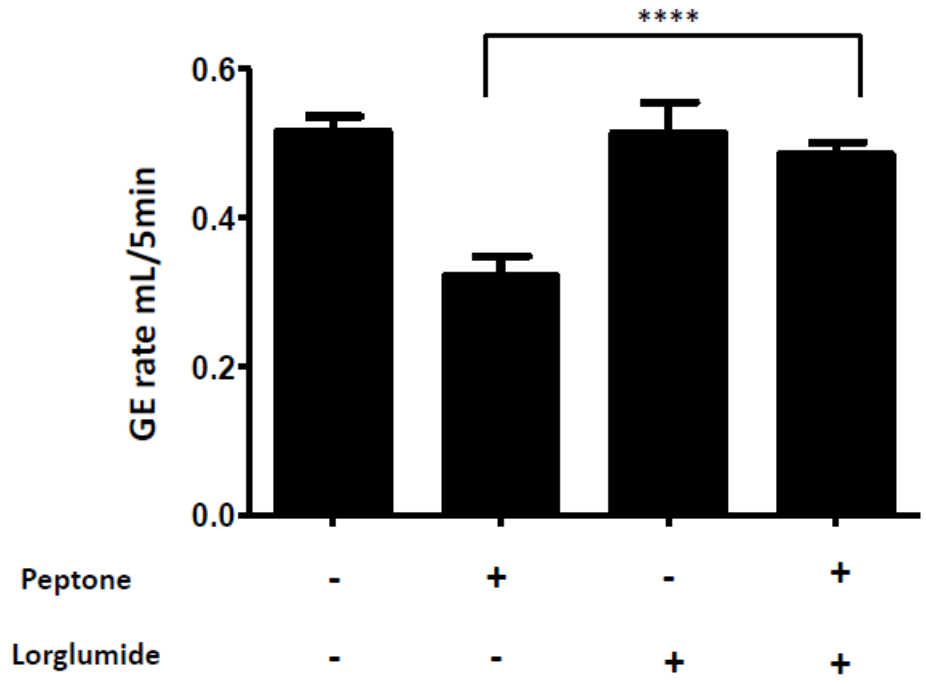

Figure 4.2

Peptone delays the rate of gastric emptying (GE), which is blocked by lorglumide

Mice that received a liquid test meal of methyl cellulose had a higher rate of gastric emptying compared with peptone test meal, as shown previously and administration of the CCK-1 receptor antagonist, lorglumide (*ip,4mg/kg*) reversed the effect by peptone. ****P= 0.0004 when comparing saline and lorglumide pre-treated C57BL/6 mice that were both given a peptone test meal, one-way ANOVA with Bonferroni post-test; n=3-6.

4.3.2 Peptone does not cause delayed gastric emptying in PAI-1-HKβ mice

In PAI-1-HKβ mice, peptone did not inhibit gastric emptying compared with methyl cellulose (methyl cellulose gastric emptying rate (GER) 0.43 ± 0.01 vs peptone GER 0.52 ± 0.007ml/5mins in PAI-1-HKβ mice; P=0.0002) but emptied at a higher rate than that of peptone in C57BL/6 mice (Figure 4.3).

4.3.3 Camostat mesilate (FOY-305) delays gastric emptying of methyl cellose

FOY-305 is a protease inhibitor that can increase plasma concentrations of CCK by suppressing the negative feedback control of CCK secretion exerted by pancreatic proteases (Goke *et al*., 1986, Liddle *et al*., 1984). In this study, prior gavage of FOY-305, significantly delayed gastric emptying of methyl cellulose in C57BL/6 but not PAI-1-HKβ mice (Figure 4.4).

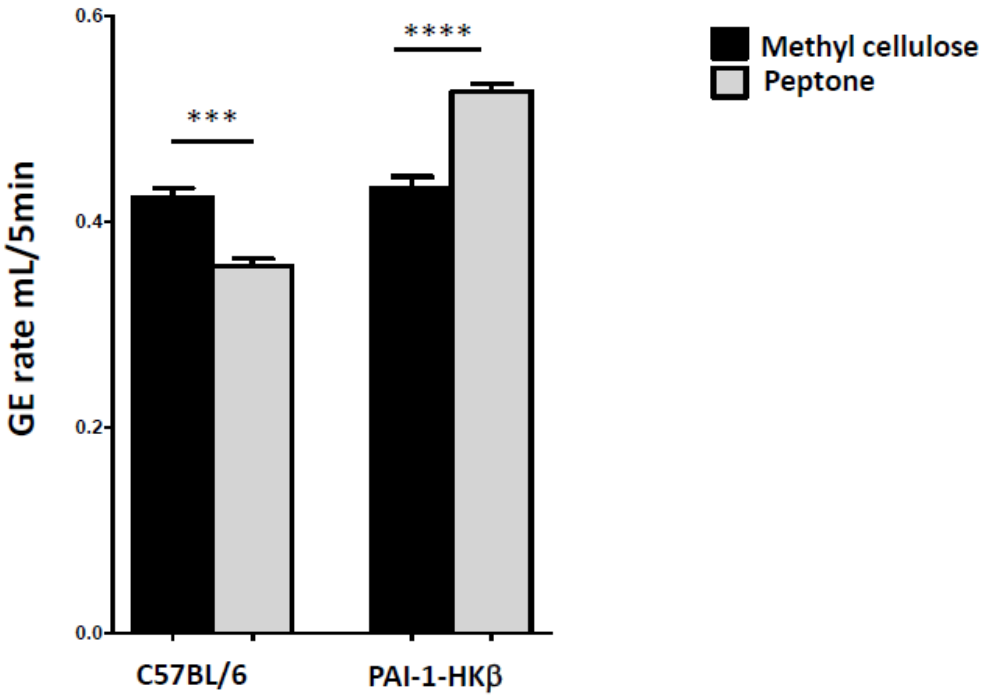

Figure 4.3

Peptone delays gastric emptying (GE) in wild type C57BL/6 but not in PAI-1-HKβ mice

PAI-1-HKβ mice that were gavaged with a liquid test meal of methyl cellulose had a similar rate of gastric emptying to that of C57BL/6 mice. However, with a peptone test meal, the PAI-1-HKβ mice showed an increase in the rate of gastric emptying. ****P= 0.0002 when comparing peptone with methyl cellulose-treated PAI-1HKβ mice, unpaired t-test n=5-17, pooled controls.

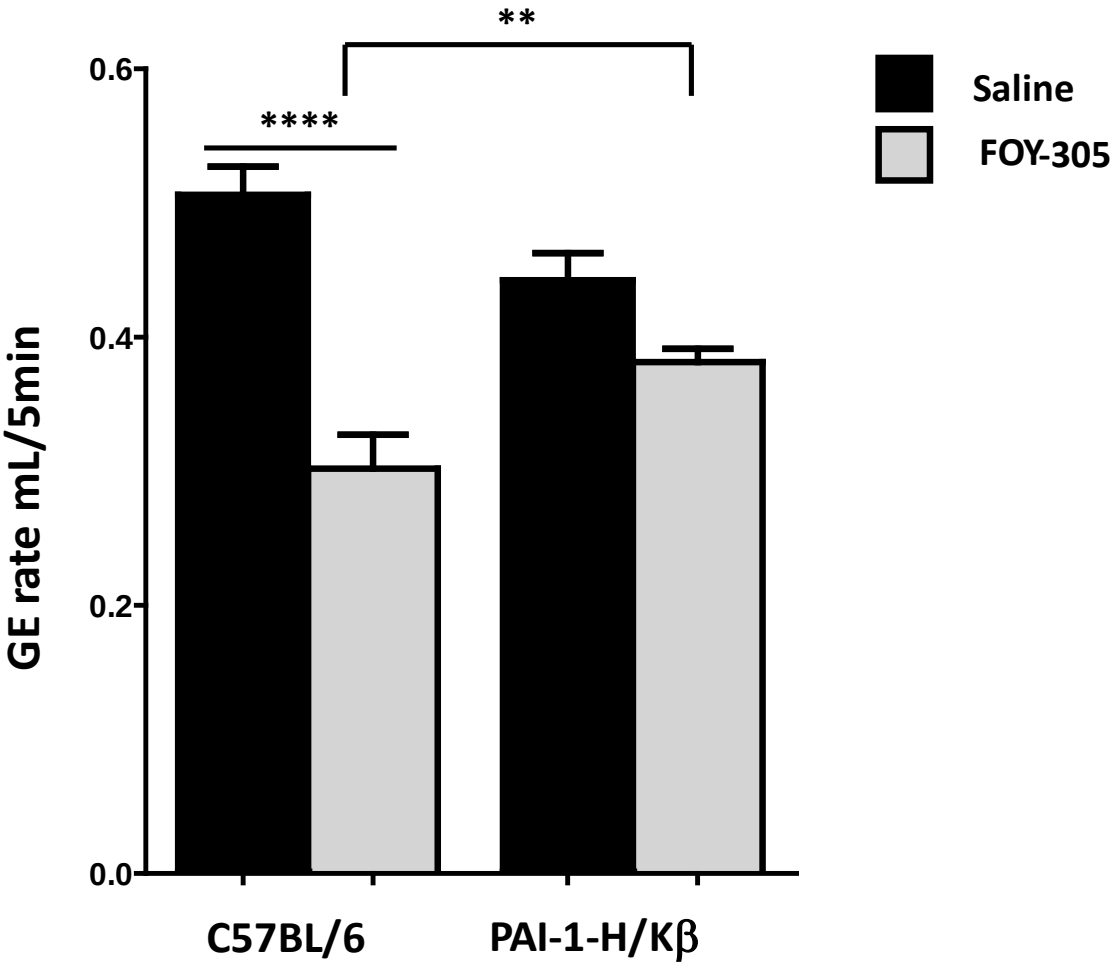

Figure 4.4

Oral preload of FOY-305 (Camostat mesilate) significantly delays GE rate in wild type C57BL/6 but not in PAI-1-HKβ mice

Pre-treatment by gavage 5 min prior to test meals with the CCK-releasing agent, camostat mesylate (FOY-305, 100 mg/kg) significantly delays gastric emptying (GE) of methyl cellulose in C57BL/6 but not in PAI-1-HKβ mice. ****P= 0.0003 when comparing vehicle pre-treatments with FOY-305 in C57BL/6 mice, **P= 0.0078 when comparing FOY-305- treated C57BL/6 and PAI-1-HKβ mice, one-way ANOVA, Bonferroni post-test n= 4-7.

4.3.4 PAI-1 reverses the action of exogenous CCK on gastric emptying

Previous experimental findings have indicated attenuation by PAI-1 of endogenous CCK-mediated effects on gut-brain signalling via the vagus (Kenny *et al*., 2013a). It was therefore considered important to determine whether PAI-1-HKβ mice were also resistant to the effects of exogenous CCK on gastric empting, since these effects are known to be mediated via vagal afferent neurons. The action of CCK8s (2.5nmol/kg, ip) on gastric emptying was investigated by administration prior to gavage of methyl cellulose. Ip. CCK8s produced an approximately 50% inhibition of emptying of methyl cellulose in C57BL/6 mice while in PAI-1-HKβ mice there was no significant difference in emptying after administration of either vehicle or CCK8s (Figure 4.5). To extend these findings, the effect of exogenous PAI-1 (2.5nmol/kg, ip) on the inhibition of gastric emptying by exogenous CCK was examined. In C57BL/6 mice, exogenous PAI-1 accelerated the emptying of a peptone test meal (Figure 4.6). Furthermore, the inhibition of gastric emptying by *ip* CCK8s was partially reversed by prior administration of PAI-1 (Figure 4.7). The data indicate that PAI-1 inhibits the effects of endogenous as well as exogenous CCK on gastric emptying.

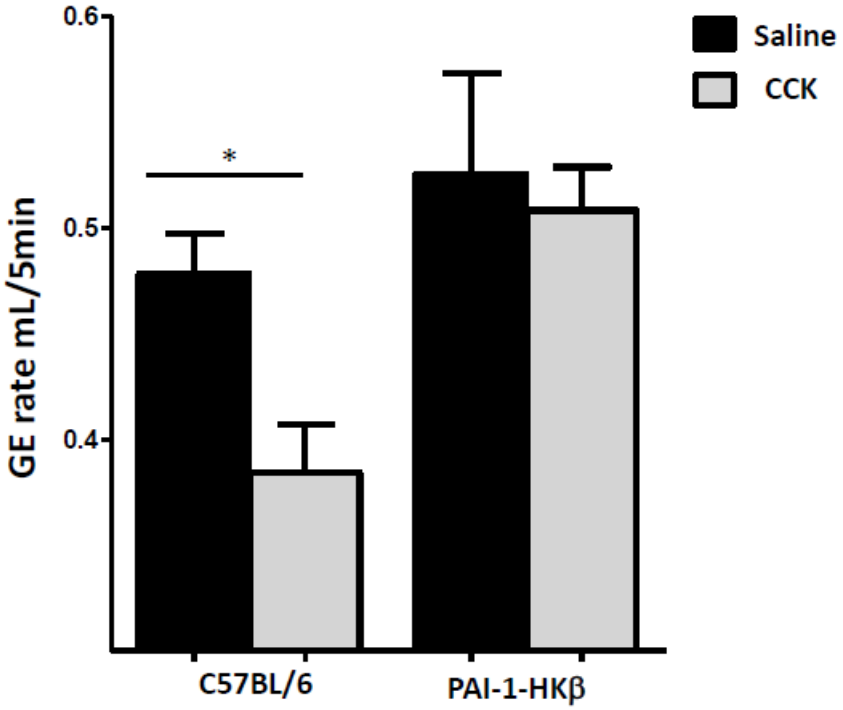

Figure 4.5

Exogenous CCK delays gastric emptying (GE) rate in wild type C57BL/6 but not in PAI-1-HKβ mice

Mice received either *ip* saline or CCK (2.5nmol/kg) prior to gavage with a liquid test meal of methyl cellulose. CCK-treated C57BL/6 mice exhibited decreased gastric emptying compared to saline-treated mice. In contrast, PAI-1-H/Kβ mice showed no significant difference between treatments. *P= 0.0139 when comparing CCK-treated C57BL/6 to CCK-treated PAI-1-H/Kβ mice, unpaired t-test n= 4-5.

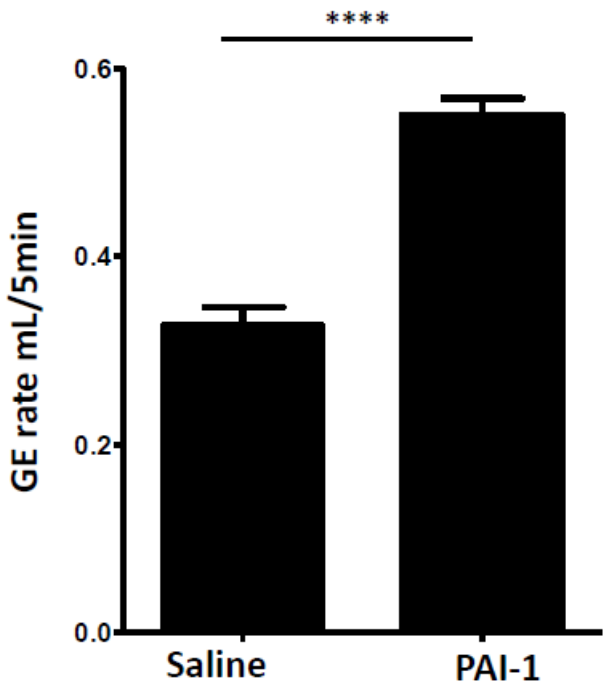

Figure 4.6

Exogenous PAI-1 attenuates peptone-mediated delayed gastric emptying (GE) in C57BL/6 mice.

C57BL/6 mice received either saline or PAI-1 *ip* prior to a liquid test meal of peptone. PAI-1-treated mice (2.5nmol/kg) showed an increase in the rate of gastric emptying compared to those treated with saline. ****P= <0.0001 when comparing saline-treated and PAI-1-treated mice receiving a peptone test meal, unpaired t-test; n= 4-8.

4.3.5 Increased endogenous gastric PAI-1 attenuates CCK-mediated gastric emptying

The expression of PAI-1 has been shown to be highly regulated (Loskutoff *et al*., 1993); moreover, elevated plasma gastrin concentrations are associated with an increase of expression of gastric PAI-1. Therefore, it was considered possible that prior administration of gastrin to increase gastric PAI-1 levels might influence CCK-mediated gastric emptying. There was increased plasma PAI-1 in C57BL/6 mice 6 h following ip administration of G17ns (Figure 4.8) compared with saline (saline: 4.6 ± 0.6 ng/ml; G17ns: 9.8 ± 1.0 ng/ml; p=0.0016, n=6). There was no significant difference in plasma PAI-1 after treatment with CCK8s. In C57BL/6 mice, prior treatment with G17ns significantly reversed the effect of subsequent administration of CCK8s in delaying gastric emptying of methyl cellulose (Figure 4.9). There was a stark contrast, however, in PAI-$1^{-/-}$ mice in which CCK inhibition of gastric emptying was maintained after prior treatment with G17ns indicating a role for endogenous PAI-1 in mediating the effect of gastrin.

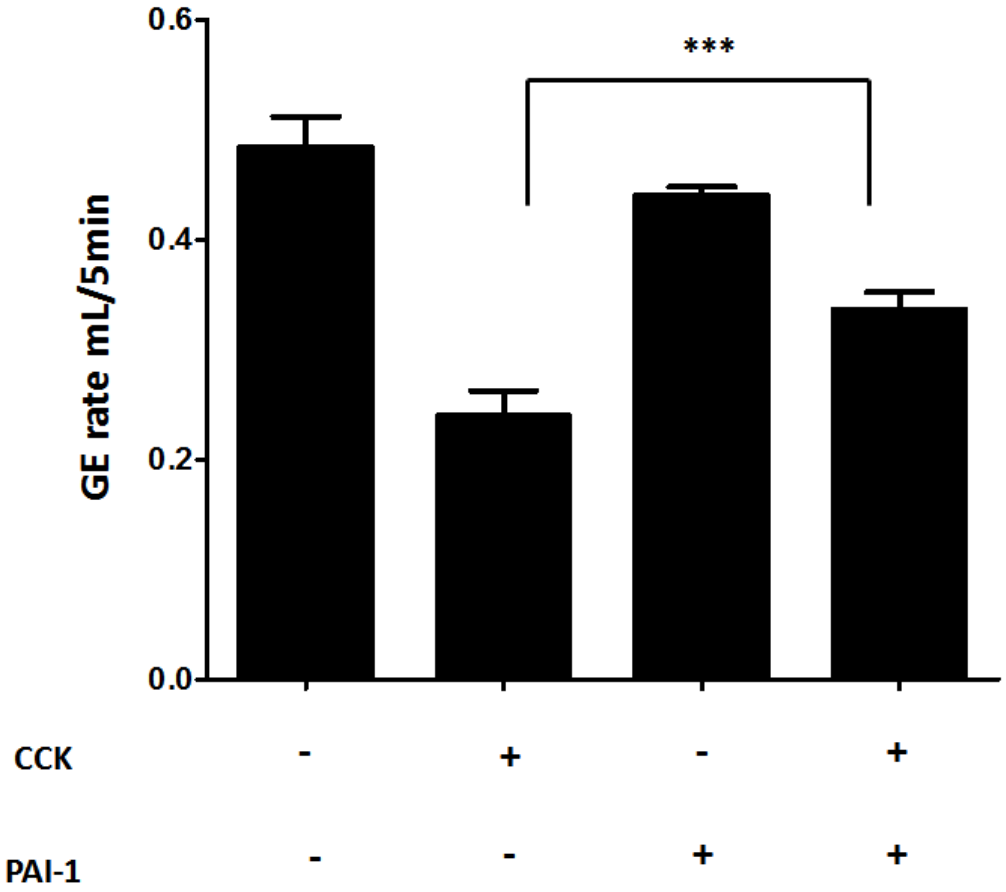

Figure 4.7

Exogenous CCK delays gastric emptying (GE) in wild type C57BL/6 mice, exogenous PAI-1 reverses this effect

Animals were given combination of *ip* injections of saline, CCK or PAI-1 prior to a liquid test meal of methyl cellulose. Mice receiving a combination of CCK (*ip; 2.5nmol/kg*) and PAI-1 (*ip 2.5nmol/kg*), had a significantly higher gastric emptying when compared to CCK treatment alone. ***P= 0.0001 when comparing CCK-treated and CCK plus PAI-1-treated mice, one-way ANOVA with Bonferroni post-test; n=5.

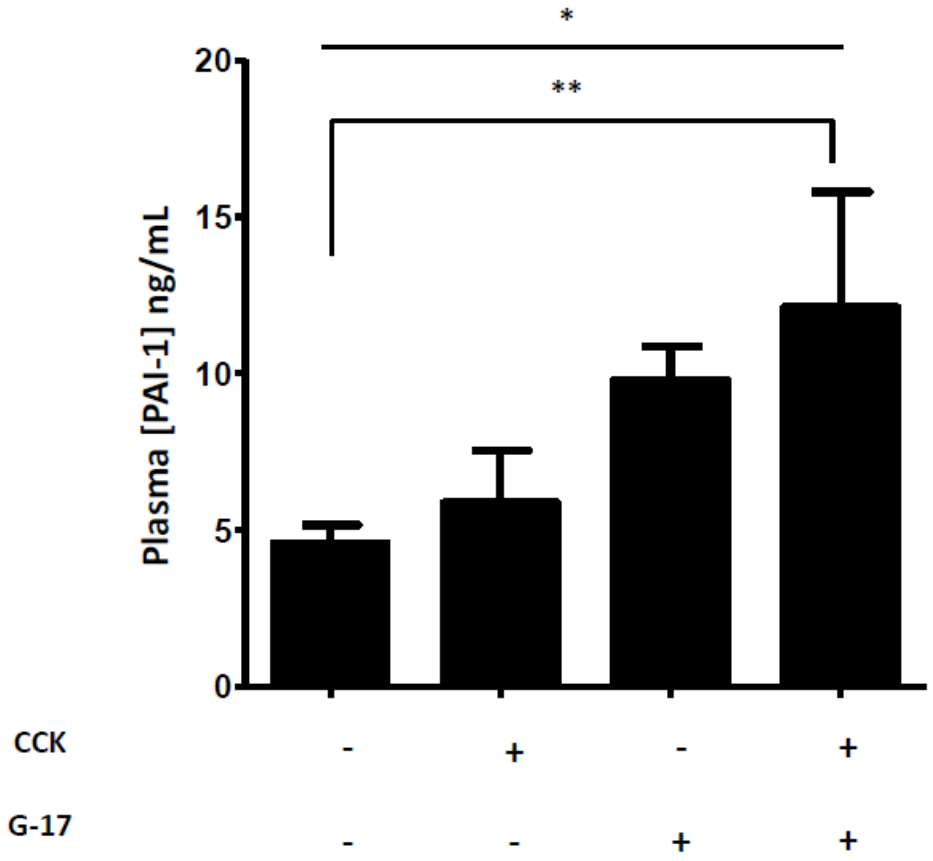

Figure 4.8

Gastrin (G-17ns) increases plasma PAI-1 concentrations in wild type C57BL/6

Pre-treatment with G17ns (20 nmol/kg, *ip*, 6 h previously) significantly increased plasma PAI-1 in C57BL/6 mice (*P= 0.0464 when comparing all groups and **P= 0.0062 when comparing saline-treated controls to G-17 plus CCK-treated C57BL/6 mice; with one-way ANOVA with Bonferroni post-test; n=5-6).

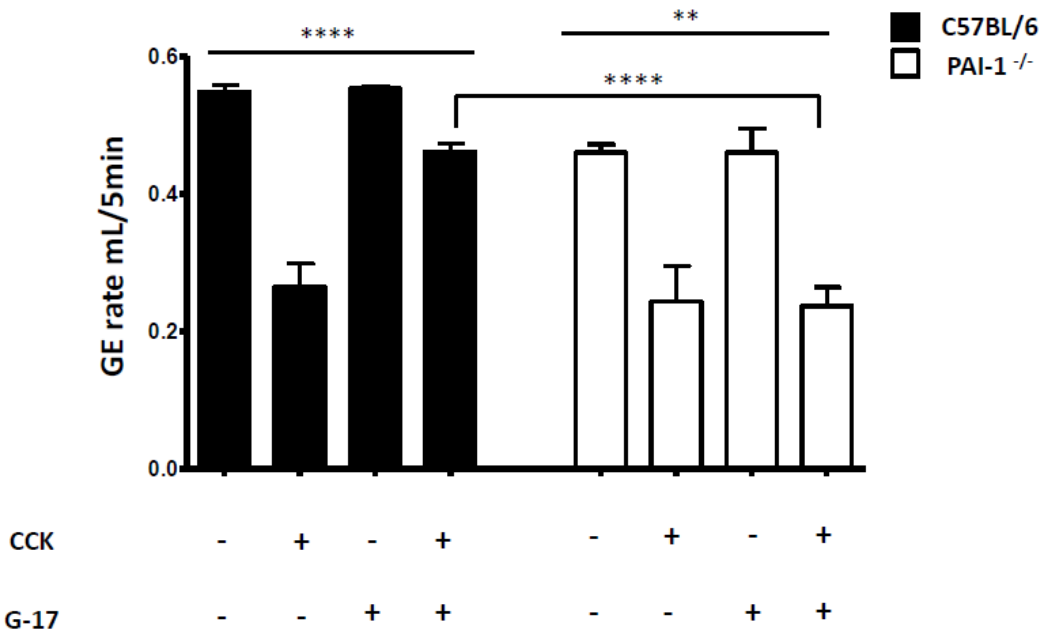

Figure 4.9

Gastrin (G-17ns) increases gastric emptying in wild type C57BL/6 but not in PAI-1 $^{-/-}$ mice

Mice of both C57BL/6 and PAI-1$^{-/-}$ strains pre-treated with saline showed similar inhibition of gastric emptying of methyl cellulose in response to CCK However, after pre-treatment with G-17ns, C57BL/6 mice showed no inhibition of gastric emptying in response to CCK while there was still a significant decrease in gastric emptying in PAI-1$^{-/-}$ mice. ****P=< 0.0001 comparing all treatment groups of C57BL/6 mice, **p= 0.0074 when comparing all treatment groups of PAI-1$^{-/-}$ mice, respectively; one-way analysis of variance; ****P= <0.0001 when comparing gastrin-treated C57BL/6 to PAI-1$^{-/-}$ mice when given CCK, two-way ANOVA with Bonferroni post-test; n=3-7.

4.3.6 Expression of neuropeptides in the gastric corpus of C57BL/6 and PAI-1HKβ mice

The myenteric plexus is responsible for the motor innervation of the stomach. The results presented above raise the possibility that in PAI-1HKβ mice insensitivity to the effects of CCK on gastric emptying might be attributable to changes in the pattern of innervation of the stomach. To examine this possibility, a pilot study using immunofluorescence to examine the neuropeptide innervation of the stomach was conducted in C57BL/6 and PAI-1HKβ mice. Similar observations were made for all three neuropeptides (CGRP, Substance P and Met-enkephalin) indicating normal innervation in the stomachs of PAI-1HKβ mice (figure 4.10).

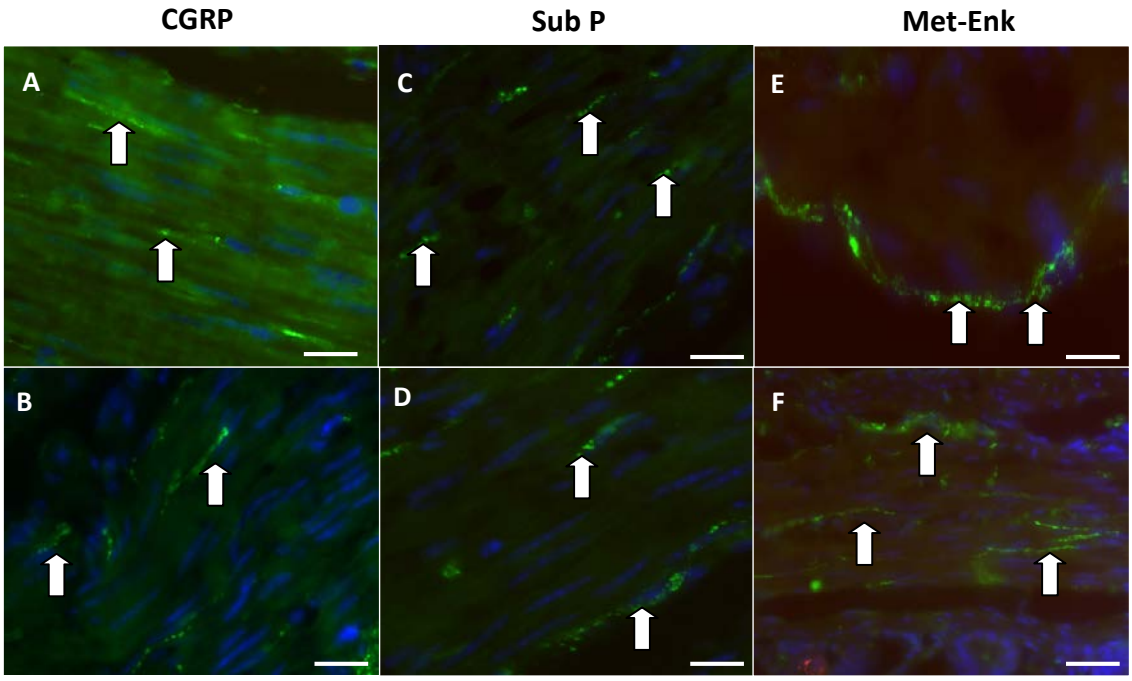

Fig 4.10

Expression of neuropeptides in the smooth muscle/myenteric plexus of C57BL/6 and PAI-1HKβ mice

Arrows highlight the expression of neuropeptides within the smooth muscle/ myenteric plexus. CGRP is expressed in C57BL/6 (A) and PAI-1-HKβ mice (B). Similar expression of Substance P in stomachs of both strains (C, D) and expression of Met-enkephalin in both strains (E,F) was observed (n=1).

4.4 Discussion

The results presented in this chapter contribute further evidence to indicate that PAI-1 can influence the actions of CCK. The experiments were based on the concept that CCK stimulates vagal afferent neurons to delay nutrient delivery to the small intestine via inhibition of both gastric emptying and food intake. Since previous studies (Kenny *et al*., 2013a) had indicated that PAI-1 inhibited the satiety effect of CCK, the present experiments were designed to investigate the effect of gastric PAI-1 on CCK-dependent delays in gastric emptying. The data indicate that both exogenous and endogenous PAI-1 are able to reverse inhibition of gastric emptying either endogenous or exogenous CCK.

The results in this chapter have been obtained using methods to investigate gastric emptying adapted from those first reported by Debas *et al.* (Debas *et al*., 1975). Gavaging fully-conscious animals has advantages: it is efficient and quick to perform. However, the limitation of only using one animal per observation can be costly in terms of animal numbers. There have been several studies, many of which conducted in humans, to measure gastric emptying that have been adapted for the use in animal experiments (Symonds *et al*., 2000, Schoonjans *et al*., 2002) and may be used as alternatives. These methods include firstly, radioscintigraphy, which is considered to be the gold standard for measuring gastric emptying in humans (Heading *et al*., 1976) and secondly, the ^{13}C-octanoic breath test that is widely applied in clinical settings following the first reported results from Ghoos *et al* (Ghoos *et al*., 1993). The latter method has been described as well-suited for repetitive measures and for use in children as no radioactive materials are used

(Schoonjans *et al*., 2002). Another non-invasive technique to consider is MRI (de Zwart and de Roos, 2010) although this could not be conducted in conscious animals due to the production of artefacts from the moving targets.

Debas *et al.* first reported that CCK played an important physiological role in the delay of gastric emptying (Debas *et al*., 1975). Gastric emptying is dependent on the resistance to flow at the pylorus and on the pressure gradient within the main body of the stomach. CCK relaxes the stomach via a vago-vagal reflex that results in relaxation of the gastric corpus thereby delaying the delivery of nutrients to the intestine (Zittel et al., 1999b, Schwartz et al., 1993b, Schwartz et al., 1993a). Thus, animal studies show the effects of CCK on gastric emptying and feeding are blocked by vagal afferent damage either by capsaicin or surgical intervention (Moran *et al*., 1997). Furthermore, in human studies, vagotomy disrupts the effects of nutrients on gastric emptying, motor functions of the stomach, and proximal gastric relaxation - all of which are dependent to some extent on the actions of CCK (Thompson *et al*., 1982, Lal *et al*., 2004). Receptors located in the small intestine are sensitive to dietary protein and which activates mechanisms that delayed gastric emptying (Green *et al*., 1988) (Liddle *et al*., 1986b, Forster and Dockray, 1992, Debas *et al*., 1975). Evidence for the involvement of CCK-1 receptors in energy homeostasis has been reported in studies using CCK and CCK-1 receptor null mice (Lo *et al*., 2008, Bi *et al*., 2004, Donovan *et al*., 2007). Additionally, CCK-1 receptors antagonists (Lotti *et al*., 1987), including lorglumide, where the normal delay of gastric emptying following a protein-richest meal is attenuated. Furthermore, it is considered that CCK release is suppressed by trypsin so that trypsin inhibitors can increase plasma CCK concentrations (Rausch *et al*., 1987). Thus, the use of the trypsin

inhibitor, FOY-305 (camostat mesilate) has provided an additional line of evidence in comparing gastric emptying in wild type and PAI-1-HKβ mice.

Obese individuals, especially in Western cultures are more likely to consume energy-dense foods with high fat content. Studies into the effects of a high fat diet have shown acceleration in the rate of gastric emptying (Cunningham *et al*., 1991, Castiglione *et al*., 2002). Furthermore, studies in obese subjects and animal models have demonstrated decreased sensitivity to CCK-induced gastric emptying (Little *et al*., 2007, Covasa and Ritter, 2000). As previously discussed, PAI-1-HKβ mice display a moderately obese phenotype; with increased hyperphagia, adiposity and body weight (Kenny *et al*., 2013a). These results support evidence that in obesity, the action of CCK on gastric emptying is altered by the presence of PAI-1. The question remains whether the resistance to CCK on gastric emptying in PAI-1-HKβ mice is a consequence of their obesity.

There has been no previous link between increased gastric PAI-1 and the disruption of gastric emptying, there is evidence associating high gastrin levels with an increase of PAI-1 (Norsett *et al*., 2011). Interestingly, studies into patients with Zollinger-Ellison syndrome, who suffer from upregulated release of gastrin (Dockray *et al*., 1975), have also presented with symptoms of rapid gastric emptying (Quigley, 1996, Dubois *et al*., 1977). In the current study, PAI-1HKβ mice have demonstrated insensitivity to CCK-induced delay in gastric emptying. Such desensitivity to CCK in obesity is still not yet fully understood, although many potential mechanisms have been implicated, most notably changes

in sensitivity of vagal afferent neurons. In this case, PAI-1 appears to be acting as a prokinetic, much in the way ghrelin has been described (Levin *et al*., 2006).

Overall, the work described in this chapter indicates that PAI-1 suppresses the effect of CCK on gastric emptying. A variety of gut signals can influence gastric emptying, including GLP-1 and PYY_{3-36} which can delay (Dockray, 2009a), and ghrelin which can accelerate gastric emptying, respectively (Dockray, 2009a, Bassil *et al*., 2006, Edholm *et al*., 2004, Fujino *et al*., 2003, Kamiji *et al*., 2009). Manipulating the expression of PAI-1 in the stomach has been useful in determining the effects on the actions of CCK, which may lead to future studies into other hormones released by the stomach and how they interact with PAI-1. Moreover, the clinical implications for patients suffering from gastric dumping, obesity and diabetes are important if PAI-1 is indeed considered a prokinetic like ghrelin (Bassil *et al*., 2006, Murray *et al*., 2005). It is known that ghrelin, which is also released from the stomach (Kojima *et al*., 1999, Asakawa *et al*., 2005) can influence the effects of CCK on both food intake and gastric emptying (Asakawa *et al*., 2005). Unlike other peripheral signals in appetite control, ghrelin increases appetite and body fat accumulation (Abizaid and Horvath, 2012). Recent evidence has emerged that ghrelin can inhibit CCK-induced effects in vagal afferent neurons (de Lartigue et al., 2007a), similar to the inhibition in studies into PAI-1 (Kenny *et al*., 2013a). The role that PAI-1 plays centrally is an important next step in this present study; the effects of peripheral and circulating PAI-1 on hypothalamic orexigenic neurons associated with ghrelin (Lawrence *et al*., 2002) would provide valuable insights into PAI-1 and energy balance.

4.5 Conclusions

1. Delayed gastric emptying occurs following protein rich gastric test meals an upon stimulation with oral pre-loads of FOY-305 in C57BL/6 mice

2. CCK8s *ip* (2.5nmol/kg) inhibits gastric emptying of a non-nutrient containing test meal in C57BL/6 mice via the CCK_1 receptor.

3. Both endogenous and exogenous PAI-1 attenuated the effect of CCK on gastric emptying. An increase in PAI-1 occurred following administration of gastrin, which also attenuated CCK-mediated delayed gastric emptying.

4. A pilot study indicated that both C57BL/6 and PAI-1HKβ mice have similar vagal afferent innervation to the stomach.

Chapter 5

PAI-1 stimulates food intake via a separate mechanism to ghrelin.

5.1. Introduction

Exogenous administration of recombinant PAI-1 was shown in Chapters 3 and 4 to decrease brainstem fos responses to feeding and to CCK, and to supress CCK-inhibition of food intake and gastric emptying in C57BL/6 mice.; Taken together with the observation that mice overexpressing PAI-1 in the stomach are hyperphagic (Kenny *et al*., 2013a), it would appear that PAI-1 is an orexigen. Orexigens provide the drive within humans and animals to feed. Several molecules induce feeding, including orexin and anadamide. However, the major and only gut hormone known to stimulate hunger is ghrelin. Ghrelin acts by stimulating neurons associated with appetite located in the ARC. Peripheral ghrelin works via a mechanism can be observed in the ventromedial ARC neurons (Figure 5.1) using fos expression as a marker for activation (Kobelt *et al*., 2008).

The GSH-1 receptors, by which ghrelin acts, are also co-localised with CCK-1 receptors in certain populations of vagal afferent neurons (Burdyga *et al*., 2006). The release of ghrelin can influence gene expression mediated by CCK in these neurons. As noted earlier, PAI-1 may inhibit satiety via a vagally-mediated pathway, and therefore potentially increases food intake via a similar mechanism to ghrelin. PAI-$1^{-/-}$ mice lack PAI-1, and have been shown to be resistant to diet-induced obesity, and potentially could be resistant to orexigenic signals produced by ghrelin and other appetite-stimulating peptides.

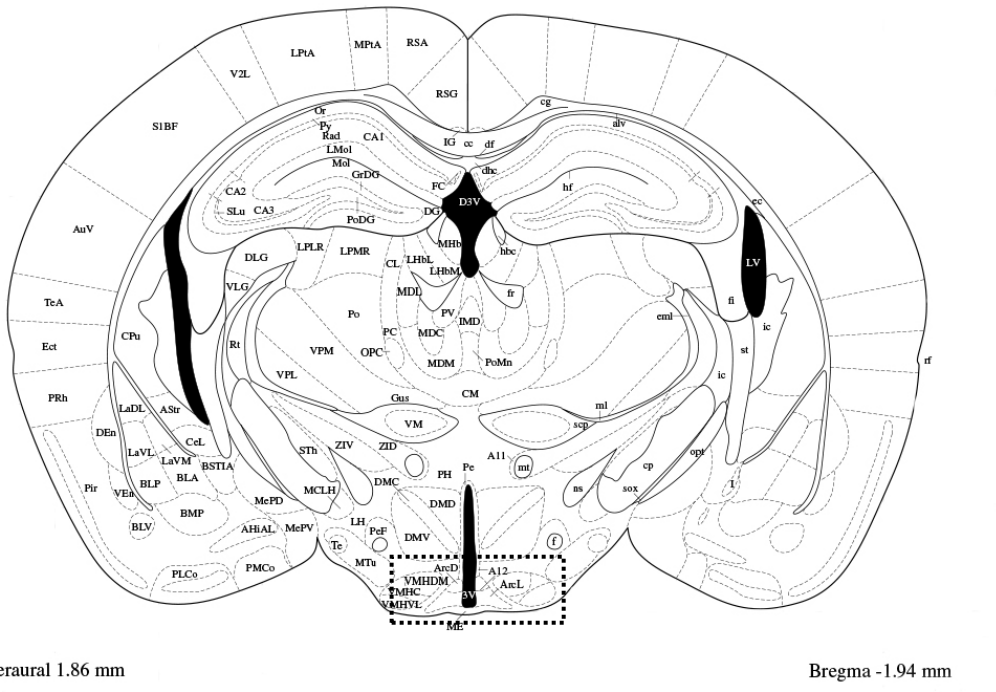

Figure 5.1

Hypothalamic Neuroanatomy

Figure displaying some of the hypothalamic nuclei involved in feeding and appetite regulation. ARC is highlighted both left and right of the 3rd ventricle (3V) and above the median eminence (ME), adapted from Paxinos and Franklin. Permission was granted for this figure by Elsevier. Copyright Elsevier (Paxinos & Franklin, 2001).

5.1.1 Aims

1. To test the hypothesis that PAI-1 stimulates food intake in both C57BL/6 and PAI-$1^{-/-}$ mice and to determine whether this is influenced by nutritional status (fasted or fed *ad libitum*).
2. To determine whether the effect of ghrelin on food intake is preserved in PAI-$1^{-/-}$ mice.
3. To elucidate the neuronal responses to PAI-1 in CNS regions influenced by orexigenic stimulation.

5.2 Materials and methods

5.2.1 Animals

Wild type (C57BL/6) and PAI-1 null (PAI-$1^{-/-}$) mice were either fed *ad libitum* or fasted for 6 to 24 hours prior to procedures with full access to water. Mice were not subjected to fasting for more than twice per week.

5.2.2 Arcuate nucleus fos labelling

Mice were anesthetised and perfused transcardially 90 min after ip. administration of ghrelin, PAI-1 or saline. Neurons located in the arcuate nucleus with nuclear black brown staining at the plane of view were counted as positive. Generally, 4-6 sections per animal were quantified and the mean number of fos-positive neurons was determined.

5.2.3 Feeding behaviour

Mice received *ip* injections of orexigenic agents: ghrelin (3nmol/kg), orexin-A (10nmol/kg), anandamide (14nmol/kg) and stable recombinant PAI-1 (2.5nmol/kg). Food intake was noted after 30 and 60 minutes.

5.3 Results

5.3.1 PAI-1 stimulates food intake in fasted mice

The present experiments were designed to test the effects of PAI-1 on food intake of C57BL/6 and PAI-$1^{-/-}$ mice, in both fed and fasted states. Fasted mice of both strains exhibited significantly increased food intake 30 minutes after PAI-1 (2.5nmol/kg) compared with saline treatment (Fig 5.1). There was a slight but not statistically significant increase of food intake in fed *ad libitum* mice of both strains when treated with PAI-1.

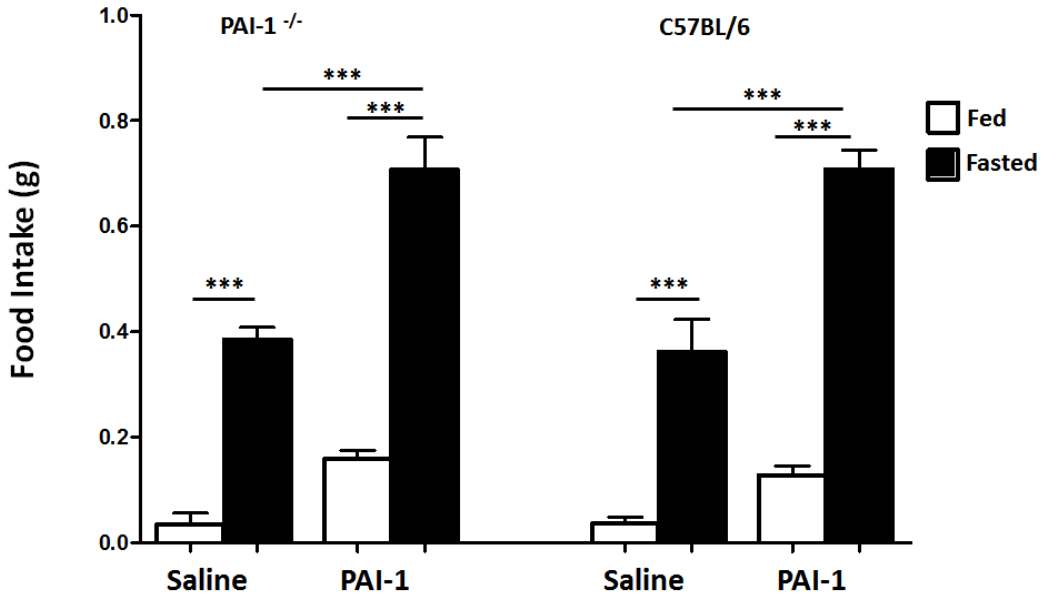

Figure 5.2

PAI-1 increased food intake in fasted C57BL/6 and PAI-1 null mice but not in fed *ad libitum* mice

Both C57BL/6 and PAI-1 null mice that were fasted for 24 hours exhibited a significant increase in food intake 30 minutes after PAI-1 (2.5 nmol/kg) compared to vehicle-treated mice *** P = 0.0001 when comparing fed *ad libitum* to fasted saline-treated PAI-$1^{-/-}$ mice, fasted saline-treated to fasted PAI-1-treated PAI-$1^{-/-}$ mice, fed *ad libitum* to fasted saline-treated C57BL/6 mice, fasted saline-treated C57BL/6 to fasted PAI-1-treated C57BL/6 mice; two-way ANOVA as genotype not relevant, with Bonferroni post-test, n=6).

5.3.2 Ghrelin and other orexigens stimulate food intake independently of PAI-1

Because both PAI-1 and ghrelin stimulate food intake it was considered important to determine whether the action of ghrelin depends upon the presence of endogenous PAI-1. To test this, fasted and fed *ad libitum* PAI-$1^{-/-}$ and C57BL/6 mice were treated with ghrelin and food intake monitored. In fed *ad libitum* PAI-$1^{-/-}$ mice, ghrelin strongly stimulated food intake compared with saline. There was also stimulation by ghrelin in fed C57BL/6 mice although the response was smaller than in PAI-$1^{-/-}$ mice. In fasted mice, food intake was not significantly different after ghrelin compared with saline and was similar in the two strains, presumably reflecting a strong orexigenic drive after fasting. Nevertheless, the data indicate that PAI-1 is not required for the orexigenic action of ghrelin (Figure 5.2).

In other experiments, additional orexigens including orexin-A and anandamide were tested with the same protocol, and were shown to elicit weak orexigenic responses, but again these were not dependent on PAI-1 expression since there was no difference in response in C57BL/6 and PAI-1 null mice (Fig 5.3 and 5.4).

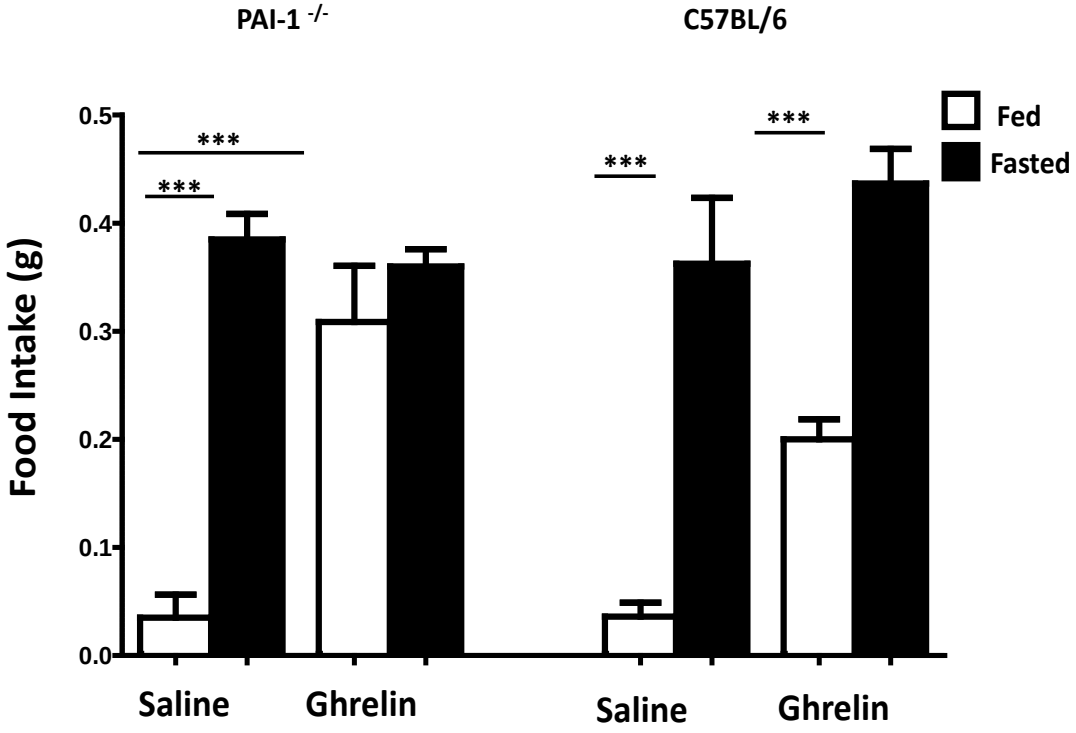

Figure 5.3

PAI-1 is not required for the orexigenic effect of ghrelin

Both C57BL/6 and PAI-$1^{-/-}$ mice fed *ad libitum* exhibited a significant increase in food intake 30 minutes after ip ghrelin (10µg/mouse); ghrelin did not stimulate additional food intake compared to saline in mice refed after fasting for 24 h. (*** P = <0.001 when comparing fed *ad libitum* to fasted saline-treated PAI-$1^{-/-}$ mice; *** P = <0.001 when comparing fed *ad libitum* saline-treated and ghrelin-treated PAI-$1^{-/-}$ mice; ***P = <0.001 when comparing fed *ad libitum* saline-treated C57BL/6 to fed *ad libitum* ghrelin-treated C57BL/6 mice; no significant difference between strains treated with ghrelin; two-way ANOVA, Bonferroni post-test; n=4-7).

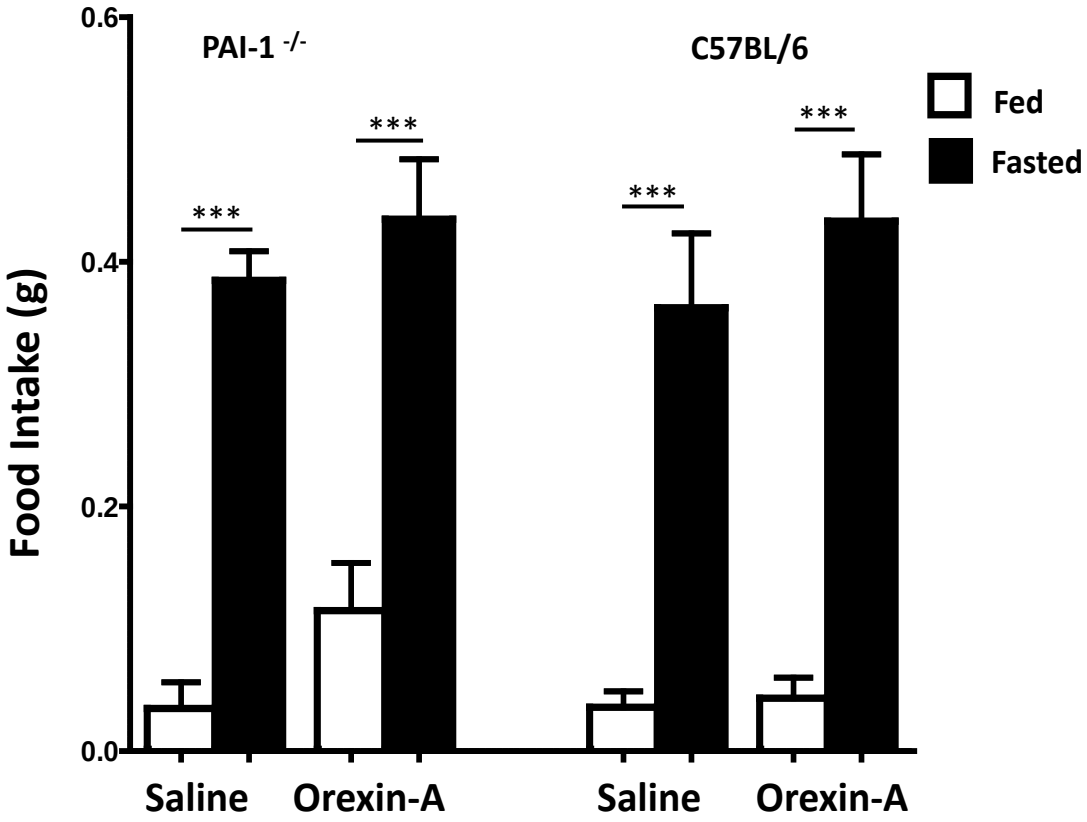

Figure 5.4

Orexin-A does not induce a strong orexigenic effect in fed *ad libitum* mice, and does not require PAI-1 to induce an orexigenic effect in fasted mice

Fed *ad libitum* mice of both strains treated with orexin-A had a small increase in food intake. Fasted mice had a larger increase in food in both C57BL/6 and PAI-$1^{-/-}$ ***P = 0.001 when comparing fed *ad libitum* C57BL/6 and PAI-$1^{-/-}$ mice in both treatment groups; no significant difference between strains treated with orexin-A; two-way ANOVA, Bonferroni post-test; n=4-6).

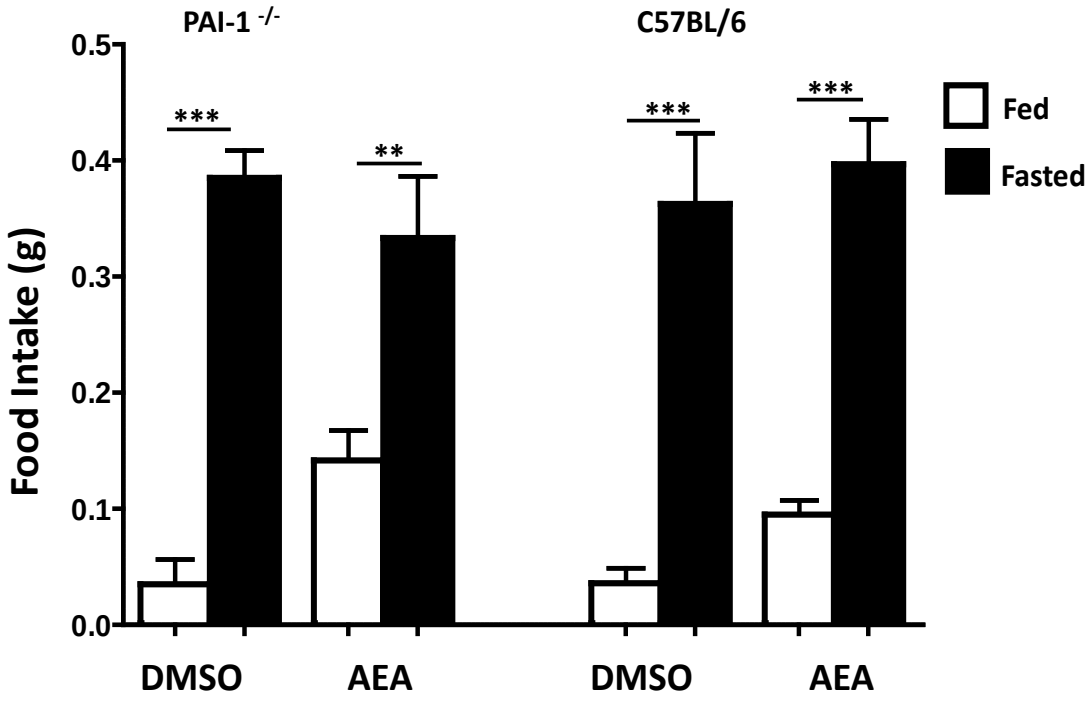

Figure 5.5

Anandamide induces a mild orexigenic effect in fed *ad libitum* mice, independently of PAI-1

Fed *ad libitum* mice of both strains treated with anandamide had a small increase in food intake. Fasted mice had a larger increase in food in both C57BL/6 and PAI-$1^{-/-}$. *** P = 0.001 when comparing fed *ad libitum* with fasted vehicle-treated PAI-$1^{-/-}$ mice. (**P = 0.0093 when comparing fed *ad libitum* saline-treated C57BL/6 to fed *ad libitum* Anandamide-treated C57BL/6 mice, two-way ANOVA, Bonferroni post-test; n=4-6).

5.3.3 PAI-1 and ghrelin stimulate food intake via a separate pathways

Systemic administration of ghrelin stimulates neurons in the arcuate nucleus of the hypothalamus (Date *et al*., 2001, Kobelt *et al*., 2008), which can be observed using fos as a marker (Hewson and Dickson, 2000, Lawrence *et al*., 2002). To determine whether PAI-1 also activated arcute neurons, *ip* injections of ghrelin (3nmol/kg) and PAI-1(2.5nmol/kg) were administered to fasted C57BL/6 and PAI-$1^{-/-}$ mice, and the hypothalamus processed for quantification of fos. Both C57BL/6 and PAI-$1^{-/-}$ mice treated with ghrelin exhibited a significant increase in fos expression in the arcuate nucleus (Figure 5.5 and figure 5.6). However, PAI-1 did not stimulate fos expression, indicating a different central mechanism for its effect on food intake.

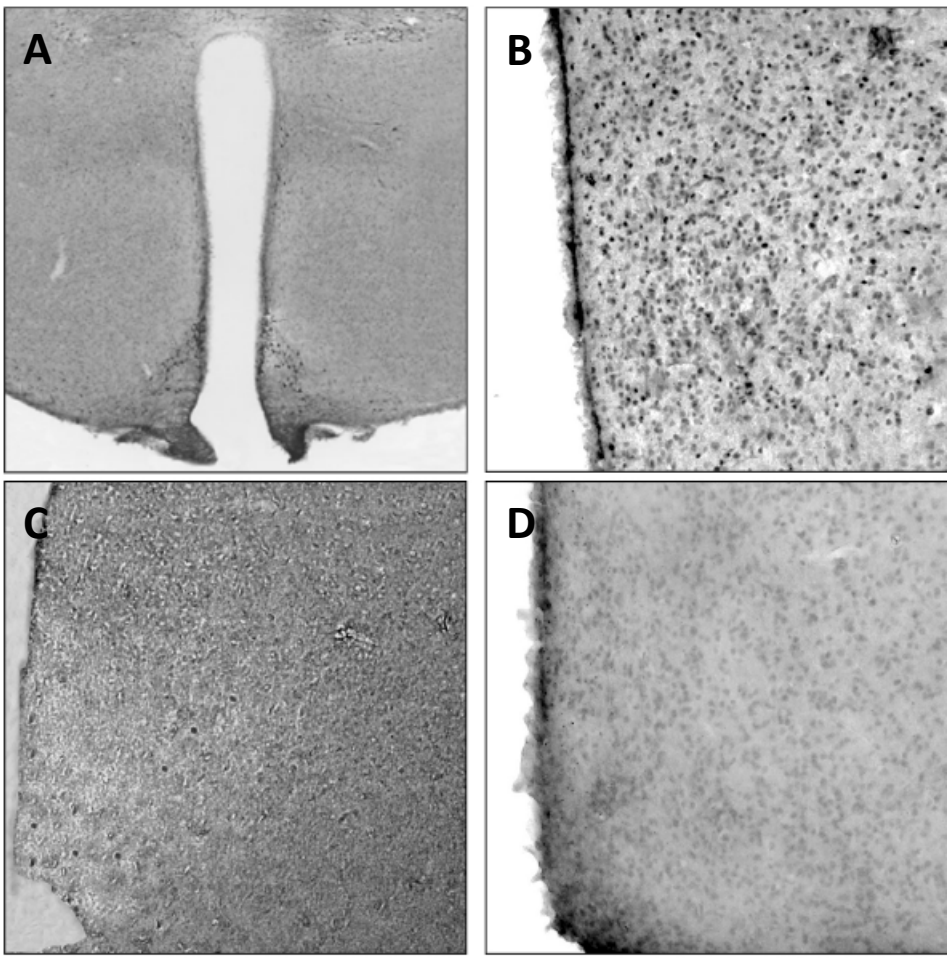

Figure 5.6

Ghrelin stimuates fos expression the arcuate nucleus of C57BL/6 mice

Example of a full section of the arcuate nucleus (bilateral; A) with widespread fos expression in the arcuate nucleus following *ip* ghrelin (B;10µg/mouse) compared to low fos expression in sections following *ip* PAI-1 (2.5nmol/kg; C) and ip saline (D). Representative of 4-6 sections from 4-7 animals.

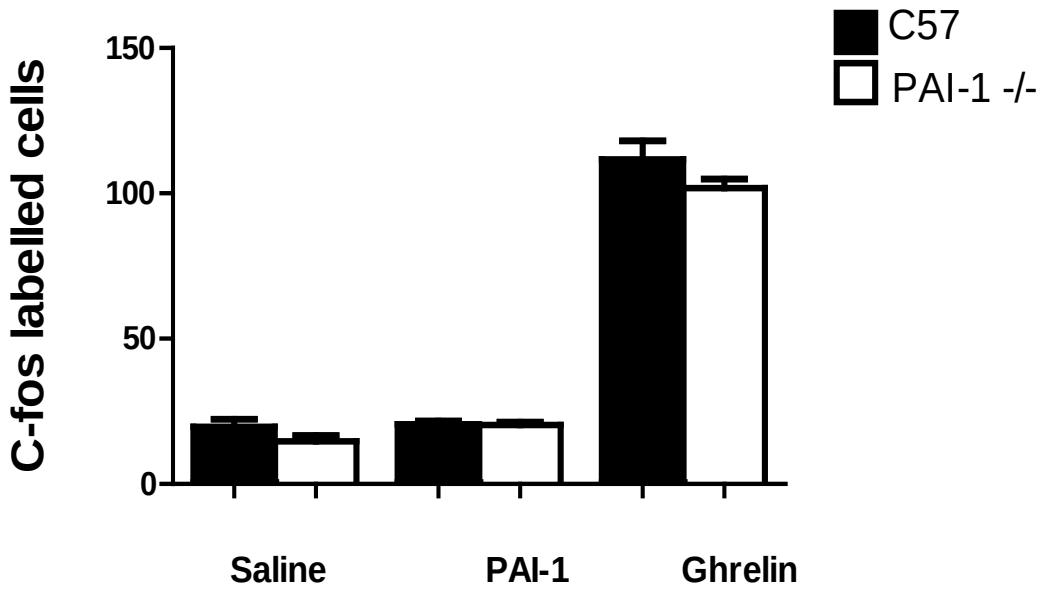

Figure 5.7

PAI-1 does not stimulate orexigenic neurons in the arcuate nucleus

Quantification of fos-positive labelled cells in the arcuate nucleus (bilateral) in response to PAI-1 and Ghrelin in C57BL/6 and PAI-$1^{-/-}$ mice, showed no significant difference between strains. In addition, when compared to vehicle, PAI-1 had no significant difference in the level of fos stimulation in both strains (Two-way ANOVA, n= 4-6).

5.3.4 PAI-1 stimulates food intake following a short term fast

The orexigenic effect of PAI-1 described above was seen after 24 h fasting and there was a relatively weak effect on mice fed *ad libitum*. To determine whether there might be a physiological role for PAI-1 in regulating food intake after a fast, C57BL/6 and PAI-1 $^{-/-}$ male mice were fasted for 6 h prior to a period re-feeding. In mice fasted for 24 h there was no difference in food intake 30 and 60 after refeeding in the two strains. After fasting for 6 h, food intake was less in both strains compared with 24 h fasting. Importantly, however, there were differences between the two strains. The mean weight of food at 30 minutes in C57BL/6 mice was 0.20 ± 0.03 g and 0.05 ± 0.004 g at 30 and 60 min respectively, compared with a mean food weight of 0.12 ± 0.03 and 0.04g ± 0.03 and 30 at 60 minutes, respectively, in PAI-1$^{-/-}$ mice ($p=0.0105$ C57BL/6 compared to PAI-1$^{-/-}$; unpaired t test, n=6-10; figure 5.7). A short term fast may therefore stimulate a PAI-1-mediated orexigenic response.

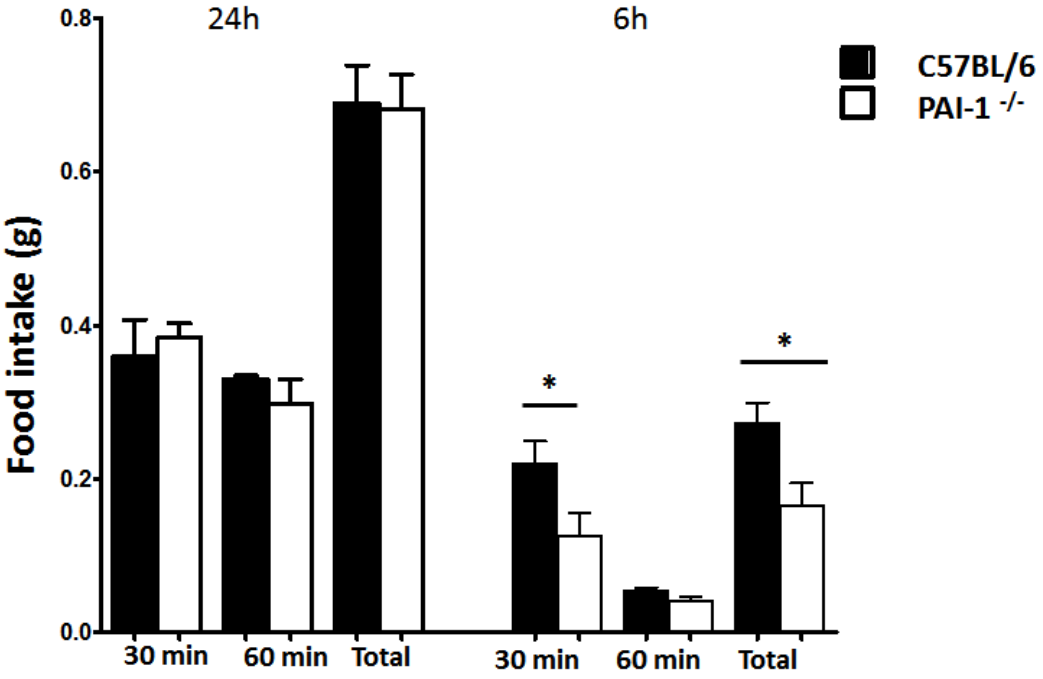

Figure 5.8

Food intake data following short term fasting in C57BL/6 and PAI-1 $^{-/-}$ male mice

PAI-1$^{-/-}$ and C57BL/6 mice have a similar food intake profile following a 24h fast. However, following a 6h fast, behavioural differences between strains are observed. * p= 0.0145 C57BL/6 compared to PAI-1 $^{-/-}$ food intake after 30 minutes and total food intake; unpaired t-test; n=6-10.

5.4 Discussion

The results in this chapter provide further insight into the mechanisms by which PAI-1 regulates food intake. It is well known that ghrelin stimulates food intake even in fed *ad libitum* mice (Toshinai *et al*., 2006). However, this is not the case with PAI-1 which has little effect in mice fed *ad libitum* but acts to increase, still further, food intake in mice reintroduced to food after a period of fasting. The orexigenic effect of ghrelin was similar in wild type and $PAI^{-/-}$ mice and so not dependent on PAI-1 expression; moreover while ghrelin increased arcuate fos labelling, PAI-1 did not. The data suggest that PAI-1 and ghrelin act through different mechanisms, and that PAI-1 may have a physiological role after a relatively short fast.

Several feeding behaviour studies have made use of metabolic cages which can monitor a range of parameters in rodents, including food intake, energy expenditure and water consumption. In addition to overall food intake, mice also visit the food hoppers to take small bouts, that are not necessarily due to hunger signals alone (Atalayer and Rowland, 2011). Such equipment can be useful when measuring parameters over long periods of time. In this present study, methods using a computerised metabolic cage system were initially sought, although in practice, it was found not to accurately reflect food intake in mice. Mice are known to be 'grazers' taking several bouts of food throughout the day (Atalayer and Rowland, 2011), and consequently, the food pellets crumbled outside of the hopper, making it impossible to record the correct weight of food consumed. This behaviour was considered to be linked to boredom or the need to gnaw, so

wooden blocks were introduced, but had no positive effect on animal behaviour. Recent evidence suggests that mice may not readily acclimatise to metabolic cages so that aberrant behaviours are observed (Kalliokoski *et al*., 2013). The current method of weighing food hoppers in home cages, at specific time points, reflects normal mouse behaviour with minimal intervention. The method is well-recognised and has been used extensively by others (Kenny *et al*., 2013a, Dakin *et al*., 2001). However, a disadvantage of the method is long-term monitoring of food intake is more difficult. At present, overall 24 hour food intake can be measured but the amount and duration of 'meals' or 'bouts' is unknown. However, all experiments in this study were designed to investigate short-term effects of systemic administration of orexigenic peptides on feeding, and this method proved to be adequate. Another disadvantage is not having an automated system to monitor water intake, as some studies have reported changes in water intake following weight loss (Thanos *et al*., 2012). However, the main aim of this study was to investigate the effects of orexigens on short-term food intake in the absence of endogenous PAI-1.

Experiments in this chapter were designed to investigate specific questions about the role of PAI-1 in the stimulation of food intake, and to determine if the presence of PAI-1 could influence the responses to other orexigens, Previous studies indicate that PAI-1, both endogenous and exogenous, increases food intake and attenuates the normal physiological responses to CCK (Kenny *et al*., 2013a, Gamble *et al*., 2013). The results obtained from feeding studies showed a weak effect on previously fed C57BL/6 mice. However, there was a substantial orexigenic drive produced in fasted C57BL/6 mice. Interestingly, the same effect was observed in the PAI-$1^{-/-}$ mice, indicating that this effect is not dependent on

the presence of endogenous PAI-1. Mice lacking PAI-1 were generated and investigated by Carmeliet *et al.* (Carmeliet *et al.*, 1993b). Since then, other studies have reported PAI-$1^{-/-}$ mice are resistant to diet-induced obesity and insulin resistance (Ma *et al.*, 2004). In direct contrast, other groups have found that PAI-1 deficiency had no overall effect on diet-induced obesity and overexpression of PAI-1 attenuated obesity following a high fat diet (Ma *et al.*, 2004). However, slight variations in the genetic strains used to generate the mice, combined with a difference in food formulation could account for such discrepancies. The present studies indicate that PAI-$1^{-/-}$ mice respond similarly to C57BL/6 mice when receiving *ip* PAI-1.

Ghrelin was first discovered as a stomach-derived hormone by Kojima *et al.* (Kojima *et al.*, 1999). It is well established that gastric expression and circulating levels of ghrelin are increased by fasting (Asakawa *et al.*, 2001). Ghrelin can encourage adiposity, increase gastric emptying and gastric acid secretions (Cota, 2007, Tschop *et al.*, 2000). Experiments in fed rodents that received peripheral administration of ghrelin increased their feeding to a level similarly observed following a 24h fast (Tschop *et al.*, 2000). Consistent with this, a significant increase of food intake in fed *ad libitum* C57BL/6 mice following *ip* ghrelin, was observed in current studies. Furthermore, fed *ad libitum* PAI-$1^{-/-}$ mice had a substantial increase in food intake following *ip* ghrelin. These results indicate that the orexigenic drive induced by ghrelin is not dependent upon the presence of PAI-1.

Other orexigenic peptides have since been discovered. Firstly, orexin-A along with orexin-B was described by Sakurai *et al.* (Sakurai *et al*., 1998). These novel neuropeptides were capable of stimulating food intake when centrally administered to rodents. More recently, orexin can positively modulate spontaneous physical activity in rodents (Perez-Leighton *et al*., 2012). Perez-Leighton *et al.* found that orexin-treated rats developed a resistance to diet-induced obesity also had an increase in physical activity. Interestingly, they claimed that central administration of orexin-A increased physical activity and helped to combat obesity. However, high variability can occur in animal models of diet-induced obesity. Secondly, anandamide (AEA), was first identified by Devane *et al.* as an endogenous ligand for the cannabinoid receptor (Devane *et al*., 1992). Recent evidence suggests that endocannabinoids like anandamide stimulate orexigenic pathways (Cota, 2007, Di Marzo and Matias, 2005). Elevated levels of anandamide have been observed in obese patients, although a direct link between high circulating levels and obesity is yet to be discovered (Gatta-Cherifi *et al*., 2012). The group suggests that anandamide is similar to ghrelin that acts as a meal initiator (Gatta-Cherifi *et al*., 2012) and that deregulation of both orexigenic and anorectic pathways can lead to depressed satiety and obesity (le Roux *et al*., 2006). Both orexigens were found to stimulate food intake, to a lesser degree than with ghrelin, in both fasted C57BL/6 and PAI-$1^{-/-}$ mice, but had a much weaker effect compared to ghrelin in fed *ad libitum* mice of both strains. This is consistent with the idea that PAI-1 is not required for the stimulation of orexigenic pathways.

Ghrelin induced the biggest increase in food intake in these experiments; working via a specific pathway that can easily be observed and investigated in mice (Scott

et al., 2007). It stimulates food intake and increases fat mass by acting on the neuropeptide Y/agouti-related peptide population of neurons within the arcuate nucleus of the hypothalamus (Cone *et al*., 2001). Activation of such neurons enabled the observation of the effect of ghrelin in the arcuate nucleus in our test animals. Interestingly, when PAI-1 was administered using the same protocol, it did not produce widespread fos expression in the arcuate, and expression levels were comparable to that of saline-treated mice. The results confirm reports in published literature that ghrelin produces widespread activation of such neurons in C57BL6 mice. Moreover, having a similar response in both strains demonstrates that ghrelin-induced stimulation of food intake is not mediated via a PAI-1-dependent signalling pathway. This is compatible with the idea that the hyperphagia reported in PAI-1HKβ mice is not due to the stimulation of appetite, but by potentially inhibiting satiety signals. However, the control of food intake appears to be influenced by the presence of PAI-1. PAI-1 may not be required to maintain food intake via an orexgenic pathway, but potentially works via another mechanism of control. Interestingly, when a short term fast is introduced, we begin to observe behavioural differences between the wild type and PAI $1^{-/-}$ mice. This indicates that PAI-1 may play a role in the control of food intake following short term food withdrawal. Moreover, the previous results suggest that this is not due to differences in sensitivity to orexigens.

The clinical implications from these results reinforce previous data that PAI-1 is associated with obesity, but that the inhibition of PAI-1 may not necessarily attenuate hyperphagia via pathways involved in hunger. It is important to establish if PAI-1-HKβ mice can restore all aspects of food intake control such as

gastric emptying and fos expression in the NTS following diet-induced weight loss. If PAI-1 is behaving as an anti-satiety factor, it is plausible that it could attenuate the effects of additional satiety hormones acting via vagal pathways. Experiments were designed to address these hypotheses and are discussed in the next chapter.

5.5 Conclusions

- Exogenous PAI-1 stimulates food intake in fasted C57BL/6 and PAI-$1^{-/-}$ but not in fed *ad libitum* mice.
- Ghrelin stimulated food intake in fed *ad libitum* C57BL/6 and PAI-$1^{-/-}$ mice.
- Unlike ghrelin, *ip* PAI-1 did not stimulate fos expression in neurons in the arcuate nucleus.
- Short term fasting highlighted feeding behavioural differences between C57BL/6 and PAI-$1^{-/-}$ mice, indicating a role for PAI-1 in stimulating food intake after a short fast.
- This study suggests that PAI-1 and ghrelin stimulate food intake via separate pathways.

Chapter 6

Effect of satiety factors in PAI-1HKβ and PAI-1 $^{-/- tg}$ $^{+/+}$ and PAI-1$^{-/- tg +/-}$ mice.

6.1 Introduction

PAI-1 is positively correlated with an increase in body fat and leptin. BMI has been implicated as a major determinant of plasma PAI-1 levels (Mavri *et al*., 1999). Dieting and weight loss have been shown to reduce PAI-1 levels, and improve insulin and glucose levels (Mavri *et al*., 1999). By reducing the body weight of PAI-1HKβ via a restricted diet, it is possible to observe the return of physiological responses to CCK that have previously been attenuated (chapter 3 and 4).

Gut hormones play a major role in the homeostatic control food intake and energy expenditure. CCK is the key satiety hormone that acts via the vagus to regulate food intake. Several other satiety peptides act via their cognate receptors expressed by vagal afferent neurons. Animal studies have shown that diet-induced obesity can attenuate these signals in vagal afferent neurons, as well as altering nutrient detection and gut peptide secretion (Ukkola *et al*., 2011, Kentish *et al*., 2013, Daly *et al*., 2011, de Lartigue *et al*., 2011a). Exposing PAI-1HKβ mice to other anorectic peptides could provide mechanistic insights into the action of PAI-1 in feeding and obesity.

As previously demonstrated, the PAI-1 has a moderately obese phenotype as a result of targeted overexpression of PAI-1 in gastric parietal cells (Kenny *et al*., 2013a). However, these mice express wild type PAI-1 in both stromal and epithelial cells of the stomach. The transgene in the PAI-1HKβ mice is only

expressed in the parietal cells of the stomach. The generation of a mouse line with expression of the transgene only would allow investigations in to the role of gastric PAI-1 expression in satiety.

6.1.1 Aims

1. To determine whether CCK resistance in PAI-1-HKβ mice is a consequence or cause of obesity by studies of NTS fos labelling and gastric emptying after calorie-restriction.
2. To compare the effects on food intake in PAI-1-HKβ mice of a variety of satiety factors.
3. To investigate whether the obese phenotype in PAI-1HKβ mice is solely attributable to the transgene by studies in transgenic mice null for wild type PAI-1 expression.

6.2 Materials and Methods

6.2.1 Body weight and calorie restriction

The body weight of PAI-1-HKβ and C57BL/6 mice were recorded daily for five days. Food consumption for PAI-1-HKβ mice was restricted to 3.5g per day for four weeks.

Brainstem fos expression and gastric emptying following CCK8s ip, (2.5nmol/kg) were determined as described in Chapter 2.

6.2.2 Food intake studies

Food intake was measured directly in all experiments. In some experiments, food intake was measured at 30 and 60 minutes following ip CCK8s (2.5nmol/kg), exendin-4 (0.75nmol/kg), oxyntomodulin (3nmol/kg) or OEA (5mg/kg) in both PAI-1-HKβ and C57BL/6 mice. In other experiments, transgenic mice of both genders and null for wild type PAI-1 (described in chapter 2) were monitored using the same protocol.

6.3 Results

6.3.1 Insensitivity to CCK is reversed in PAI-1HKβ mice following weight reduction by caloric restriction

PAI-1-HKβ mice display a modest obese phenotype (Kenny *et al*., 2013a) and this phenotype was confirmed in the mice used for this study (Figure 6.1). PAI-1-H/Kβ mice also consumed on average 14% more than C57BL6 mice when monitored over 24 hours (Figure 6.1).

When PAI-1-HKβ mice were entered into a restricted diet plan that matched their daily food intake to that of C57BL/6 mice (3 and 3.5g) there was a mean weight loss of 12% (Figure 6.1). Pair-fed PAI-1- H/Kβ mice then received CCK8s and fos expression was determined. There was no significant difference between the number of fos-positive neurons within the NTS of saline-treated and CCK-treated PAI-1-HKβ mice; moreover, fos-labelled cells were significantly fewer in pair-fed mice than in C57BL/6 mice (Figure 6.2).

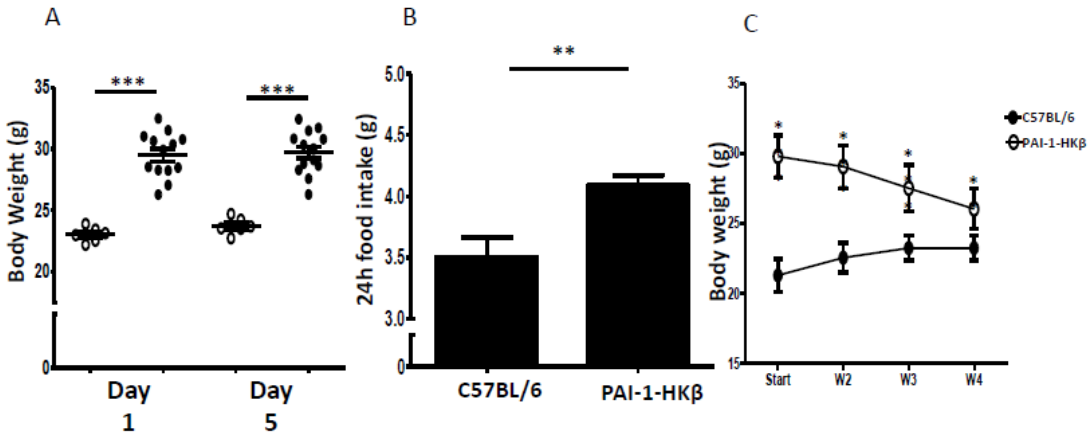

Figure 6.1

PAI-1-H/Kβ male mice have increased body weight and are hyperphagic but lose body weight when pair fed with C57BL/6 mice

PAI-1-H/Kβ mice weighed more than C57BL/6 (A; ***P < 0.0001 C57BL/6 compared to PAI-1-H/Kβ, unpaired t test. SYMBOLS: Open circles, C57BL/6; filled circles, PAI-1-H/Kβ). PAI-1-HKβ mice consumed more food than C57BL/6 over a 24 hour period (B;**P= 0.0057 for C57BL/6 food intake compared to PAI-1-H/Kβ food intake, unpaired t-test). PAI-1-H/Kβ mice pair-fed with C57BL/6 mice lost weight over a 4 week period (C); one-way ANOVA with Bonferroni post tests showed a significant difference in body weight between strains (* = $p<0.05$ comparing mean C57BL/6 body weight to PAI-1-H/Kβ mice, week 1 (start) - week 3; ** = $p<0.01$ comparing C57BL/6 mean body weights to PAI-1-H/Kβ mice, week 4; n=6-9)

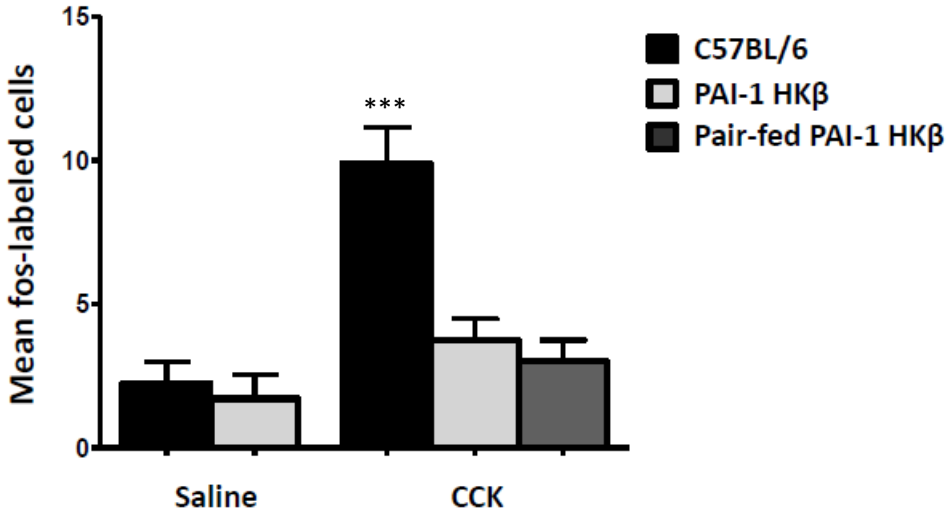

Figure 6.2 Diet-induced weight loss in PAI-1HKβ mice does not restore brainstem responses to CCK

PAI-1H/Kβ mice that were pair fed with C57BL/6 mice did not exhibit increased fos-positive neurons in the NTS following *ip* CCK8s (*2.5nmol/kg*). ***p = 0.0001 one-way ANOVA comparing both groups of PAI-1HKβ with C57BL/6 mice all treated with CCK. Bonferroni post-test showed no significance between the pair-fed and fed *ad libitum* PAI-1HKβ mice; n=5-12.

Gastric emptying assays were then performed to identify functional changes in response to CCK in pair-fed PAI-1H/Kβ mice (Figure 6.3). As previously reported (Chapter 4), PAI-1HKβ mice are resistant to inhibition of gastric emptying in response to CCK. In comparison, the pair-fed group showed a significant decrease in the rate of gastric emptying to CCK, similar to C57BL/6 mice. Thus, CCK8s–treated C57BL/6 and fed *ad libitum* PAI-1HKβ exhibited mean emptying rates of 0.17 ± 0.02 and 0.41 ± 0.01 mL, respectively, compared to 0.24 ± 0.04 mL in pair-fed PAI-1HKβ mice; Bonferroni post test showed no significant difference between pair-fed PAI-1-HKβ and C57BL/6 mice n=4-6.

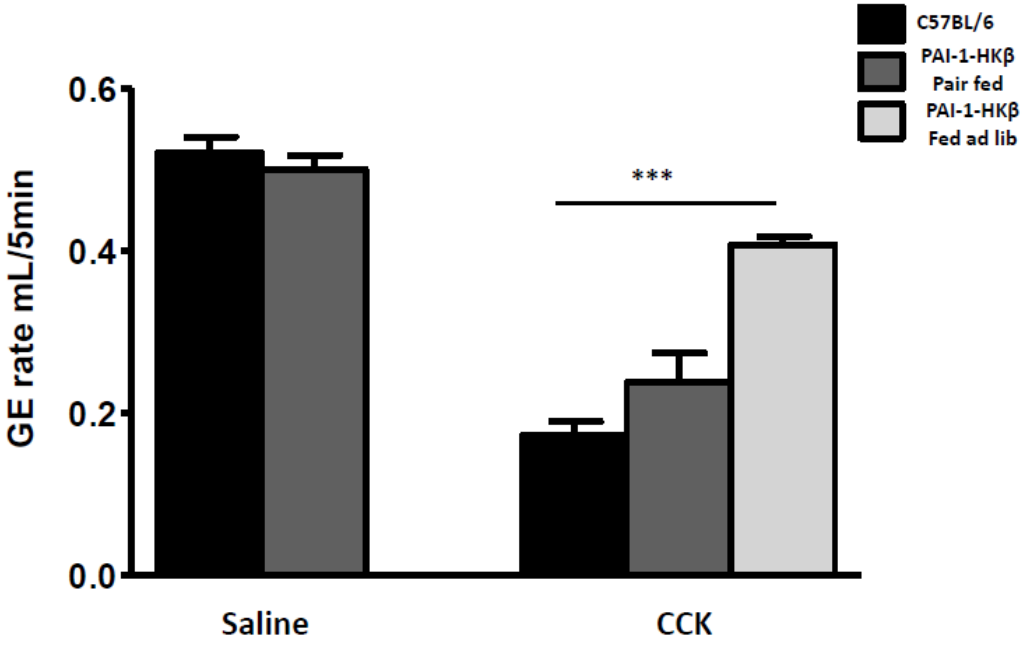

Figure 6.3

CCK (2.5nmol/kg) delays gastric emptying in calorie-restricted PAI-1-H/Kβ mice.

PAI-1-HKβ mice that were pair fed with C57BL/6 mice regain gastric responses to CCK (***p= 0.0002 one-way ANOVA. Bonferroni post test showed no significant difference between pair-fed PAI-1-H/Kβ and C57BL/6 mice, n=4-6).

6.3.2 PAI-1-H/Kβ mice are resistant to several satiety hormones

In order to determine whether the resistance of PAI-1-H/Kβ mice to CCK applied to other satiety hormones, the effects of several other gut-derived satiety factors were examined. Comparisons of food intake in PAI-1-H/Kβ and C57BL/6 mice that had been fasted for 24 h were monitored at 30 minutes as intake at 60 minutes proved uninformative. Vehicle–treated C57BL/6 mice consumed 0.38 ± 0.01 g compared with 0.49 ± 0.04 g over 30 minutes in PAI-1-H/Kβ mice. CCK inhibited consumption in C57BL/6 mice by over 65% (0.13 ± 0.02 g) compared with approximately 12% inhibition in PAI-1-H/Kβ mice (0.43 ± 0.07 g) (**** p= <0.0001 comparing C57BL/6 and PAI-1-H/Kβ CCK-treated mice; ANOVA, Bonferroni post-test, n=6-12).

The GLP-1 agonist, exendin-4, inhibited food intake in C57BL/6 mice by 60% at 30 minutes (0.15g ± 0.03), compared with approximately 25% inhibition (0.37g ± 0.04) at 30 minutes in the PAI-1-H/Kβ mice (** p =<0.001 comparing C57BL/6 to PAI-1-H/Kβ exendin-4-treated mice, ANOVA, Bonferroni post-test n=6) (Figure 6.4).

Administration of proglucagon gene product, oxyntomodulin demonstrated a significant difference between the two strains, with an inhibition in C57BL/6 mice of 48% at 30 minutes (0.20g ± 0.04) for C57BL/6 mice, compared with no inhibition (0.54g ± 0.08) at 30 minutes in the PAI-1-H/Kβ mice (Figure 6.4)(**p= <0.001 C57BL/6 compared to PAI-1-H/Kβ oxyntomodulin-treated mice; ANOVA with Bonferroni post-test, n=7.

Oleoylethanolamide (OEA) also highlighted significant behavioural differences between the two strains. Vehicle–treated C57BL/6 mice consumed in 30 minutes,

0.42 ± 0.06 g compared with 0.63 ± 0.05 g in PAI-1-H/Kβ mice. OEA inhibited consumption in C57BL/6 mice by over 45% (0.23 ± 0.03 g) compared with no inhibition in PAI-1-H/Kβ mice (0.75 ± 0.08 g) at 30 minutes (Figure 6.5)(**** p= <0.0001 comparing C57BL/6 and PAI-1-H/Kβ CCK-treated mice; ANOVA, Bonferroni post-test, n=6.

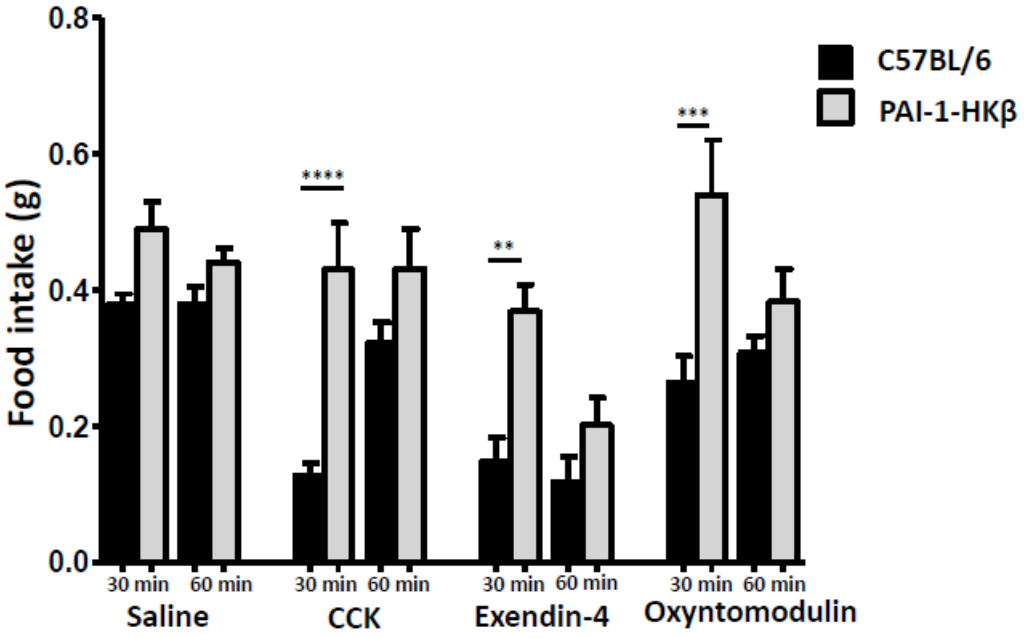

Figure 6.4

PAI-1HKβ mice are resistant to the satiety effects of three anorexigenic peptides

Food intake in PAI-1HKβ mice following *ip* injections of CCK, exending-4 and oxyntomodulin was greater than in wild type mice. Two-way ANOVA was used to highlight differences between strain and treatments and showed significant differences in food intake between strains treated with anorectic peptides; one-way ANOVA with Bonferroni post tests showed significant increases in food intake when comparing PAI-1-HKβ to C57BL/6 mice (**** p=<0.0001 comparing C57BL/6 and PAI-1-HKβ CCK-treated mice;. ** p=0.001 comparing C57BL/6 to PAI-1HKβ exendin-4-treated mice; *** p= 0.0041 C57BL/6 compared to PAI-1HKβ oxyntomodulin-treated mice; n=6-12).

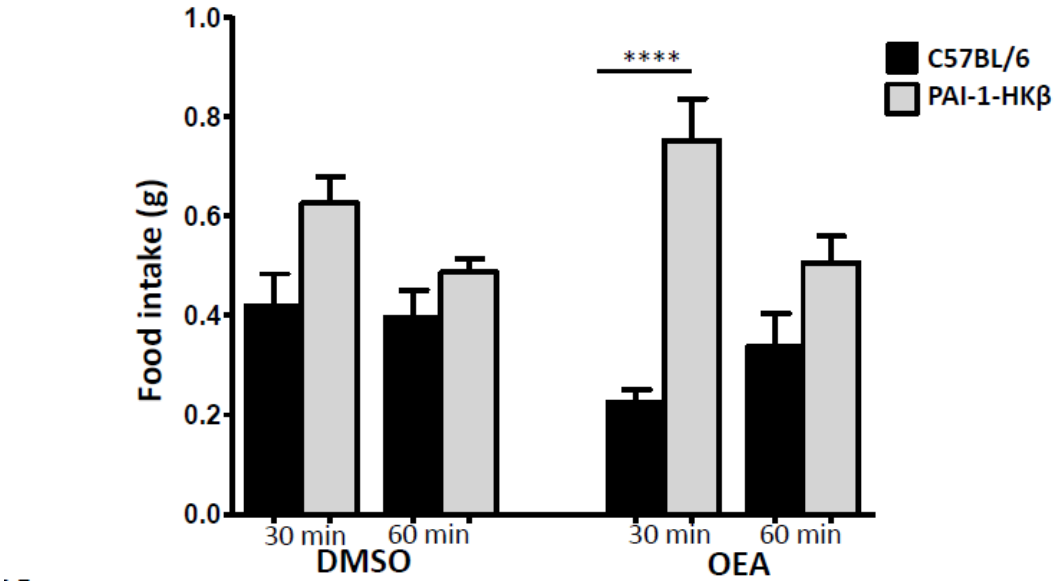

Figure 6.5

Oleoylethanolamide (OEA) reduces food intake in C57BL/6 mice but not in PAI-1HKβ

Insensitivity to OEA was observed in the PAI-1HKβ mice. Two-way ANOVA showed a significant difference between strains (****p= <0.0001; Bonferroni post-test highlighted the biggest difference was when comparing C57BL/6 to PAI-1HKβ mice treated with OEA at 30 minute time point; n=6).

6.3.3 PAI-1 $^{-/-\ tg\ +/+}$ and PAI-1 $^{-/-\ tg\ +/-}$ male and female mice respond to CCK

Body weights of PAI-$1^{-/-}$ and PAI-$1^{-/-\ tg\ +/+}$ and PAI-$1^{-/-\ tg\ +/-}$ were not significantly different to those of C57BL/6 mice (Figure 6.6). Food intake over 30 min following ip. CCK8s (Figure 6.7) in C57BL/6 and PAI-$1^{-/-}$ male mice was similar (0.13 ± 0.02 compared with 0.07 ± 0.02, respectively; n=4-6). Moreover, food intake (Figure 6.8) was inhibited by CCK8s in both homozygous and heterozygous transgenic mice that were also null for wild type PAI-1.

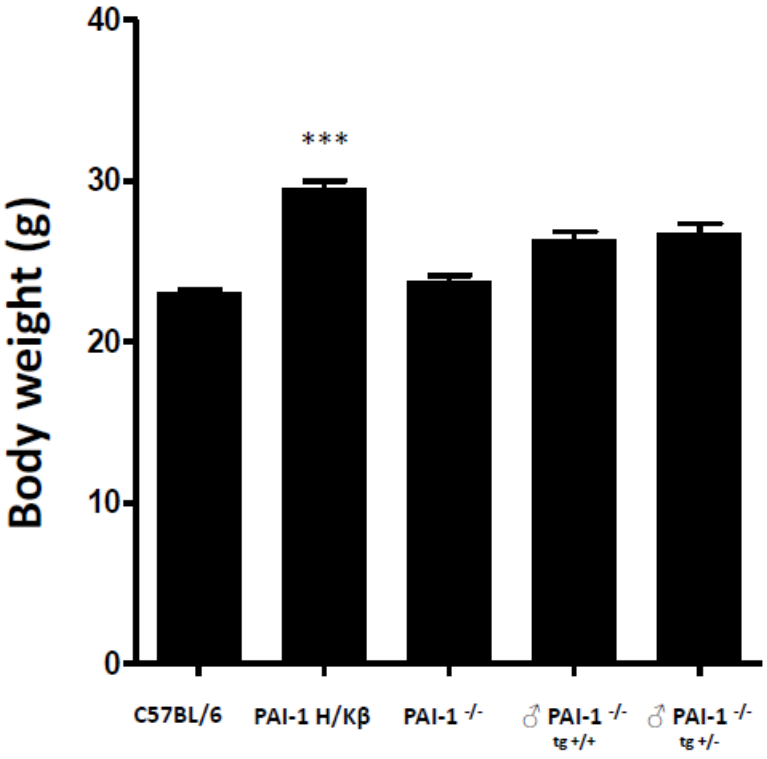

Figure 6.6 Comparative body weights of all transgenic mice

The weights of male mice (PAI-1HKβ, PAI-1 $^{-/-}$, PAI-1 $^{-/-\ tg\ +/+}$ and PAI-1 $^{-/-\ tg\ +/-}$) of 10-12 weeks of age were monitored and compared to C57BL/6 male mice. PAI-1HKβ mice are obese compared to C57BL/6 mice ***p= <0.0001 one way ANOVA; Bonferroni showed no significant weight differences between the other PAI-1 transgenics when compared to C57BL/6 mice, n=3-5.

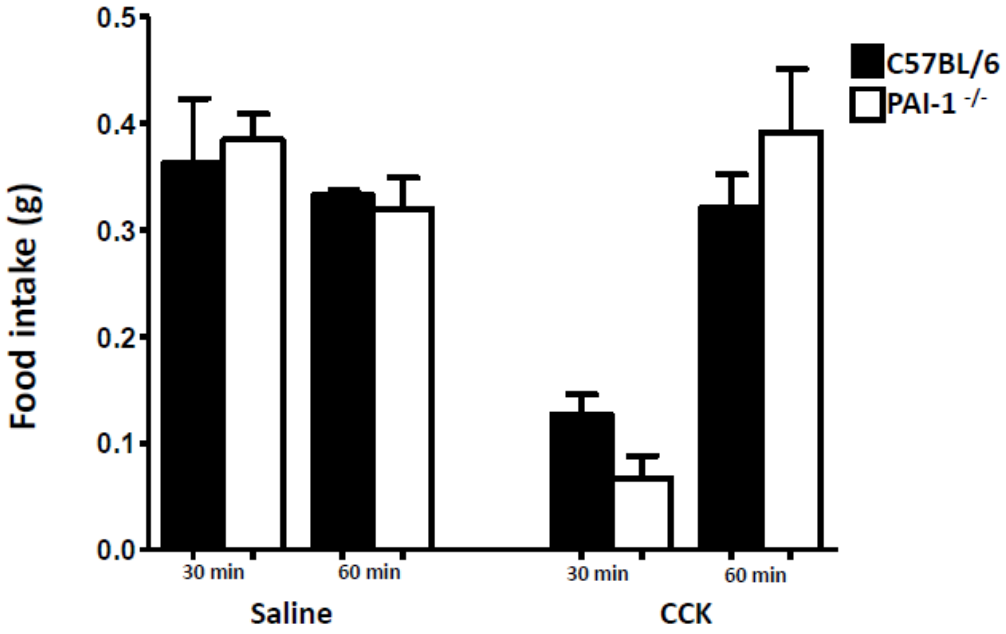

Figure 6.7

Food intake data following CCK compared to saline administration after a 24 hour fast in C57BL/6 and PAI-1 $^{-/-}$ male mice

PAI-1$^{-/-}$ mice respond to CCK following a 24 hour fast, consuming less food than C57BL/6 mice at 30 minutes. However, this was not significant. n=4-6

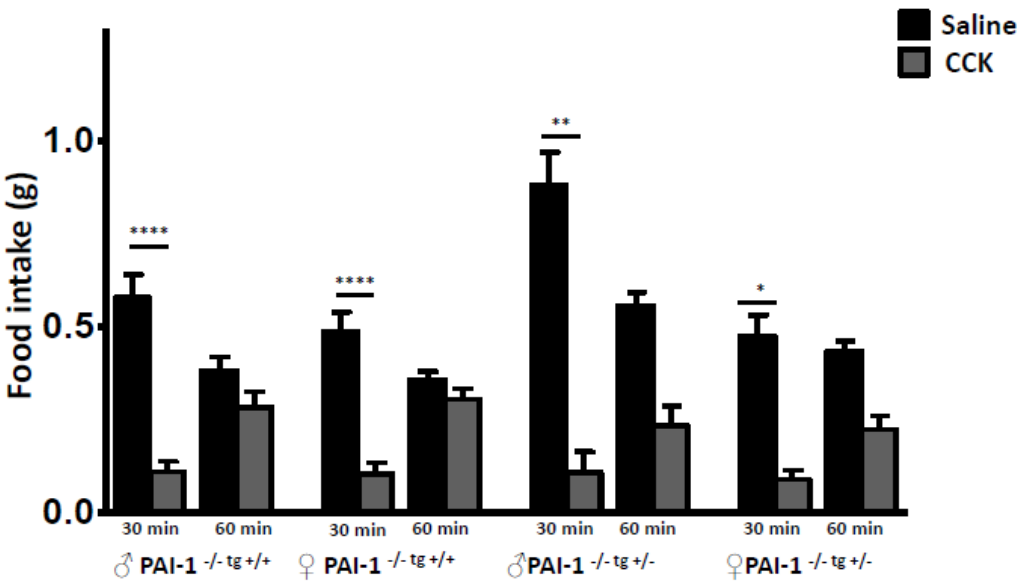

Figure 6.8

PAI-1HKβ transgenic mice that are null for wild type PAI-1 respond to CCK

Mice injected with CCK consumed less food than mice treated with saline following a 24 hour fast. One-way ANOVA with Bonferroni post-tests showed ****p<0.0001 saline-treated compared to CCK-treated PAI-1 $^{-/-\ tg\ +/+}$ male mice, unpaired t test, n=11. ****p<0.0001 saline-treated compared to CCK-treated PAI-1 $^{-/-\ tg\ +/+}$ female mice, unpaired t test, n=12. ** p= 0.0012 saline-treated compared to CCK-treated PAI-1 $^{-/-\ tg\ +/-}$ male mice, unpaired t-test, n=3. *p= 0.0117 saline-treated compared to CCK-treated PAI-1 $^{-/-\ tg\ +/-}$ female mice, unpaired t-test corrected for number of comparisons, n=5.

6.4 Discussion

The experiments discussed in this chapter were designed to determine the physiological basis of the hyperphagic and obese phenotype in PAI-1HKβ mice. Insensitivity to CCK is one of the features of obesity (Lawton *et al*., 1993, de Lartigue *et al*., 2012), therefore it becomes relevant to ask whether the phenotype of PAI-1-HKβ mice is a cause of CCK resistance or a consequence of it. By maintaining PAI-1HKβ mice on a calorie-restricted diet (matched food intake to C57BL/6 mice) it was possible to examine this question. In previous food intake studies in pair-fed PAI-1HKβ mice, a partial restoration in CCK sensitivity was reported (Kenny *et al*., 2013a). In this current study, PAI-1HKβ mice did lost body weight and after 4 weeks were only marginally heavier than wild type mice. However, brainstem fos responses to CCK were still significantly depressed in calorie-restricted PAI-1HKβ mice. In contrast, gastric emptying assays revealed restoration of sensitivity to CCK in pair-fed PAI-1HKβ mice. From these data, we can conclude that CCK resistance observed in the PAI-1HKβ mice can be partly attributed to their obesity.

The next experiments were designed to investigate the response of PAI-1HKβ mice to other satiety hormones (exendin-4, oxyntomodulin and OEA) known to exhibit CCK-like actions. The response of C57BL/6 mice was as expected, with an inhibition of food intake following *ip* injections of the three hormones. However, consistent with the CCK data, PAI-1HKβ mice were insensitive to these hormones. The final set of experiments examined whether the phenotype of the PAI-1HKβ mice was solely attributable to the expression of the transgene or whether both transgene and wild type gene were required. The results clearly

indicate that both transgene and wild type gene are required for inhibition of food intake by CCK.

Analogues of anandamide were first investigated by Koga *et al.* (Koga *et al*., 1997). One particular molecule which is chemically related to anandamide, oleoylethanolamide (OEA), was found to bind to peroxisome proliferator-activated receptor alpha (PPARα) leading to a reduction in food intake and increased lipolysis (Tourino *et al*., 2010). OEA was best described by Fonseca *et al.* in terms of appetite regulation (de Fonseca *et al*., 2001). They observed that fasting reduced synthesis of OEA in the small intestine. Peripheral administration of OEA caused a significant decrease in food intake, which was not observed following central administration (de Fonseca *et al*., 2001). Oleoylethanolamide has also been reported to bind to both GPR119 and TRPV1 receptors (Overton *et al*., 2006, Wang *et al*., 2005). Both of these receptors are expressed in vagal afferents, and therefore potential targets for the reduction of food intake.

Oxyntomodulin (OXM) was first described by Bataille *et al.* (Bataille *et al*., 1981) following the isolation of the peptide from pig intestines. Although evidence suggesting the existence of OXM appeared as early as 1968, when it was discovered that there were two gut peptides that showed glucagon-like immunoreactivity (Unger *et al*., 1968). OXM, a product of the proglucagon gene, inhibits gastric acid secretion, pancreatic enzyme secretion and food intake (Dakin *et al*., 2001). It is a dual agonist at both the GLP-1 and glucagon receptors (Dakin *et al*., 2001), making it an attractive potential therapy for type 2 diabetes and obesity (Pocai, 2012). Agonists of the GLP-1 receptor have become the subject of

huge therapeutic interest, for the most part for their role in diabetes (Meier, 2012, Wilding, 2007). However, evidence has also been reported on how they are able to lower post-prandial blood glucose through inhibition of gastric emptying (Wilding, 2007). The experiments from this study have demonstrated that PAI-1-HKβ mice are also resistant to the satiety effects of OXM.

Exendin-4 was first described by Eng *et al.* following work conducted on the beaded lizard's venom (Raufman *et al*., 1991, Eng *et al*., 1992). Exendin-4 was first isolated from the venomous saliva of the Gila monster (Raufman *et al*., 1982) and differs from exenedin-3 by two amino acid substitutions (Eng *et al*., 1992). This allows exendin-4 to interact exclusively with the GLP-1 receptor to increase cAMP (Eng *et al*., 1992). In addition to exendin-4's anti-diabetic properties, reduction of food intake has also been reported (de Fonseca *et al*., 2000). Exendin-4 is known to delay gastric emptying and decrease appetite (Wettergren *et al*., 1993, de Fonseca *et al*., 2000). De Fonseca *et al.* reported weight loss in Zucker obese rats following treatment with exendin-4. Moreover, peripheral administration produced better results than centrally administered exendin-4 (de Fonseca *et al*., 2000). Exendin-4 also has a longer duration of action than endogenous GLP-1 (Wilding, 2007), allowing it to inhibit food intake for up to 4 hours (de Fonseca *et al*., 2000). In the present study, the results were consistent with reports that suggest that exendin-4 has a more pronounced effect (Dakin *et al*., 2001) on the inhibition of food intake in C57BL/6 mice than OXM and OEA. However, compatible with the findings throughout this thesis, PAI-1-HKβ mice were resistant to exendin-4, even at a low dose of 0.175nmol/kg.

Overall, the resistance to a variety of satiety hormones in PAI-1-HKβ mice provides evidence that elevated gastric PAI-1 disrupts satiety signalling. GLP-1

agonists are already well established as a therapy for type II diabetes. The additional benefit of losing weight provides hopes for future anti-obesity drugs. However, it is important to note that not all patients who are obese have type II diabetes, although could potentially be at risk of developing this in the future. PAI-1-HKβ mice, however do not exhibit a diabetic phenotype (Kenny *et al*., 2013a). Whether the obese model we present closely resembles the moderate obese population is yet to be confirmed.

As discussed throughout this thesis, the moderate obesity phenotype is an unexpected outcome in the PAI-1-HKβ model. Interestingly, the insensitivity of PAI-1-HKβ mice to CCK requires both the wild type and transgene of PAI-1. Following experiments in mice possessing only the transgene, their feeding behaviour following CCK was comparable to the response observed in PAI-$1^{-/-}$ mice. In chapter 5, the PAI-$1^{-/-}$ mice were investigated with particular emphasis on the orexigenic pathways. In this chapter, their food intake behaviour was monitored in response to CCK. The results obtained from this study demonstrate that PAI-$1^{-/-}$ mice are sensitive to CCK.

Gastric PAI-1 may indeed play a vital part in the development of obesity, but appears to depend upon the additional presence of wild type PAI-1 released from either parietal cells or other sources, possibly from adipose tissue. The insensitivity to satiety factors in this model suggests that PAI-1 may play a pivotal role in the development of obesity. Particularly when these mice are diet-controlled to lose weight, they are able to regain some homeostatic aspects of energy balance.

6.5 Conclusions

1. Restriction of food promoted weight loss in the PAI-1-HKβ mice which resulted in the return of normal physiological responses in CCK-induced gastric emptying.
2. CCK-induced fos expression in the NTS was not restored in the PAI-1-HKβ mice following weight loss.
3. PAI-1-HKβ mice are not only insensitive to the satiety effects of CCK, but other hormones including exendin-4, oxyntomodulin and OEA.
4. The presence of the wild type PAI-1 gene appears to be required for the resistance to CCK exhibited by PAI-1-HKβ mice.

Chapter 7

Discussion

7.1 Major findings

The major finding of this thesis is that PAI-1 suppresses the effects of the satiety hormone, CCK. Transgenic mice over-expressing PAI-1 in gastric parietal cells exhibited a decrease in brainstem neuronal fos-labelling following administration of exogenous CCK-8s. Furthermore, a decrease in brainstem responses was also observed in previously fasted PAI-1HKβ mice that were re-fed to induce the release of post-prandial CCK. In addition to the inhibitory effect of endogenous PAI-1 on CCK, brainstem responses were also decreased in C57BL/6 mice treated with exogenous PAI-1 following feeding. Experiments using mice without the receptor which binds PAI-1 (uPAR), showed that this receptor was required for the inhibition by PAI-1 of brain stem responses to CCK.

In addition to inhibition of food intake, CCK also delays gastric emptying via vagal afferent neurons. The present experiments showed that both exogenous and endogenous PAI-1 attenuated CCK's ability to delay gastric emptying in mice. Furthermore, administration of gastrin increased plasma PAI-1 concentration in C57BL/6 mice, which also attenuated the effects of CCK.

In feeding studies, PAI-1 administration increased food intake dramatically in fasted C57BL/6 and PAI-$1^{-/-}$ mice. The effect was substantially attenuated in mice fed *ad libitum*. PAI-$1^{-/-}$ mice exhibited a normal physiological response to ghrelin in both feeding and hypothalamic fos expression studies indicating that PAI-1 is not required for the orexigenic response to ghrelin. Short-term food withdrawal highlighted behavioural differences between C57BL/6 and PAI-$1^{-/-}$ mice, suggesting PAI-1 may have a role in the maintenance of food intake.

Finally, there is the question of whether PAI-1 was a cause of the obese phenotype or a consequence of it in PAI-1-HKβ mice. When the latter were entered onto a calorie-restricted feeding study, there was weight loss and increased effects of CCK in delaying gastric emptying. In further experiments to characterise the phenotype of the PAI-1HKβ mice, feeding studies showed that the mice have a blunted response to other gut-derived satiety factors that act on vagal afferents. Overall, this thesis describes how PAI-1 expression can act to attenuate the physiological mechanism of satiety via the vagus.

A limitation from these present studies using PAI-1-HKβ mice is that targeted overexpression to parietal cells may not completely mimic physiological levels of PAI-1. By utilising an inducible model, this limitation may be overcome. The tetracycline-dependent regulatory system allows precise control of gene expression (Urlinger *et al*., 2000). With this approach, the transgene allows the expression of the tetracycline-controlled transactivator (tTA) and one other gene of interest, and is controlled by a synthetic tTA-dependent promoter. This allows the activation of the tTA promotor and transcription of the gene of interest in the absence of tetracycline and its analogues. When tetracycline is introduced, this causes a conformational change of tTA, inhibiting its interaction with DNA, and subsequent gene expression. Genes can be then induced specifically. For example, Blondeau *et al.* employed an approach by where activation of the reporter gene occurred specifically in Pdx1- or Insulin1- expressing cells in the pancreas (Blondeau *et al*., 2002). This approach could potentially be adapted to study the PAI-1HKβ mice. Thus, with inducible expression of PAI-1 in gastric parietal cells that accurately mimic physiological process, further studies into disease states are possible.

Nutrient content, inflammation and the microflora of the gut can all influence the development of obesity. Vagal afferent neurons are known to alter their neurochemical phenotype in response to nutrient status (Dockray, 2013). Fasting rats for over 6 hours decreases the expression of cocaine-amphetamine-regulated transcript (CART) and Y2 receptors in vagal afferent neurons. In contrast, CB1 receptor expression is increased and this phenotype can be reversed by CCK (Dockray, 2013). Recent animal studies have shown that high-fat diets that promote obesity are capable of 'locking' the neurochemical make-up of the neurons into a 'fasted' state. This results in insensitivity to satiety hormones such as CCK and leptin and also gastric distension. The cell bodies contained within the nodose ganglion have increased CB1 and MCH1 receptor expression and decreased Y2 and leptin receptors (Daly *et al*., 2011, de Lartigue *et al*., 2011a, de Lartigue *et al*., 2012, Kentish *et al*., 2013). Furthermore, leptin resistance appears to occur in vagal afferent neurons, before it occurs in the hypothalamus. Changes in microbiota that causes intestinal inflammation are considered to be capable of desensitising vagal afferents to leptin (de Lartigue *et al*., 2011b, Raybould, 2012). With this in mind, future work would benefit from studies of the nodose ganglia of the PAI-1HKβ mice. This would establish whether the hyperphagic behaviour observed in PAI-1HKβ mice could be attributed to a 'locked' fasting-type of neurochemical phenotype. This would reinforce previous data showing that PAI-1 plays a major role in the development of obesity.

PAI-1 can influence the physiological responses involved in the regulation of energy homeostasis. Plasma PAI-1 and gastric PAI-1 are known to be increased in obesity (Landin *et al*., 1990, Shimomura *et al*., 1996) and *Helicobacter* infection (Kenny *et al*., 2008, Keates *et al*., 2008), respectively. Furthermore,

there is evidence that obesity can attenuate the ability of CCK to inhibit gastric emptying (Little *et al*., 2007, Covasa, 2010). This is consistent with the findings discussed in this thesis of the PAI-1HKβ model. The implications point towards PAI-1 acting as a gastric factor that is capable of suppressing not only the satiety effects of CCK, but also other satiety peptides that can act via vagal afferents. The mechanism by which PAI-1 acts is considered to be via a paracrine action on nearby vagal afferents. It is well established that food inhibition by CCK via vagal afferent neurons is potentiated by leptin (de Lartigue *et al*., 2010, Barrachina *et al*., 1997a). The results from this investigation suggest that PAI-1 and leptin are both adipokines expressed in the stomach and adipose tissue, but act via opposite mechanisms; where PAI-1 suppresses vagal responses to CCK, and indeed other satiety peptides. The orexigen, ghrelin similarly promotes feeding, adiposity and inhibits the effects of CCK on vagal afferent neurons (Cummings and Overduin, 2007, Date *et al*., 2002a, Burdyga *et al*., 2006). However the present data suggests that PAI-1 does not act via a similar pathway to ghrelin since PAI-1 (unlike ghrelin) did not increase fos in ARC neurons). Instead, it seems possible that PAI-1 is exerting its effects via vagal afferent neurons, but not through stimulation of NPY/AgRP neurons located in the arcuate.

PAI-1 appears to be a gastric factor expressed in addition to ghrelin, whose function is to maintain food intake in circumstances of infection and inflammation. *Helicobacter* infection in humans has been linked to depressed ghrelin levels (Nweneka and Prentice, 2011), and in mice, it has the ability to disrupt feeding patterns (Bercik *et al*., 2009). Furthermore, it has been shown that during cases of increased inflammation in the stomach, proinflammatory cytokines can enhance satiety factors such as CCK (McDermott *et al*., 2006), and

the present data suggest PAI-1 acts as a defence mechanism to protect nutrient delivery in such pathological circumstances.

Multiple mechanisms have been implicated in the development of obesity in animals including changes is the sensitivity and the neurochemical phenotype of vagal afferent neurons, inflammation and infection. It is known that PAI-1 is increased in obesity and these present studies are compatible with the idea that PAI-1 causes insensitivity to CCK. However, a component of these present investigations have relied upon exogenous PAI-1 which is a long lasting human recombinant form of the molecule and future work including dose-response studies would be beneficial.

The present data indicate that PAI-1 is a previously unsuspected anti-satiety gastric factor that modulates vagal afferent signalling to maintain nutrient delivery to the gut by opposing interactions between CCK and other satiety factors at the level of the vagus. This work will provide potential new prospects for the prevention and treatment of obesity.

Chapter 8

References

ABBOTT, C. R., MONTEIRO, M., SMALL, C. J., SAJEDI, A., SMITH, K. L., PARKINSON, J. R., GHATEI, M. A. & BLOOM, S. R. 2005. The inhibitory effects of peripheral administration of peptide YY(3-36) and glucagon-like peptide-1 on food intake are attenuated by ablation of the vagal-brainstem-hypothalamic pathway. *Brain Res,* 1044, 127-31.

ABIZAID, A. & HORVATH, T. L. 2012. Ghrelin and the central regulation of feeding and energy balance. *Indian J Endocrinol Metab,* 16, S617-26.

ADRIAN, T. E., BESTERMAN, H. S., MALLINSON, C. N., GREENBERG, G. R. & BLOOM, S. R. 1979. Inhibition of secretin stimulated pancreatic secretion by pancreatic polypeptide. *Gut,* 20, 37-40.

ADRIAN, T. E., BLOOM, S. R., BRYANT, M. G., POLAK, J. M., HEITZ, P. H. & BARNES, A. J. 1976. Distribution and release of human pancreatic polypeptide. *Gut,* 17, 940-44.

ADRIAN, T. E., FERRI, G. L., BACARESE-HAMILTON, A. J., FUESSL, H. S., POLAK, J. M. & BLOOM, S. R. 1985. Human distribution and release of a putative new gut hormone, peptide YY. *Gastroenterology,* 89, 1070-7.

AHERN, G. P. 2003. Activation of TRPV1 by the satiety factor oleoylethanolamide. *J Biol Chem,* 278, 30429-34.

ALESSI, M. C., PEIRETTI, F., MORANGE, P., HENRY, M., NALBONE, G. & JUHAN-VAGUE, I. 1997. Production of plasminogen activator inhibitor 1 by human adipose tissue: possible link between visceral fat accumulation and vascular disease. *Diabetes,* 46, 860-7.

ALIPOUR, A., ELTE, J. W., VAN ZAANEN, H. C., RIETVELD, A. P. & CABEZAS, M. C. 2007. Postprandial inflammation and endothelial dysfuction. *Biochem Soc Trans,* 35, 466-9.

ANGEL, P. & KARIN, M. 1991. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim Biophys Acta,* 1072, 129-57.

ARIYASU, H., TAKAYA, K., TAGAMI, T., OGAWA, Y., HOSODA, K., AKAMIZU, T., SUDA, M., KOH, T., NATSUI, K., TOYOOKA, S., SHIRAKAMI, G., USUI, T.,

SHIMATSU, A., DOI, K., HOSODA, H., KOJIMA, M., KANGAWA, K. & NAKAO, K. 2001. Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. ***J Clin Endocrinol Metab,*** **86, 4753-8.**

ARONNE, L. J., TONSTAD, S., MORENO, M., GANTZ, I., ERONDU, N., SURYAWANSHI, S., MOLONY, C., SIEBERTS, S., NAYEE, J., MEEHAN, A. G., SHAPIRO, D., HEYMSFIELD, S. B., KAUFMAN, K. D. & AMATRUDA, J. M. 2010. A clinical trial assessing the safety and efficacy of taranabant, a CB1R inverse agonist, in obese and overweight patients: a high-dose study. ***Int J Obes (Lond),*** **34, 919-35.**

ASAKAWA, A., INUI, A., FUJIMIYA, M., SAKAMAKI, R., SHINFUKU, N., UETA, Y., MEGUID, M. M. & KASUGA, M. 2005. Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin. ***Gut,*** **54, 18-24.**

ASAKAWA, A., INUI, A., KAGA, T., YUZURIHA, H., NAGATA, T., UENO, N., MAKINO, S., FUJIMIYA, M., NIIJIMA, A., FUJINO, M. A. & KASUGA, M. 2001. Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. ***Gastroenterology,*** **120, 337-45.**

ASAKAWA, A., INUI, A., UENO, N., FUJIMIYA, M., FUJINO, M. A. & KASUGA, M. 1999. Mouse pancreatic polypeptide modulates food intake, while not influencing anxiety in mice. ***Peptides,*** **20, 1445-8.**

ASAKAWA, A., INUI, A., YUZURIHA, H., UENO, N., KATSUURA, G., FUJIMIYA, M., FUJINO, M. A., NIIJIMA, A., MEGUID, M. M. & KASUGA, M. 2003. Characterization of the effects of pancreatic polypeptide in the regulation of energy balance. ***Gastroenterology,*** **124, 1325-36.**

ASHBY, D. & BLOOM, S. R. 2007. Recent progress in PYY research--an update report for 8th NPY meeting. ***Peptides,*** **28, 198-202.**

ASTRUP, A., CARRARO, R., FINER, N., HARPER, A., KUNESOVA, M., LEAN, M. E., NISKANEN, L., RASMUSSEN, M. F., RISSANEN, A., ROSSNER, S., SAVOLAINEN, M. J., VAN GAAL, L. & INVESTIGATORS, N. N. 2012. Safety, tolerability and sustained weight loss over 2 years with the once-daily human GLP-1 analog, liraglutide. ***Int J Obes (Lond),*** **36, 843-54.**

ASTRUP, A., GREENWAY, F. L., LING, W., PEDICONE, L., LACHOWICZ, J., STRADER, C. D., KWAN, R. & ECOPIPAM OBESITY STUDY, G. 2007. Randomized

controlled trials of the D1/D5 antagonist ecopipam for weight loss in obese subjects. *Obesity (Silver Spring),* 15, 1717-31.

ATALAYER, D. & ROWLAND, N. E. 2011. Structure of motivation using food demand in mice. *Physiol Behav,* 104, 15-9.

AZUMA, T., SUTO, H., ITO, Y., OHTANI, M., DOJO, M., KURIYAMA, M. & KATO, T. 2001. Gastric leptin and Helicobacter pylori infection. *Gut,* 49, 324-9.

BABU, G. N. & VIJAYAN, E. 1983. Plasma gonadotropin, prolactin levels and hypothalamic tyrosine hydroxylase activity following intraventricular bombesin and secretin in ovariectomized conscious rats. *Brain Res Bull,* 11, 25-9.

BACKHED, F., DING, H., WANG, T., HOOPER, L. V., KOH, G. Y., NAGY, A., SEMENKOVICH, C. F. & GORDON, J. I. 2004. The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci U S A,* 101, 15718-23.

BACKHED, F., MANCHESTER, J. K., SEMENKOVICH, C. F. & GORDON, J. I. 2007. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. *Proc Natl Acad Sci U S A,* 104, 979-84.

BAGGIO, L. L. & DRUCKER, D. J. 2007. Biology of incretins: GLP-1 and GIP. *Gastroenterology,* 132, 2131-57.

BARRACHINA, M. D., MARTINEZ, V., WANG, L., WEI, J. Y. & TACHE, Y. 1997a. Synergistic interaction between leptin and cholecystokinin to reduce short-term food intake in lean mice. *Proc Natl Acad Sci U S A,* 94, 10455-60.

BARRACHINA, M. D., MARTINEZ, V., WANG, L. X., WEI, J. Y. & TACHE, Y. 1997b. Synergistic interaction between leptin and cholecystokinin to reduce short-term food intake in lean mice. *Proceedings of the National Academy of Sciences of the United States of America,* 94, 10455-10460.

BASSIL, A. K., DASS, N. B. & SANGER, G. J. 2006. The prokinetic-like activity of ghrelin in rat isolated stomach is mediated via cholinergic and tachykininergic motor neurones. *European Journal of Pharmacology,* 544, 146-152.

BATAILLE, D., GESPACH, C., COUDRAY, A. M. & ROSSELIN, G. 1981. ENTEROGLUCAGON - A SPECIFIC EFFECT ON GASTRIC GLANDS ISOLATED

FROM THE RAT FUNDUS - EVIDENCE FOR AN OXYNTOMODULIN ACTION. ***Bioscience Reports,*** **1, 151-155.**

BATTERHAM, R. L., COHEN, M. A., ELLIS, S. M., LE ROUX, C. W., WITHERS, D. J., FROST, G. S., GHATEI, M. A. & BLOOM, S. R. 2003. Inhibition of food intake in obese subjects by peptide YY3-36. ***N Engl J Med,*** **349, 941-8.**

BATTERHAM, R. L., COWLEY, M. A., SMALL, C. J., HERZOG, H., COHEN, M. A., DAKIN, C. L., WREN, A. M., BRYNES, A. E., LOW, M. J., GHATEI, M. A., CONE, R. D. & BLOOM, S. R. 2002. Gut hormone PYY(3-36) physiologically inhibits food intake. ***Nature,*** **418, 650-4.**

BAWAB, W., GESPACH, C., MARIE, J. C., CHASTRE, E. & ROSSELIN, G. 1988. Pharmacology and molecular identification of secretin receptors in rat gastric glands. ***Life Sci,*** **42, 791-8.**

BAYLISS, W. M. & STARLING, E. H. 1902. The mechanism of pancreatic secretion. ***Journal of Physiology-London,*** **28, 325-353.**

BERCIK, P., VERDU, E. F., FOSTER, J. A., LU, J., SCHARRINGA, A., KEAN, I., WANG, L., BLENNERHASSETT, P. & COLLINS, S. M. 2009. Role of gut-brain axis in persistent abnormal feeding behavior in mice following eradication of Helicobacter pylori infection. ***Am J Physiol Regul Integr Comp Physiol,*** **296, R587-94.**

BERGLUND, M. M., HIPSKIND, P. A. & GEHLERT, D. R. 2003. Recent developments in our understanding of the physiological role of PP-fold peptide receptor subtypes. ***Exp Biol Med (Maywood),*** **228, 217-44.**

BESCHORNER, R., SCHLUESENER, H. J., NGUYEN, T. D., MAGDOLEN, V., LUTHER, T., PEDAL, I., MATTERN, R., MEYERMANN, R. & SCHWAB, J. M. 2000. Lesion-associated accumulation of uPAR/CD87- expressing infiltrating granulocytes, activated microglial cells/macrophages and upregulation by endothelial cells following TBI and FCI in humans. ***Neuropathol Appl Neurobiol,*** **26, 522-7.**

BI, S., SCOTT, K. A., KOPIN, A. S. & MORAN, T. H. 2004. Differential roles for cholecystokinin a receptors in energy balance in rats and mice. ***Endocrinology,*** **145, 3873-80.**

BINDER, B. R., CHRIST, G., GRUBER, F., GRUBIC, N., HUFNAGL, P., KREBS, M., MIHALY, J. & PRAGER, G. W. 2002. Plasminogen activator inhibitor 1: physiological and pathophysiological roles. ***News Physiol Sci,*** **17, 56-61.**

BISHOP, L., DIMALINE, R., BLACKMORE, C., DEAVALL, D., DOCKRAY, G. J. & VARRO, A. 1998. Modulation of the cleavage of the gastrin precursor by prohormone phosphorylation. ***Gastroenterology,*** **115, 1154-62.**

BJERKNES, M. & CHENG, H. 2001. Modulation of specific intestinal epithelial progenitors by enteric neurons. ***Proc Natl Acad Sci U S A,*** **98, 12497-502.**

BLACKMORE, C. G., VARRO, A., DIMALINE, R., BISHOP, L., GALLACHER, D. V. & DOCKRAY, G. J. 2001. Measurement of secretory vesicle pH reveals intravesicular alkalinization by vesicular monoamine transporter type 2 resulting in inhibition of prohormone cleavage. ***J Physiol,*** **531, 605-17.**

BLONDEAU, B., AVRIL, I., DUCHENE, B. & BREANT, B. 2002. Endocrine pancreas development is altered in foetuses from rats previously showing intra-uterine growth retardation in response to malnutrition. ***Diabetologia,*** **45, 394-401.**

BLUMBERG, S., SCHROEDER, M., MALKESMAN, O., TORREGROSSA, A. M., SMITH, G. P. & WELLER, A. 2007. Gastric preloads of corn oil and mineral oil produce different patterns of increases of c-Fos-like immunoreacitve cells in the brain of 9-12 day-old rats. ***Brain Research,*** **1134, 140-147.**

BORNSTEIN, S. R., PREAS, H. L., CHROUSOS, G. P. & SUFFREDINI, A. F. 1998. Circulating leptin levels during acute experimental endotoxemia and antiinflammatory therapy in humans. ***J Infect Dis,*** **178, 887-90.**

BOSWELL, T. & LI, Q. 1998. Cholecystokinin induces Fos expression in the brain of the Japanese quail. ***Hormones and Behavior,*** **34, 56-66.**

BRIGGS, D. I., ENRIORI, P. J., LEMUS, M. B., COWLEY, M. A. & ANDREWS, Z. B. 2010. Diet-induced obesity causes ghrelin resistance in arcuate NPY/AgRP neurons. ***Endocrinology,*** **151, 4745-55.**

BROLIN, R. E. 2002. Bariatric surgery and long-term control of morbid obesity. ***JAMA,*** **288, 2793-6.**

BROLIN, R. E., LAMARCA, L. B., KENLER, H. A. & CODY, R. P. 2002. Malabsorptive gastric bypass in patients with superobesity. ***J Gastrointest Surg,*** **6, 195-203; discussion 204-5.**

BROMER, W. W., SINN, L. G., STAUB, A. & BEHRENS, O. K. 1957. The amino acid sequence of glucagon. ***Diabetes,*** **6, 234-8.**

BROTHERS, S. P. & WAHLESTEDT, C. 2010. Therapeutic potential of neuropeptide Y (NPY) receptor ligands. ***EMBO Mol Med,*** **2, 429-39.**

BROWN, J. C. 1971. A gastric inhibitory polypeptide. I. The amino acid composition and the tryptic peptides. ***Can J Biochem,*** **49, 255-61.**

BROWNING, K. N. & TRAVAGLI, R. A. 2009. Modulation of inhibitory neurotransmission in brainstem vagal circuits by NPY and PYY is controlled by cAMP levels. ***Neurogastroenterol Motil,*** **21, 1309-e126.**

BUNCK, M. C., DIAMANT, M., CORNER, A., ELIASSON, B., MALLOY, J. L., SHAGINIAN, R. M., DENG, W., KENDALL, D. M., TASKINEN, M. R., SMITH, U., YKI-JARVINEN, H. & HEINE, R. J. 2009. One-year treatment with exenatide improves beta-cell function, compared with insulin glargine, in metformin-treated type 2 diabetic patients: a randomized, controlled trial. ***Diabetes Care,*** **32, 762-8.**

BURDYGA, G., DE LARTIGUE, G., RAYBOULD, H. E., MORRIS, R., DIMALINE, R., VARRO, A., THOMPSON, D. G. & DOCKRAY, G. J. 2008. Cholecystokinin regulates expression of Y2 receptors in vagal afferent neurons serving the stomach. ***J Neurosci,*** **28, 11583-92.**

BURDYGA, G., LAL, S., SPILLER, D., JIANG, W., THOMPSON, D., ATTWOOD, S., SAEED, S., GRUNDY, D., VARRO, A., DIMALINE, R. & DOCKRAY, G. J. 2003. Localization of orexin-1 receptors to vagal afferent neurons in the rat and humans. ***Gastroenterology,*** **124, 129-39.**

BURDYGA, G., LAL, S., VARRO, A., DIMALINE, R., THOMPSON, D. G. & DOCKRAY, G. J. 2004. Expression of cannabinoid CB1 receptors by vagal afferent neurons is inhibited by cholecystokinin. ***J Neurosci,*** **24, 2708-15.**

BURDYGA, G., SPILLER, D., MORRIS, R., LAL, S., THOMPSON, D. G., SAEED, S., DIMALINE, R., VARRO, A. & DOCKRAY, G. J. 2002. Expression of the leptin receptor in rat and human nodose ganglion neurones. ***Neuroscience,*** **109, 339-47.**

BURDYGA, G., VARRO, A., DIMALINE, R., THOMPSON, D. G. & DOCKRAY, G. J. 2006. Ghrelin receptors in rat and human nodose ganglia: putative role in regulating CB-1 and MCH receptor abundance. ***Am J Physiol Gastrointest Liver Physiol,*** **290, G1289-97.**

BURMAN, P., MARDH, S., NORBERG, L. & KARLSSON, F. A. 1989. Parietal cell antibodies in pernicious anemia inhibit H+, K+-adenosine triphosphatase, the proton pump of the stomach. ***Gastroenterology,*** **96, 1434-8.**

BUSE, J. B., HENRY, R. R., HAN, J., KIM, D. D., FINEMAN, M. S., BARON, A. D. & EXENATIDE-113 CLINICAL STUDY, G. 2004. Effects of exenatide (exendin-4) on glycemic control over 30 weeks in sulfonylurea-treated patients with type 2 diabetes. ***Diabetes Care,*** **27, 2628-35.**

BUYSE, M., OVESJO, M. L., GOIOT, H., GUILMEAU, S., PERANZI, G., MOIZO, L., WALKER, F., LEWIN, M. J., MEISTER, B. & BADO, A. 2001. Expression and regulation of leptin receptor proteins in afferent and efferent neurons of the vagus nerve. ***Eur J Neurosci,*** **14, 64-72.**

CALEGARI, V. C., TORSONI, A. S., VANZELA, E. C., ARAUJO, E. P., MORARI, J., ZOPPI, C. C., SBRAGIA, L., BOSCHERO, A. C. & VELLOSO, L. A. 2011. Inflammation of the hypothalamus leads to defective pancreatic islet function. ***J Biol Chem,*** **286, 12870-80.**

CALLES-ESCANDON, J., BALLOR, D., HARVEY-BERINO, J., ADES, P., TRACY, R. & SOBEL, B. 1996. Amelioration of the inhibition of fibrinolysis in elderly, obese subjects by moderate energy intake restriction. ***Am J Clin Nutr,*** **64, 7-11.**

CAMMISOTTO, P. G., LEVY, E., BUKOWIECKI, L. J. & BENDAYAN, M. 2010. Cross-talk between adipose and gastric leptins for the control of food intake and energy metabolism. ***Prog Histochem Cytochem,*** **45, 143-200.**

CANI, P. D., AMAR, J., IGLESIAS, M. A., POGGI, M., KNAUF, C., BASTELICA, D., NEYRINCK, A. M., FAVA, F., TUOHY, K. M., CHABO, C., WAGET, A., DELMEE, E., COUSIN, B., SULPICE, T., CHAMONTIN, B., FERRIERES, J., TANTI, J. F., GIBSON, G. R., CASTEILLA, L., DELZENNE, N. M., ALESSI, M. C. & BURCELIN, R. 2007. Metabolic endotoxemia initiates obesity and insulin resistance. ***Diabetes,*** **56, 1761-72.**

CANI, P. D., LECOURT, E., DEWULF, E. M., SOHET, F. M., PACHIKIAN, B. D., NASLAIN, D., DE BACKER, F., NEYRINCK, A. M. & DELZENNE, N. M. 2009. Gut microbiota fermentation of prebiotics increases satietogenic and incretin gut peptide

production with consequences for appetite sensation and glucose response after a meal. *Am J Clin Nutr,* 90, 1236-43.

CANI, P. D., MONTOYA, M. L., NEYRINCK, A. M., DELZENNE, N. M. & LAMBERT, D. M. 2004. Potential modulation of plasma ghrelin and glucagon-like peptide-1 by anorexigenic cannabinoid compounds, SR141716A (rimonabant) and oleoylethanolamide. *Br J Nutr,* 92, 757-61.

CARMELIET, P., KIECKENS, L., SCHOONJANS, L., REAM, B., VAN NUFFELEN, A., PRENDERGAST, G., COLE, M., BRONSON, R., COLLEN, D. & MULLIGAN, R. C. 1993a. Plasminogen activator inhibitor-1 gene-deficient mice. I. Generation by homologous recombination and characterization. *J Clin Invest,* 92, 2746-55.

CARMELIET, P., STASSEN, J. M., SCHOONJANS, L., REAM, B., VAN DEN OORD, J. J., DE MOL, M., MULLIGAN, R. C. & COLLEN, D. 1993b. Plasminogen activator inhibitor-1 gene-deficient mice. II. Effects on hemostasis, thrombosis, and thrombolysis. *J Clin Invest,* 92, 2756-60.

CASTIGLIONE, K. E., READ, N. W. & FRENCH, S. J. 2002. Adaptation to high-fat diet accelerates emptying of fat but not carbohydrate test meals in humans. *Am J Physiol Regul Integr Comp Physiol,* 282, R366-71.

CATALAND, S., CROCKETT, S. E., BROWN, J. C. & MAZZAFERRI, E. L. 1974. Gastric inhibitory polypeptide (GIP) stimulation by oral glucose in man. *J Clin Endocrinol Metab,* 39, 223-8.

CHAUDHRI, O. B., PARKINSON, J. R., KUO, Y. T., DRUCE, M. R., HERLIHY, A. H., BELL, J. D., DHILLO, W. S., STANLEY, S. A., GHATEI, M. A. & BLOOM, S. R. 2006. Differential hypothalamic neuronal activation following peripheral injection of GLP-1 and oxyntomodulin in mice detected by manganese-enhanced magnetic resonance imaging. *Biochem Biophys Res Commun,* 350, 298-306.

CHEN, D. Y., DEUTSCH, J. A., GONZALEZ, M. F. & GU, Y. 1993. The induction and suppression of c-fos expression in the rat brain by cholecystokinin and its antagonist L364,718. *Neurosci Lett,* 149, 91-4.

CHENG, C. Y., CHU, J. Y. & CHOW, B. K. 2011. Central and peripheral administration of secretin inhibits food intake in mice through the activation of the melanocortin system. *Neuropsychopharmacology,* 36, 459-71.

CHO, Y. M. & KIEFFER, T. J. 2010. K-cells and glucose-dependent insulinotropic polypeptide in health and disease. ***Vitam Horm,*** **84, 111-50.**

COLEMAN, D. L. 1982. Diabetes-obesity syndromes in mice. ***Diabetes,*** **31, 1-6.**

COLLEN, D. & LIJNEN, H. R. 2004. Tissue-type plasminogen activator: a historical perspective and personal account. ***J Thromb Haemost,*** **2, 541-6.**

CONE, R. D. 1999. The Central Melanocortin System and Energy Homeostasis. ***Trends Endocrinol Metab,*** **10, 211-216.**

CONE, R. D., COWLEY, M. A., BUTLER, A. A., FAN, W., MARKS, D. L. & LOW, M. J. 2001. The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis. ***Int J Obes Relat Metab Disord,*** **25 Suppl 5, S63-7.**

COTA, D. 2007. CB1 receptors: emerging evidence for central and peripheral mechanisms that regulate energy balance, metabolism, and cardiovascular health. ***Diabetes Metab Res Rev,*** **23, 507-17.**

COTA, D. 2008. The role of the endocannabinoid system in the regulation of hypothalamic-pituitary-adrenal axis activity. ***J Neuroendocrinol,*** **20 Suppl 1, 35-8.**

COTA, D., TSCHOP, M. H., HORVATH, T. L. & LEVINE, A. S. 2006. Cannabinoids, opioids and eating behavior: the molecular face of hedonism? ***Brain Res Rev,*** **51, 85-107.**

COVASA, M. 2010. Deficits in gastrointestinal responses controlling food intake and body weight. ***Am J Physiol Regul Integr Comp Physiol,*** **299, R1423-39.**

COVASA, M. & RITTER, R. C. 2000. Adaptation to high-fat diet reduces inhibition of gastric emptying by CCK and intestinal oleate. ***Am J Physiol Regul Integr Comp Physiol,*** **278, R166-70.**

COWLEY, M. A., SMART, J. L., RUBINSTEIN, M., CERDAN, M. G., DIANO, S., HORVATH, T. L., CONE, R. D. & LOW, M. J. 2001. Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. ***Nature,*** **411, 480-4.**

CUMMINGS, D. E., FOSTER-SCHUBERT, K. E. & OVERDUIN, J. 2005. Ghrelin and energy balance: focus on current controversies. ***Curr Drug Targets,*** **6, 153-69.**

CUMMINGS, D. E. & OVERDUIN, J. 2007. Gastrointestinal regulation of food intake. ***J Clin Invest,*** **117, 13-23.**

CUMMINGS, D. E., OVERDUIN, J. & FOSTER-SCHUBERT, K. E. 2004. Gastric bypass for obesity: mechanisms of weight loss and diabetes resolution. ***J Clin Endocrinol Metab,*** **89, 2608-15.**

CUMMINGS, D. E., WEIGLE, D. S., FRAYO, R. S., BREEN, P. A., MA, M. K., DELLINGER, E. P. & PURNELL, J. Q. 2002. Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery. ***N Engl J Med,*** **346, 1623-30.**

CUNNINGHAM, K. M., HOROWITZ, M. & READ, N. W. 1991. The effect of short-term dietary supplementation with glucose on gastric emptying in humans. ***Br J Nutr,*** **65, 15-9.**

DAKIN, C. L., GUNN, I., SMALL, C. J., EDWARDS, C. M., HAY, D. L., SMITH, D. M., GHATEI, M. A. & BLOOM, S. R. 2001. Oxyntomodulin inhibits food intake in the rat. ***Endocrinology,*** **142, 4244-50.**

DAKIN, C. L., SMALL, C. J., BATTERHAM, R. L., NEARY, N. M., COHEN, M. A., PATTERSON, M., GHATEI, M. A. & BLOOM, S. R. 2004. Peripheral oxyntomodulin reduces food intake and body weight gain in rats. ***Endocrinology,*** **145, 2687-95.**

DALY, D. M., PARK, S. J., VALINSKY, W. C. & BEYAK, M. J. 2011. Impaired intestinal afferent nerve satiety signalling and vagal afferent excitability in diet induced obesity in the mouse. ***J Physiol,*** **589, 2857-70.**

DAMHOLT, A. B., BUCHAN, A. M., HOLST, J. J. & KOFOD, H. 1999. Proglucagon processing profile in canine L cells expressing endogenous prohormone convertase 1/3 and prohormone convertase 2. ***Endocrinology,*** **140, 4800-8.**

DATE, Y., MURAKAMI, N., TOSHINAI, K., MATSUKURA, S., NIIJIMA, A., MATSUO, H., KANGAWA, K. & NAKAZATO, M. 2002a. The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. ***Gastroenterology,*** **123, 1120-8.**

DATE, Y., NAKAZATO, M., HASHIGUCHI, S., DEZAKI, K., MONDAL, M. S., HOSODA, H., KOJIMA, M., KANGAWA, K., ARIMA, T., MATSUO, H., YADA, T. & MATSUKURA, S. 2002b. Ghrelin is present in pancreatic alpha-cells of humans and rats and stimulates insulin secretion. ***Diabetes,*** **51, 124-9.**

DATE, Y., NAKAZATO, M., MURAKAMI, N., KOJIMA, M., KANGAWA, K. & MATSUKURA, S. 2001. Ghrelin acts in the central nervous system to stimulate gastric acid secretion. ***Biochem Biophys Res Commun,*** **280, 904-7.**

DATE, Y., TOSHINAI, K., KODA, S., MIYAZATO, M., SHIMBARA, T., TSURUTA, T., NIIJIMA, A., KANGAWA, K. & NAKAZATO, M. 2005. Peripheral interaction of ghrelin with cholecystokinin on feeding regulation. ***Endocrinology,*** **146, 3518-3525.**

DAY, J. W., OTTAWAY, N., PATTERSON, J. T., GELFANOV, V., SMILEY, D., GIDDA, J., FINDEISEN, H., BRUEMMER, D., DRUCKER, D. J., CHAUDHARY, N., HOLLAND, J., HEMBREE, J., ABPLANALP, W., GRANT, E., RUEHL, J., WILSON, H., KIRCHNER, H., LOCKIE, S. H., HOFMANN, S., WOODS, S. C., NOGUEIRAS, R., PFLUGER, P. T., PEREZ-TILVE, D., DIMARCHI, R. & TSCHOP, M. H. 2009. A new glucagon and GLP-1 co-agonist eliminates obesity in rodents. ***Nat Chem Biol,*** **5, 749-57.**

DE FONSECA, F. R., NAVARRO, M., ALVAREZ, E., RONCERO, I., CHOWEN, J. A., MAESTRE, O., GOMEZ, R., MUNOZ, R. M., ENG, J. & BLAZQUEZ, E. 2000. Peripheral versus central effects of glucagon-like peptide-1 receptor agonists on satiety and body weight loss in Zucker obese rats. ***Metabolism-Clinical and Experimental,*** **49, 709-717.**

DE FONSECA, F. R., NAVARRO, M., GOMEZ, R., ESCUREDO, L., NAVA, F., FU, J., MURILLO-RODRIGUEZ, E., GIUFFRIDA, A., LOVERME, J., GAETANI, S., KATHURIA, S., GALL, C. & PIOMELLI, D. 2001. An anorexic lipid mediator regulated by feeding. ***Nature,*** **414, 209-212.**

DE LARTIGUE, G., BARBIER DE LA SERRE, C., ESPERO, E., LEE, J. & RAYBOULD, H. E. 2011a. Diet-induced obesity leads to the development of leptin resistance in vagal afferent neurons. ***Am J Physiol Endocrinol Metab,*** **301, E187-95.**

DE LARTIGUE, G., BARBIER DE LA SERRE, C., ESPERO, E., LEE, J. & RAYBOULD, H. E. 2012. Leptin resistance in vagal afferent neurons inhibits cholecystokinin signaling and satiation in diet induced obese rats. ***PLoS One,*** **7, e32967.**

DE LARTIGUE, G., DE LA SERRE, C. B. & RAYBOULD, H. E. 2011b. Vagal afferent neurons in high fat diet-induced obesity; intestinal microflora, gut inflammation and cholecystokinin. ***Physiol Behav,*** **105, 100-5.**

DE LARTIGUE, G., DIMALINE, R., VARRO, A. & DOCKRAY, G. J. 2007a. Cocaine- and amphetamine-regulated transcript: Stimulation of expression in rat vagal afferent

neurons by cholecystokinin and suppression by ghrelin. *Journal of Neuroscience,* 27, 2876-2882.

DE LARTIGUE, G., DIMALINE, R., VARRO, A. & DOCKRAY, G. J. 2007b. Cocaine- and amphetamine-regulated transcript: stimulation of expression in rat vagal afferent neurons by cholecystokinin and suppression by ghrelin. *J Neurosci,* 27, 2876-82.

DE LARTIGUE, G., LUR, G., DIMALINE, R., VARRO, A., RAYBOULD, H. & DOCKRAY, G. J. 2010. EGR1 Is a target for cooperative interactions between cholecystokinin and leptin, and inhibition by ghrelin, in vagal afferent neurons. *Endocrinology,* 151, 3589-99.

DE LEON, M., NAHIN, R. L., MOLINA, C. A., DE LEON, D. D. & RUDA, M. A. 1995. Comparison of c-jun, junB, and junD mRNA expression and protein in the rat dorsal root ganglia following sciatic nerve transection. *J Neurosci Res,* 42, 391-401.

DE ZWART, I. M. & DE ROOS, A. 2010. MRI for the evaluation of gastric physiology. *Eur Radiol,* 20, 2609-16.

DEACON, C. F. 2004. Circulation and degradation of GIP and GLP-1. *Horm Metab Res,* 36, 761-5.

DEBAS, H. T., FAROOQ, O. & GROSSMAN, M. I. 1975. Inhibition of gastric emptying is a physiological action of cholecystokinin. *Gastroenterology,* 68, 1211-7.

DEFRONZO, R. A., RATNER, R. E., HAN, J., KIM, D. D., FINEMAN, M. S. & BARON, A. D. 2005. Effects of exenatide (exendin-4) on glycemic control and weight over 30 weeks in metformin-treated patients with type 2 diabetes. *Diabetes Care,* 28, 1092-100.

DELZENNE, N. M. & CANI, P. D. 2011. Interaction between obesity and the gut microbiota: relevance in nutrition. *Annu Rev Nutr,* 31, 15-31.

DELZENNE, N. M., NEYRINCK, A. M., BACKHED, F. & CANI, P. D. 2011. Targeting gut microbiota in obesity: effects of prebiotics and probiotics. *Nat Rev Endocrinol,* 7, 639-46.

DEVANE, W. A., HANUS, L., BREUER, A., PERTWEE, R. G., STEVENSON, L. A., GRIFFIN, G., GIBSON, D., MANDELBAUM, A., ETINGER, A. & MECHOULAM, R. 1992. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science,* 258, 1946-9.

DHANVANTARI, S., SEIDAH, N. G. & BRUBAKER, P. L. 1996. Role of prohormone convertases in the tissue-specific processing of proglucagon. ***Mol Endocrinol,*** **10, 342-55.**

DI MARZO, V., GOPARAJU, S. K., WANG, L., LIU, J., BATKAI, S., JARAI, Z., FEZZA, F., MIURA, G. I., PALMITER, R. D., SUGIURA, T. & KUNOS, G. 2001. Leptin-regulated endocannabinoids are involved in maintaining food intake. ***Nature,*** **410, 822-5.**

DI MARZO, V. & MATIAS, I. 2005. Endocannabinoid control of food intake and energy balance. ***Nat Neurosci,*** **8, 585-9.**

DOCKRAY, G., DIMALINE, R. & VARRO, A. 2005. Gastrin: old hormone, new functions. ***Pflugers Arch,*** **449, 344-55.**

DOCKRAY, G. J. 2004. Clinical endocrinology and metabolism. Gastrin. ***Best Pract Res Clin Endocrinol Metab,*** **18, 555-68.**

DOCKRAY, G. J. 2009a. Cholecystokinin and gut-brain signalling. ***Regul Pept,*** **155, 6-10.**

DOCKRAY, G. J. 2009b. The versatility of the vagus. ***Physiol Behav,*** **97, 531-6.**

DOCKRAY, G. J. 2013. Enteroendocrine cell signalling via the vagus nerve. ***Curr Opin Pharmacol.***

DOCKRAY, G. J., MOORE, A., VARRO, A. & PRITCHARD, D. M. 2012. Gastrin receptor pharmacology. ***Curr Gastroenterol Rep,*** **14, 453-9.**

DOCKRAY, G. J., WALSH, J. H. & PASSARO, E. 1975. RELATIVE ABUNDANCE OF BIG AND LITTLE GASTRINS IN TUMORS AND BLOOD OF PATIENTS WITH ZOLLINGER-ELLISON SYNDROME. ***Gut,*** **16, 353-358.**

DONATH, M. Y., BONI-SCHNETZLER, M., ELLINGSGAARD, H., HALBAN, P. A. & EHSES, J. A. 2010. Cytokine production by islets in health and diabetes: cellular origin, regulation and function. ***Trends Endocrinol Metab,*** **21, 261-7.**

DONOVAN, M. J., PAULINO, G. & RAYBOULD, H. E. 2007. CCK(1) receptor is essential for normal meal patterning in mice fed high fat diet. ***Physiol Behav,*** **92, 969-74.**

DRAVIAM, E. J., GOMEZ, G., HASHIMOTO, T., MIYASHITA, T., HILL, F. L., UCHIDA, T., SINGH, P., GREELEY, G. H., JR. & THOMPSON, J. C. 1991.

Characterization of secretin release in response to food and intraduodenal administration of fat and hydrochloric acid. *Dig Dis Sci,* 36, 513-9.

DRUCKER, D. J. 2007. The role of gut hormones in glucose homeostasis. *J Clin Invest,* 117, 24-32.

DRUCKER, D. J. & NAUCK, M. A. 2006. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet,* 368, 1696-705.

DUBOIS, A., EERDEWEGH, P. V. & GARDNER, J. D. 1977. Gastric emptying and secretion in Zollinger-Ellison syndrome. *J Clin Invest,* 59, 255-63.

DUPRE, J., ROSS, S. A., WATSON, D. & BROWN, J. C. 1973. Stimulation of insulin secretion by gastric inhibitory polypeptide in man. *J Clin Endocrinol Metab,* 37, 826-8.

EBERLEIN, G. A., EYSSELEIN, V. E. & GOEBELL, H. 1988. Cholecystokinin-58 is the major molecular form in man, dog and cat but not in pig, beef and rat intestine. *Peptides,* 9, 993-8.

EDHOLM, T., LEVIN, F., HELLSTROM, P. M. & SCHMIDT, P. T. 2004. Ghrelin stimulates motility in the small intestine of rats through intrinsic cholinergic neurons. *Regulatory Peptides,* 121, 25-30.

EDKINS, J. S. 1906. The chemical mechanism of gastric secretion. *J, Physiol, (Lond.),* 34, 133-144.

EHSES, J. A., BONI-SCHNETZLER, M., FAULENBACH, M. & DONATH, M. Y. 2008. Macrophages, cytokines and beta-cell death in Type 2 diabetes. *Biochem Soc Trans,* 36, 340-2.

EHSES, J. A., PERREN, A., EPPLER, E., RIBAUX, P., POSPISILIK, J. A., MAOR-CAHN, R., GUERIPEL, X., ELLINGSGAARD, H., SCHNEIDER, M. K., BIOLLAZ, G., FONTANA, A., REINECKE, M., HOMO-DELARCHE, F. & DONATH, M. Y. 2007. Increased number of islet-associated macrophages in type 2 diabetes. *Diabetes,* 56, 2356-70.

EITZMAN, D. T., KRAUSS, J. C., SHEN, T., CUI, J. & GINSBURG 1996a. Lack of plasminogen activator inhibitor-1 effect in a transgenic mouse model of metastatic melanoma. *Blood,* 87, 4718-22.

EITZMAN, D. T., MCCOY, R. D., ZHENG, X., FAY, W. P., SHEN, T., GINSBURG, D. & SIMON, R. H. 1996b. Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack or overexpress the murine plasminogen activator inhibitor-1 gene. ***J Clin Invest,*** **97, 232-7.**

ELLIOTT, R. M., MORGAN, L. M., TREDGER, J. A., DEACON, S., WRIGHT, J. & MARKS, V. 1993. Glucagon-like peptide-1 (7-36)amide and glucose-dependent insulinotropic polypeptide secretion in response to nutrient ingestion in man: acute post-prandial and 24-h secretion patterns. ***J Endocrinol,*** **138, 159-66.**

EMILSSON, V., THORLEIFSSON, G., ZHANG, B., LEONARDSON, A. S., ZINK, F., ZHU, J., CARLSON, S., HELGASON, A., WALTERS, G. B., GUNNARSDOTTIR, S., MOUY, M., STEINTHORSDOTTIR, V., EIRIKSDOTTIR, G. H., BJORNSDOTTIR, G., REYNISDOTTIR, I., GUDBJARTSSON, D., HELGADOTTIR, A., JONASDOTTIR, A., JONASDOTTIR, A., STYRKARSDOTTIR, U., GRETARSDOTTIR, S., MAGNUSSON, K. P., STEFANSSON, H., FOSSDAL, R., KRISTJANSSON, K., GISLASON, H. G., STEFANSSON, T., LEIFSSON, B. G., THORSTEINSDOTTIR, U., LAMB, J. R., GULCHER, J. R., REITMAN, M. L., KONG, A., SCHADT, E. E. & STEFANSSON, K. 2008. Genetics of gene expression and its effect on disease. ***Nature,*** **452, 423-8.**

ENG, J. 1992. Exendin peptides. ***Mt Sinai J Med,*** **59, 147-9.**

ENG, J., KLEINMAN, W. A., SINGH, L., SINGH, G. & RAUFMAN, J. P. 1992. ISOLATION AND CHARACTERIZATION OF EXENDIN-4, AN EXENDIN-3 ANALOG, FROM HELODERMA-SUSPECTUM VENOM - FURTHER EVIDENCE FOR AN EXENDIN RECEPTOR ON DISPERSED ACINI FROM GUINEA-PIG PANCREAS. ***Journal of Biological Chemistry,*** **267, 7402-7405.**

ERICKSON, L. A., FICI, G. J., LUND, J. E., BOYLE, T. P., POLITES, H. G. & MAROTTI, K. R. 1990. Development of venous occlusions in mice transgenic for the plasminogen activator inhibitor-1 gene. ***Nature,*** **346, 74-76.**

FABBRINI, E., MAGKOS, F., MOHAMMED, B. S., PIETKA, T., ABUMRAD, N. A., PATTERSON, B. W., OKUNADE, A. & KLEIN, S. 2009. Intrahepatic fat, not visceral fat, is linked with metabolic complications of obesity. ***Proc Natl Acad Sci U S A,*** **106, 15430-5.**

FAGGIONI, R., FANTUZZI, G., FULLER, J., DINARELLO, C. A., FEINGOLD, K. R. & GRUNFELD, C. 1998. IL-1 beta mediates leptin induction during inflammation. ***Am J Physiol,*** **274, R204-8.**

FAHRENKRUG, J. 1993. Transmitter role of vasoactive intestinal peptide. *Pharmacol Toxicol,* 72, 354-63.

FAVRETTI, F., ASHTON, D., BUSETTO, L., SEGATO, G. & DE LUCA, M. 2009. The gastric band: first-choice procedure for obesity surgery. *World J Surg,* 33, 2039-48.

FAY, W. P., PARKER, A. C., CONDREY, L. R. & SHAPIRO, A. D. 1997. Human plasminogen activator inhibitor-1 (PAI-1) deficiency: characterization of a large kindred with a null mutation in the PAI-1 gene. *Blood,* 90, 204-8.

FLORIDON, C., NIELSEN, O., HOLUND, B., SUNDE, L., WESTERGAARD, J. G., THOMSEN, S. G. & TEISNER, B. 1999. Localization and significance of urokinase plasminogen activator and its receptor in placental tissue from intrauterine, ectopic and molar pregnancies. *Placenta,* 20, 711-21.

FOEKENS, J. A., PETERS, H. A., LOOK, M. P., PORTENGEN, H., SCHMITT, M., KRAMER, M. D., BRUNNER, N., JANICKE, F., MEIJER-VAN GELDER, M. E., HENZEN-LOGMANS, S. C., VAN PUTTEN, W. L. & KLIJN, J. G. 2000. The urokinase system of plasminogen activation and prognosis in 2780 breast cancer patients. *Cancer Res,* 60, 636-43.

FORSTER, E. R. & DOCKRAY, G. J. 1992. THE ROLE OF CHOLECYSTOKININ IN INHIBITION OF GASTRIC-EMPTYING BY PEPTONE IN THE RAT. *Experimental Physiology,* 77, 693-700.

FORSTER, E. R., GREEN, T., ELLIOT, M., BREMNER, A. & DOCKRAY, G. J. 1990. Gastric emptying in rats: role of afferent neurons and cholecystokinin. *Am J Physiol,* 258, G552-6.

FOX, J. G., ROGERS, A. B., IHRIG, M., TAYLOR, N. S., WHARY, M. T., DOCKRAY, G., VARRO, A. & WANG, T. C. 2003. Helicobacter pylori-associated gastric cancer in INS-GAS mice is gender specific. *Cancer Res,* 63, 942-50.

FRASER, K. A., RAIZADA, E. & DAVISON, J. S. 1995. Oral-pharyngeal-esophageal and gastric cues contribute to meal-induced c-fos expression. *Am J Physiol,* 268, R223-30.

FREMEAU, R. T., JR., JENSEN, R. T., CHARLTON, C. G., MILLER, R. L., O'DONOHUE, T. L. & MOODY, T. W. 1983. Secretin: specific binding to rat brain membranes. *J Neurosci,* 3, 1620-5.

FRIED, M., ERLACHER, U., SCHWIZER, W., LOCHNER, C., KOERFER, J., BEGLINGER, C., JANSEN, J. B., LAMERS, C. B., HARDER, F., BISCHOF-DELALOYE, A. & ET AL. 1991. Role of cholecystokinin in the regulation of gastric emptying and pancreatic enzyme secretion in humans. Studies with the cholecystokinin-receptor antagonist loxiglumide. ***Gastroenterology,*** **101, 503-11.**

FRISARD, M. I., MCMILLAN, R. P., MARCHAND, J., WAHLBERG, K. A., WU, Y., VOELKER, K. A., HEILBRONN, L., HAYNIE, K., MUOIO, B., LI, L. & HULVER, M. W. 2010. Toll-like receptor 4 modulates skeletal muscle substrate metabolism. ***Am J Physiol Endocrinol Metab,*** **298, E988-98.**

FU, J., GAETANI, S., OVEISI, F., LO VERME, J., SERRANO, A., RODRIGUEZ DE FONSECA, F., ROSENGARTH, A., LUECKE, H., DI GIACOMO, B., TARZIA, G. & PIOMELLI, D. 2003. Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha. ***Nature,*** **425, 90-3.**

FUJINO, K., INUI, A., ASAKAWA, A., KIHARA, N., FUJIMURA, M. & FUJIMIYA, M. 2003. Ghrelin induces fasted motor activity of the gastrointestinal tract in conscious fed rats. ***Journal of Physiology-London,*** **550, 227-240.**

FURET, J. P., KONG, L. C., TAP, J., POITOU, C., BASDEVANT, A., BOUILLOT, J. L., MARIAT, D., CORTHIER, G., DORE, J., HENEGAR, C., RIZKALLA, S. & CLEMENT, K. 2010. Differential adaptation of human gut microbiota to bariatric surgery-induced weight loss: links with metabolic and low-grade inflammation markers. ***Diabetes,*** **59, 3049-57.**

GAMBLE, J., KENNY, S. & DOCKRAY, G. J. 2013. Plasminogen activator inhibitor (PAI)-1 suppresses inhibition of gastric emptying by cholecystokinin (CCK) in mice. ***Regul Pept.***

GARDINER, J. V., CAMPBELL, D., PATTERSON, M., KENT, A., GHATEI, M. A., BLOOM, S. R. & BEWICK, G. A. 2010. The hyperphagic effect of ghrelin is inhibited in mice by a diet high in fat. ***Gastroenterology,*** **138, 2468-76, 2476 e1.**

GATTA-CHERIFI, B., MATIAS, I., VALLEE, M., TABARIN, A., MARSICANO, G., PIAZZA, P. V. & COTA, D. 2012. Simultaneous postprandial deregulation of the orexigenic endocannabinoid anandamide and the anorexigenic peptide YY in obesity. ***International Journal of Obesity,*** **36, 880-885.**

GEARY, N., LE SAUTER, J. & NOH, U. 1993. Glucagon acts in the liver to control spontaneous meal size in rats. ***Am J Physiol,*** **264, R116-22.**

GESPACH, C., BATAILLE, D., VAUCLIN, N., ROSSELIN, G., MORODER, L. & WUNSCH, E. 1981. Secretin binding sites coupled with adenylate cyclase in rat fundic membranes. ***Peptides,*** **2 Suppl 2, 247-51.**

GEURTS, L., LAZAREVIC, V., DERRIEN, M., EVERARD, A., VAN ROYE, M., KNAUF, C., VALET, P., GIRARD, M., MUCCIOLI, G. G., FRANCOIS, P., DE VOS, W. M., SCHRENZEL, J., DELZENNE, N. M. & CANI, P. D. 2011. Altered gut microbiota and endocannabinoid system tone in obese and diabetic leptin-resistant mice: impact on apelin regulation in adipose tissue. ***Front Microbiol,*** **2, 149.**

GHAMARI-LANGROUDI, M., COLMERS, W. F. & CONE, R. D. 2005. PYY3-36 inhibits the action potential firing activity of POMC neurons of arcuate nucleus through postsynaptic Y2 receptors. ***Cell Metab,*** **2, 191-9.**

GHOOS, Y. F., MAES, B. D., GEYPENS, B. J., MYS, G., HIELE, M. I., RUTGEERTS, P. J. & VANTRAPPEN, G. 1993. Measurement of gastric emptying rate of solids by means of a carbon-labeled octanoic acid breath test. ***Gastroenterology,*** **104, 1640-7.**

GIBBS, J., YOUNG, R. C. & SMITH, G. P. 1973. Cholecystokinin elicits satiety in rats with open gastric fistulas. ***Nature,*** **245, 323-5.**

GOKE, B., PRINTZ, H., KOOP, I., RAUSCH, U., RICHTER, G., ARNOLD, R. & ADLER, G. 1986. Endogenous CCK release and pancreatic growth in rats after feeding a proteinase inhibitor (camostate). ***Pancreas,*** **1, 509-15.**

GOLDSTONE, A. P., UNMEHOPA, U. A., BLOOM, S. R. & SWAAB, D. F. 2002. Hypothalamic NPY and agouti-related protein are increased in human illness but not in Prader-Willi syndrome and other obese subjects. ***J Clin Endocrinol Metab,*** **87, 927-37.**

GOMEZ, R., NAVARRO, M., FERRER, B., TRIGO, J. M., BILBAO, A., DEL ARCO, I., CIPPITELLI, A., NAVA, F., PIOMELLI, D. & RODRIGUEZ DE FONSECA, F. 2002. A peripheral mechanism for CB1 cannabinoid receptor-dependent modulation of feeding. ***J Neurosci,*** **22, 9612-7.**

GRANDT, D., SCHIMICZEK, M., STRUK, K., SHIVELY, J., EYSSELEIN, V. E., GOEBELL, H. & REEVE, J. R., JR. 1994. Characterization of two forms of peptide YY, PYY(1-36) and PYY(3-36), in the rabbit. ***Peptides,*** **15, 815-20.**

GREEN, T., DIMALINE, R., PEIKIN, S. & DOCKRAY, G. J. 1988. Action of the cholecystokinin antagonist L364,718 on gastric emptying in the rat. ***Am J Physiol,*** **255, G685-9.**

GREENBERG, G. R., MCCLOY, R. F., CHADWICK, V. S., ADRIAN, T. E., BARON, J. H. & BLOOM, S. R. 1979. Effect of bovine pancreatic polypeptide on basal pancreatic and biliary outputs in man. ***Dig Dis Sci,*** **24, 11-4.**

GREGORY, R. A. & TRACY, H. J. 1964. The constitution and properties of two gastrins extracted from hog antral mucosa. . ***Gut,*** **5, 103-14.**

GRILL, H. J. & HAYES, M. R. 2009. The nucleus tractus solitarius: a portal for visceral afferent signal processing, energy status assessment and integration of their combined effects on food intake. ***Int J Obes (Lond),*** **33 Suppl 1, S11-5.**

GRILL, H. J. & HAYES, M. R. 2012. Hindbrain neurons as an essential hub in the neuroanatomically distributed control of energy balance. ***Cell Metab,*** **16, 296-309.**

GRILL, H. J. & KAPLAN, J. M. 2002. The neuroanatomical axis for control of energy balance. ***Front Neuroendocrinol,*** **23, 2-40.**

GRILL, H. J. & SMITH, G. P. 1988. Cholecystokinin decreases sucrose intake in chronic decerebrate rats. ***Am J Physiol,*** **254, R853-6.**

GUO, Y., MA, L., ENRIORI, P. J., KOSKA, J., FRANKS, P. W., BROOKSHIRE, T., COWLEY, M. A., SALBE, A. D., DELPARIGI, A. & TATARANNI, P. A. 2006. Physiological evidence for the involvement of peptide YY in the regulation of energy homeostasis in humans. ***Obesity (Silver Spring),*** **14, 1562-70.**

GUTIERREZ, J. A., SOLENBERG, P. J., PERKINS, D. R., WILLENCY, J. A., KNIERMAN, M. D., JIN, Z., WITCHER, D. R., LUO, S., ONYIA, J. E. & HALE, J. E. 2008. Ghrelin octanoylation mediated by an orphan lipid transferase. ***Proc Natl Acad Sci U S A,*** **105, 6320-5.**

HAINER, V. & HAINEROVA, I. A. 2012. Do we need anti-obesity drugs? ***Diabetes Metab Res Rev,*** **28 Suppl 2, 8-20.**

HALAAS, J. L., GAJIWALA, K. S., MAFFEI, M., COHEN, S. L., CHAIT, B. T., RABINOWITZ, D., LALLONE, R. L., BURLEY, S. K. & FRIEDMAN, J. M. 1995.

Weight-reducing effects of the plasma protein encoded by the obese gene. *Science,* 269, 543-6.

HALES, C. N., BARKER, D. J., CLARK, P. M., COX, L. J., FALL, C., OSMOND, C. & WINTER, P. D. 1991. Fetal and infant growth and impaired glucose tolerance at age 64. *BMJ,* 303, 1019-22.

HANIF, M. W. & KUMAR, S. 2002. Pharmacological management of obesity. *Expert Opin Pharmacother,* 3, 1711-8.

HASHIMOTO, H., YAMAMOTO, K., HAGIGARA, N., OGAWA, N., NISHINO, A., AINO, H., NOGI, H., IMANISHI, K., MATSUDA, T. & BABA, A. 1996. cDNA cloning of a mouse pituitary adenylate cyclase-activating polypeptide receptor. *Biochim Biophys Acta,* 1281, 129-33.

HEADING, R. C., TOTHILL, P., MCLOUGHLIN, G. P. & SHEARMAN, D. J. 1976. Gastric emptying rate measurement in man. A double isotope scanning technique for simultaneous study of liquid and solid components of a meal. *Gastroenterology,* 71, 45-50.

HEALTH AND SOCIAL CARE INFORMATION CENTRE, L. S. 2013. Statistics on Obesity,

Physical Activity and Diet:

England, 2013. 1-120.

HEKMAN, C. M. & LOSKUTOFF, D. J. 1988. Kinetic analysis of the interactions between plasminogen activator inhibitor 1 and both urokinase and tissue plasminogen activator. *Arch Biochem Biophys,* 262, 199-210.

HELDSINGER, A., GRABAUSKAS, G., SONG, I. & OWYANG, C. 2011. Synergistic interaction between leptin and cholecystokinin in the rat nodose ganglia is mediated by PI3K and STAT3 signaling pathways: implications for leptin as a regulator of short term satiety. *J Biol Chem,* 286, 11707-15.

HELLEMANS, J., VANTRAPPEN, G. & BLOOM, S. R. 1976. Endogenous motilin and the LES pressure. *Scand J Gastroenterol Suppl,* 39, 67-73.

HERRMANN, C., GOKE, R., RICHTER, G., FEHMANN, H. C., ARNOLD, R. & GOKE, B. 1995. Glucagon-like peptide-1 and glucose-dependent insulin-releasing polypeptide plasma levels in response to nutrients. *Digestion,* 56, 117-26.

HEWSON, A. K. & DICKSON, S. L. 2000. Systemic administration of ghrelin induces Fos and Egr-1 proteins in the hypothalamic arcuate nucleus of fasted and fed rats. *J Neuroendocrinol,* 12, 1047-9.

HILDEBRANDT, M. A., HOFFMANN, C., SHERRILL-MIX, S. A., KEILBAUGH, S. A., HAMADY, M., CHEN, Y. Y., KNIGHT, R., AHIMA, R. S., BUSHMAN, F. & WU, G. D. 2009. High-fat diet determines the composition of the murine gut microbiome independently of obesity. *Gastroenterology,* 137, 1716-24 e1-2.

HOLST, J. J. 2007. The physiology of glucagon-like peptide 1. *Physiol Rev,* 87, 1409-39.

HOLZER, P., REICHMANN, F. & FARZI, A. 2012. Neuropeptide Y, peptide YY and pancreatic polypeptide in the gut-brain axis. *Neuropeptides,* 46, 261-74.

HOTAMISLIGIL, G. S. 2006. Inflammation and metabolic disorders. *Nature,* 444, 860-7.

HOTAMISLIGIL, G. S., ARNER, P., CARO, J. F., ATKINSON, R. L. & SPIEGELMAN, B. M. 1995. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest,* 95, 2409-15.

HOTTA, K., FUNAHASHI, T., ARITA, Y., TAKAHASHI, M., MATSUDA, M., OKAMOTO, Y., IWAHASHI, H., KURIYAMA, H., OUCHI, N., MAEDA, K., NISHIDA, M., KIHARA, S., SAKAI, N., NAKAJIMA, T., HASEGAWA, K., MURAGUCHI, M., OHMOTO, Y., NAKAMURA, T., YAMASHITA, S., HANAFUSA, T. & MATSUZAWA, Y. 2000. Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arterioscler Thromb Vasc Biol,* 20, 1595-9.

HUAI, Q., ZHOU, A., LIN, L., MAZAR, A. P., PARRY, G. C., CALLAHAN, J., SHAW, D. E., FURIE, B., FURIE, B. C. & HUANG, M. 2008. Crystal structures of two human vitronectin, urokinase and urokinase receptor complexes. *Nat Struct Mol Biol,* 15, 422-3.

HUANG, W., METLAKUNTA, A., DEDOUSIS, N., ZHANG, P., SIPULA, I., DUBE, J. J., SCOTT, D. K. & O'DOHERTY, R. M. 2010. Depletion of liver Kupffer cells prevents the development of diet-induced hepatic steatosis and insulin resistance. *Diabetes,* 59, 347-57.

IGGO, A. 1955. Tension receptors in the stomach and the urinary bladder. *J Physiol,* 128, 593-607.

IMERYUZ, N., YEGEN, B. C., BOZKURT, A., COSKUN, T., VILLANUEVA-PENACARRILLO, M. L. & ULUSOY, N. B. 1997. Glucagon-like peptide-1 inhibits gastric emptying via vagal afferent-mediated central mechanisms. *Am J Physiol,* 273, G920-7.

INGALLS, A. M., DICKIE, M. M. & SNELL, G. D. 1950. Obese, a new mutation in the house mouse. *J Hered,* 41, 317-8.

IRWIN, N. & FLATT, P. R. 2009. Evidence for beneficial effects of compromised gastric inhibitory polypeptide action in obesity-related diabetes and possible therapeutic implications. *Diabetologia,* 52, 1724-31.

ISHIHARA, T., SHIGEMOTO, R., MORI, K., TAKAHASHI, K. & NAGATA, S. 1992. Functional expression and tissue distribution of a novel receptor for vasoactive intestinal polypeptide. *Neuron,* 8, 811-9.

IVY, A. C. & OLDBERG, E. 1928. A HORMONE MECHANISM FOR GALL-BLADDER CONTRACTION AND EVACUATION. *American Journal of Physiology -- Legacy Content,* 86, 599-613.

IWAI, K., FUKUOKA, S., FUSHIKI, T., TSUJIKAWA, M., HIROSE, M., TSUNASAWA, S. & SAKIYAMA, F. 1987. Purification and sequencing of a trypsin-sensitive cholecystokinin-releasing peptide from rat pancreatic juice. Its homology with pancreatic secretory trypsin inhibitor. *J Biol Chem,* 262, 8956-9.

JAMSHIDI, N. & TAYLOR, D. A. 2001. Anandamide administration into the ventromedial hypothalamus stimulates appetite in rats. *Br J Pharmacol,* 134, 1151-4.

JOHNS, C. E., NEWTON, J. L., WESTLEY, B. R. & MAY, F. E. 2006. Human pancreatic polypeptide has a marked diurnal rhythm that is affected by ageing and is associated with the gastric TFF2 circadian rhythm. *Peptides,* 27, 1341-8.

JORPES, J. E., MUTT, V., MAGNUSSON, S. & STEELE, B. B. 1962. Amino acid composition and N-terminal amino acid sequence of porcine secretin. *Biochem Biophys Res Commun,* 9, 275-9.

JOY, S. V., RODGERS, P. T. & SCATES, A. C. 2005. Incretin mimetics as emerging treatments for type 2 diabetes. *Ann Pharmacother,* 39, 110-8.

JUHAN-VAGUE, I. & ALESSI, M. C. 1997. PAI-1, obesity, insulin resistance and risk of cardiovascular events. ***Thromb Haemost,*** **78, 656-60.**

JUNG, S. Y., SONG, H. S., PARK, S. Y., CHUNG, S. H. & KIM, Y. J. 2011. Pyruvate promotes tumor angiogenesis through HIF-1-dependent PAI-1 expression. ***Int J Oncol,*** **38, 571-6.**

KALLIOKOSKI, O., JACOBSEN, K. R., DARUSMAN, H. S., HENRIKSEN, T., WEIMANN, A., POULSEN, H. E., HAU, J. & ABELSON, K. S. 2013. Mice do not habituate to metabolism cage housing--a three week study of male BALB/c mice. ***PLoS One,*** **8, e58460.**

KALRA, S. P., DUBE, M. G., PU, S., XU, B., HORVATH, T. L. & KALRA, P. S. 1999. Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. ***Endocr Rev,*** **20, 68-100.**

KAMEGAI, J., TAMURA, H., SHIMIZU, T., ISHII, S., SUGIHARA, H. & WAKABAYASHI, I. 2001. Chronic central infusion of ghrelin increases hypothalamic neuropeptide Y and Agouti-related protein mRNA levels and body weight in rats. ***Diabetes,*** **50, 2438-43.**

KAMIJI, M. M., TRONCON, L. E. A., SUEN, V. M. M. & DE OLIVEIRA, R. B. 2009. Gastrointestinal transit, appetite, and energy balance in gastrectomized patients. ***American Journal of Clinical Nutrition,*** **89, 231-239.**

KANG, Y. M., MA, Y., ZHENG, J. P., ELKS, C., SRIRAMULA, S., YANG, Z. M. & FRANCIS, J. 2009. Brain nuclear factor-kappa B activation contributes to neurohumoral excitation in angiotensin II-induced hypertension. ***Cardiovasc Res,*** **82, 503-12.**

KEATES, A. C., TUMMALA, S., PEEK, R. M., JR., CSIZMADIA, E., KUNZLI, B., BECKER, K., CORREA, P., ROMERO-GALLO, J., PIAZUELO, M. B., SHETH, S., KELLY, C. P., ROBSON, S. C. & KEATES, S. 2008. Helicobacter pylori infection stimulates plasminogen activator inhibitor 1 production by gastric epithelial cells. ***Infect Immun,*** **76, 3992-9.**

KENDALL, D. M., RIDDLE, M. C., ROSENSTOCK, J., ZHUANG, D., KIM, D. D., FINEMAN, M. S. & BARON, A. D. 2005. Effects of exenatide (exendin-4) on glycemic control over 30 weeks in patients with type 2 diabetes treated with metformin and a sulfonylurea. ***Diabetes Care,*** **28, 1083-91.**

KENNEY, A. M. & KOCSIS, J. D. 1997. Timing of c-jun protein induction in lumbar dorsal root ganglia after sciatic nerve transection varies with lesion distance. *Brain Res,* 751, 90-5.

KENNY, S., DUVAL, C., SAMMUT, S. J., STEELE, I., PRITCHARD, D. M., ATHERTON, J. C., ARGENT, R. H., DIMALINE, R., DOCKRAY, G. J. & VARRO, A. 2008. Increased expression of the urokinase plasminogen activator system by Helicobacter pylori in gastric epithelial cells. *Am J Physiol Gastrointest Liver Physiol,* 295, G431-41.

KENNY, S., GAMBLE, J., LYONS, S., VLATKOVIC, N., DIMALINE, R., VARRO, A. & DOCKRAY, G. J. 2013a. Gastric Expression of Plasminogen Activator Inhibitor (PAI)-1 Is Associated with Hyperphagia and Obesity in Mice. *Endocrinology,* 154, 718-26.

KENNY, S., STEELE, I., LYONS, S., MOORE, A. R., MURUGESAN, S. V., TISZLAVICZ, L., DIMALINE, R., PRITCHARD, D. M., VARRO, A. & DOCKRAY, G. J. 2013b. The role of plasminogen activator inhibitor-1 in gastric mucosal protection. *Am J Physiol Gastrointest Liver Physiol,* 304, G814-22.

KENTISH, S. J., O'DONNELL, T. A., ISAACS, N. J., YOUNG, R. L., LI, H., HARRINGTON, A. M., BRIERLEY, S. M., WITTERT, G. A., BLACKSHAW, L. A. & PAGE, A. J. 2013. Gastric vagal afferent modulation by leptin is influenced by food intake status. *J Physiol,* 591, 1921-34.

KIEFFER, T. J. & HABENER, J. F. 1999. The glucagon-like peptides. *Endocr Rev,* 20, 876-913.

KIM, M. S., LEE, K. Y. & CHEY, W. Y. 1979. Plasma secretin concentrations in fasting and postprandial states in dog. *Am J Physiol,* 236, E539-44.

KIM, W. & EGAN, J. M. 2008. The role of incretins in glucose homeostasis and diabetes treatment. *Pharmacol Rev,* 60, 470-512.

KIMBALL CP, M. J. 1923. Aqueous extracts of pancreas. III: Some precipitation reaction of insulin. *J. Biol. Chem.,* 58, 337.

KIMMEL, J. R., POLLOCK, H. G. & HAZELWOOD, R. L. 1968. Isolation and characterization of chicken insulin. *Endocrinology,* 83, 1323-30.

KIRCHGESSNER, A. L. & LIU, M. 1999. Orexin synthesis and response in the gut. *Neuron,* 24, 941-51.

KIRKHAM, T. C., WILLIAMS, C. M., FEZZA, F. & DI MARZO, V. 2002. Endocannabinoid levels in rat limbic forebrain and hypothalamus in relation to fasting, feeding and satiation: stimulation of eating by 2-arachidonoyl glycerol. *Br J Pharmacol,* 136, 550-7.

KJOLLER, L. & HALL, A. 2001. Rac mediates cytoskeletal rearrangements and increased cell motility induced by urokinase-type plasminogen activator receptor binding to vitronectin. *J Cell Biol,* 152, 1145-57.

KOBELT, P., WISSER, A. S., STENGEL, A., GOEBEL, M., INHOFF, T., NOETZEL, S., VEH, R. W., BANNERT, N., VAN DER VOORT, I., WIEDENMANN, B., KLAPP, B. F., TACHE, Y. & MONNIKES, H. 2008. Peripheral injection of ghrelin induces Fos expression in the dorsomedial hypothalamic nucleus in rats. *Brain Res,* 1204, 77-86.

KODA, S., DATE, Y., MURAKAMI, N., SHIMBARA, T., HANADA, T., TOSHINAI, K., NIIJIMA, A., FURUYA, M., INOMATA, N., OSUYE, K. & NAKAZATO, M. 2005. The role of the vagal nerve in peripheral PYY3-36-induced feeding reduction in rats. *Endocrinology,* 146, 2369-75.

KOEGLER, F. H., ENRIORI, P. J., BILLES, S. K., TAKAHASHI, D. L., MARTIN, M. S., CLARK, R. L., EVANS, A. E., GROVE, K. L., CAMERON, J. L. & COWLEY, M. A. 2005. Peptide YY(3-36) inhibits morning, but not evening, food intake and decreases body weight in rhesus macaques. *Diabetes,* 54, 3198-204.

KOGA, D., SANTA, T., FUKUSHIMA, T., HOMMA, H. & IMAI, K. 1997. Liquid chromatographic-atmospheric pressure chemical ionization mass spectrometric determination of anandamide and its analogs in rat brain and peripheral tissues. *Journal of Chromatography B,* 690, 7-13.

KOHLER, H. P. & GRANT, P. J. 2000. Plasminogen-activator inhibitor type 1 and coronary artery disease. *N Engl J Med,* 342, 1792-801.

KOJIMA, M., HOSODA, H., DATE, Y., NAKAZATO, M., MATSUO, H. & KANGAWA, K. 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature,* 402, 656-60.

KOJIMA, S., UENO, N., ASAKAWA, A., SAGIYAMA, K., NARUO, T., MIZUNO, S. & INUI, A. 2007. A role for pancreatic polypeptide in feeding and body weight regulation. ***Peptides,*** **28, 459-63.**

KORNER, J., INABNET, W., CONWELL, I. M., TAVERAS, C., DAUD, A., OLIVERO-RIVERA, L., RESTUCCIA, N. L. & BESSLER, M. 2006. Differential effects of gastric bypass and banding on circulating gut hormone and leptin levels. ***Obesity (Silver Spring),*** **14, 1553-61.**

KORNER, J., INABNET, W., FEBRES, G., CONWELL, I. M., MCMAHON, D. J., SALAS, R., TAVERAS, C., SCHROPE, B. & BESSLER, M. 2009. Prospective study of gut hormone and metabolic changes after adjustable gastric banding and Roux-en-Y gastric bypass. ***Int J Obes (Lond),*** **33, 786-95.**

KOSTELI, A., SUGARU, E., HAEMMERLE, G., MARTIN, J. F., LEI, J., ZECHNER, R. & FERRANTE, A. W., JR. 2010. Weight loss and lipolysis promote a dynamic immune response in murine adipose tissue. ***J Clin Invest,*** **120, 3466-79.**

KREMER, M., HINES, I. N., MILTON, R. J. & WHEELER, M. D. 2006. Favored T helper 1 response in a mouse model of hepatosteatosis is associated with enhanced T cell-mediated hepatitis. ***Hepatology,*** **44, 216-27.**

LABURTHE, M., COUVINEAU, A., GAUDIN, P., MAORET, J. J., ROUYER-FESSARD, C. & NICOLE, P. 1996. Receptors for VIP, PACAP, secretin, GRF, glucagon, GLP-1, and other members of their new family of G protein-linked receptors: structure-function relationship with special reference to the human VIP-1 receptor. ***Ann N Y Acad Sci,*** **805, 94-109; discussion 110-1.**

LABURTHE, M., COUVINEAU, A. & MARIE, J. C. 2002. VPAC receptors for VIP and PACAP. ***Receptors Channels,*** **8, 137-53.**

LAL, S., MCLAUGHLIN, J., BARLOW, J., D'AMATO, M., GIACOVELLI, G., VARRO, A., DOCKRAY, G. J. & THOMPSON, D. G. 2004. Cholecystokinin pathways modulate sensations induced by gastric distension in humans. ***Am J Physiol Gastrointest Liver Physiol,*** **287, G72-9.**

LAMBERS, J. W., CAMMENGA, M., KONIG, B. W., MERTENS, K., PANNEKOEK, H. & VAN MOURIK, J. A. 1987. Activation of human endothelial cell-type plasminogen activator inhibitor (PAI-1) by negatively charged phospholipids. ***J Biol Chem,*** **262, 17492-6.**

LAMBERT, D. M. & MUCCIOLI, G. G. 2007. Endocannabinoids and related N-acylethanolamines in the control of appetite and energy metabolism: emergence of new molecular players. *Curr Opin Clin Nutr Metab Care,* 10, 735-44.

LANDIN, K., STIGENDAL, L., ERIKSSON, E., KROTKIEWSKI, M., RISBERG, B., TENGBORN, L. & SMITH, U. 1990. Abdominal obesity is associated with an impaired fibrinolytic activity and elevated plasminogen activator inhibitor-1. *Metabolism,* 39, 1044-8.

LARHAMMAR, D. 1996. Structural diversity of receptors for neuropeptide Y, peptide YY and pancreatic polypeptide. *Regul Pept,* 65, 165-74.

LARSSON, L. I., SUNDLER, F. & HAKANSON, R. 1975. Immunohistochemical localization of human pancreatic polypeptide (HPP) to a population of islet cells. *Cell Tissue Res,* 156, 167-71.

LASSMANN, V., VAGUE, P., VIALETTES, B. & SIMON, M. C. 1980. Low plasma levels of pancreatic polypeptide in obesity. *Diabetes,* 29, 428-30.

LAUFFER, L. M., IAKOUBOV, R. & BRUBAKER, P. L. 2009. GPR119 is essential for oleoylethanolamide-induced glucagon-like peptide-1 secretion from the intestinal enteroendocrine L-cell. *Diabetes,* 58, 1058-66.

LAVILLE, M. & DISSE, E. 2009. Bariatric surgery for diabetes treatment: why should we go rapidly to surgery. *Diabetes Metab,* 35, 562-3.

LAWRENCE, C. B., SNAPE, A. C., BAUDOIN, F. M. & LUCKMAN, S. M. 2002. Acute central ghrelin and GH secretagogues induce feeding and activate brain appetite centers. *Endocrinology,* 143, 155-62.

LAWRENCE, D., STRANDBERG, L., GRUNDSTROM, T. & NY, T. 1989. Purification of active human plasminogen activator inhibitor 1 from Escherichia coli. Comparison with natural and recombinant forms purified from eucaryotic cells. *Eur J Biochem,* 186, 523-33.

LAWTON, C. L., BURLEY, V. J., WALES, J. K. & BLUNDELL, J. E. 1993. Dietary fat and appetite control in obese subjects: weak effects on satiation and satiety. *Int J Obes Relat Metab Disord,* 17, 409-16.

LE QUELLEC, A., KERVRAN, A., BLACHE, P., CIURANA, A. J. & BATAILLE, D. 1992. Oxyntomodulin-like immunoreactivity: diurnal profile of a new potential enterogastrone. ***J Clin Endocrinol Metab,*** **74, 1405-9.**

LE ROUX, C. W., BATTERHAM, R. L., AYLWIN, S. J., PATTERSON, M., BORG, C. M., WYNNE, K. J., KENT, A., VINCENT, R. P., GARDINER, J., GHATEI, M. A. & BLOOM, S. R. 2006. Attenuated peptide YY release in obese subjects is associated with reduced satiety. ***Endocrinology,*** **147, 3-8.**

LE ROUX, C. W., WELBOURN, R., WERLING, M., OSBORNE, A., KOKKINOS, A., LAURENIUS, A., LONROTH, H., FANDRIKS, L., GHATEI, M. A., BLOOM, S. R. & OLBERS, T. 2007. Gut hormones as mediators of appetite and weight loss after Roux-en-Y gastric bypass. ***Ann Surg,*** **246, 780-5.**

LE SAUTER, J. & GEARY, N. 1990. Redundant vagal mediation of the synergistic satiety effect of pancreatic glucagon and cholecystokinin in sham feeding rats. ***J Auton Nerv Syst,*** **30, 13-22.**

LEPAGE, W. A. 1901. ***Journal de Physiologie*** **3, 335.**

LESUK, A., TERMINIELLO, L. & TRAVER, J. H. 1965. Crystalline Human Urokinase: Some Properties. ***Science,*** **147, 880-2.**

LEVIN, E. G. & SANTELL, L. 1987. Conversion of the active to latent plasminogen activator inhibitor from human endothelial cells. ***Blood,*** **70, 1090-8.**

LEVIN, F., EDHOLM, T., SCHMIDT, P. T., GRYBÄCK, P., JACOBSSON, H., DEGERBLAD, M., HÖYBYE, C., HOLST, J. J., REHFELD, J. F., HELLSTRÖM, P. M. & NÄSLUND, E. 2006. Ghrelin Stimulates Gastric Emptying and Hunger in Normal-Weight Humans. ***Journal of Clinical Endocrinology & Metabolism,*** **91, 3296-3302.**

LI, P., CHANG, T. M. & CHEY, W. Y. 1998. Secretin inhibits gastric acid secretion via a vagal afferent pathway in rats. ***Am J Physiol,*** **275, G22-8.**

LI, R. L., SHERBET, D. P., ELSBERND, B. L., GOLDSTEIN, J. L., BROWN, M. S. & ZHAO, T. J. 2012. Profound hypoglycemia in starved, ghrelin-deficient mice is caused by decreased gluconeogenesis and reversed by lactate or fatty acids. ***J Biol Chem,*** **287, 17942-50.**

LI, Z., SOLOSKI, M. J. & DIEHL, A. M. 2005. Dietary factors alter hepatic innate immune system in mice with nonalcoholic fatty liver disease. *Hepatology,* 42, 880-5.

LIDDLE, R. A., GOLDFINE, I. D. & WILLIAMS, J. A. 1984. Bioassay of plasma cholecystokinin in rats: effects of food, trypsin inhibitor, and alcohol. *Gastroenterology,* 87, 542-9.

LIDDLE, R. A., GREEN, G. M., CONRAD, C. K. & WILLIAMS, J. A. 1986a. Proteins but not amino acids, carbohydrates, or fats stimulate cholecystokinin secretion in the rat. *Am J Physiol,* 251, G243-8.

LIDDLE, R. A., MORITA, E. T., CONRAD, C. K. & WILLIAMS, J. A. 1986b. Regulation of gastric emptying in humans by cholecystokinin. *J Clin Invest,* 77, 992-6.

LIMONGI, P., RESNATI, M., HERNANDEZ-MARRERO, L., CREMONA, O., BLASI, F. & FAZIOLI, F. 1995. Biosynthesis and apical localization of the urokinase receptor in polarized MDCK epithelial cells. *FEBS Lett,* 369, 207-11.

LIN, T. M. & CHANCE, R. E. 1974. Candidate hormones of the gut. VI. Bovine pancreatic polypeptide (BPP) and avian pancreatic polypeptide (APP). *Gastroenterology,* 67, 737-8.

LIN, Z., JIANG, L., YUAN, C., JENSEN, J. K., ZHANG, X., LUO, Z., FURIE, B. C., FURIE, B., ANDREASEN, P. A. & HUANG, M. 2011. Structural basis for recognition of urokinase-type plasminogen activator by plasminogen activator inhibitor-1. *J Biol Chem,* 286, 7027-32.

LIOU, A. P., LU, X., SEI, Y., ZHAO, X., PECHHOLD, S., CARRERO, R. J., RAYBOULD, H. E. & WANK, S. 2011. The G-protein-coupled receptor GPR40 directly mediates long-chain fatty acid-induced secretion of cholecystokinin. *Gastroenterology,* 140, 903-12.

LITTLE, C. C. 1913. "Yellow" and "Agouti" Factors in Mice. *Science,* 38, 205.

LITTLE, T. J., HOROWITZ, M. & FEINLE-BISSET, C. 2007. Modulation by high-fat diets of gastrointestinal function and hormones associated with the regulation of energy intake: implications for the pathophysiology of obesity. *Am J Clin Nutr,* 86, 531-41.

LIU, J., DIVOUX, A., SUN, J., ZHANG, J., CLEMENT, K., GLICKMAN, J. N., SUKHOVA, G. K., WOLTERS, P. J., DU, J., GORGUN, C. Z., DORIA, A., LIBBY, P.,

BLUMBERG, R. S., KAHN, B. B., HOTAMISLIGIL, G. S. & SHI, G. P. 2009. Genetic deficiency and pharmacological stabilization of mast cells reduce diet-induced obesity and diabetes in mice. ***Nat Med,*** **15, 940-5.**

LIU, Y. L., FORD, H. E., DRUCE, M. R., MINNION, J. S., FIELD, B. C., SHILLITO, J. C., BAXTER, J., MURPHY, K. G., GHATEI, M. A. & BLOOM, S. R. 2010. Subcutaneous oxyntomodulin analogue administration reduces body weight in lean and obese rodents. ***Int J Obes (Lond),*** **34, 1715-25.**

LO, C. M., SAMUELSON, L. C., CHAMBERS, J. B., KING, A., HEIMAN, J., JANDACEK, R. J., SAKAI, R. R., BENOIT, S. C., RAYBOULD, H. E., WOODS, S. C. & TSO, P. 2008. Characterization of mice lacking the gene for cholecystokinin. ***Am J Physiol Regul Integr Comp Physiol,*** **294, R803-10.**

LOFTUS, T. M. 1999. An adipocyte-central nervous system regulatory loop in the control of adipose homeostasis. ***Semin Cell Dev Biol,*** **10, 11-8.**

LOMAX, A. E. & VANNER, S. J. 2010. Presynaptic inhibition of neural vasodilator pathways to submucosal arterioles by release of purines from sympathetic nerves. ***Am J Physiol Gastrointest Liver Physiol,*** **298, G700-5.**

LOSKUTOFF, D. J. & EDGINGTON, T. S. 1981. An inhibitor of plasminogen activator in rabbit endothelial cells. ***J Biol Chem,*** **256, 4142-5.**

LOSKUTOFF, D. J., LINDERS, M., KEIJER, J., VEERMAN, H., VAN HEERIKHUIZEN, H. & PANNEKOEK, H. 1987. Structure of the human plasminogen activator inhibitor 1 gene: nonrandom distribution of introns. ***Biochemistry,*** **26, 3763-8.**

LOSKUTOFF, D. J. & QUIGLEY, J. P. 2000. PAI-1, fibrosis, and the elusive provisional fibrin matrix. ***J Clin Invest,*** **106, 1441-3.**

LOSKUTOFF, D. J. & SAMAD, F. 1998. The adipocyte and hemostatic balance in obesity: studies of PAI-1. ***Arterioscler Thromb Vasc Biol,*** **18, 1-6.**

LOSKUTOFF, D. J., SAWDEY, M., KEETON, M. & SCHNEIDERMAN, J. 1993. Regulation of PAI-1 gene expression in vivo. ***Thromb Haemost,*** **70, 135-7.**

LOTTI, V. J., PENDLETON, R. G., GOULD, R. J., HANSON, H. M., CHANG, R. S. & CLINESCHMIDT, B. V. 1987. In vivo pharmacology of L-364,718, a new potent nonpeptide peripheral cholecystokinin antagonist. ***J Pharmacol Exp Ther,*** **241, 103-9.**

LUCKMAN, S. M. 1992. Fos-like immunoreactivity in the brainstem of the rat following peripheral administration of cholecystokinin. ***J Neuroendocrinol,*** **4, 149-52.**

LUMENG, C. N., BODZIN, J. L. & SALTIEL, A. R. 2007. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. ***J Clin Invest,*** **117, 175-84.**

LUMENG, C. N. & SALTIEL, A. R. 2011. Inflammatory links between obesity and metabolic disease. ***J Clin Invest,*** **121, 2111-7.**

LUND, P. K., GOODMAN, R. H. & HABENER, J. F. 1981. Pancreatic pre-proglucagons are encoded by two separate mRNAs. ***J Biol Chem,*** **256, 6515-8.**

LUND, P. K., GOODMAN, R. H., JACOBS, J. W. & HABENER, J. F. 1980. Glucagon precursors identified by immunoprecipitation of products of cell-free translation of messenger RNA. ***Diabetes,*** **29, 583-6.**

LUNDBERG, J. M., TERENIUS, L., HOKFELT, T., MARTLING, C. R., TATEMOTO, K., MUTT, V., POLAK, J., BLOOM, S. & GOLDSTEIN, M. 1982. Neuropeptide Y (NPY)-like immunoreactivity in peripheral noradrenergic neurons and effects of NPY on sympathetic function. ***Acta Physiol Scand,*** **116, 477-80.**

LUPU, F., BERGONZELLI, G. E., HEIM, D. A., COUSIN, E., GENTON, C. Y., BACHMANN, F. & KRUITHOF, E. K. 1993. Localization and production of plasminogen activator inhibitor-1 in human healthy and atherosclerotic arteries. ***Arterioscler Thromb,*** **13, 1090-100.**

MA, L. J., MAO, S. L., TAYLOR, K. L., KANJANABUCH, T., GUAN, Y., ZHANG, Y., BROWN, N. J., SWIFT, L. L., MCGUINNESS, O. P., WASSERMAN, D. H., VAUGHAN, D. E. & FOGO, A. B. 2004. Prevention of obesity and insulin resistance in mice lacking plasminogen activator inhibitor 1. ***Diabetes,*** **53, 336-46.**

MACDONALD, P. E., EL-KHOLY, W., RIEDEL, M. J., SALAPATEK, A. M., LIGHT, P. E. & WHEELER, M. B. 2002a. The multiple actions of GLP-1 on the process of glucose-stimulated insulin secretion. ***Diabetes,*** **51 Suppl 3, S434-42.**

MACDONALD, P. E., SALAPATEK, A. M. & WHEELER, M. B. 2002b. Glucagon-like peptide-1 receptor activation antagonizes voltage-dependent repolarizing K(+) currents in beta-cells: a possible glucose-dependent insulinotropic mechanism. ***Diabetes,*** **51 Suppl 3, S443-7.**

MACFARLANE, R. G. & PILLING, J. 1947. Fibrinolytic activity of normal urine. *Nature,* 159, 779.

MACLAGAN, N. F. 1937. The role of appetite in the control of body weight. *J Physiol,* 90, 385-94.

MALIK, S., MCGLONE, F., BEDROSSIAN, D. & DAGHER, A. 2008. Ghrelin modulates brain activity in areas that control appetitive behavior. *Cell Metab,* 7, 400-9.

MASUDA, Y., TANAKA, T., INOMATA, N., OHNUMA, N., TANAKA, S., ITOH, Z., HOSODA, H., KOJIMA, M. & KANGAWA, K. 2000. Ghrelin stimulates gastric acid secretion and motility in rats. *Biochem Biophys Res Commun,* 276, 905-8.

MATIAS, I., GONTHIER, M. P., PETROSINO, S., DOCIMO, L., CAPASSO, R., HOAREAU, L., MONTELEONE, P., ROCHE, R., IZZO, A. A. & DI MARZO, V. 2007. Role and regulation of acylethanolamides in energy balance: focus on adipocytes and beta-cells. *Br J Pharmacol,* 152, 676-90.

MATSON, C. A., REID, D. F., CANNON, T. A. & RITTER, R. C. 2000. Cholecystokinin and leptin act synergistically to reduce body weight. *Am J Physiol Regul Integr Comp Physiol,* 278, R882-90.

MATSON, C. A. & RITTER, R. C. 1999. Long-term CCK-leptin synergy suggests a role for CCK in the regulation of body weight. *Am J Physiol,* 276, R1038-45.

MATSON, C. A., WIATER, M. F., KUIJPER, J. L. & WEIGLE, D. S. 1997. Synergy between leptin and cholecystokinin (CCK) to control daily caloric intake. *Peptides,* 18, 1275-8.

MAVRI, A., STEGNAR, M., KREBS, M., SENTOCNIK, J. T., GEIGER, M. & BINDER, B. R. 1999. Impact of adipose tissue on plasma plasminogen activator inhibitor-1 in dieting obese women. *Arterioscler Thromb Vasc Biol,* 19, 1582-7.

MCCLEAN, P. L., IRWIN, N., CASSIDY, R. S., HOLST, J. J., GAULT, V. A. & FLATT, P. R. 2007. GIP receptor antagonism reverses obesity, insulin resistance, and associated metabolic disturbances induced in mice by prolonged consumption of high-fat diet. *Am J Physiol Endocrinol Metab,* 293, E1746-55.

MCDERMOTT, J. R., LESLIE, F. C., D'AMATO, M., THOMPSON, D. G., GRENCIS, R. K. & MCLAUGHLIN, J. T. 2006. Immune control of food intake: enteroendocrine

cells are regulated by CD4+ T lymphocytes during small intestinal inflammation. *Gut,* 55, 492-7.

MCLAUGHLIN, J., GRAZIA LUCA, M., JONES, M. N., D'AMATO, M., DOCKRAY, G. J. & THOMPSON, D. G. 1999. Fatty acid chain length determines cholecystokinin secretion and effect on human gastric motility. *Gastroenterology,* 116, 46-53.

MEDEIROS, M. D. & TURNER, A. J. 1994. Processing and metabolism of peptide-YY: pivotal roles of dipeptidylpeptidase-IV, aminopeptidase-P, and endopeptidase-24.11. *Endocrinology,* 134, 2088-94.

MEERAN, K., O'SHEA, D., EDWARDS, C. M., TURTON, M. D., HEATH, M. M., GUNN, I., ABUSNANA, S., ROSSI, M., SMALL, C. J., GOLDSTONE, A. P., TAYLOR, G. M., SUNTER, D., STEERE, J., CHOI, S. J., GHATEI, M. A. & BLOOM, S. R. 1999. Repeated intracerebroventricular administration of glucagon-like peptide-1-(7-36) amide or exendin-(9-39) alters body weight in the rat. *Endocrinology,* 140, 244-50.

MEIER, J. J. 2012. GLP-1 receptor agonists for individualized treatment of type 2 diabetes mellitus. *Nature Reviews Endocrinology,* 8, 728-742.

MERCER, J. G., HOGGARD, N., WILLIAMS, L. M., LAWRENCE, C. B., HANNAH, L. T. & TRAYHURN, P. 1996. Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization. *FEBS Lett,* 387, 113-6.

MEYRE, D., PROULX, K., KAWAGOE-TAKAKI, H., VATIN, V., GUTIERREZ-AGUILAR, R., LYON, D., MA, M., CHOQUET, H., HORBER, F., VAN HUL, W., VAN GAAL, L., BALKAU, B., VISVIKIS-SIEST, S., PATTOU, F., FAROOQI, I. S., SAUDEK, V., O'RAHILLY, S., FROGUEL, P., SEDGWICK, B. & YEO, G. S. 2010. Prevalence of loss-of-function FTO mutations in lean and obese individuals. *Diabetes,* 59, 311-8.

MICHEL, M. C., BECK-SICKINGER, A., COX, H., DOODS, H. N., HERZOG, H., LARHAMMAR, D., QUIRION, R., SCHWARTZ, T. & WESTFALL, T. 1998. XVI. International Union of Pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. *Pharmacol Rev,* 50, 143-50.

MILLER, J. L., JAMES, G. A., GOLDSTONE, A. P., COUCH, J. A., HE, G., DRISCOLL, D. J. & LIU, Y. 2007. Enhanced activation of reward mediating prefrontal

regions in response to food stimuli in Prader-Willi syndrome. *J Neurol Neurosurg Psychiatry,* 78, 615-9.

MIX, H., WIDJAJA, A., JANDL, O., CORNBERG, M., KAUL, A., GOKE, M., BEIL, W., KUSKE, M., BRABANT, G., MANNS, M. P. & WAGNER, S. 2000. Expression of leptin and leptin receptor isoforms in the human stomach. *Gut,* 47, 481-6.

MIYAWAKI, K., YAMADA, Y., BAN, N., IHARA, Y., TSUKIYAMA, K., ZHOU, H., FUJIMOTO, S., OKU, A., TSUDA, K., TOYOKUNI, S., HIAI, H., MIZUNOYA, W., FUSHIKI, T., HOLST, J. J., MAKINO, M., TASHITA, A., KOBARA, Y., TSUBAMOTO, Y., JINNOUCHI, T., JOMORI, T. & SEINO, Y. 2002. Inhibition of gastric inhibitory polypeptide signaling prevents obesity. *Nat Med,* 8, 738-42.

MORAN-RAMOS, S., TOVAR, A. R. & TORRES, N. 2012. Diet: friend or foe of enteroendocrine cells--how it interacts with enteroendocrine cells. *Adv Nutr,* 3, 8-20.

MORAN, G. W., LESLIE, F. C., LEVISON, S. E., WORTHINGTON, J. & MCLAUGHLIN, J. T. 2008. Enteroendocrine cells: neglected players in gastrointestinal disorders? *Therap Adv Gastroenterol,* 1, 51-60.

MORAN, T. H., BALDESSARINI, A. R., SALORIO, C. F., LOWERY, T. & SCHWARTZ, G. J. 1997. Vagal afferent and efferent contributions to the inhibition of food intake by cholecystokinin. *Am J Physiol,* 272, R1245-51.

MORAN, T. H., NORGREN, R., CROSBY, R. J. & MCHUGH, P. R. 1990. Central and peripheral vagal transport of cholecystokinin binding sites occurs in afferent fibers. *Brain Res,* 526, 95-102.

MORIARTY, P., DIMALINE, R., THOMPSON, D. G. & DOCKRAY, G. J. 1997. Characterization of cholecystokininA and cholecystokininB receptors expressed by vagal afferent neurons. *Neuroscience,* 79, 905-13.

MORINO, P., HERRERA-MARSCHITZ, M., CASTEL, M. N., UNGERSTEDT, U., VARRO, A., DOCKRAY, G. & HOKFELT, T. 1994. Cholecystokinin in cortico-striatal neurons in the rat: immunohistochemical studies at the light and electron microscopical level. *Eur J Neurosci,* 6, 681-92.

MOUNIEN, L., BIZET, P., BOUTELET, I., GOURCEROL, G., FOURNIER, A., VAUDRY, H. & JEGOU, S. 2006. Pituitary adenylate cyclase-activating polypeptide

directly modulates the activity of proopiomelanocortin neurons in the rat arcuate nucleus. *Neuroscience,* 143, 155-63.

MUNDINGER, T. O., CUMMINGS, D. E. & TABORSKY, G. J., JR. 2006. Direct stimulation of ghrelin secretion by sympathetic nerves. *Endocrinology,* 147, 2893-901.

MUNROE, D. G., GUPTA, A. K., KOOSHESH, F., VYAS, T. B., RIZKALLA, G., WANG, H., DEMCHYSHYN, L., YANG, Z. J., KAMBOJ, R. K., CHEN, H., MCCALLUM, K., SUMNER-SMITH, M., DRUCKER, D. J. & CRIVICI, A. 1999. Prototypic G protein-coupled receptor for the intestinotrophic factor glucagon-like peptide 2. *Proc Natl Acad Sci U S A,* 96, 1569-73.

MURPHY, E. F., COTTER, P. D., HEALY, S., MARQUES, T. M., O'SULLIVAN, O., FOUHY, F., CLARKE, S. F., O'TOOLE, P. W., QUIGLEY, E. M., STANTON, C., ROSS, P. R., O'DOHERTY, R. M. & SHANAHAN, F. 2010. Composition and energy harvesting capacity of the gut microbiota: relationship to diet, obesity and time in mouse models. *Gut,* 59, 1635-42.

MURPHY, K. G. & BLOOM, S. R. 2006. Gut hormones and the regulation of energy homeostasis. *Nature,* 444, 854-9.

MURPHY, M. C., CHAPMAN, C., LOVEGROVE, J. A., ISHERWOOD, S. G., MORGAN, L. M., WRIGHT, J. W. & WILLIAMS, C. M. 1996. Meal frequency; does it determine postprandial lipaemia? *Eur J Clin Nutr,* 50, 491-7.

MURRAY, C. D. R., MARTIN, N. M., PATTERSON, M., TAYLOR, S. A., GHATEI, M. A., KAMM, M. A., JOHNSTON, C., BLOOM, S. R. & EMMANUEL, A. V. 2005. Ghrelin enhances gastric emptying in diabetic gastroparesis: a double blind, placebo controlled, crossover study. *Gut,* 54, 1693-1698.

MUTT, V. & JORPES, E. 1971. Hormonal polypeptides of the upper intestine. *Biochem J,* 125, 57P-58P.

MUTT, V., JORPES, J. E. & MAGNUSSON, S. 1970. Structure of porcine secretin. The amino acid sequence. *Eur J Biochem,* 15, 513-9.

MUTT, V. & SAID, S. I. 1974. Structure of the porcine vasoactive intestinal octacosapeptide. The amino-acid sequence. Use of kallikrein in its determination. *Eur J Biochem,* 42, 581-9.

NAIR, K. S. 1987. Hyperglucagonemia increases resting metabolic rate in man during insulin deficiency. *J Clin Endocrinol Metab,* 64, 896-901.

NAKAZATO, M., MURAKAMI, N., DATE, Y., KOJIMA, M., MATSUO, H., KANGAWA, K. & MATSUKURA, S. 2001. A role for ghrelin in the central regulation of feeding. *Nature,* 409, 194-8.

NAR, H., BAUER, M., STASSEN, J. M., LANG, D., GILS, A. & DECLERCK, P. J. 2000. Plasminogen activator inhibitor 1. Structure of the native serpin, comparison to its other conformers and implications for serpin inactivation. *J Mol Biol,* 297, 683-95.

NASLUND, E., BOGEFORS, J., SKOGAR, S., GRYBACK, P., JACOBSSON, H., HOLST, J. J. & HELLSTROM, P. M. 1999. GLP-1 slows solid gastric emptying and inhibits insulin, glucagon, and PYY release in humans. *Am J Physiol,* 277, R910-6.

NEARY, N. M., GOLDSTONE, A. P. & BLOOM, S. R. 2004. Appetite regulation: from the gut to the hypothalamus. *Clin Endocrinol (Oxf),* 60, 153-60.

NIELSEN, S. & JENSEN, M. D. 1997. Obesity and cardiovascular disease: is body structure a factor? *Curr Opin Lipidol,* 8, 200-4.

NORDSTROM, S. M., CARLETON, S. M., CARSON, W. L., EREN, M., PHILLIPS, C. L. & VAUGHAN, D. E. 2007. Transgenic over-expression of plasminogen activator inhibitor-1 results in age-dependent and gender-specific increases in bone strength and mineralization. *Bone,* 41, 995-1004.

NORSETT, K. G., STEELE, I., DUVAL, C., SAMMUT, S. J., MURUGESAN, S. V., KENNY, S., RAINBOW, L., DIMALINE, R., DOCKRAY, G. J., PRITCHARD, D. M. & VARRO, A. 2011. Gastrin stimulates expression of plasminogen activator inhibitor-1 in gastric epithelial cells. *Am J Physiol Gastrointest Liver Physiol,* 301, G446-53.

NOZAKI, S., NAKATA, R., MIZUMA, H., NISHIMURA, N., WATANABE, Y., KOHASHI, R. & WATANABE, Y. 2002. In vitro autoradiographic localization of (125)i-secretin receptor binding sites in rat brain. *Biochem Biophys Res Commun,* 292, 133-7.

NWENEKA, C. V. & PRENTICE, A. M. 2011. Helicobacter pylori infection and circulating ghrelin levels - a systematic review. *BMC Gastroenterol,* 11, 7.

NYKJAER, A., MOLLER, B., TODD, R. F., 3RD, CHRISTENSEN, T., ANDREASEN, P. A., GLIEMANN, J. & PETERSEN, C. M. 1994. Urokinase receptor. An activation antigen in human T lymphocytes. ***J Immunol,*** **152, 505-16.**

O'RAHILLY, S. & FAROOQI, I. S. 2008. Human obesity: a heritable neurobehavioral disorder that is highly sensitive to environmental conditions. ***Diabetes,*** **57, 2905-10.**

O'ROURKE, R. W., METCALF, M. D., WHITE, A. E., MADALA, A., WINTERS, B. R., MAIZLIN, II, JOBE, B. A., ROBERTS, C. T., JR., SLIFKA, M. K. & MARKS, D. L. 2009. Depot-specific differences in inflammatory mediators and a role for NK cells and IFN-gamma in inflammation in human adipose tissue. ***Int J Obes (Lond),*** **33, 978-90.**

OHMURA, K., ISHIMORI, N., OHMURA, Y., TOKUHARA, S., NOZAWA, A., HORII, S., ANDOH, Y., FUJII, S., IWABUCHI, K., ONOE, K. & TSUTSUI, H. 2010. Natural killer T cells are involved in adipose tissues inflammation and glucose intolerance in diet-induced obese mice. ***Arterioscler Thromb Vasc Biol,*** **30, 193-9.**

OKANO-MATSUMOTO, S., MCROBERTS, J. A., TACHE, Y. & ADELSON, D. W. 2011. Electrophysiological evidence for distinct vagal pathways mediating CCK-evoked motor effects in the proximal versus distal stomach. ***J Physiol,*** **589, 371-93.**

ORSKOV, C., RABENHOJ, L., WETTERGREN, A., KOFOD, H. & HOLST, J. J. 1994. Tissue and plasma concentrations of amidated and glycine-extended glucagon-like peptide I in humans. ***Diabetes,*** **43, 535-9.**

OSAWA, H. 2008. Ghrelin and Helicobacter pylori infection. ***World J Gastroenterol,*** **14, 6327-33.**

OVERTON, H. A., BABBS, A. J., DOEL, S. M., FYFE, M. C., GARDNER, L. S., GRIFFIN, G., JACKSON, H. C., PROCTER, M. J., RASAMISON, C. M., TANG-CHRISTENSEN, M., WIDDOWSON, P. S., WILLIAMS, G. M. & REYNET, C. 2006. Deorphanization of a G protein-coupled receptor for oleoylethanolamide and its use in the discovery of small-molecule hypophagic agents. ***Cell Metab,*** **3, 167-75.**

PAINTAL, A. S. 1973. Vagal sensory receptors and their reflex effects. ***Physiol Rev,*** **53, 159-227.**

PASSARO, E., JR., DEBAS, H., OLDENDORF, W. & YAMADA, T. 1982. Rapid appearance of intraventricularly administered neuropeptides in the peripheral circulation. ***Brain Res,*** **241, 335-40.**

PATTERSON, L. M., ZHENG, H. & BERTHOUD, H. R. 2002. Vagal afferents innervating the gastrointestinal tract and CCKA-receptor immunoreactivity. *Anat Rec,* 266, 10-20.

PAUWELS, S., DESMOND, H., DIMALINE, R. & DOCKRAY, G. J. 1986. Identification of progastrin in gastrinomas, antrum, and duodenum by a novel radioimmunoassay. *J Clin Invest,* 77, 376-81.

PAVLOV, I. P. 1901. Le Travail des Glandes Digestives. *Paris: Masson et Cie, 1901.*

PAXINOS G, F. K. 2001. The Mouse Brain in Stereotaxic

Coordinates, 2nd edn.

PELLETIER, M. J., CHAYVIALLE, J. A. & MINAIRE, Y. 1978. Uneven and transient secretin release after a liquid test meal. *Gastroenterology,* 75, 1124-32.

PELLEYMOUNTER, M. A., CULLEN, M. J., BAKER, M. B., HECHT, R., WINTERS, D., BOONE, T. & COLLINS, F. 1995. Effects of the obese gene product on body weight regulation in ob/ob mice. *Science,* 269, 540-3.

PEPPER, M. S., SAPPINO, A. P., STOCKLIN, R., MONTESANO, R., ORCI, L. & VASSALLI, J. D. 1993. Upregulation of urokinase receptor expression on migrating endothelial cells. *J Cell Biol,* 122, 673-84.

PEREZ-LEIGHTON, C. E., BOLAND, K., TESKE, J. A., BILLINGTON, C. & KOTZ, C. M. 2012. Behavioral responses to orexin, orexin receptor gene expression, and spontaneous physical activity contribute to individual sensitivity to obesity. *American Journal of Physiology-Endocrinology and Metabolism,* 303, E865-E874.

PEREZ-TILVE, D., HEPPNER, K., KIRCHNER, H., LOCKIE, S. H., WOODS, S. C., SMILEY, D. L., TSCHOP, M. & PFLUGER, P. 2011. Ghrelin-induced adiposity is independent of orexigenic effects. *FASEB J,* 25, 2814-22.

PETERSEN, G., SORENSEN, C., SCHMID, P. C., ARTMANN, A., TANG-CHRISTENSEN, M., HANSEN, S. H., LARSEN, P. J., SCHMID, H. H. & HANSEN, H. S. 2006. Intestinal levels of anandamide and oleoylethanolamide in food-deprived rats are regulated through their precursors. *Biochim Biophys Acta,* 1761, 143-50; discussion 141-2.

PFLUGER, P. T., KAMPE, J., CASTANEDA, T. R., VAHL, T., D'ALESSIO, D. A., KRUTHAUPT, T., BENOIT, S. C., CUNTZ, U., ROCHLITZ, H. J., MOEHLIG, M., PFEIFFER, A. F., KOEBNICK, C., WEICKERT, M. O., OTTO, B., SPRANGER, J. & TSCHOP, M. H. 2007. Effect of human body weight changes on circulating levels of peptide YY and peptide YY3-36. ***J Clin Endocrinol Metab,*** **92, 583-8.**

PLESNER, T., RALFKIAER, E., WITTRUP, M., JOHNSEN, H., PYKE, C., PEDERSEN, T. L., HANSEN, N. E. & DANO, K. 1994. Expression of the receptor for urokinase-type plasminogen activator in normal and neoplastic blood cells and hematopoietic tissue. ***Am J Clin Pathol,*** **102, 835-41.**

PLOUG, M., BEHRENDT, N., LOBER, D. & DANO, K. 1991. Protein structure and membrane anchorage of the cellular receptor for urokinase-type plasminogen activator. ***Semin Thromb Hemost,*** **17, 183-93.**

POCAI, A. 2012. Unraveling oxyntomodulin, GLP1's enigmatic brother. ***Journal of Endocrinology,*** **215, 335-346.**

PROULX, K., COTA, D., CASTANEDA, T. R., TSCHOP, M. H., D'ALESSIO, D. A., TSO, P., WOODS, S. C. & SEELEY, R. J. 2005. Mechanisms of oleoylethanolamide-induced changes in feeding behavior and motor activity. ***Am J Physiol Regul Integr Comp Physiol,*** **289, R729-37.**

PRZEMECK, S. M., VARRO, A., BERRY, D., STEELE, I., WANG, T. C., DOCKRAY, G. J. & PRITCHARD, D. M. 2008. Hypergastrinemia increases gastric epithelial susceptibility to apoptosis. ***Regul Pept,*** **146, 147-56.**

PUNJABI, M., ARNOLD, M., GEARY, N., LANGHANS, W. & PACHECO-LOPEZ, G. 2011. Peripheral glucagon-like peptide-1 (GLP-1) and satiation. ***Physiol Behav,*** **105, 71-6.**

PURKAYASTHA, S., ZHANG, H., ZHANG, G., AHMED, Z., WANG, Y. & CAI, D. 2011. Neural dysregulation of peripheral insulin action and blood pressure by brain endoplasmic reticulum stress. ***Proc Natl Acad Sci U S A,*** **108, 2939-44.**

QUIGLEY, E. M. M. 1996. Gastric and small intestinal motility in health and disease. ***Gastroenterology Clinics of North America,*** **25, 113-&.**

RAUFMAN, J. P., JENSEN, R. T., SUTLIFF, V. E., PISANO, J. J. & GARDNER, J. D. 1982. Actions of Gila monster venom on dispersed acini from guinea pig pancreas. ***Am J Physiol,*** **242, G470-4.**

RAUFMAN, J. P., SINGH, L. & ENG, J. 1991. Exendin-3, a novel peptide from Heloderma horridum venom, interacts with vasoactive intestinal peptide receptors and a newly described receptor on dispersed acini from guinea pig pancreas. Description of exendin-3(9-39) amide, a specific exendin receptor antagonist. *J Biol Chem,* 266, 2897-902.

RAUSCH, U., ADLER, G., WEIDENBACH, H., WEIDENBACH, F., RUDOLFF, D., KOOP, I. & KERN, H. F. 1987. Stimulation of pancreatic secretory process in the rat by low-molecular weight proteinase inhibitor. I. Dose-response study on enzyme content and secretion, cholecystokinin release and pancreatic fine structure. *Cell Tissue Res,* 247, 187-93.

RAYBOULD, H. E. 2012. Gut microbiota, epithelial function and derangements in obesity. *J Physiol,* 590, 441-6.

RAYBOULD, H. E., GAYTON, R. J. & DOCKRAY, G. J. 1988. Mechanisms of action of peripherally administered cholecystokinin octapeptide on brain stem neurons in the rat. *J Neurosci,* 8, 3018-24.

RAYBOULD, H. E. & TACHE, Y. 1988. Cholecystokinin inhibits gastric motility and emptying via a capsaicin-sensitive vagal pathway in rats. *Am J Physiol,* 255, G242-6.

RAYBOULD, H. E., ZITTEL, T. T., HOLZER, H. H., LLOYD, K. C. & MEYER, J. H. 1994. Gastroduodenal sensory mechanisms and CCK in inhibition of gastric emptying in response to a meal. *Dig Dis Sci,* 39, 41S-43S.

REIDELBERGER, R. D., HERNANDEZ, J., FRITZSCH, B. & HULCE, M. 2004. Abdominal vagal mediation of the satiety effects of CCK in rats. *Am J Physiol Regul Integr Comp Physiol,* 286, R1005-12.

REIMANN, F. 2010. Molecular mechanisms underlying nutrient detection by incretin-secreting cells. *Int Dairy J,* 20, 236-242.

RENTSCH, J., LEVENS, N. & CHIESI, M. 1995. Recombinant ob-gene product reduces food intake in fasted mice. *Biochem Biophys Res Commun,* 214, 131-6.

RIEDY, C. A., CHAVEZ, M., FIGLEWICZ, D. P. & WOODS, S. C. 1995. Central insulin enhances sensitivity to cholecystokinin. *Physiol Behav,* 58, 755-60.

RINAMAN, L., BAKER, E. A., HOFFMAN, G. E., STRICKER, E. M. & VERBALIS, J. G. 1998. Medullary c-Fos activation in rats after ingestion of a satiating meal. ***American Journal of Physiology-Regulatory Integrative and Comparative Physiology,*** **275, R262-R268.**

ROGERS, R. C. & HERMANN, G. E. 2008. Mechanisms of action of CCK to activate central vagal afferent terminals. ***Peptides,*** **29, 1716-1725.**

ROLLS, B. J., ROE, L. S. & MEENGS, J. S. 2007. The effect of large portion sizes on energy intake is sustained for 11 days. ***Obesity (Silver Spring),*** **15, 1535-43.**

ROMER, J., LUND, L. R., ERIKSEN, J., PYKE, C., KRISTENSEN, P. & DANO, K. 1994. The receptor for urokinase-type plasminogen activator is expressed by keratinocytes at the leading edge during re-epithelialization of mouse skin wounds. ***J Invest Dermatol,*** **102, 519-22.**

SAID, S. I. & MUTT, V. 1970a. Polypeptide with broad biological activity: isolation from small intestine. ***Science,*** **169, 1217-8.**

SAID, S. I. & MUTT, V. 1970b. Potent peripheral and splanchnic vasodilator peptide from normal gut. ***Nature,*** **225, 863-4.**

SAKURAI, T., AMEMIYA, A., ISHII, M., MATSUZAKI, I., CHEMELLI, R. M., TANAKA, H., WILLIAMS, S. C., RICHARDSON, J. A., KOZLOWSKI, G. P., WILSON, S., ARCH, J. R. S., BUCKINGHAM, R. E., HAYNES, A. C., CARR, S. A., ANNAN, R. S., MCNULTY, D. E., LIU, W. S., TERRETT, J. A., ELSHOURBAGY, N. A., BERGSMA, D. J. & YANAGISAWA, M. 1998. Orexins and orexin receptors: A family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. ***Cell,*** **92, 573-585.**

SAMAD, F. & LOSKUTOFF, D. J. 1996. Tissue distribution and regulation of plasminogen activator inhibitor-1 in obese mice. ***Mol Med,*** **2, 568-82.**

SARRAF, P., FREDERICH, R. C., TURNER, E. M., MA, G., JASKOWIAK, N. T., RIVET, D. J., 3RD, FLIER, J. S., LOWELL, B. B., FRAKER, D. L. & ALEXANDER, H. R. 1997. Multiple cytokines and acute inflammation raise mouse leptin levels: potential role in inflammatory anorexia. ***J Exp Med,*** **185, 171-5.**

SAVAGE, A. P., ADRIAN, T. E., CAROLAN, G., CHATTERJEE, V. K. & BLOOM, S. R. 1987. Effects of peptide YY (PYY) on mouth to caecum intestinal transit time and on the rate of gastric emptying in healthy volunteers. ***Gut,*** **28, 166-70.**

SAWDEY, M. S. & LOSKUTOFF, D. J. 1991. Regulation of murine type 1 plasminogen activator inhibitor gene expression in vivo. Tissue specificity and induction by lipopolysaccharide, tumor necrosis factor-alpha, and transforming growth factor-beta. ***J Clin Invest,*** **88, 1346-53.**

SCHIRRA, J., NICOLAUS, M., ROGGEL, R., KATSCHINSKI, M., STORR, M., WOERLE, H. J. & GOKE, B. 2006. Endogenous glucagon-like peptide 1 controls endocrine pancreatic secretion and antro-pyloro-duodenal motility in humans. ***Gut,*** **55, 243-51.**

SCHJOLDAGER, B., MORTENSEN, P. E., MYHRE, J., CHRISTIANSEN, J. & HOLST, J. J. 1989. Oxyntomodulin from distal gut. Role in regulation of gastric and pancreatic functions. ***Dig Dis Sci,*** **34, 1411-9.**

SCHMIDT, P. T., NASLUND, E., GRYBACK, P., JACOBSSON, H., HARTMANN, B., HOLST, J. J. & HELLSTROM, P. M. 2003. Peripheral administration of GLP-2 to humans has no effect on gastric emptying or satiety. ***Regul Pept,*** **116, 21-5.**

SCHNEIDERMAN, J., SAWDEY, M. S., KEETON, M. R., BORDIN, G. M., BERNSTEIN, E. F., DILLEY, R. B. & LOSKUTOFF, D. J. 1992. Increased type 1 plasminogen activator inhibitor gene expression in atherosclerotic human arteries. ***Proc Natl Acad Sci U S A,*** **89, 6998-7002.**

SCHOONJANS, R., VAN VLEM, B., VAN HEDDEGHEM, N., VANDAMME, W., VANHOLDER, R., LAMEIRE, N., LEFEBVRE, R. & DE VOS, M. 2002. The 13C-octanoic acid breath test: validation of a new noninvasive method of measuring gastric emptying in rats. ***Neurogastroenterol Motil,*** **14, 287-93.**

SCHUBERT, M. L. 2002. Gastric secretion. ***Curr Opin Gastroenterol,*** **18, 639-49.**

SCHWARTZ, G. J., BERKOW, G., MCHUGH, P. R. & MORAN, T. H. 1993a. Gastric branch vagotomy blocks nutrient and cholecystokinin-induced suppression of gastric emptying. ***Am J Physiol,*** **264, R630-7.**

SCHWARTZ, G. J., MCHUGH, P. R. & MORAN, T. H. 1993b. GASTRIC LOADS AND CHOLECYSTOKININ SYNERGISTICALLY STIMULATE RAT GASTRIC VAGAL AFFERENTS. ***American Journal of Physiology,*** **265, R872-R876.**

SCHWARTZ, G. J., MCHUGH, P. R. & MORAN, T. H. 1993c. Gastric loads and cholecystokinin synergistically stimulate rat gastric vagal afferents. ***Am J Physiol,*** **265, R872-6.**

SCHWARTZ, M. W., SEELEY, R. J., CAMPFIELD, L. A., BURN, P. & BASKIN, D. G. 1996. Identification of targets of leptin action in rat hypothalamus. ***J Clin Invest,*** **98, 1101-6.**

SCHWARTZ, M. W., WOODS, S. C., PORTE, D., JR., SEELEY, R. J. & BASKIN, D. G. 2000. Central nervous system control of food intake. ***Nature,*** **404, 661-71.**

SCOTT, R. B., KIRK, D., MACNAUGHTON, W. K. & MEDDINGS, J. B. 1998. GLP-2 augments the adaptive response to massive intestinal resection in rat. ***Am J Physiol,*** **275, G911-21.**

SCOTT, V., MCDADE, D. M. & LUCKMAN, S. M. 2007. Rapid changes in the sensitivity of arcuate nucleus neurons to central ghrelin in relation to feeding status. ***Physiol Behav,*** **90, 180-5.**

SHEN, X., CARLSTROM, M., BORNIQUEL, S., JADERT, C., KEVIL, C. G. & LUNDBERG, J. O. 2013. Microbial regulation of host hydrogen sulfide bioavailability and metabolism. ***Free Radic Biol Med,*** **60, 195-200.**

SHIMOMURA, I., FUNAHASHI, T., TAKAHASHI, M., MAEDA, K., KOTANI, K., NAKAMURA, T., YAMASHITA, S., MIURA, M., FUKUDA, Y., TAKEMURA, K., TOKUNAGA, K. & MATSUZAWA, Y. 1996. Enhanced expression of PAI-1 in visceral fat: possible contributor to vascular disease in obesity. ***Nat Med,*** **2, 800-3.**

SHIODA, S., SHUTO, Y., SOMOGYVARI-VIGH, A., LEGRADI, G., ONDA, H., COY, D. H., NAKAJO, S. & ARIMURA, A. 1997. Localization and gene expression of the receptor for pituitary adenylate cyclase-activating polypeptide in the rat brain. ***Neurosci Res,*** **28, 345-54.**

SHIVERS, B. D., GORCS, T. J., GOTTSCHALL, P. E. & ARIMURA, A. 1991. Two high affinity binding sites for pituitary adenylate cyclase-activating polypeptide have different tissue distributions. ***Endocrinology,*** **128, 3055-65.**

SHUGHRUE, P. J., LANE, M. V. & MERCHENTHALER, I. 1996. Glucagon-like peptide-1 receptor (GLP1-R) mRNA in the rat hypothalamus. ***Endocrinology,*** **137, 5159-62.**

SIDENIUS, N., ANDOLFO, A., FESCE, R. & BLASI, F. 2002. Urokinase regulates vitronectin binding by controlling urokinase receptor oligomerization. ***J Biol Chem,*** **277, 27982-90.**

SLOMKO, H., HEO, H. J. & EINSTEIN, F. H. 2012. Minireview: Epigenetics of obesity and diabetes in humans. ***Endocrinology,*** **153, 1025-30.**

SLOTH, B., HOLST, J. J., FLINT, A., GREGERSEN, N. T. & ASTRUP, A. 2007. Effects of PYY1-36 and PYY3-36 on appetite, energy intake, energy expenditure, glucose and fat metabolism in obese and lean subjects. ***Am J Physiol Endocrinol Metab,*** **292, E1062-8.**

SMITH, G. P. & GIBBS, J. 1985. The satiety effect of cholecystokinin. Recent progress and current problems. ***Ann N Y Acad Sci,*** **448, 417-23.**

SMITH, G. P., GIBBS, J., JEROME, C., PI-SUNYER, F. X., KISSILEFF, H. R. & THORNTON, J. 1981. The satiety effect of cholecystokinin: a progress report. ***Peptides,*** **2 Suppl 2, 57-9.**

SMITH, G. P., JEROME, C. & NORGREN, R. 1985. Afferent axons in abdominal vagus mediate satiety effect of cholecystokinin in rats. ***Am J Physiol,*** **249, R638-41.**

SOLBERG, H., PLOUG, M., HOYER-HANSEN, G., NIELSEN, B. S. & LUND, L. R. 2001. The murine receptor for urokinase-type plasminogen activator is primarily expressed in tissues actively undergoing remodeling. ***J Histochem Cytochem,*** **49, 237-46.**

SOUTH, E. H. & RITTER, R. C. 1988. Capsaicin application to central or peripheral vagal fibers attenuates CCK satiety. ***Peptides,*** **9, 601-12.**

SOWDEN, G. L., DRUCKER, D. J., WEINSHENKER, D. & SWOAP, S. J. 2007. Oxyntomodulin increases intrinsic heart rate in mice independent of the glucagon-like peptide-1 receptor. ***Am J Physiol Regul Integr Comp Physiol,*** **292, R962-70.**

SPEAKMAN, J., HAMBLY, C., MITCHELL, S. & KROL, E. 2007. Animal models of obesity. ***Obes Rev,*** **8 Suppl 1, 55-61.**

SPEAKMAN, J. R. 2007. A nonadaptive scenario explaining the genetic predisposition to obesity: the "predation release" hypothesis. ***Cell Metab,*** **6, 5-12.**

STEELE, I. A., DIMALINE, R., PRITCHARD, D. M., PEEK, R. M., JR., WANG, T. C., DOCKRAY, G. J. & VARRO, A. 2007. Helicobacter and gastrin stimulate Reg1 expression in gastric epithelial cells through distinct promoter elements. ***Am J Physiol Gastrointest Liver Physiol,*** **293, G347-54.**

STEIN, B., ANGEL, P., VAN DAM, H., PONTA, H., HERRLICH, P., VAN DER EB, A. & RAHMSDORF, H. J. 1992. Ultraviolet-radiation induced c-jun gene transcription: two AP-1 like binding sites mediate the response. ***Photochem Photobiol,*** **55, 409-15.**

STEINER, T. S., MANGEL, A. W., MCVEY, D. C. & VIGNA, S. R. 1993. Secretin receptors mediating rat forestomach relaxation. ***Am J Physiol,*** **264, G863-7.**

STEVENSON, R. W., STEINER, K. E., DAVIS, M. A., HENDRICK, G. K., WILLIAMS, P. E., LACY, W. W., BROWN, L., DONAHUE, P., LACY, D. B. & CHERRINGTON, A. D. 1987. Similar dose responsiveness of hepatic glycogenolysis and gluconeogenesis to glucagon in vivo. ***Diabetes,*** **36, 382-9.**

STRIFFLER, J., CARDELL, E. L. & CARDELL, R. R., JR. 1981. Effects of glucagon on hepatic glycogen and smooth endoplasmic reticulum. ***Am J Anat,*** **160, 363-79.**

SUN, Y. X., TSUBOI, K., ZHAO, L. Y., OKAMOTO, Y., LAMBERT, D. M. & UEDA, N. 2005. Involvement of N-acylethanolamine-hydrolyzing acid amidase in the degradation of anandamide and other N-acylethanolamines in macrophages. ***Biochim Biophys Acta,*** **1736, 211-20.**

SUZUKI, K., JAYASENA, C. N. & BLOOM, S. R. 2011. The gut hormones in appetite regulation. ***J Obes,*** **2011, 528401.**

SVOBODA, M., TASTENOY, M., VERTONGEN, P. & ROBBERECHT, P. 1994. Relative quantitative analysis of glucagon receptor mRNA in rat tissues. ***Mol Cell Endocrinol,*** **105, 131-7.**

SYMONDS, E. L., BUTLER, R. N. & OMARI, T. I. 2000. Assessment of gastric emptying in the mouse using the [13C]-octanoic acid breath test. ***Clin Exp Pharmacol Physiol,*** **27, 671-5.**

SYMONDS, M. E., SEBERT, S. & BUDGE, H. 2011. The obesity epidemic: from the environment to epigenetics - not simply a response to dietary manipulation in a thermoneutral environment. *Front Genet,* 2, 24.

SZECOWKA, J., TATEMOTO, K., RAJAMAKI, G. & EFENDIC, S. 1983. Effects of PYY and PP on endocrine pancreas. *Acta Physiol Scand,* 119, 123-6.

TACK, J., DEPOORTERE, I., BISSCHOPS, R., DELPORTE, C., COULIE, B., MEULEMANS, A., JANSSENS, J. & PEETERS, T. 2006. Influence of ghrelin on interdigestive gastrointestinal motility in humans. *Gut,* 55, 327-33.

TADROSS, J. A. & LE ROUX, C. W. 2009. The mechanisms of weight loss after bariatric surgery. *Int J Obes (Lond),* 33 Suppl 1, S28-32.

TAMURA, H., KAMEGAI, J., SHIMIZU, T., ISHII, S., SUGIHARA, H. & OIKAWA, S. 2002. Ghrelin stimulates GH but not food intake in arcuate nucleus ablated rats. *Endocrinology,* 143, 3268-75.

TANAKA, T., KATSUMA, S., ADACHI, T., KOSHIMIZU, T. A., HIRASAWA, A. & TSUJIMOTO, G. 2008. Free fatty acids induce cholecystokinin secretion through GPR120. *Naunyn Schmiedebergs Arch Pharmacol,* 377, 523-7.

TANG-CHRISTENSEN, M., VRANG, N. & LARSEN, P. J. 2001. Glucagon-like peptide containing pathways in the regulation of feeding behaviour. *Int J Obes Relat Metab Disord,* 25 Suppl 5, S42-7.

TANG, W. W., FENG, L., LOSKUTOFF, D. J. & WILSON, C. B. 2000. Glomerular extracellular matrix accumulation in experimental anti-GBM Ab glomerulonephritis. *Nephron,* 84, 40-8.

TARTAGLIA, L. A., DEMBSKI, M., WENG, X., DENG, N., CULPEPPER, J., DEVOS, R., RICHARDS, G. J., CAMPFIELD, L. A., CLARK, F. T., DEEDS, J., MUIR, C., SANKER, S., MORIARTY, A., MOORE, K. J., SMUTKO, J. S., MAYS, G. G., WOOL, E. A., MONROE, C. A. & TEPPER, R. I. 1995. Identification and expression cloning of a leptin receptor, OB-R. *Cell,* 83, 1263-71.

TATEMOTO, K. & MUTT, V. 1980. Isolation of two novel candidate hormones using a chemical method for finding naturally occurring polypeptides. *Nature,* 285, 417-8.

TATEMOTO, K., SIIMESMAA, S., JORNVALL, H., ALLEN, J. M., POLAK, J. M., BLOOM, S. R. & MUTT, V. 1985. Isolation and characterization of neuropeptide Y from porcine intestine. ***FEBS Lett,*** **179, 181-4.**

TAYLOR, I. L., IMPICCIATORE, M., CARTER, D. C. & WALSH, J. H. 1978. Effect of atropine and vagotomy on pancreatic polypeptide response to a meal in dogs. ***Am J Physiol,*** **235, E443-7.**

THALER, J. P. & SCHWARTZ, M. W. 2010. Minireview: Inflammation and obesity pathogenesis: the hypothalamus heats up. ***Endocrinology,*** **151, 4109-15.**

THANOS, P. K., SUBRIZE, M., DELIS, F., COONEY, R. N., CULNAN, D., SUN, M. J., WANG, G. J., VOLKOW, N. D. & HAJNAL, A. 2012. Gastric Bypass Increases Ethanol and Water Consumption in Diet-Induced Obese Rats. ***Obesity Surgery,*** **22, 1884-1892.**

THEODORAKIS, M. J., CARLSON, O., MICHOPOULOS, S., DOYLE, M. E., JUHASZOVA, M., PETRAKI, K. & EGAN, J. M. 2006. Human duodenal enteroendocrine cells: source of both incretin peptides, GLP-1 and GIP. ***Am J Physiol Endocrinol Metab,*** **290, E550-9.**

THOGERSEN, A. M., JANSSON, J. H., BOMAN, K., NILSSON, T. K., WEINEHALL, L., HUHTASAARI, F. & HALLMANS, G. 1998. High plasminogen activator inhibitor and tissue plasminogen activator levels in plasma precede a first acute myocardial infarction in both men and women: evidence for the fibrinolytic system as an independent primary risk factor. ***Circulation,*** **98, 2241-7.**

THOMPSON, D. G., RITCHIE, H. D. & WINGATE, D. L. 1982. Patterns of small intestinal motility in duodenal ulcer patients before and after vagotomy. ***Gut,*** **23, 517-23.**

THOMPSON, J. C., FENDER, H. R., RAMUS, N. I., VILLAR, H. V. & RAYFORD, P. L. 1975. Cholecystokinin metabolism in man and dogs. ***Ann Surg,*** **182, 496-504.**

TOSHINAI, K., YAMAGUCHI, H., SUN, Y., SMITH, R. G., YAMANAKA, A., SAKURAI, T., DATE, Y., MONDAL, M. S., SHIMBARA, T., KAWAGOE, T., MURAKAMI, N., MIYAZATO, M., KANGAWA, K. & NAKAZATO, M. 2006. Des-acyl ghrelin induces food intake by a mechanism independent of the growth hormone secretagogue receptor. ***Endocrinology,*** **147, 2306-14.**

TOURINO, C., OVEISI, F., LOCKNEY, J., PIOMELLI, D. & MALDONADO, R. 2010. FAAH deficiency promotes energy storage and enhances the motivation for food. ***International Journal of Obesity,*** **34, 557-568.**

TSCHOP, M., CASTANEDA, T. R., JOOST, H. G., THONE-REINEKE, C., ORTMANN, S., KLAUS, S., HAGAN, M. M., CHANDLER, P. C., OSWALD, K. D., BENOIT, S. C., SEELEY, R. J., KINZIG, K. P., MORAN, T. H., BECK-SICKINGER, A. G., KOGLIN, N., RODGERS, R. J., BLUNDELL, J. E., ISHII, Y., BEATTIE, A. H., HOLCH, P., ALLISON, D. B., RAUN, K., MADSEN, K., WULFF, B. S., STIDSEN, C. E., BIRRINGER, M., KREUZER, O. J., SCHINDLER, M., ARNDT, K., RUDOLF, K., MARK, M., DENG, X. Y., WHITCOMB, D. C., HALEM, H., TAYLOR, J., DONG, J., DATTA, R., CULLER, M., CRANEY, S., FLORA, D., SMILEY, D. & HEIMAN, M. L. 2004. Physiology: does gut hormone PYY3-36 decrease food intake in rodents? ***Nature,*** **430, 1 p following 165; discussion 2 p following 165.**

TSCHOP, M., SMILEY, D. L. & HEIMAN, M. L. 2000. Ghrelin induces adiposity in rodents. ***Nature,*** **407, 908-13.**

TSCHOP, M., WAWARTA, R., RIEPL, R. L., FRIEDRICH, S., BIDLINGMAIER, M., LANDGRAF, R. & FOLWACZNY, C. 2001. Post-prandial decrease of circulating human ghrelin levels. ***J Endocrinol Invest,*** **24, RC19-21.**

TSIGOS, C., HAINER, V., BASDEVANT, A., FINER, N., FRIED, M., MATHUS-VLIEGEN, E., MICIC, D., MAISLOS, M., ROMAN, G., SCHUTZ, Y., TOPLAK, H., ZAHORSKA-MARKIEWICZ, B. & OBESITY MANAGEMENT TASK FORCE OF THE EUROPEAN ASSOCIATION FOR THE STUDY OF, O. 2008. Management of obesity in adults: European clinical practice guidelines. ***Obes Facts,*** **1, 106-16.**

TSUKIYAMA, K., YAMADA, Y., YAMADA, C., HARADA, N., KAWASAKI, Y., OGURA, M., BESSHO, K., LI, M., AMIZUKA, N., SATO, M., UDAGAWA, N., TAKAHASHI, N., TANAKA, K., OISO, Y. & SEINO, Y. 2006. Gastric inhibitory polypeptide as an endogenous factor promoting new bone formation after food ingestion. ***Mol Endocrinol,*** **20, 1644-51.**

TUCCI, S. A., ROGERS, E. K., KORBONITS, M. & KIRKHAM, T. C. 2004. The cannabinoid CB1 receptor antagonist SR141716 blocks the orexigenic effects of intrahypothalamic ghrelin. ***Br J Pharmacol,*** **143, 520-3.**

TURNBAUGH, P. J., BACKHED, F., FULTON, L. & GORDON, J. I. 2008. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. ***Cell Host Microbe,*** **3, 213-23.**

TURNBAUGH, P. J., LEY, R. E., MAHOWALD, M. A., MAGRINI, V., MARDIS, E. R. & GORDON, J. I. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. ***Nature,*** **444, 1027-31.**

TURTON, M. D., O'SHEA, D., GUNN, I., BEAK, S. A., EDWARDS, C. M., MEERAN, K., CHOI, S. J., TAYLOR, G. M., HEATH, M. M., LAMBERT, P. D., WILDING, J. P., SMITH, D. M., GHATEI, M. A., HERBERT, J. & BLOOM, S. R. 1996. A role for glucagon-like peptide-1 in the central regulation of feeding. ***Nature,*** **379, 69-72.**

UHE, A. M., SZMUKLER, G. I., COLLIER, G. R., HANSKY, J., O'DEA, K. & YOUNG, G. P. 1992. Potential regulators of feeding behavior in anorexia nervosa. ***Am J Clin Nutr,*** **55, 28-32.**

UKKOLA, O. H., PUURUNEN, V. P., PIIRA, O. P., NIVA, J. T., LEPOJARVI, E. S., TULPPO, M. P. & HUIKURI, H. V. 2011. High serum fasting peptide YY (3-36) is associated with obesity-associated insulin resistance and type 2 diabetes. ***Regul Pept,*** **170, 38-42.**

ULRICH, C. D., 2ND, WOOD, P., HADAC, E. M., KOPRAS, E., WHITCOMB, D. C. & MILLER, L. J. 1998. Cellular distribution of secretin receptor expression in rat pancreas. ***Am J Physiol,*** **275, G1437-44.**

UNGER, R. H., OHNEDA, A., VALVERDE, I., EISENTRAUT, A. M. & EXTON, J. 1968. Characterization of the responses of circulating glucagon-like immunoreactivity to intraduodenal and intravenous administration of glucose. ***J Clin Invest,*** **47, 48-65.**

URLINGER, S., BARON, U., THELLMANN, M., HASAN, M. T., BUJARD, H. & HILLEN, W. 2000. Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. ***Proc Natl Acad Sci U S A,*** **97, 7963-8.**

USDIN, T. B., BONNER, T. I. & MEZEY, E. 1994. Two receptors for vasoactive intestinal polypeptide with similar specificity and complementary distributions. ***Endocrinology,*** **135, 2662-80.**

USDIN, T. B., MEZEY, E., BUTTON, D. C., BROWNSTEIN, M. J. & BONNER, T. I. 1993. Gastric inhibitory polypeptide receptor, a member of the secretin-vasoactive intestinal peptide receptor family, is widely distributed in peripheral organs and the brain. ***Endocrinology,*** **133, 2861-70.**

VAISSE, C., HALAAS, J. L., HORVATH, C. M., DARNELL, J. E., JR., STOFFEL, M. & FRIEDMAN, J. M. 1996. Leptin activation of Stat3 in the hypothalamus of wild-type and ob/ob mice but not db/db mice. ***Nat Genet,*** **14, 95-7.**

VARMA, V., YAO-BORENGASSER, A., RASOULI, N., NOLEN, G. T., PHANAVANH, B., STARKS, T., GURLEY, C., SIMPSON, P., MCGEHEE, R. E., JR., KERN, P. A. & PETERSON, C. A. 2009. Muscle inflammatory response and insulin resistance: synergistic interaction between macrophages and fatty acids leads to impaired insulin action. ***Am J Physiol Endocrinol Metab,*** **296, E1300-10.**

VERBALIS, J. G., STRICKER, E. M., ROBINSON, A. G. & HOFFMAN, G. E. 1991. Cholecystokinin activates C-fos expression in hypothalamic oxytocin and corticotropin-releasing hormone neurons. ***J Neuroendocrinol,*** **3, 205-13.**

VERDICH, C., FLINT, A., GUTZWILLER, J. P., NASLUND, E., BEGLINGER, C., HELLSTROM, P. M., LONG, S. J., MORGAN, L. M., HOLST, J. J. & ASTRUP, A. 2001a. A meta-analysis of the effect of glucagon-like peptide-1 (7-36) amide on ad libitum energy intake in humans. ***J Clin Endocrinol Metab,*** **86, 4382-9.**

VERDICH, C., TOUBRO, S., BUEMANN, B., LYSGARD MADSEN, J., JUUL HOLST, J. & ASTRUP, A. 2001b. The role of postprandial releases of insulin and incretin hormones in meal-induced satiety--effect of obesity and weight reduction. ***Int J Obes Relat Metab Disord,*** **25, 1206-14.**

VETTOR, R., FABRIS, R., PAGANO, C. & FEDERSPIL, G. 2002. Neuroendocrine regulation of eating behavior. ***J Endocrinol Invest,*** **25, 836-54.**

VIANNA, C. R., DONATO, J., JR., ROSSI, J., SCOTT, M., ECONOMIDES, K., GAUTRON, L., PIERPONT, S., ELIAS, C. F. & ELMQUIST, J. K. 2012. Cannabinoid receptor 1 in the vagus nerve is dispensable for body weight homeostasis but required for normal gastrointestinal motility. ***J Neurosci,*** **32, 10331-7.**

VILLANUEVA, E. C. & MYERS, M. G., JR. 2008. Leptin receptor signaling and the regulation of mammalian physiology. ***Int J Obes (Lond),*** **32 Suppl 7, S8-12.**

VRIEZE, A., VAN NOOD, E., HOLLEMAN, F., SALOJARVI, J., KOOTTE, R. S., BARTELSMAN, J. F., DALLINGA-THIE, G. M., ACKERMANS, M. T., SERLIE, M. J., OOZEER, R., DERRIEN, M., DRUESNE, A., VAN HYLCKAMA VLIEG, J. E., BLOKS, V. W., GROEN, A. K., HEILIG, H. G., ZOETENDAL, E. G., STROES, E. S., DE VOS, W. M., HOEKSTRA, J. B. & NIEUWDORP, M. 2012. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. *Gastroenterology,* 143, 913-6 e7.

WANG, L., BARACHINA, M. D., MARTINEZ, V., WEI, J. Y. & TACHE, Y. 2000. Synergistic interaction between CCK and leptin to regulate food intake. *Regul Pept,* 92, 79-85.

WANG, L., CARDIN, S., MARTINEZ, V., TACHE, Y. & LLOYD, K. C. 1999. Duodenal loading with glucose induces fos expression in rat brain: selective blockade by devazepide. *Am J Physiol,* 277, R667-74.

WANG, X., MIYARES, R. L. & AHERN, G. P. 2005. Oleoylethanolamide excites vagal sensory neurones, induces visceral pain and reduces short-term food intake in mice via capsaicin receptor TRPV1. *J Physiol,* 564, 541-7.

WANG, Y. H., TACHE, Y., SHEIBEL, A. B., GO, V. L. & WEI, J. Y. 1997. Two types of leptin-responsive gastric vagal afferent terminals: an in vitro single-unit study in rats. *Am J Physiol,* 273, R833-7.

WANK, S. A. 1995. Cholecystokinin receptors. *Am J Physiol,* 269, G628-46.

WASSERMAN, D. H., SPALDING, J. A., LACY, D. B., COLBURN, C. A., GOLDSTEIN, R. E. & CHERRINGTON, A. D. 1989. Glucagon is a primary controller of hepatic glycogenolysis and gluconeogenesis during muscular work. *Am J Physiol,* 257, E108-17.

WEI, Y., WALTZ, D. A., RAO, N., DRUMMOND, R. J., ROSENBERG, S. & CHAPMAN, H. A. 1994. Identification of the urokinase receptor as an adhesion receptor for vitronectin. *J Biol Chem,* 269, 32380-8.

WETTERGREN, A., SCHJOLDAGER, B., MORTENSEN, P. E., MYHRE, J., CHRISTIANSEN, J. & HOLST, J. J. 1993. Truncated GLP-1 (proglucagon 78-107-amide) inhibits gastric and pancreatic functions in man. *Dig Dis Sci,* 38, 665-73.

WHO 2000. Obesity: preventing and managing the global epidemic: report of WHO consultation. ***Geneva, Tech Rep Ser,*** **894, 1-253.**

WILDING, J. P. H. 2007. Treatment strategies for obesity. ***Obesity Reviews,*** **8, 137-144.**

WILLIAMS, J. R. 1951. The fibrinolytic activity of urine. ***Br J Exp Pathol,*** **32, 530-7.**

WYNNE, K., FIELD, B. C. & BLOOM, S. R. 2010. The mechanism of action for oxyntomodulin in the regulation of obesity. ***Curr Opin Investig Drugs,*** **11, 1151-7.**

WYNNE, K., PARK, A. J., SMALL, C. J., PATTERSON, M., ELLIS, S. M., MURPHY, K. G., WREN, A. M., FROST, G. S., MEERAN, K., GHATEI, M. A. & BLOOM, S. R. 2005. Subcutaneous oxyntomodulin reduces body weight in overweight and obese subjects: a double-blind, randomized, controlled trial. ***Diabetes,*** **54, 2390-5.**

XIAO, Q., BOUSHEY, R. P., DRUCKER, D. J. & BRUBAKER, P. L. 1999. Secretion of the intestinotropic hormone glucagon-like peptide 2 is differentially regulated by nutrients in humans. ***Gastroenterology,*** **117, 99-105.**

YAMAMOTO, K. & LOSKUTOFF, D. J. 1996. Fibrin deposition in tissues from endotoxin-treated mice correlates with decreases in the expression of urokinase-type but not tissue-type plasminogen activator. ***J Clin Invest,*** **97, 2440-51.**

YAMATO, E., IKEGAMI, H., TAKEKAWA, K., FUJISAWA, T., NAKAGAWA, Y., HAMADA, Y., UEDA, H. & OGIHARA, T. 1997. Tissue-specific and glucose-dependent expression of receptor genes for glucagon and glucagon-like peptide-1 (GLP-1). ***Horm Metab Res,*** **29, 56-9.**

YANG, Y., CHEN, M., GEORGESON, K. E. & HARMON, C. M. 2007. Mechanism of oleoylethanolamide on fatty acid uptake in small intestine after food intake and body weight reduction. ***Am J Physiol Regul Integr Comp Physiol,*** **292, R235-41.**

YUNG, W. H., LEUNG, P. S., NG, S. S., ZHANG, J., CHAN, S. C. & CHOW, B. K. 2001. Secretin facilitates GABA transmission in the cerebellum. ***J Neurosci,*** **21, 7063-8.**

YUSTA, B., HUANG, L., MUNROE, D., WOLFF, G., FANTASKE, R., SHARMA, S., DEMCHYSHYN, L., ASA, S. L. & DRUCKER, D. J. 2000. Enteroendocrine localization of GLP-2 receptor expression in humans and rodents. ***Gastroenterology,*** **119, 744-55.**

ZEYDA, M., GOLLINGER, K., KRIEHUBER, E., KIEFER, F. W., NEUHOFER, A. & STULNIG, T. M. 2010. Newly identified adipose tissue macrophage populations in obesity with distinct chemokine and chemokine receptor expression. ***Int J Obes (Lond),*** **34, 1684-94.**

ZHANG, X., ZHANG, G., ZHANG, H., KARIN, M., BAI, H. & CAI, D. 2008. Hypothalamic IKKbeta/NF-kappaB and ER stress link overnutrition to energy imbalance and obesity. ***Cell,*** **135, 61-73.**

ZHANG, Y., PROENCA, R., MAFFEI, M., BARONE, M., LEOPOLD, L. & FRIEDMAN, J. M. 1994. Positional cloning of the mouse obese gene and its human homologue. ***Nature,*** **372, 425-32.**

ZITTEL, T. T., GLATZLE, J., KREIS, M. E., STARLINGER, M., EICHNER, M., RAYBOULD, H. E., BECKER, H. D. & JEHLE, E. C. 1999a. C-fos protein expression in the nucleus of the solitary tract correlates with cholecystokinin dose injected and food intake in rats. ***Brain Res,*** **846, 1-11.**

ZITTEL, T. T., GLATZLE, J., KREIS, M. E., STARLINGER, M., EICHNER, M., RAYBOULD, H. E., BECKER, H. D. & JEHLE, E. C. 1999b. C-fos protein expression in the nucleus of the solitary tract correlates with cholecystokinin dose injected and food intake in rats. ***Brain Research,*** **846, 1-11.**