# Modulation of neuropeptide W on gastric vagal afferents

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## ABSTRACT

**Background:** Gastric vagal afferents play an important role in the regulation of food intake in response to mechanical stimuli. In the stomach neuropeptide W (NPW) is secreted from G-cells. It is known that NPW is involved in central regulation of food intake and energy homeostasis, however, whether NPW can modulate gastric vagal afferents mechanosensitivity and how this role changes in different nutritional states, such as obesity, is not known. Furthermore, the role of different macronutrients in NPW expression and secretion in the stomach is not clear.

**Aims:** This thesis aims to determine:

1) The modulatory effect of NPW on gastric vagal afferent mechanosensitivity under different states of nutrition including food restriction and high-fat diet-induced obese mice.
2) The modulatory effect of NPW on gastric vagal afferent mechanosensitivity in mice of different age and gender.
3) The macronutrients responsible for regulation of gastric NPW.

**Methods:** An *in vitro* electrophysiology preparation was used to determine the effect of NPW on the mechanosensitivity of gastric mucosal and tension receptors in C57BL/6 mice fed *ad libitum*, fasted overnight, or fed with a high-fat diet. Expression of NPW in the gastric mucosa and GPR7 in the whole nodose ganglia was determined by quantitative RT-PCR (QRT-PCR). Expression of GPR7 in gastric vagal afferent neurons was determined by retrograde tracing and QRT-PCR. Plasma NPW levels were determined in healthy lean subjects after nutrient intake. Plasma and gastric NPW levels were determined in mice after feeding with different nutrients. Primary cell cultures of

mouse gastric antral mucosal cells were used to investigate the signalling pathway of NPW expression.

**Results:** In 20-week-old adult mice NPW selectively inhibited the responses of gastric vagal tension receptors to stretch. The inhibitory effect of NPW on gastric vagal tension receptors was gender consistent, but not observed in younger mice, high-fat diet-fed mice or food restricted mice. Protein and glucose intake increased gastric NPW transcript and protein levels in mice but had no effect on plasma NPW levels in human and mice. Protein and glucose are stimulants of gastric NPW expression, via distinct mechanisms.

**Conclusion:** NPW modulates mechanosensitivity of gastric vagal afferents; an effect related to feeding status, age and gender. Gastric NPW is regulated by specific nutrients.

## STATEMENT

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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## CONFERENCE PROCEEDINGS

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**Li H.**, Kentish S.J., O'Donnell T.A., Wittert G., Blackshaw L.A., Page A.J. (2012) The role of gastric neuropeptide W is regulated by food intake. Australian and New Zealand Obesity Society 2012, Auckland, New Zealand.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AgRP | agouti-related peptide |
| α-MSH | α-melanocyte stimulating hormone |
| ARC | arcuate nucleus |
| BMI | body mass index |
| CART | cocaine and amphetamine regulated transcript |
| CaSR | calcium sensing receptor |
| CB1 receptor | cannabinoid receptor type 1 |
| CCK | cholecystokinin |
| CNS | central nervous system |
| CRH | corticotropin-releasing hormone |
| DH | dorsal hypothalamus |
| DMN | dorsomedial nucleus |
| DMV | dorsal motor nucleus of vagus |
| DRN | dorsal raphe nucleus |
| EW | Edinger-Westphal nucleus |
| GI | gastrointestinal |
| GLP-1 | glucagon-like peptide-1 |
| GPCRs | G-protein-coupled receptors |
| GPR7 | G-protein-coupled receptor 7 |
| GPR8 | G-protein-coupled receptor 8 |
| GHRH | growth-hormone-releasing hormone |
| GHSR | growth hormone secretagogue receptor |
| HFD | high-fat diet |
| HPA axis | hypothalamus-pituitary-adrenal axis |

| | |
|---|---|
| HPT axis | hypothalamus-pituitary-thyroid axis |
| I.c.v. | intracerebroventricular |
| I.p. | intraperitoneal |
| IGLEs | intraganglionic laminar endings |
| IMAs | intramuscular arrays |
| LHA | lateral hypothalamic area |
| MCH | melanin-concentrating hormone |
| MCH1R | melanin-concentrating hormone-1 receptor |
| NPB | neuropeptide B |
| NPW | neuropeptide W |
| NPY | neuropeptide Y |
| NTS | nucleus tractus solitaries |
| PAG | periaqueductal gray |
| POMC | pro-opiomelanocortin |
| PVN | paraventricular nucleus |
| PYY | peptide YY |
| SON | supraoptic nucleus |
| SCN | suprachiasmatic nucleus |
| TRH | thyrotropin-releasing hormone |
| VMN | ventral medial nucleus |
| VTA | ventral tegmental area |

# CHAPTER 1. GENERAL INTRODUCTION

## 1.1 Obesity

There has been a growing concern about obesity worldwide. According to the World Health Organization, global obesity has nearly doubled since 1980. In 2008 35% of adults, aged 20 and over, were overweight (body mass index (BMI) $\geq$ 25) and 11% were obese (BMI $\geq$ 30). In the United States, the prevalence of obesity was 35.5% among adult men and 35.8% among adult women in 2009-2010 [1]. Once considered as a problem only in high-income countries, obesity has extended to low-income and middle-income countries such as Brazil and Africa that typically had very low incidence of obesity [2, 3]. The prevalence of overweight and obesity in children and adolescent has also dramatically increased over the past decades. In 2009-2010, the prevalence of adolescent obesity was approaching 20% in the United States [4].

Obesity increases the risk of a number of medical conditions including type II diabetes mellitus, cardiovascular disease, respiratory conditions and numerous types of cancer [3]. Many of these diseases can be prevented or ameliorated by loss of body weight in obese or overweight patients [5, 6]. Each year at least 2.8 million adults die as a result of being overweight or obese. The enormous health, economic and social burden caused by obesity support it as a major health problem and lead to the urgent need for the prevention and treatment of obesity.

Current strategies for the management of obesity include low-calorie diet, physical activity, and behaviour modification. These approaches have been shown to be effective in the short-term reduction of body weight. However, diet and physical strategies alone are less likely to achieve weight control in the long term due to the difficulty in maintenance and associated rebound weight gain [7, 8]. Several anti-obesity drugs have been developed, mainly targeting appetite or energy absorption. However, the role of

these drugs in weight loss is not optimistic [9]. Phetermine, an appetite suppressant, is one of the most commonly used anti-obesity medicines in the United States. It can induce an 8-10% weight loss, but is associated with elevation in heart rate and blood pressure. Orlistat, an inhibitor of dietary fat absorption, is less effective in weight loss, with only 15-30% of patients achieving > 5% weight loss after 1 year of therapy, and is associated with gastrointestinal side effects. Due to the lack of efficiency and associated side effects, the acceptance and usage of anti-obesity drugs are limited. Bariatric surgery is currently the most effective treatment for weight loss in obese individuals. It results in sustained weight loss and significantly improved health risks [10, 11]. However, its cost, associated mortality, and selection criteria only for patients with morbid obesity make it impractical to prevent the increasing prevalence of obesity [12]. The development of new anti-obesity treatments is therefore important. Understanding the mechanisms of appetite regulation and the development of obesity are critical for future development of effective anti-obesity treatments.

### 1.2 Appetite regulation

The mechanism of obesity is not fully understood, but based on the law of thermodynamics, obesity and overweight conditions are the result of a higher energy input than energy output. Excessive energy consumption is mainly stored in the form of body fat, leading to obesity and overweight conditions. Therefore, regulating food intake is an important target for maintaining normal body weight.

Individual meal intake varies based on a wide range of factors including the availability and palatability of foods, mood, environmental factors and lifestyle factors. However in general, in response to food intake, satiety perception is gradually developed and meal intake is terminated, while with prolonged fasting periods, hunger signals gradually

accumulate and initiate food intake [13]. In the long run, food intake over a period is well controlled under normal physiological conditions. Body weight is stabilized at a specific level by regulation of food intake and energy expenditure, reflecting a well biologically controlled process protecting normal body weight [14, 15].

Discoveries over the past few decades have identified several factors and their signalling pathways that regulate short-term and long-term food intake and energy homeostasis. The central nervous system (CNS) is a key site in the appetite regulatory system, which receives, integrates and processes a wide range of signals, to regulate food intake. Dysfunction of some of these signalling pathways has been observed in the obese state, suggesting crucial mechanisms of disrupted appetite regulation. These mechanisms might represent potential targets for therapeutic interventions in obesity [16].

### 1.2.1 Neuronal circuits in the central nervous system

The CNS receives diverse vascular and afferent neural signals from the periphery regarding energy storage and nutritional status, and responds to regulate feeding behaviour and energy expenditure. The main regions within the CNS responsible for the regulation of food intake include the hypothalamus and the nucleus tractus solitarius (NTS) in the brainstem. Other areas such as amygdala, the prefrontal cortex, septal nuclei and the area postrema also participate in the regulation of food intake [17-19]. In the hypothalamus, several regions play essential roles in the regulation of energy homeostasis, including arcuate nucleus (ARC), paraventricular nucleus (PVN), lateral hypothalamic area (LHA), ventral medial nucleus (VMN) and dorsomedial nucleus (DMN), which are illustrated in Figure 1.1.

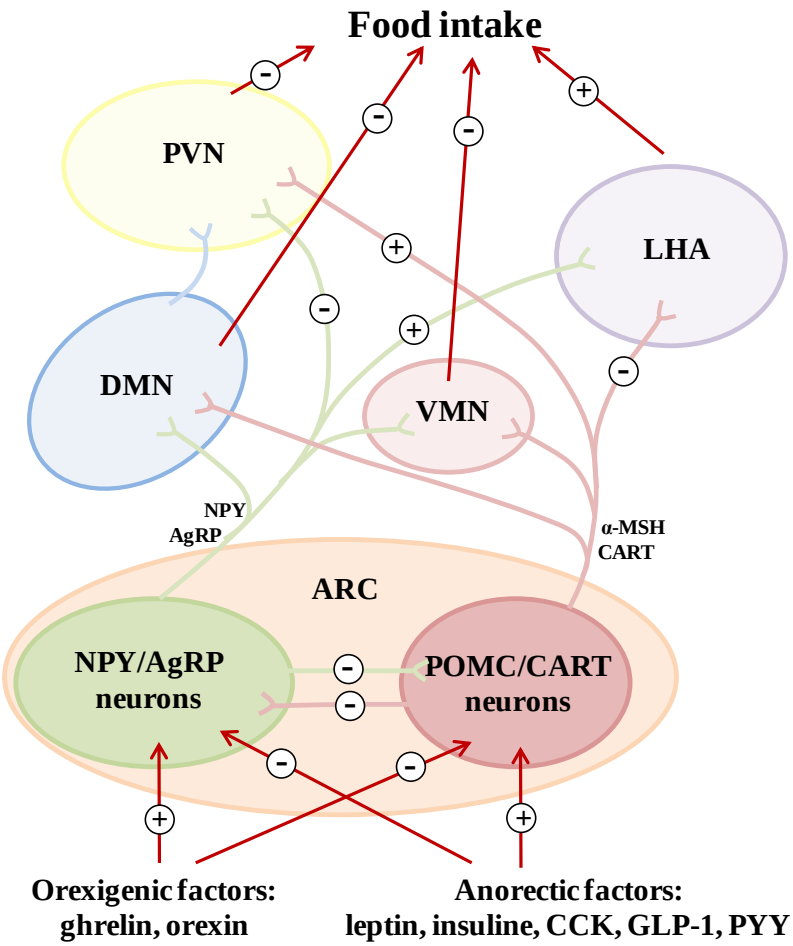


**Figure 1.1 Major hypothalamic regions involved in the control of food intake and energy homeostasis**

The major hypothalamic nuclei controlling food intake include arcuate nucleus (ARC), paraventricular nucleus (PVN), lateral hypothalamic area (LHA), ventral medial nucleus (VMN) and the dorsomedial nucleus (DMN). The ARC is the major sites receiving hormonal inputs through the circulation (other hypothalamic nuclei also directly receive hormonal signals; not shown here). The ARC contains neuropeptide Y/ agouti-related peptide (NPY/AgRP) containing neurons and pro-opiomelanocortin/cocaine and amphetamine regulated transcript (POMC/CART) containing neurons. NPY/AgRP neurons are stimulated by circulating orexigenic factors and inhibited by anorectic factors; a reverse effect is found with POMC/CART neurons. NPY/AgRP neurons have extensive projections to the PVN, LHA, DMN and the VMN to exert orexigenic effect by releasing NPY and AgRP. POMC/CART neurons directly regulate the PVN, LHA, VMN and the DMN by releasing α-melanocyte stimulating hormone (α-MSH) and CART, which exert anorectic effect.

The ARC, an elongate collection of neurons adjacent to the floor of the third ventricle with a weak blood-brain barrier, occupies approximately one-half of the length of the hypothalamus [20]. It is important in receiving hormonal signals from the circulation [20]. Two adjacent and distinct subsets of neurons are localized in the ARC, the neurons containing neuropeptide Y (NPY) and agouti-related peptide (AgRP), and the neurons containing pro-opiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) [21, 22]. Both of these neurons are major sites for transducing information from circulating hormones. NPY/AgRP neurons are stimulated by the orexigenic factor ghrelin [23], and inhibited by the anorectic factors leptin, insulin and peptide YY3-36 (PYY3-36) [24, 25]. These neurons release NPY and AgRP that stimulate food intake via distinct mechanisms. In contrast, POMC/CART neurons are stimulated by the anorectic factors leptin and PYY3-36 [25], and inhibited by the orexigenic factor ghrelin [26]. These neurons release α-melanocyte stimulating hormone (α-MSH) and CART which decrease food intake. It is important to note that NPY/AgRP neurons and POMC/CART neurons also project directly to each other to suppress their activity [27, 28].

From the ARC, signals are transmitted via nerves to other hypothalamic areas including the PVN, LHA, DMN and VMN [29, 30]. PVN neurons are stimulated by POMC/CART signalling and inhibited by NPY/AgRP signalling and signal to other brain regions including the sympathetic nervous system and the thyroid axis. Several neuropeptides synthesized in the PVN can inhibit food intake, for example, corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH) [31-33]. LHA neurons are stimulated by NPY signalling, inhibited by POMC/CART signalling and release melanin-concentrating hormone (MCH) and orexins to increase food intake [34]. DMN lesion results in hyperphagia and obesity [35]. The DMN

contains NPY neurons as well as NPY terminals and α-MSH terminals originating from the ARC [36, 37]. Lesioning of the VMN results in rapid-onset hyperphagia and obesity, more dramatically than DMN lesioning [38]. The VMN receives projections from the NPY/AgRP and POMC/CART neurons of the ARC [39]. Although the ARC neurons are major targets for circulating hormones, other hypothalamic neurons can also be directly regulated by circulating hormones but generally to a lesser extent [16, 20]. Different populations of these hypothalamic nuclei have specific efferent projections to the spinal cord, brain stem, medial eminence, pituitary and other brain areas to regulate motor and autonomic responses, the endocrine system and cognitive, reward and emotion systems [39, 40].

There is extensive reciprocal communications between the hypothalamus and the brain stem. The information received by these regions is integrated to regulate food intake [41]. In the brain stem, the NTS, area postrema and dorsal motor nucleus of the vagus (DMV) are all involved in the regulation of energy homeostasis [42, 43]. Vagal afferents endings, conveying a variety of signals from the gastrointestinal (GI) tract and abdominal viscera, terminate in the NTS nucleus in the brain stem. Via second-order sensory neurons within the NTS, these signals are further spread to adjacent areas in the brain stem and other brain areas including the hypothalamus. DMV is the primary location of vagal efferent motor neurons, which mediate GI reflexes in response to food intake.

### 1.2.2 Peripheral appetite regulatory signals

Peripheral appetite regulatory signals are mainly generated from adipose tissue, the pancreas and the GI tract. The signals from adipose tissue and the pancreas are major signals reflecting energy storage, and therefore are responsible for long-term regulation

of food intake. GI signals (nutrients, mechanical stimulants, and hormones) generated before or post food ingestion are important in reflecting acute nutritional status, which are responsible for the regulation of short-term food intake. These signals are transmitted via both the blood circulation and afferent neuronal pathways to the CNS, as illustrated in Figure 1.2.

#### 1.2.2.1 Adipose signals

The "adipose negative-feedback" model of energy homeostasis was introduced more than 50 years ago. Two hormones insulin and leptin, released from the pancreas and adipose tissue respectively, are considered as adipose signals [20]. Both insulin and leptin circulate at levels proportional to the body fat mass [44, 45]. They enter the CNS via the blood circulation and activate their corresponding receptors expressed in the brain neurons controlling food intake [46, 47]. These signals inform the CNS of the information about body fat mass, and then the brain regulates energy homeostasis to stabilize body fat stores. Central administration of these hormones inhibits food intake [48, 49], while deficiency of these hormones within the CNS exerts opposite effects [50, 51]. In human and rodents, obesity is associated with both insulin resistance and leptin resistance [52-54]. The reduced neuronal signalling by these hormones induces hyperphagia and weight gain, which may contribute to the development of obesity [55, 56].

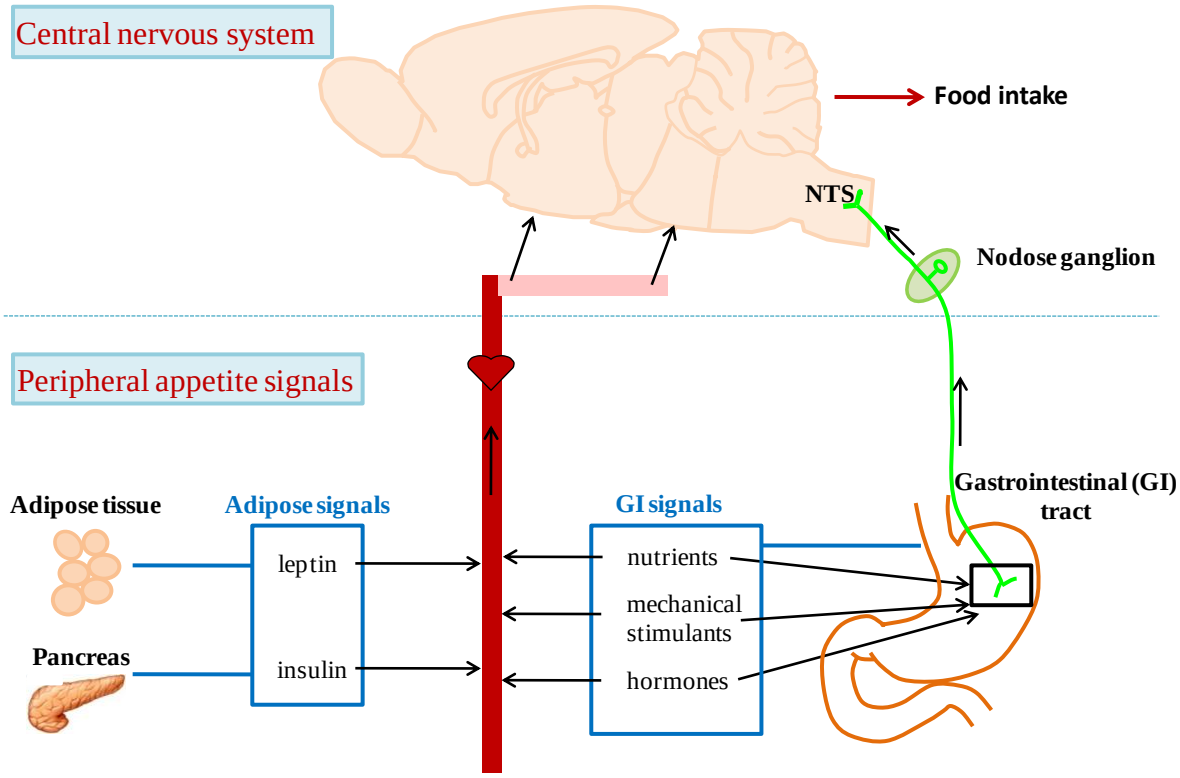


**Figure 1.2 Central nervous system sensing of peripheral signals involved in the regulation of food intake**

The central nervous system (CNS) receives and integrates peripheral appetite regulatory signals via the blood circulation and afferent neural pathways (mainly vagal afferents), to stimulate or inhibit food intake. Two hormones insulin and leptin, released from the pancreas and adipose tissue respectively, are considered as adipose signals. These signals enter the CNS via the blood circulation sending information about body fat mass. This is important in the long-term regulation of energy homeostasis to stabilize body fat store. Gastrointestinal (GI) signals are generated upon food consumption within GI tract. These signals are initiated by nutrients, mechanical stimulants, and hormones, and sent to the CNS via the blood circulation or to the nucleus tractus solitaries (NTS), in the brainstem, via vagal afferents innervating the GI tract. These signals are important in the short-term regulation of food intake.

### 1.2.2.2 Gastrointestinal signals

The GI tract is the first organ in the process of food intake. In addition to food digestion and absorption, the GI tract also generates signals to regulate short-term food intake. These signals mainly arise from the upper GI tract, i.e., the stomach and small intestine, and consist of detection of either specific nutrients and/or mechanical stimuli with the subsequent release of GI hormones and/or activation of neural pathways. These signals are then transferred to the CNS via the blood circulation or/and neural pathway, as illustrated in Figure 1.2.

#### 1.2.2.2.1 Nutrients

***Nutrient induced GI hormone release*:** As digested food progresses through the GI tract, the enteroendocrine cells scattered along the epithelium can be activated and secret a group of GI hormones, some of which are peripheral appetite regulators. The physiological and pathological functions of these hormones are discussed in detail in section 1.2.2.2.2. The secretion of these hormones could be stimulated by several factors, including mechanical stimulation, nutrients, other hormones and neural pathways [57]. Among them, macronutrients (i.e. protein, carbohydrate and lipid) are generally major stimulants. Protein breakdown products are important in stimulating hormones release from the stomach, while lipid is more effective in releasing hormones in the small intestine [58, 59]. The luminal nutrients can be directly monitored by nutrients sensors located on the endocrine cells along GI tract. Calcium sensing receptors (CaSR), GPRC6A and T1R1/T1R3 are receptors for protein hydrolysate and amino acids. Glucose sensors include T1R2/T1R3, and fatty acid sensors include GPR41, GPR40, GPR43, GPR84, GPR120 and GPR119 [60]. The origination and

nutritional stimulants of some important GI hormones, as well as the nutrients sensors expressed on the secreting cells are listed in Table 1.1 [58, 60-63].

***Nutrient regulation of vagal afferent pathways*:** Absorbed nutrients can directly stimulate GI vagal afferents. It has been demonstrated that breakdown products of carbohydrates, protein and fats activate intestinal vagal afferents, which are important in inducing satiation [64-66]. Intraduodenal infusion of macronutrients induces the expression of c-fos in vagal afferent terminals within the NTS and area postrema of the brain stem [67]. Furthermore, subdiaphragmatic vagotomy or abdominal afferent denervation largely or completely blocks the inhibitory effect of intestinally infused carbohydrates and fatty acids on food intake [68, 69]. Therefore, nutrients can regulate food intake partly via vagal afferents.

***Nutrient sensing in the CNS*:** Some nutrients such as free fatty acids can directly exert an anorectic effect in brain areas involved in energy homeostasis. Long-chain fatty acid oleic acid has a potent effect on hypothalamic neurons and likely on other CNS neurons by favouring intracellular accumulation of long chain fatty acyl-CoA, which in turn activates a chain of neuronal events, including inhibition of the expression of NPY in the hypothalamus, to inhibit food intake [70, 71]. This process could be a mechanism by which the CNS maintains energy and metabolic homeostasis in response to increased nutrient signals from the circulation. Inhibition of this signalling in the hypothalamus induce a rapid increase in food intake and body weight, further confirming that the central nutrient-sensing pathway is essential in controlling food intake and energy homeostasis [72].

**Table 1.1 Nutritional regulation of gastrointestinal hormones secretion**

| Hormone | Origination | Cell type | Nutritional stimuli | Nutrients sensors |
|---|---|---|---|---|
| leptin | Stomach | Chief, P | ? | ? |
| gastrin | Stomach | G | protein*, lipid, carbohydrate | GPRC6A, CaSR, GPR40 |
| PYY | small intestine | L | lipid*, carbohydrate, protein | GPR41, GPR43, GPR40, GPR119 |
| GLP-1 | small intestine | L | lipid*, carbohydrate*, protein | T1R2/ T1R3, GPR43, GPR40, GPR120 |
| CCK | small intestine | I | lipid*, protein*, carbohydrate | T1R2, GPR93, CaSR, GPR41, GPR40, GPR120 |
| GIP | small intestine | K | lipid*, carbohydrate*, protein | T1R2/T1R3, GPR40, GPR120, GPR119 |

Star designates major nutritional stimulus of the GI hormone. Question mark designates the corresponding information is not reported.

#### 1.2.2.2.2 Gastrointestinal hormones

The GI tract is the biggest endocrine organ within the body and releases more than 20 different hormones. Some of these hormones are stimulated in response to food intake [73, 74], as discussed in section 1.2.2.2.1, while others are induced by prolonged fasting but inhibited after food intake, such as ghrelin and orexin-A [75-77]. These hormones are involved in multiple physiological roles locally and distally, including regulation of food intake and GI functions such as gastric acid secretion, gastrointestinal motility and mucosal cell proliferation [78]. Many GI hormones are critical in the regulation of short-term food intake and energy homeostasis. Feeding stimulated hormones are usually satiety factors released to terminate a meal, while fasting induced hormones generally induce food intake. These hormones communicate with the CNS to control food intake through both hormonal and neuronal pathways. The hormonal pathway includes a direct action of these hormones, released into the circulation, on parts of the CNS involved in controlling food intake. The neuronal pathway primarily consists of vagal afferent nerves that innervate the GI tract. These nerves receive hormone signals and relay this information to the NTS in the brain stem where this information is ultimately transmitted to the hypothalamus. Some GI hormones are also expressed in the CNS controlling energy homeostasis, and the appetite regulatory actions of these hormones within the periphery are usually integrated with their functions in the CNS. In obesity, the secretion and appetite regulation of some hormones are changed, which may contribute to or compensate for the obese phenotype [79, 80]. The physiological functions of some important GI hormones and their roles in obesity are listed in Table 1.2.

**Table 1.2 Role of gastrointestinal hormones in food intake and obesity**

| Gastrointestinal hormones | Effect on food intake | Gastrointestinal function | Levels in obesity | Sensitivity in obesity |
|---|---|---|---|---|
| CCK | ↓[81] | gut motility↓[82], gastric empty↓[83], gallbladder contraction↑[84], pancreatic secretion↑[85] | ↑[86, 87] | ↔[88, 89] |
| GLP-1 | ↓[90] | GI motality↓[91], gastric empty↓[92], gastric acid secretion↓[93] | ↓[94] | ↔[95] |
| PYY3-36 | ↓[25] | Gastric empty↓[96] , gastric acid secretion↓[93] | ↓[97] | ↔[98] |
| leptin | ↓[99] | Mucosal cell proliferation↑[100], modulate immune function[101] | ↑[102] | ↓[103] |
| ghrelin | ↑[104] | motility↑ [105], acid secretion↑[106] | fasting↓[107] fed↑[108] | ↑[109] |

Arrows designates direction of the GI hormone function, and changes in level and sensitivity of GI hormone in obesity.

***Cholecystokinin*:** Cholecystokinin (CCK) is the most studied satiety hormone. It is secreted from I cells in the duodenal and jejuna mucosa in response to food intake [110]. Following secretion, CCK regulates multiple gastrointestinal functions including gut motility, gastric emptying, contraction of the gallbladder, and pancreatic enzyme secretion [82, 83]. Central and peripheral administration of CCK inhibits acute food intake [111, 112], but chronic administration of CCK has no effect on weight loss [113], suggesting CCK as a short-term satiety factor. Intestinal vagal afferent express CCK1 receptor and vagotomy eliminates the anorectic effects of CCK [114], suggesting CCK elicits anorexia via vagal afferent pathways. In obese subjects, plasma CCK levels are higher compared with lean subjects at fasting levels [86] and following nutrient ingestion [87]. The anorectic effect of exogenous CCK in obese subjects does not differ from that observed in lean subjects [89], suggesting the sensitivity to the satiation effect of CCK is not changed in the obese state. Taken together, in the obese state the satiation exerted by CCK would be increased rather than decreased.

***Glucagon-like peptide-1*:** Glucagon-like peptide-1 (GLP-1) is an incretin secreted from L cells in the ileum and colon in response to food intake. It plays multiple gastrointestinal functions including inhibition of GI motility, GI secretion and gastric emptying [91, 92]. Peripheral administration of GLP-1 inhibits food intake, which is probably related to its inhibitory effects on GI motility and gastric empty. Peripheral GLP-1 can cross the blood-brain barrier [115], and exert satiety effects via its receptors located in the hypothalamus [116, 117]. Centrally administration of GLP-1 also induce conditioned taste aversions [118] and endocrine and behavioural responses to stress [119], which are indirectly related to the control of food intake. In obese subjects, GLP-1 secretion is reduced, which may account for the reduced satiation in the obese state

[94]. A similar role of GLP-1 in food intake and gastric empty was observed in obese rodents and humans [95, 120].

***Peptide YY*:** PYY is secreted from L cells in the distal ileum and colon in response to food intake [121]. PYY3-36 is reported to reduce food intake in monkeys and humans [25, 96], although its role in rodents is not consistent [122]. Besides regulation of food intake, PYY also inhibits gastric empty and gastric secretion [93, 96]. In high-fat induced obese mice and obese patients, the anorectic effect of PYY is not changed, however, its circulating levels are reduced, which may contribute to the hyperphagia in obesity [97, 98].

***Leptin*:** Leptin is traditionally known to be released from adipocytes and responsible for long-term regulation of energy homeostasis. However, in the GI tract, leptin is also secreted from chief cells and P cells within the gastric mucosa in response to food intake [117, 123]. In the GI tract, leptin has multiple complex roles including the regulation of GI motility, nutrient absorption, mucosal cell proliferation and immune function [124]. Leptin receptor is also expressed in the nodose ganglia and leptin induced satiation is mediated by vagal afferents [125]. Leptin exerts multiple functions centrally and peripherally to control body weight, including inhibition of food intake and reducing body fat stores [126]. In the obese state, the levels of leptin in the circulation and stomach are increased, however, leptin resistance prevent the normal function of leptin [102, 103].

***Ghrelin*:** Ghrelin is a peptide secreted from X/A cells in the gastric fundus in fasting condition. Exogenous ghrelin increases food intake when administered centrally or peripherally, suggesting a role in initiating food intake [104]. In addition, ghrelin also stimulates GI motility, gastric acid secretion, and induces adiposity [105, 106, 127].

Ghrelin regulates energy homeostasis through direct modulation of the CNS as well as at the gastric level via vagal afferent nerves. In obese subjects, plasma ghrelin levels are decreased [107]. However, postprandial suppression of ghrelin levels are reduced in the obese state [108, 128-130], and obese subjects are more sensitive to ghrelin induced food intake [109], which may be responsible for the hyperphagia in obesity.

#### 1.2.2.2.3 Sensory innervation of the gastrointestinal tract

The GI tract is innervated by intrinsic and extrinsic sensory neurons, as illustrated in Figure 1.3. Intrinsic sensory neurons include neurons located in the myenteric plexus and the submucosal plexus, which innervate both mucosal and muscular layers of the GI tract [131]. They sense chemical and mechanical stimuli and provide information to the enteric nervous system to operate autonomic control of gut motility and secretion. Extrinsic sensory neurons include the vagus nerve and splanchnic mesenteric nerves, which convey information from the GI tract to the brain. Their nerve fibres supply different layers of the GI tract and respond to changes in chemical and mechanical environment in the GI lumen [132]. Vagal afferents are the primary neural signalling pathway involved in food intake, while the splanchnic afferents are mainly involved in signalling pain responses with limited evidence for a role in food intake regulation [133]. There are abundant synapses between enteric neurons and extrinsic sensory neurons, suggesting a direct means of communication between these nerves.The anatomy and function of GI vagal afferents in food intake and energy homeostasis is discussed in detail in section 1.3.

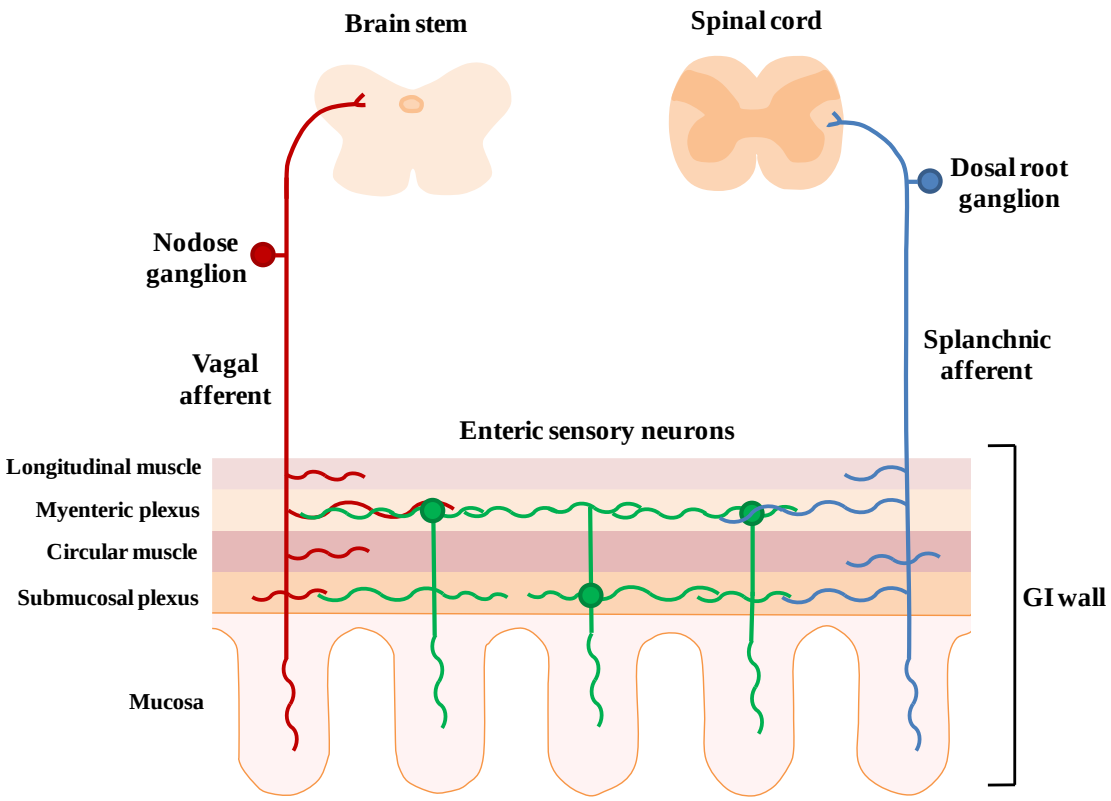


**Figure 1.3 Innervation of the gastrointestinal tract by intrinsic and extrinsic sensory neurons**

Gastrointestinal (GI) tract is innervated by both intrinsic and extrinsic sensory neurons. Intrinsic sensory neurons have their cell bodies in the myenteric plexus or submucosal plexus. Extrinsic sensory neurons include vagal afferents and splanchnic afferents. Vagal afferents have their cell bodies in the nodose ganglia and jugular ganglia (not shown), with axons projecting to the GI wall and brain stem. Splanchnic afferents have their cell bodies in the dosal root ganglion, and nerve endings in the GI wall and spinal cord. Extrinsic sensory nerve endings spread throughout the gut and make synaptic contact with enteric neurons.

## 1.3 Gastrointestinal vagal afferents

The involvement of vagal afferents in sensation and signalling GI information to the CNS has been appreciated for over a century. It is now clear that vagal afferents play important roles in energy homeostasis and GI functions in response to sensing mechanical stimuli (distension and contraction), chemicals (such as nutrients) and hormones. Vagal afferent satiety signals from the GI tract involve integration of signals from the stomach and small intestine. Gastric vagal afferents mainly respond to mechanical stimuli, and the small intestine is more important in sensing nutrients and releasing appetite regulatory hormones to regulate vagal afferents.

### 1.3.1 Anatomy of gastrointestinal vagal afferents

The vagus nerve is the longest carnial nerve, which has the widest distribution but predominantly supplies the thoracic and abdominal cavities. The vagal nerve contains both afferents and efferents, with the ratio of afferents to efferents between 8:1 and 10:1 for most mammalian species studied [134]. Neurons of vagal afferent nerves are localized in the nodose ganglia and jugular ganglia, which are bipolar. The peripheral nerve fibres innervate the oesophagus, lower airways, heart, aorta with cervical and thoracic branches, and the entire gastrointestinal tract, liver, portal vein, biliary system and pancreas via abdominal branches [135-137]. The central nerve fibres terminate on NTS neurons in the brain stem, ultimately transmitting to the hypothalamus and other brain areas [138]. The anatomy of vagal afferents innervating the GI tract described in both rodents and human, are generally similar [138, 139].

***Vagus nerve exit from the brain*:** The vagus nerve exits the brain stem through the medulla between the medullary pyramid and the inferior cerebellar peduncle. It leaves

the cranium through the jugular foramen. Immediately outside the cranium lie the two sensory ganglia of the vagus nerve, the jugular ganglia (superior) and more distally the nodose ganglia (inferior). The peripheral fibres separate into several branches on the way down to the abdomen.

***Vagus nerve branches into the gastrointestinal tract*:** After passing through the diaphragm, along the oesophagus, the left and right vagus become contiguous with the anterior and posterior vagal trunks. The anterior trunk, which is contiguous with the left vagus, branches into hepatic, ventral gastric and ventral celiac branches. The hepatic branch is then divided into a branch supplying the gastric antrum and pyloric sphincter, and a branch supplying the proximal duodenum and head of the pancreas [135, 140]. The ventral (anterior) and dorsal (posterior) gastric nerves are then formed from the oesophageal plexus. The ventral gastric is formed mainly from the left vagus, and the dorsal gastric nerves mainly from the right vagus. The ventral gastric branch supplies the ventral side of the stomach and the proximal duodenum. The dorsal branch supplies the dorsal side of the stomach and proximal duodenum. The ventral and dorsal celiac branches distribute to the small and large intestines along the superior mesenteric artery and its mesenteric offshoots.

***Vagal nerve endings in the gastrointestinal tract*:** In the GI tract, at least three morphological types of terminals are present: intraganglionic laminar endings (IGLEs), intramuscular arrays (IMAs) and mucosal vagal afferents. Studies have demonstrated a high similarity in the ending morphology and regional distribution of IGLEs and IMAs among closely related species, i.e., rat and different strains of mouse [138, 140-142].

IGLEs are abundant throughout the stomach and small intestine. They surround neurons of the myenteric plexus and lie between the longitudinal and circular muscle [138, 141].

Individual axons typically terminate in several separate but closely distributed IGLEs, which associate with neurons of adjacent myenteric ganglia [143]. About a half to one third of neurons in the gastric myenteric plexus receive at least one IGLE. IGLEs are found throughout different regions of the stomach in mouse, and the distribution of IGLEs ipsilateral to the injected nodose [143]. The peak IGLEs density was observed in the corpus/antrum near the pylorus of both mouse and rats [137, 143]. Given the good position to detect the intra-mural tension/shearing forces between the orthogonal smooth muscle layers, IGLEs represent a class of mechanosensor that responds to muscle tension generated by stretch [144].

IMAs are located in the circular and longitudinal muscle, consisting of several long and straight axons running parallel to smooth muscle fibres, interconnected by oblique or perpendicular connecting branches [142, 143]. They are most frequently encountered in the longitudinal muscle layer. Single afferents typically form arrays of telodendria in only one of the muscle layers, i.e. circular and longitudinal smooth muscle layers, but a few afferents supply both layers [143]. Most IMAs are simple, consisting of one or two processes, while some are composed of multiple interconnected elements of varying length [143]. Within the muscle sheet, IMAs form appositions consisting of spine-like processes or varicosities, with interstitial cells of Cajal and smooth muscle fibres [142, 143]. In both mouse and rat, IMAs are located predominantly in the forestomach, with the densest innervations of longitudinal IMAs near the greater curvature of the forestomach, and densest innervations of circular IMAs near the lesser curvature of the forestomach, however, IMAs are also present in the distal antrum and pylorus [143]. In the small intestine, IMAs are much less frequent than in the stomach [138]. The morphological features of IMAs suggest they are more likely to detect stretch.

Vagal afferents are moderately abundant in the stomach and most abundant in the proximal duodenum, and becoming relatively scarce in the distal small intestine [138]. Mucosal vagal afferents penetrate the muscle layer and the submucosa with endings located in the lamina propria of crypts and villi [145]. They respond to light stroking as well as luminal and arterially delivered nutrients and hormones, and therefore are both mechanosensors and chemosensors.

### 1.3.2 Intestinal vagal afferents satiety signals

Vagal afferents innervating the intestine are densely distributed in the duodenum and less densely distributed in the ileum and distal intestine [146]. Intestinal vagal afferents endings are sensitive to a wide range of food intake induced luminal stimuli, including mechanical stimuli and chemical stimuli [64, 66]. Evidence suggests that intestinal vagal afferents are responsible for the GI satiety signalling. Exposures of the small intestine to nutrients, such as glucose and lipid induce inhibition of food intake [67, 147]. This effect could be largely or completely blocked by vagal afferent denervation, suggesting the involvement of intestinal vagal afferents in postprandial satiety signalling [68, 69].

Evidence suggests intestinal vagal afferents satiety signals are related to hormones released by gut epithelia cells. Receptors for these hormones are identified in vagal afferent neurons in the nodose ganglia of humans and rodents, such as CCK, glucagon-like peptide-1 (GLP-1), and PYY3-36 [148-152]. The receptors expressed in vagal afferent neuron cell bodies can be transported via the axons to the vagal afferent terminals [150, 153], where they can be modulated by locally released hormones. Previous studies have shown that exogenous GLP-1 and CCK stimulate the discharge of vagal afferents [154, 155]. Subdiaphragmatic vagal deafferentation inhibits the CCK or

GLP-1 receptor agonist induced reduction in food intake [156, 157]. Furthermore, CCK receptor antagonists block the nutrient induced inhibition of food intake [158]. The evidence suggests that hormones released from the small intestine are responsible for the intestinal vagal afferents satiety signals.

### 1.3.3 Gastric vagal afferents satiety signals

Perception of fullness following food intake largely depend on the activation of gastric vagal afferents by mechanical distension. Rats with closed pyloric cuffs (to confine gastric preload to the stomach) demonstrated dose-dependent depression of food intake in response to gastric preload, an effect attenuated by selective vagotomy [159]. Load-sensitive gastric vagal afferents are not responsive to chemical composition of saline, carbohydrate, protein or fat solution [160], suggesting nutrients are less important in vagal afferent satiety signalling. In bariatric surgery, one mechanism for the weight loss is that the resulting restricted stomach enables the same load of food to exaggeratedly activate mechanoreceptive vagal afferents, signalling fullness to the CNS [161, 162]. Correspondingly, gastric vagal electrical stimulation in rats significantly reduced food intake and body weight [163]. Gastric vagal stimulation has been investigated and used successfully to treat obesity in humans [164]. Together, the evidence strongly suggests that gastric mechanosensitive vagal afferents play a key role in satiety signalling.

There are two functional types of mechanosensitive vagal afferent innervating the stomach, mucosal receptors and tension receptors, according to the location of their receptive fields [165-167]. The typical responses of these two receptors in mouse are shown in Figure 1.4.

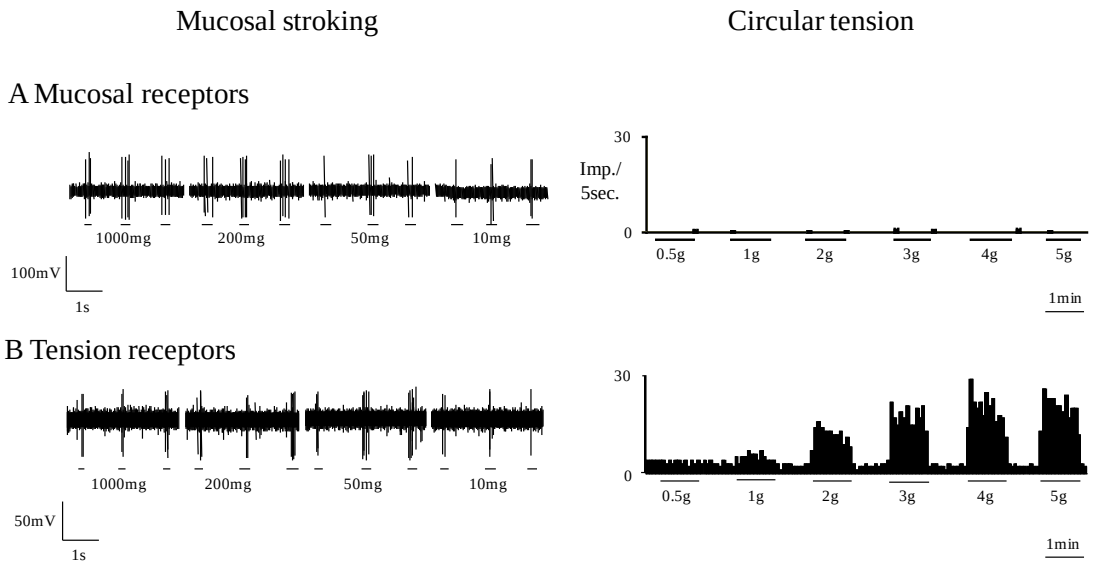


**Figure 1.4 Response of mouse gastro-oesophageal vagal afferents to mucosal stroking with calibrated von Frey hairs and circular tension [166]**

Typical response of a mucosal receptor (A) and a tension receptor (B) to mucosal stroking (left; raw trace) and circular tension (right; spike frequency). Mucosal receptors generate a burst of action potentials in responses to each mucosal stroke with a calibrated von Fry hair from 10 mg to 1000 mg, but has no responses to circular tension from 1 to 5 g. Tension receptor shows response to mucosal stroking, as well as slow-adapting tension dependent responses to circular stretch (0.5-5 g).

Mucosal receptors, located in the mucosal layer, are generally silent at rest and sensitive to light stroking of the mucosa. In mouse and ferret, mucosal receptors generate a burst of action potentials in response to light mechanical stroking (10 mg-1000 mg) over the receptive field [165, 166]. They are insensitive to contraction and distension of the gastric wall [166]. In the stomach, food particles are triturated into small particles before entering the duodenum. The function of mucosal receptors might be to selectively distinguish food particle size and thus provide negative feedback on gastric emptying [168-170].

Tension receptors, located in the muscular layer, often have a resting discharge. They show slowly adapting tension dependent responses to contraction and distension of the gastric wall [165, 166, 171]. In mouse stomach, tension receptors sense both mucosal stroking and circular tension [166], which could be due to the disturbance of tension receptors by light stroking through the thin gastric wall. They sense and transfer distension signals to the CNS to induce satiation and terminate food intake, and also trigger reflexes that control GI function [60, 172].

Hormones produced in the mucosa of the stomach have been shown to modulate the response of gastric vagal afferent mechanosensitive fibres to mechanical stimulation. Typical examples are ghrelin and leptin. Ghrelin released from exocrine X/A cells in the stomach is known to initiate food intake [173]. In the stomach, ghrelin containing cells are found in close a proximity to vagal afferent endings [174]. The receptor for ghrelin, GHSR, is expressed in the vagal afferent neurons specifically innervating the stomach [175, 176]. *In vitro* electrophysiological studies have shown that ghrelin selectively inhibits the response of gastric vagal afferents to circular tension, but has no effect on mucosal stroking sensitive vagal afferents [176]. Furthermore, blockade of

gastric vagal afferents *in vivo* abolishes ghrelin-induced feeding, suggesting gastric vagal afferents are the major signalling pathway of ghrelin regulated food intake [153, 177].

In the stomach, leptin is expressed in the mucosal chief cells and parietal cells [123, 178, 179]. Leptin receptor is expressed in vagal afferents neurons in both rodents and humans [148, 149], especially those innervating the stomach [180]. Evidence from several studies indicates that leptin selectively activate gastric vagal afferents [180-182], suggesting gastric released leptin is also involved in short-term regulation of food intake via gastric vagal afferents. This finding is confirmed by an *in vivo* study in rats, which demonstrates that the reduction in food intake induced by leptin infusion is abolished by vagotomy or capsaicin denervation of vagal afferent [183]. A recent study revealed the second messenger pathway involved in leptin signalling [180]. The inhibition of leptin on gastric tension receptors after fasting and diet-induced obesity was mediated through phosphatidylinositol 3-kinase-dependent activation of large-conductance calcium-activated potassium channels [180]. The excitatory effect of leptin on gastric mucosal vagal afferents at fed status was mediated by phospholipase C-dependent activation of canonical transient receptor potential channels [180].

### 1.3.4 Modulation of vagal afferents signals by food intake

The vagal afferent signalling pathway is regulated by short-term food intake, which is reflected in function and molecular expression. Firstly, the response of vagal afferents to circular tension is reduced after short-term fasting, which is observed in vagal afferents innervating the stomach and oesophagus [174]. This reduced response of vagal afferents could be a physiological adaptive response to a fast, which would facilitate food intake by inhibiting vagal afferent satiety signals.

Similarly, the response of vagal afferents to hormones can also be changed to promote food intake during fasting, and can be reflected in changes in levels of receptor expression within vagal afferent neurons, as shown in Table 1.3. A short-term fast increases the expression of receptors for orexigenic factors, such as cannabinoid receptor type 1 (CB1 receptor), melanin-concentrating hormone-1 receptor (MCH1R) and growth hormone secretagogue receptor (GHSR) in nodose ganglia of vagal afferent nerves innervating the upper GI tract [174, 184-186], although no change was observed in the expression of GHSR and orexin-A receptors in other studies [184, 187]. This up-regulation of receptors suggests increased orexigenic signalling during fasting, which is compatible with the effects in the CNS. On the other hand, the receptor for the anorectic factor PYY is reduced after fasting, however, a similar reduction is not observed with leptin receptor and CCK receptor [149]. The changes in receptors expression after short-term fasting could be restored by refeeding [149], suggesting that the neurochemical phenotype of vagal afferent innervating the upper GI tract is reversibly dependent on food intake. These changes could be party related to the interactions between different hormones, as a wide range of receptors are co-expressed in the nodose ganglia. For example, the anorectic hormone CCK decreases expression of CB1 receptor and MCH1R in nodose ganglia [184, 185], while orexin-A and ghrelin inhibit the vagal afferent response to CCK [188]. Therefore, the down-regulated MCH1 and CB1 receptors after food intake could be due to the increased CCK expression in response to food intake. In addition, in the fasted status CCK signalling is inhibited by the increased orexin-A and ghrelin expression.

**Table 1.3 Regulation of the expression of hormone receptors in the nodose ganglion by an overnight fast or a chronic high-fat diet**

| Hormones receptors in nodose | Regulation by fasting | Regulation by HFD |
|---|---|---|
| **Receptors for orexigenic hormones** | | |
| CB1R (cannabinoid) | ↑[184] | ↑[189], ↓[190] |
| MCH1R (MCH) | ↑[185] | |
| GHSR (ghrelin) | ↑[174, 186], ↔[187] | ↑[189], ↔ [174] |
| Orexin-A receptor (orexin-A) | ↔ [184] | ↓[190] |
| **Receptors for anorectic hormones** | | |
| Y2 receptor (NPY) | ↓[191] | ↔[189] |
| CCK1R (CCK) | ↔ [184] | ↑[189], ↓[190, 192] |
| Leptin receptor (leptin) | ↑[149] | ↔[189], ↓[190] |

The bracket refers to ligand for the receptor. Arrows designates direction of the receptor expression regulated by fasting or a chronic HFD.

The modulation of gastric vagal afferents by ghrelin and leptin is regulated by food intake. The inhibition of ghrelin on gastric mucosal sensitive vagal afferents is observed after food deprivation but not in rodents fed *ad libitum* [174, 180]. In contrast, the activation of leptin on gastric mucosal receptors is observed in fed *ad libitum* status but abolished after fasting. In addition, the inhibitory effect of ghrelin on tension sensitive vagal afferents is increased after fasting compared to fed *ad libitum* status [174, 180], while leptin inhibits tension sensitive vagal afferents after fasting but not at fed *ad libitum* status. This evidence is consistent with the orexigenic phenotype after food deprivation and increased satiation after food intake, which may be related to the changes in the expression of their receptors in corresponding vagal afferent neurons [174, 180]. Thus, gastric hormones–vagal afferent signalling pathway could be regulated by nutritional status.

### 1.3.5 Vagal afferents signals in obesity

The vagal afferent signalling pathway can also be altered by chronic high-fat diet (HFD) feeding induced obesity. A reduction in the response of vagal afferents to circular tension was observed in the stomach, oesophagus and jejunum of HFD fed mice, suggesting a systemic reduction of GI vagal afferent mechanosensitivity to stretch after HFD feeding [174, 192]. Furthermore, there are also changes in the response of GI vagal afferents to appetite regulating hormones. Jejunal afferents demonstrate attenuated responses to the satiety mediator CCK in HFD fed mice [192]. This finding is consistent with the reduced satiety effect of CCK *in vivo* in HFD fed rodents [190, 193]. Correspondingly, nutrient induced c-fos expression in the NTS neurons is reduced in HFD fed mice [190, 194]. In the stomach, the role of ghrelin on gastric vagal afferents in mice fed a chronic HFD is similar with its role in the fasted status,

suggesting ghrelin-gastric vagal afferent signalling may contribute to the orexigenic phenotype in obesity [174]. Evidence shows that leptin resistance in gastric vagal afferent is observed in rats fed a chronic HFD, which may be responsible for the reduced anorectic signalling in CNS [195, 196]. Therefore, after chronic HFD feeding, the vagal afferent signalling pathway regulated by hormones is changed and favours a stimulation of orexigenic signals and a reduction in anorectic signals, which may ultimately contribute to hyperphagia and the development of obesity.

## 1.4 Identification of NPW

The super-family of G-protein-coupled receptors (GPCRs) are a family of seven transmembrane proteins, with more than 800 members in the human genome [197]. This family of receptors mediates most of the physiological responses to environmental stimulants, hormones and neurotransmitters, and thus has been found to control diverse physiological processes including metabolism, homeostasis, growth and reproduction [198, 199]. Several members of GPCRs have been identified as important regulators of food intake and energy homeostasis, including ghrelin, orexins/hypocretins and MCH [200, 201]. Each discovery of these new ligands improves our understanding of the development of obesity, and offers key potential new targets for therapeutic application [202, 203]. Therefore, searching for endogenous ligands of orphan GPCRs and illustrating their functions in the energy homeostasis is essential for understanding and controlling obesity.

G-protein-coupled receptor 7 (GPR7) and G-protein-coupled receptor 8 (GPR8) are structurally highly related orphan GPCRs, which share 64% amino acid identity with each other and show high similarity to the opioid and somatostatin receptor families [204]. Both GPR7 and GPR8 are highly conserved among species, but GPR8 is absent

in rodents and only found in higher evolutionary mammals such as human, shrew, lemur and rabbits [205]. In 2002/3, neuropeptide W (NPW) and neuropeptide B (NPB) were identified as endogenous ligands for GPR7 and GPR8 [206-209]. NPB is a brominated peptide with 29 amino acid residues which is 61% amino acid identical to NPW [209]. NPW shows much higher potency to activate GPR8 compared to NPB, whereas GPR7 has slightly higher potency for NPB [208]. Binding experiments show that NPW and NPB have similar binding affinities for GPR7 [207, 210]. There are two forms of NPW, NPW23 which contain 23 amino acids and NPW30 with 30 amino acids. The amino acid sequence of NPW23 is identical to that of the N-terminal 23 residues of NPW30. Their amino acid sequences are highly conserved among species, i.e. human, porcine, rat and mouse [206]. *In vitro*, NPW23 and NPW30 show similar tendency to activate both GPR7 and GPR8, but NPW23 appear to be slightly more potent than NPW30 in this regard [206]. *In vivo* study shows that administration of NPW23 or NPW30 into the rat CNS induced similar potency on feeding behaviour [211, 212].

## 1.5 Distribution of NPW and its receptors

### 1.5.1 Distribution of NPW and its receptors in the central nervous system

The expression of NPW and its receptors GPR7 and GPR8 is distributed in several regions in the CNS of rat, mouse and human, which is summarized in Table 1.4.

**Table 1.4 Distribution of NPW and its receptors GPR7 and GPR8 in the central nervous system**

| Nucleus | NPW | GPR7 | GPR8 | Major functions the nucleus involved in |
|---|---|---|---|---|
| Hypothalamus | PCR [213] | PCR, ISH, ir cells, ir process, LB [208, 209, 213, 214] | | temperature, hunger, thirst, sleep, circadian cycles |
| Paraventricular nucleus | PCR, ir cells, ir process [215-219] | ISH [205] | | endocrine hormones release |
| Ventromedial nucleus | PCR, ir cells [218, 219] | ISH [204, 205] | | satiety and neuroendocrine control |
| Lateral hypothalamus | PCR, ir cells, ir process [215, 217-220] | | | thirst and hunger |
| Arcuate nucleus | PCR, ir cells, ir process [215, 217-219] | ISH [204, 205, 208, 210] | | growth hormone-releasing hormone, feeding, dopamine |
| Suprachiasmatic nucelus | | ISH, LB [204, 205, 208, 210, 214] | | vasopressin release, circadian rhythms |
| Dorsomedial nucleus | ir cells, ir process [215, 218, 220] | ISH [205, 208 , 210] | | blood pressure, heart rate, GI stimulation |
| Supraoptic nucleus | ir cells, ir process [215, 216] | ISH [205] | | oxytocin and vasopressin release |
| Anterior hypothalamic nucleus | | ISH [208] | | thermoregulation, panting, sweating, thyrotropin inhibition |
| Posterior hypothalamus nucleus | ir cells, ir process [218-220] | | | blood pressure, pupillary dilation, shivering, |
| Parvocellular Neurons | ir cells [215] | | | endocrine hormones release |
| Accessory neurosecretory nuclei | ir cells [215] | | | |
| Median eminence | ir cells, ir process [215, 216] | | | endocrine hormones release |
| Cerebral Cortex | | PCR [209] | | |
| Claustrum | | ISH [208] | | communication between two hemisphere |
| dorsal endopiriform nucleus | | ISH [208] | | |
| Frontal cortex | | NB [204] | NB [204] | reward, attention, memory, planning, and motivation |
| Parietal cortex | PCR [207] | | PCR [207] | sensory information integration |
| Hippocampus | PCR [207] | PCR [207, 209] | PCR [207] | memory and spatial navigation |
| dentate gyrus | | ISH [205] | | |
| CA1 Fields of Amnon's horn | ir cells [220] | ISH [204, 205, 208] | | |

**Table continued on next page**

**Continued from previous page**

| Nucleus | NPW | GPR7 | GPR8 | Major functions the nucleus involved in |
|---|---|---|---|---|
| Brain stem | | PCR [209] | | cardiovascular and respiratory system, pain sensitivity, alertness, awareness, and consciousness |
| Dorsal raphe nucleus | ir cells, ISH [208, 214, 219] | LB [214] | | |
| Ventral tegmental area | ISH, ir cells [208, 214] | ISH [204, 208] | | |
| Edinger-Westphal nucleus | ISH, ir cells [208, 218] | | | |
| periaqueductal gray | ISH, ir cells, ir process [208, 218, 219] | LB [214] | | |
| Olfactory cortex | | ISH [205] | | sense chemical signals |
| Amygdala | PCR, ir process [207, 215, 218, 220] | ISH, PCR, ir cells, LB [205, 207, 208, 210, 214] | PCR [207] | process memory and emotional reactions |
| Islands of calleja | ir cells [218] | ISH [205] | | regulate reward pathway |
| Thalamus | | PCR, LB [207, 209, 214] | PCR [207] | sensory and motor, consciousness, sleep, and alertness |
| Spinal cord | | PCR [209] | | sensory and motor, certain reflexes |
| Cerebellum | PCR, ir cells [207, 216] | PCR, NB [204, 207, 209] | PCR [207] | motor control |
| Corpus callosum | PCR [207] | PCR [207] | PCR [207] | interhemispheric communication |
| Substantia nigra | PCR [207] | PCR [207] | PCR [207] | reward, addiction, and movement |
| Caudate nucleus | | | PCR [207] | learning and memmory |
| Choroid plexus | PCR [207] | PCR [207] | | |
| Supratrigeminal nucleus | | ISH [208] | | receives sensory input |

PCR designates mRNA expression of target gene was detected using RT-PCR; ISH designates mRNA expression of target gene using in situ hybridization; ir cells designates immunoreactive cells of target protein using immunohistochemistry; ir process designates immunoreactive fibres of target protein using immunohistochemistry; NB designates mRNA expression of target gene using northern blot; LB designates NPW (ligand of GPR7) binding sites which suggest the expression of GPR7 protein in the binding sites. In this table, distribution of NPW and its receptors was not distinguished among species, i.e. mouse, rat and human.

In human brain, RT-PCR analysis has shown that NPW mRNA is present in several brain areas including parietal cortex, hippocampus, amygdala, substantia nigra, choroid plexus and cerebellum, but was not present in other regions including the hypothalamus, thalamus, spinal cord and dorsal root ganglia [207]. In mouse brain, in situ hybridization studies identified the expression of NPW mRNA in a few confined areas in the brain stem, comprising the ventral tegmental area (VTA), periaqueductal gray (PAG), Edinger-Westphal nucleus (EW) and dorsal raphe nucleus (DRN), particularly the dorsal part of the DRN [208]. These results were confirmed by immunohistochemical studies in rat brain, where NPW immunoreactive cells were found confined to the midbrain including the EW nucleus, VTA and lateral PAG, but not the hypothalamus [220]. In contrast, another report has demonstrated a dense expression of NPW immunoreactive cells in the PVN, supraoptic nucleus (SON) and median eminence of the hypothalamus, as well as the cerebellum in rat [216]. Similarly, it is also reported that NPW immunoreactive cells are expressed in hypothalamic regions including the PVN, parvocellular neurons, SON, dorsal hypothalamus (DH), LHA, and accessory neurosecretory nuclei, and the density appear to be higher in male rats compare to female rats [215]. The labelled NPW immunoreactive processes are distributed in a broader pattern including the PVN, SON, ARC, retrochiasmatic nucleus, LHA, median eminence and DH regions throughout the hypothalamus, as well as the amygdala, hippocampus, spetum, hypothalamus, thalamus, and brain stem [215, 220]. Further studies using colchicine pretreated rats, where the number and density of NPW immunoreactivity were increased, also detected NPW immunoreactive cells and fibres in several hypothalamic regions including the ARC, VMN, PVN, posterior hypothalamus and LHA, and other regions such as the amygdala, PAG, EW nucleus and DRN [217-219]. The expression of NPW mRNA was also found in the PVN, LHA,

ARC and VMN of rat hypothalamus using RT-PCR analysis [218, 219]. The controversial results among studies may be due to different origin and specificity of antibodies, and the low expression of NPW in some brain regions may make it difficult to be detected with some techniques. However, in general, NPW is abundantly expressed in several brain regions including the hypothalamus, brain stem and amygdala.

The pattern of distribution of GPR7 in the CNS appears to partly overlap with that of NPW. In situ hybridization analysis has revealed that in mouse brain GPR7 mRNA is expressed in the hippocampus, brain stem and hypothalamus [204], and in rat brain it was detected in the hypothalamus, cerebral cortex and hippocampus [205]. In both mice and rats a dense expression of GPR7 was observed in the hypothalamus, although the expression patterns appear to be more widespread in rat compared to mouse [205]. In the rat hypothalamus GPR7 was detected in the PVN, DMN and SON, in addition to the suprachiasmatic nucleus (SCN), ARC and VMN as seen in the mouse [204]. In rats, the brain area expressing the highest level of GPR7 mRNA is the amygdala, SCN and the ventral tuberomamillary nuclei, followed by moderate expression in the dorsal endopiriform, dorsal tenia tecta, bed nucleus and the red nucleus, and low expression in the olfactory bulb, parastrial nucleus, hypothalamus, laterodorsal tegmentum, superior colliculus, locus coeruleus, and the nucleus of the solitary tract [210]. These findings were confirmed and extended by another report on GPR7 mRNA distribution in mouse brain using *in situ* hybridization, which detected the expression of GPR7 in other regions including the claustrum, dorsal endopiriform nucleus, amygdala, magnocellular preoptic nucleus, SCN, anterior hypothalamic area posterior part, subiculum, VTA, and supratrigeminal nucleus [208]. RT-PCR analysis demonstrated a high expression of GPR7 mRNA in rat hypothalamus, as well as in the brain stem, cerebral cortex,

striatum, hippocampus, thalamus, midbrain, cerebellum, medulla oblongata and the spinal cord [209]. NPW binding sites were identified in the amygdala, thalamic and hypothalamic nuclei in rat and mouse brain, suggesting the expression of GPR7 protein in these regions, which was confirmed by a immunohistochemical study [214].

The expression of GPR7 and GPR8 in human brain is less well studied. In human brain, RT-PCR results indicated that GPR8 was more widely expressed than GPR7 in the CNS [207]. Both GPR7 and GPR8 are highly expressed in the hippocampus, amygdala, corpus callosum, thalamus, and substantia nigra, but additional sites are positive for GPR8, such as the parietal cortex, caudate nucleus and cerebellum [207]. Both GPR7 and GPR8 expression was not detected in human hypothalamus, consistent to findings from northern blot analysis [204]. However the northern blot study suggests a more restricted distribution of GPR8 mRNA expression, present in the frontal cortex but not in the cerebellum, hippocampus, hypothalamus, pons, putamen, and thalamus regions in human [204]. Although high GPR7 expression is detected in various brain regions, its distribution pattern is more restricted than other related receptors such as opioid, somatostatin, and nociceptin receptor [205, 207]. The distribution of NPW and its receptors GPR7 and GPR8 in the brain suggests that the role of NPW in the CNS may be involved in the regulation of energy homeostasis, neuroendocrine system, memory and learning.

### 1.5.2 Distribution of NPW and its receptors in periphery

Expression of NPW is widely distributed in the peripheral tissues of humans and rodents. In humans, NPW mRNA is highly expressed in the progenital system, including the testis, uterus, ovary and placenta, gastrointestinal tract, including stomach and rectum, kidney, adrenal gland, thyroid gland, pancreas, spleen, lymph node, blood

leukocyte, trachea, liver, skin and skeletal muscle [207]. In rats, NPW immunoreactive cells are present in the pituitary, parathyroids gland, thyroid gland, pancreatic islets, adrenal gland, ovary and testis [215, 216]. Similarly, in rats NPW mRNA was detected in the anterior pituitary, thyroid gland, andrenal gland, testis, ovary, stomach and pancreatic islets using RT-PCR [213, 216]. In rat adrenal gland, NPW mRNA and immunoreactive cells were found in the adrenal medulla but not in the adrenal cortex using *in situ* hybridization and immunohistochemical analysis [213]. However, RT-PCR analysis showed that NPW mRNA was expressed in both the adrenal medulla and adrenal cortex in rats [221]. In rat pancreatic islets, intense NPW immunoreactivity was observed in all of the cells of the pancreatic islets including A, B, D, and PP cells, and a less intense immunoreactivity was seen in the cytoplasm of the pancreatic follicular cells [222]. However, another report demonstrated NPW immunoreactive cells in the central portion of the pancreatic islets, positive in B cells, but not A or D cells [223]. In rat adipose tissue, NPW mRNA was identified in preadipocytes and macrophages, but not in mature adipocytes using RT-PCR [224].

In humans, the expression of GPR7 and GPR8 mRNA was less expressed and more restrictedly distributed than NPW expression being present in the pituitary gland, adrenal gland, spleen, lymph node, trachea, testis, lung, colon, rectum and skin [207, 225]. The expression of GPR7 mRNA in human pituitary is also confirmed using northern blot and in situ hybridization [204]. In rats, RT-PCR analysis shows that GPR7 mRNA is highly expressed in the progenital system including the uterus, ovary, placenta and testis, and is also present in the skin, pituitary gland, adrenal gland, pancreatic islets, thyroid gland, trachea, thymus, lung, adipocytes, and GI tract including the stomach, duodenum, jejunum, ileum, caecum, colon and rectum [209, 213, 216, 218, 224]. The distribution of NPW and its receptors in the periphery

indicates the possible involvement of NPW in the regulation of energy homeostasis, neuroendocrine system, reproductive function, immune system and respiratory system.

## 1.6 Expression and regulation of NPW in the stomach

### 1.6.1 Expression of NPW in the stomach

RT-PCR analysis has identified the expression of NPW mRNA in the stomach of both humans and rats [207, 213, 218]. Immunohistochemical analysis revealed an abundant distribution of NPW immunoreactive cells in the mucosal layer of the gastric antrum in mice, rats and humans [226]. In rodents, NPW immunoreactive cells are scattered throughout the basal part of the mucosal layer of the gastric antrum, but were not observed in the gastric fundus [226]. In human these cells are present in the middle layer of the mucosa of the gastric antral gland [226]. The content of NPW30 in rat gastric antrum is higher than that of NPW23 [226]. The plasma NPW concentration of rat gastric antral vein is higher than that of the systemic vein, suggesting the stomach is a source of circulating NPW [226].

The gastric antral mucosa has roughly equal numbers of three types of endocrine cells, gastrin (G) cells, enterochromaffin (EC) cells and somatostatin (D) cells [227]. Immunohistochemical studies revealed that NPW immunoreactive cells were identified in G cells, but were not found in EC cells or D cells [226]. NPW containing cells account for 85% and 89% of gastrin immunoreactive cells in rats and humans respectively [226]. In G cells NPW was observed in multiple, round, intermediate-to-high-density granules, which were clustered at the cell base [226]. Many studies have determined the physiological and pathological expression of NPW in the stomach, revealing that NPW expression is regulated by several factors, including physiological

development, food intake, energy homeostasis and some hormonal factors, which are discussed in details below.

### 1.6.2 Ontogeny of NPW in the stomach

The ontogeny of NPW in the stomach was studied in developing rats using RT-PCR [228]. The expression of NPW mRNA is initially detectable in the embryonic stomach at day 14, with levels gradually increasing until birth. After weaning, the expression of NPW mRNA gradually increases until adulthood [228]. The NPW immunoreactive cells in the stomach are detectable at postnatal day 1, and gradually increase during development to adulthood [228]. In the gastric antrum NPW is co-expressed with gastrin as early as postnatal day 1, suggesting a possible physiological function of NPW in the early development of gastric function. Whether gastric NPW expression is also regulated by age during adulthood is not known.

### 1.6.3 Regulation of gastric NPW by acute food intake

In the stomach, NPW is secreted in response to acute food intake. Gastric mucosal NPW mRNA and peptide levels decrease after an overnight fast in rats and reverse after refeeding [229]. Similarly, plasma NPW concentrations in the rat gastric antral vein decrease after an overnight fast and increase after refeeding [226]. The regulation of NPW levels in the systemic circulation in response to food intake is not well studied. It is reported that circulating NPW levels following an overnight fast is not changed in either leptin receptor deficient obese rats (fa/fa) or their lean littermates [230]. Taken together, this evidence suggests that food intake could induce gastric NPW secretion, but may not regulate NPW levels within the systemic circulation, which requires confirmation with further studies.

Meal-related nutrients are generally potent stimulants of GI hormones [231-234]. G cells are open-type flask-shaped cells, which make direct contact with the gastric lumen as chemosensors, similar to L cells and I cells in the small intestine [235-237]. In G cells, gastrin secretion is induced by both mechanical and chemical stimulants such as nutrients [238, 239]. Amino acids, especially the aromatic amino acids, L-phenylalanine and L-tryptophan, have been recognized as major stimuli for gastrin secretion [240, 241]. Glucose and lipid from the lumen or systemic circulation also induce gastrin release [242-244]. The role of macronutrients in gastric NPW release is unclear. It is reported that NPW levels in the gastric antral vein was not different in rats fed a high lipid diet or a high carbohydrate diet for two weeks [226]. Another report indicated that no variation of plasma NPW levels were observed in rats fed *ad libitum* with diets rich in protein, carbohydrate or fat [230]. However, these studies do not address the acute effect of macronutrients on gastric NPW expression and plasma levels. How gastric NPW secretion is regulated by food intake, whether macronutrients are responsible for NPW secretion, and the potential secretion interaction between NPW and gastrin requires further investigation.

### 1.6.4 Regulation of gastric NPW by energy homeostasis

Expression of NPW mRNA in the stomach is reduced in rats after chronic food restriction [229]. Similarly, reduced plasma NPW levels is observed in chronic food restricted fa/fa rats [230]. This evidence suggests that chronic food restriction inhibits gastric NPW expression, which is similar to the effect of acute food restriction. This effect could result directly from energy restriction or indirectly via energy restriction related factors such as stress as stress stimulated glucocorticoid which could inhibit gastric NPW expression [229]. Gastric NPW expression is also reduced in pregnant

rats, indicating that pregnant related factors also regulate gastric NPW secretion [229], which is similar to the expression of other appetite related GI hormones [245, 246]. Moreover, the plasma NPW level is higher in obese fa/fa rats compared to their lean littermates [230]. How gastric NPW is involved in disrupted energy balance especially in obesity remains to be further studied.

### 1.6.5 Regulation of gastric NPW by hormones

The direct effects of hormones on gastric NPW expression are not fully illustrated. Chronic dexamethasone or thyroid hormone administration decreases NPW mRNA expression in rat stomach, while thyroid hormone deficiency increases NPW mRNA expression [229]. The regulation of gastric NPW by these hormones could be a negative feedback as NPW has been shown to activate the hypothalamus-pituitary-adrenal (HPA) axis and hypothalamus-pituitary-thyroid (HPT) axis (see details in 1.7.3).

## 1.7 Physiological role of NPW

The wide distribution of NPW and its receptors within the CNS and periphery suggests multiple potential physiological functions of NPW. Many studies have demonstrated that NPW could be involved in energy homeostasis, neuroendocrine regulation, inflammatory pain, stress, cardiovascular functions, immune system and respiratory system [247-250]. The roles of NPW in short-term and long-term energy homeostasis and the neuroendocrine system are discussed in detail below.

### 1.7.1 NPW regulation of short-term food intake in the central nervous system

NPW and its receptors are abundantly expressed in the hypothalamus, an important brain area known to be involved in the regulation of food intake. Several studies

indicate that central administration of NPW regulates acute food intake. However, the effects are controversial, as shown in Table 1.5.

Some findings showed an early phase orexigenic role of NPW after central administration during the light phase. Intracerebroventricular (i.c.v.) administration of 10 nmol NPW23 to rats at 3 pm induced a three-fold increase in food intake within 2 hours compared to saline control rats [206]. Similarly, another study showed that i.c.v. injection of 3 nmol NPW23 at 10-11 am elicited a three-fold increase in food intake within 2 hours, but no difference was observed later (4-24 hours) [251]. Furthermore, administration of NPW into different sites of the hypothalamus including the PVN, known as a satiety centre, and the LHA, known as hunger centre, also demonstrated early phase orexigenic effects [252]. However, the orexigenic effect of PVN administration occurred earlier, lasted for 24 hours and required lower doses of NPW whereas the LHA injected group showed orexigenic effect at the beginning but over 24 hours the total food intake was decreased [252].

**Table 1.5 Acute effect of central administration of NPW on food intake**

| Drug injected | Injection site | Injection time | Effect on food intake | Ref. |
|---|---|---|---|---|
| NPW23 | lateral ventricle | light phase (3 pm) | increase in 1 and 2 h | [206] |
| NPW23 | lateral ventricle | light phase (10-11 am) | increase in 0.5, 1 and 2 h, no difference until 24 h | [251] |
| NPW23/NPW30 | lateral ventricle | dark phase (7:45 pm) | no difference in 1-2 h, decrease in 4 h until 48 h | [211] |
| anti-NPW IgG | lateral ventricle | dark phase (6 pm) | increase in 4 h and 12 h | [211] |
| NPW23 | lateral ventricle | light phase fasted (8:45 am) | decrease in 2 h | [211] |
| NPW23 | paraventricular nucleus | light phase (10-12 am) | increase in 1 h until 24 h | [252] |
| NPW23 | lateral hypothalamus | light phase (10-12 am) | increase in 2 h and 4 h, but decrease in 24 h | [252] |
| NPW | lateral ventricle | fasted for 2 h (time not mentioned) | decrease in 2 h | [219] |
| NPW23/NPW30 | lateral ventricle | dark phase (6:45pm) | no difference in 1-2h, decrease in 4h until 48 h | [212] |
| NPW23/NPW30 | lateral ventricle | light phase fasted (7:45 am) | decrease in 2 h | [212] |
| anti-NPW IgG | lateral ventricle | dark phase (6 pm) | increase in 4 h and 12 h | [212] |

In contrast, Mondal's group found an anorexic role of NPW when NPW was administed during the dark phase or at fasted status [211]. i.c.v. injection of 1-7.5 nmol NPW23 or NPW30 at the beginning of the dark phase had no effect on food intake in the first 1 to 2 hours, but inhibited food intake for up to 4 hours post administration (3-7.5 nmol NPW). Three nmol NPW induced inhibition on food intake was maintained for less than 12 hours post injection, whereas the anorectic effect of 5 and 7.5 nmol NPW was maintained for up to 48 hours post injection. This is further confirmed by the central administration of anti-NPW IgG, which induced an increase in food intake 4 and 12 hours post injection [211]. They also showed that after overnight fasting, i.c.v. injection of NPW at the beginning of the light phase inhibited food intake. Similar results were also observed in another study [212]. This complicated function of NPW is similar to previous findings with NPB [208], which shares the same receptors, GPR7 and GPR8, with NPW. Central administration of NPB during the light phase had no significant effects on feeding behaviour. However, in the dark phase, 3 nmol NPB exerted orexigenic effects within the first 2 hours, and a more significant anorexic effect after 2 hours, but 10 nmol NPB consistently inhibited food intake in all the intervals. The above evidence suggests that the feeding regulatory roles of both NPB and NPW in the CNS is complicated, could be regulated via different pathways and/or modulated by several factors including circadian regulation.

Anatomical evidence showed that GPR7 mRNA was widely distributed within the CNS including the hypothalamus (PVN, VMN, SCN, DMN, ARC, SON and anterior hypothalamic nucleus), cerebral cortex, hippocampus, and brain stem [205, 207, 208]. Central administration of NPW activates c-Fos expression in several regions of the CNS, mainly localized in the hypothalamic nucleus (i.e. PVN, ARC, LHA, VMN and DMN) and brain stem. The overall distribution of the increased c-Fos expression is

consistent with the distribution of GPR7 [252]. Based on the above evidence, the sites upon which NPW may exert its effects on feeding behaviour could be complicated.

***Anorectic pathway***: It is suggested that NPW may inhibit food intake via first-order neurons NPY/AgRP and POMC/CART neurons in the ARC, which is adjacent to the third ventricle and responds to peripheral signals [20]. NPW immunoreactive axon terminals synapse with both NPY/AgRP and POMC/CART neurons [219]. Electrophysiological studies showed that NPW dose dependently decreased firing activity of NPY/AgRP neurons, and also reduced the frequency of the spontaneous inhibitory postsynaptic current of POMC neurons, which should lead to the increased firing activity of POMC neurons [219]. In addition, *in vivo* studies found that after i.c.v. administration of NPW in rats during the dark phase, hypothalamic AgRP mRNA decreased and POMC mRNA increased [219]. Furthermore, NPW induced decrease in food intake in rats was abrogated by pretreatment with AgRP or inhibition of POMC-MC4-R signalling [219]. This evidence suggests NPW inhibits food intake, at least partly, by activating POMC/CART neurons and inhibiting NPY/AgRP neurons, similar to the role of leptin [24].

***Orexigenic pathway***: On the other hand, some evidence also suggests some potential mechanisms of orexigenic effects of NPW. In the LHA, the brain area known as a feeding centre, a close neuronal interaction between NPW containing nerve fibres and orexin- or MCH- containing neuronal cell bodies and nerve fibres was identified [217]. I.c.v. injection of NPW activates the hypothalamic cells expressing orexin, a factor known to increase the craving for food [253, 254], which is consistent with the role of ghrelin and opposite to that of leptin [252]. Furthermore, subcutaneous injection of 3 nmol NPW in rats during the light phase induced a transient reduction of blood leptin

levels within 30 minutes which was not observed after 1 hour [222], consistent to the NPW induced early phase hyperphagia in the CNS. The reduced leptin levels could arise from adipose tissue, as NPW was found to directly inhibit the secretion and expression of leptin in isolated rat adipocytes [224].

***Circadian rhythm/feeding status***: The role of NPW on food intake in the CNS is biphasic. One hypothesis is that the role of NPW on food intake may be under circadian regulation. This is generated from evidence that the orexigenic effects of NPW were observed during the light phase while the anorectic effects were observed during the dark phase. GPR7 is robustly expressed in the SCN of the hypothalamus in mice and rats [205, 208], the central region critical for the regulation of circadian rhythm. Although GPR7 -/- mice show normal circadian patterns of behaviour [255], i.c.v. administration of NPW in rats has no effect on locomotor activity during the dark phase [211, 212], but increases locomotor activity during the light phase [251, 256] raising the possibility that increased food intake during the light phase could be a reflection of increased activity. However, on the other hand, during the light phase, i.c.v. administration of NPW to overnight fasted rats inhibits food intake rather than increases food intake [211, 212], which is different from results found in fed *ad libitum* rats during light phase studies [206, 251, 252]. This evidence suggests that feeding status, rather than circadian rhythm may be of more importance in the regulation of NPW function, and these mechanisms require further investigation.

***Sites of action***: Different hypothalamus nucleus could have different effects on feeding behaviour in response to NPW. A report showed that administration of NPW into different sites of the hypothalamus, the PVN and LHA, have different effects on feeding behaviour [252]. Although both PVN and LHA injection of NPW show early

phase orexigenic effect, the effect on PVN occur earlier, require lower doses of NPW and could last for 24 hours. However, the LHA injection group showed early phase orexigenic effects but a reduction in total food intake over a 24 hour period. This evidence indicates that NPW may have different effects on different brain regions, and diffusion of NPW among brain regions may cause the biphasic effects on food intake.

***Other factors***: It is possible that the acute regulation of NPW on food intake could be related to other physiological roles of NPW in the CNS, such as anxiety or stress. In the hypothalamus, immobilization stress induced c-Fos expression in NPW containing neurons [257]. In addition, after i.c.v injection of NPW, rats were reported to be more alert or active [211, 251, 256], and the plasma prolectin and corticosterone levels increased and growth hormone levels decreased, which are neuroendocrine components of the stress response [251]. In addition, NPW administration modulates PVN neuron activity, with associated increases in body temperature, oxygen consumption, heat production, arterial blood pressure, and heart rate, which could also be a reflection of the stress response [211, 258, 259]. Stress is found to be associated with food intake in both rodents and human. In rodents, the effect of acute stress on food intake could be either anorexic or orexigenic [260-262], while chronic stress usually decreases food intake and body weight gain [263]. In humans, stress affects eating in a bidirectional way, but most individuals increase their food intake during stress in both laboratory and real-life setting [264-267]. Based on this evidence, it is possible that the inconsistency between laboratories on the central role of NPW on food intake is related to the anxiety or stress response. Similar contradictory results were also observed on the role of PYY on food intake among different laboratories [25, 122], and further evidence suggest that factors including handling, habituation and acclimatisation to experimental conditions and the absence of cage enrichment may influence the effects of GI hormones on food

intake, via stress associated changes in food intake [268]. Therefore, more studies should be included to further clarify the acute role of NPW on food intake excluding the stress factors. On the other hand, the reduced food intake was not related to taste aversion as i.c.v. injection of NPW has no effect on taste in rats [211].

In summary, current literature suggests that NPW regulates short-term food intake in the CNS, but the role is dependent on several conditions, including the light/dark phase, feeding status and NPW injection sites. The mechanisms of NPW on food intake are not fully clear. In the CNS, NPW may directly regulate brain areas controlling feeding behaviour and energy homeostasis via activation of GPR7 expressed in these neurons, or indirectly affect food intake via other central physiological roles of NPW such as regulation of circadian rhythm and stress.

### 1.7.2 Role of NPW in long-term energy homeostasis

GPR7-/- male mice develop an adult-onset moderately severe obese state, with increased food intake and decreased resting oxygen consumption, carbon dioxide production and locomotor activity, indicating that the loss of GPR7 results in dysregulation on energy homeostasis [255]. Evidence suggests that NPB, the other ligand of GPR7, could be responsible for this phenotype. NPB -/- male mice also develop an adult-onset mild obesity, indicating that depletion of NPB is sufficient to disrupt body weight [269]. However, NPW could also account for the hyperphagia and obese phenotype, as chronic administration of NPW into the CNS of rats reduces body weight and food intake and increase energy expenditure [211, 212]. Moreover, acute i.c.v. injection of NPW induces an increase in body temperature, oxygen consumption and heat production [211, 212], suggesting NPW is a catabolic factor in both acute and

chronic situations. Overall, these data suggests that NPW is a long-term anorectic and catabolic factor, despite its increased/decreased effects on short term food intake.

The mechanisms that underline NPWs anorectic and catabolic roles are not fully understood. *In vitro* studies demonstrate that NPW could enhance adipocyte lipolysis via GPR7 expressed in adipocytes, which could lead to decreased fat content in adipocytes [224]. Peripherally NPW inhibits circulating leptin levels and the expression and secretion of leptin from isolated rat adipocytes [222, 224], suggesting that leptin may not account for the anoretic and catabolic roles of NPW, but rather show an opposite effect. Similarly, GPR7 knockout induced obesity was at least partly independent on the leptin or melanocortin signalling pathway, as leptin and GPR7 double mutation mice ( ob/ob GPR7 -/- mice) and mice with deficient melanocortin and GPR7 signalling (Ay/a GPR7 -/- mice) were heavier than ob/ob and Ay/a mice [255]. Moreover, the GPR7 knockout induced hyperphagia could not be explained by the central anorectic pathway regulated by NPW (i.e., activating POMC/CART neurons and inhibiting NPY/AgRP neurons) [219], because in GPR7 -/- male mice POMC mRNA expression is increased and NPY mRNA expression is reduced [255], which are more characteristic of a lean state and contradictory to the leptin deficient state. In addition, it is not clear whether the catabolic role of NPW is related to increased locomotor activity. Some studies show that central administration of NPW in rats induces more physical activity [251, 256], and GPR7 -/- mice show reduced locomotor activity [255], while other groups show no variation in locomotor activity after i.c.v. injection of NPW to rats, although these rats tended to be more alert [211, 212].

As discussed above, NPW is an anorectic and catabolic factor in long term energy homeostasis, therefore, the following information suggest that in the obese state within

the CNS NPW could play a compensatory role. In different types of obese model including leptin-deficient ob/ob mice, leptin receptor-deficient db/db mice and diet induced obese rats, expression of NPW mRNA in the hypothalamus was increased [219]. This increase was not due to leptin deficiency induced hyperglycemia or increased body weight, but was related to leptin deficiency [219]. However, under normal physiological condition, i.p. injection of leptin to mice for 5 days or a single i.c.v. injection of leptin to rats has no effect on hypothalamic NPW expression, suggesting hypothalamic NPW expression is not affected by increased leptin levels. Similarly, plasma NPW levels were increased in leptin receptor deficient fa/fa rats, and inhibited by chronic food restriction, further suggesting the compensatory effect of NPW in leptin deficiency [230].

Although GPR7 -/- male mice develop hyperphagia and obesity, GPR7 -/- female mice show no change in phenotype even in mice fed a HFD. This suggests that GPR7 signalling related to energy homeostasis is sexually dimorphic, which is similar to the NPY Y1 receptor pathway [270]. Sex hormones estrogens and androgens are important factors in the regulation of long-term energy homeostasis. Estrogens inhibit food intake and body weight, while androgens show an increased effect [271, 272]. In rats, castration of males led to hypophagia and a reduction in weight gain, while ovariectomizing females resulted in hyperphagia and increased body weight [273, 274]. In rodents, both NPW and GPR7 are highly expressed in the ovary and testis, especially in their steroid-secreting cells. In a rat i*n vivo* study, intraperitoneal (i.p) injection of NPW evoked a significant increase in the plasma estradiol and testosterone levels [216], which may further regulate energy homeostasis. Therefore, the NPW/GPR7 pathway involved in long-term energy homeostasis may be regulated by sex hormones.

### 1.7.3 Role of NPW in neuroendocrine system

Evidence suggests that NPW is involved in the HPA axis. NPW and GPR7 are expressed in the signalling pathway of the HPA axis in humans and rodents [207, 216, 275]. In rats, i.c.v. administration of NPW increases plasma corticosterone levels [251]. Similarly, i.p. injection of NPW induces an increase in plasma ACTH and corticosterone concentrations [216]. Subcutaneous administration of NPW also raises plasma corticosterone levels while inhibiting ACTH levels [221]. Although GPR7 was found expressed in the pituitary gland, in a primary pituitary cell culture study NPW failed to stimulate ACTH release and did not interact with the CRH-stimulated ACTH release, suggesting NPW does not directly stimulate ACTH secretion in the pituitary gland [251]. In the hypothalamus, GPR7 is expressed in the PVN. Whole cell patch recordings demonstrate that NPW depolarized and directly increased the spike frequency of CRH-producing PVN neurons [276]. Furthermore, i.c.v. administration of 1 nmol NPW elevates plasma corticosterone levels in rats, and the effect is blocked by pretreatment with a CRH antagonist administered intravenously, suggesting NPW may activate the HPA axis directly from the hypothalamus[276]. Moreover, in rat adrenal zona fasciculata/reticularis (ZF/R) cell culture, NPW increases basal aldosterone secretion as well as ACTH-stimulated aldosterone secretion, and prolonged (4-day) exposure to NPW raises corticosterone secretion and increase the proliferation rate of cultured cells [275]. Similarly, in human adrenal ZF/R cell culture, NPW enhances cortisol production [225]. This evidence suggests that NPW could activate the HPA axis, by directly stimulating CRH secretion from PVN neurons and glucocorticoids secretion from the adrenal gland, but not ACTH secretion from the pituitary gland. The regulation of NPW in the HPA axis suggest the involvement of NPW in stress reactions

and physiological processes including digestion, energy storage and expenditure, the immune system, mood, emotions and sexuality.

NPW has been shown to regulate other pituitary released hormones. In rats, i.c.v. administration of NPW induces significant elevation in plasma prolactin levels [206, 251]. Plasma growth hormone levels were also up-regulated by i.c.v. injection of NPW in rats [251], however, this was not observed in another report [206]. In a primary pituitary cell culture study, NPW failed to regulate prolectin and growth hormone release, and had no interaction with growth-hormone-releasing hormone (GHRH) stimulated growth hormone secretion or TRH induced prolactin release, suggesting that NPW may not directly regulate pituitary hormone release in the gland, but via neurons in the hypothalamus [251].

Intraperitoneal injection of NPW in rats increases plasma parathyroid hormone, estradiol and testosterone levels [216], suggesting a possible role of NPW in calcium homeostasis and reproductive function. The expression of NPW and GPR7 were identified in the pancreatic islets of the rat [216, 222], raising the hypothesis that they are involved in glucose homeostasis. However, i.p. administration of NPW (2nmol/100g body weight) to rats has no effect on plasma insulin levels [216], but subcutaneous injection of higher doses of NPW (3nmol/100g body weight) induces a transient reduction in blood insulin levels, without affecting glucose levels [222]. Further *in vitro* studies have shown that NPW significantly increases insulin release in rat pancreatic islet cell culture, and NPW is co-locolized with insulin in pancreatic cells, indicating a direct stimulatory role of NPW on insulin secretion [223]. GPR7 knockout mice have increased plasma glucose and insulin levels [255], suggesting that chronic disruption of the NPW/GPR7 pathway breaks glucose homeostasis.

### 1.7.4 Role of NPW in the stomach

The physiological functions of gastric released NPW is not clear. It is known that G cells also secrete gastrin, which plays multiple functions including the regulation of gastric motility, acid secretion, cell proliferation and differentiation [277]. An unpublished observation mentioned in a report from Mondal indicates that i.p. administration of NPW in rats increases gastric acid secretion in the stomach, suggesting that NPW may regulate gastric acid secretion [226]. Another unpublished finding of Sakurai [278] suggests that food intake is not changed in mice after i.p. administration of NPW. However, this finding cannot exclude the possible regulation of NPW on food intake in the stomach, as i.p. administration of NPW could have multiple physiological roles via the circulation including central effects which as stated already are diverse. Further studies would be necessary to determine the physiological role of NPW in the stomach.

## 1.8 Aims

Food intake is regulated by a network of complicated signalling pathways in both the periphery and CNS. Gut-brain peptides are known to be a group of important regulators, existing and acting in both the CNS and the periphery and involved in controlling short-term and long-term energy homeostasis. The hormone-vagal afferent signalling pathway is essential in generating postprandial satiation. In obesity, this signalling pathway can be disrupted at different levels, including secretion/expression levels of hormones and their receptors, as well as the sensitivity of vagal afferents to these hormones. Identifying these gut-brain peptides and understanding their physiological and pathological roles would improve our understanding of energy homeostasis and therefore provide potential targets to combat the prevalence of obesity.

Existing evidence strongly suggest that NPW is a member of a growing group of gut-brain peptides involved in energy homeostasis; however, its physiological role in the stomach is not clear. In the stomach, vagal afferents send signals to the CNS in response to mechanical stimulation to regulate food intake and gastric function, and locally released peptides play important roles in regulating these signals [174, 176]. It is possible that NPW is also involved in the gastric vagal afferent signalling pathway. Therefore, this study aims to determine the modulatory role of NPW on the mechanosensitivity of gastric vagal afferents, and the expression of NPW/GPR7 in the gut-brain axis.

## 1.9 Thesis outline

This thesis contains three results chapters (chapter 3-5). Chapter 3 confirmed the expression of NPW in the stomach of C57BL/6 mice and identified the expression of GPR7 in the cell bodies of vagal afferent neurons. An *in vitro* electrophysiology preparation was used to determine the effect of NPW on the mechanosensitivity of gastric mucosal and tension receptors. This study revealed that in adult female mice NPW selectively inhibited the responses of gastric vagal tension receptors to stretch, but this role was not observed in HFD fed or food restricted mice. Chapter 4 further determined the role of NPW on gastric vagal afferent mechanosensitivity in mice of different age and gender. This study revealed that the inhibition of NPW on gastric tension receptors was not observed in young adult (8 weeks old) mice, but occurred during further growth. Also this role was not different between male and female mice. To further illustrate the physiological role of NPW in the stomach, Chapter 5 determined the regulation of gastric NPW in response to food intake. Plasma NPW levels were determined in both human and mice after nutrients intake, and also the

expression of NPW in response to nutrients was determined in primary antral mucosal cell culture. Studies showed that protein and glucose are potent stimulants for gastric NPW mRNA and protein expression.

# CHAPTER 2. MATERIALS AND METHODS

## 2.1 Introduction

This Chapter presents all materials and methods utilised throughout all the studies presented in the thesis, unless specified in other chapters. Manufactures of kits, chemicals, reagents and instruments are listed below:

ABR – Affinity BioReagents. Inc., Golden, USA

Arcturus – Arcturus, Fostercity, CA, USA

BD – Becton, Dickinson and Company, NJ, USA

BD Biosciences – BD Biosciences Pty. Ltd., NSW, Australia

Bio-Rad – Bio-Rad Laboratories, Inc., NSW, Australia

Bristol-Myers Squibb – Bristol-Myers Squibb Australia Pty. Ltd., VIC, Australia

Bulk Nutrition – Continental Health Partners Pty Ltd, VIC, Australia

Cambridge Isotope Laboratories – Cambridge Isotope Laboratories, Inc., Andover, MA, USA

Carl Zeiss – Carl Zeiss Inc., Jena, Germany

CED – Cambridge Electronic Design, Ltd., Cambridge, UK

CWE – CWE Inc., Ardmore, PA, USA

Danz Instruments – Danz Instruments service, Adelaide, SA, Australia

Europa Scientific – Europa Scientific Ltd., Crewe, UK

Graphpad – GraphPad Software, Inc., CA, USA

Hamilton – Hamilton company, NV, USA

Invitrogen – Life technologies Co., VIC, Australia

JRAK – JRAK, Melbourne, Australia

McCain Foods – McCain Foods Ltd., VIC, Australia

Merck Millipore – Merck KGaA, Darmstadt, Germany

MJ Research – MJ Research, Inc., MA, USA

MP Biomedicals – MP Biomedicals Australia Pty. Ltd.; NSW, Australia

Olympus – Olympus Australia Pty Ltd., NSW, Australia

Perkin Elmer – Perkin Elmer, Inc., VIC, Australia

Pfizer – Pfizer Inc., NSW, Australia

Pharmatel Fresenius Kabi – Pharmatel Pty Ltd., NSW, Australia

Phoenix Pharmaceuticals – Abacus ALS Australia, East Brisbane, QLD, Australia

Qiagen – Qiagen Pty Ltd., VIC, Australia

Roper Scientific – Roper Scientific, Inc., Tuscon, AZ, USA

Sigma Aldrich – Sigma-Aldrich, Co., NSW, Australia

Specialty Feeds – Specialty Feeds, Glen Forrest, WA, Australia

Thermo Fisher Scientific – Thermo Fisher Scientific Australia Pty Ltd., VIC, Australia

Tissue-Tek – Sakura Finetek, Alphen aan den Rijn, Netherlands

Tocris biosciences – Tocris biosciences, Inc., Bristol, UK

Trayslol – Bayer Australia Ltd., Pymble, Australia

USUN – USUN biomedical technology, Co. Ltd., Jiangyin, China

Vector Laboratories – Abacus ALS Australia, East Brisbane, QLD, Australia

WPI – World Precision Instruments, Inc., Sarasota, FL, USA

Yakogawa – Yokogawa Australia Pty Ltd., Tokyo, Japan

## 2.2 Mouse studies

All animal studies were performed with the approval of the animal ethics committee of the Institute of Medical and Veterinary Science and the University of Adelaide, and in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The experiments also conform to the principles of UK regulations, as described in Drummond [279]. All mice used in the studies were C57/BL6 mice. Mice were group housed under a 12 hours dark/12 hours light cycle with *ad libitum* access to water and standard laboratory diet (SLD) unless otherwise specified.

### 2.2.1 Chronic high fat diet feeding study

At 8 weeks of age, mice were either fed with a SLD (14 kJ/kg; energy from fat - 12%, protein - 23%, carbohydrate - 65%; Specialty Feeds) or a HFD (21.4 kJ/kg; energy from fat - 61%, protein - 16%, carbohydrate - 23%; Specialty Feeds) *ad libitum* for 12 weeks. In the SLD, the fat is comprised of mixed vegetable and canola oils and the sugar from wheat and barley. In the HFD, the fat is predominantly cocoa butter and the sugar is a mixture of sucrose, wheat starch and dextrinized starch. The weights of the mice were monitored weekly.

### 2.2.2 Overnight fasting study

On the day prior to experimentation, at 1600 hours mice were housed in a cage where food, bedding and nesting material were withdrawn. Coprophagia was restricted using a raised wire mesh inserted into the bottom of the mouse cages that allowed faecal pellets to drop to the cage floor. On the following morning at 0900 hours, mice were ready for different experimentation. Over the duration of the fasting period, the body weight of

mice dropped approximately 2 g and very limited food or other content was found in the stomach.

### 2.2.3 Acute nutrient feeding study

Mice were fasted at 1600 hours on the day before experiment. On the following morning at 0900-1100 hours mice were intragastric gavage fed with a bolus (200 µl) of either water or nutrient solution of whey protein (dissolved in water; Bulk Nutrition) or D-glucose (dissolved in water; Merck Millipore), or 10% intralipid (Pharmatel Fresenius Kabi). Each nutrient solution contained 0.22 kcal energy. Thirty min after gavage feeding at the previously determined peak gastrin response for nutrients [242, 280], mice were either anaesthetised (isoflurane, 1-1.5% in 15% oxygen) for collection of gastric mucosa and blood from the abdominal aorta, or perfuse fixed for immunohistochemistry.

## 2.3 Human study

Human blood samples were collected from a previously reported human study [233]. The human study protocol was approved by the Royal Adelaide Hospital Human Research Ethics Committee, and carried out in accordance with the Declaration of Helsinki. Subjects provided written, informed consent prior to their inclusion to the study. Samples of healthy lean male subjects (n = 8) were included in the current study. Each subjects attended the study on three occasions, separated by 3 - 7 days. They were provided with a standard meal (beef lasagne, 2472 kJ; McCain Foods) at 1900 hours on the evening prior to each study day (to standardize study condition) and were requested to fast after the meal. In the morning of the study day, at 0845 hours each subject was provided with a standard breakfast (to further standardize study condition), consisting

of a cup of white coffee or tea with 1 teaspoon of sugar, a glass of orange juice and slices of whole-meal toast with butter and jam, which was consumed within 15 minutes. The energy content of the breakfast represents ~10% of each subject's daily energy requirements, calculated based on Harris-Benedict equation and each subject's physical activity factor. At 1130 hours ($t$ = - 30 minutes), a 10 ml blood sample was taken. At $t$ = - 20 minutes, subjects ingested, in randomized order, either a *1*) high-fat (% energy from fat/protein/carbohydrate 55:15:30; protein content (g/kg body weight): 0.4), *2*) high-carbohydrate (30:10:60; 0.2), or *3*) high-protein (25:45:30; 1.35) test meal within 20 minutes. The energy content of each test meal comprises ~30% of each subject's estimated daily energy requirement. All test meals were prepared by the same investigator on the morning of each study day. Each test meal includes conventional pasta with a tomato-based sauce, lean beef mince, onion, olive oil and mixed dried herbs, and a vanilla yoghurt dessert. To achieve the described macronutrients composition in high-fat, high-carbohydrate and high-protein meals, whey protein isolate, pure cream, and corn flour and raw sugar was added to the test meal respectively. The ingredient content and nutrient composition of each test meal are listed in Table 2.1. Blood samples were collected from an intravenous cannula immediately after the meal and at $t$ = 15, 30, 45, 60, 75, 90, 120, and 180 minutes following the meal.

**Table 2.1 Ingredients and nutrient composition of high-fat, high-carbohydrate or high-protein meals (per 100 g) [233]**

| | High-fat meal | High-carbohydrate meal | High-protein meal |
|---|---|---|---|
| Meal ingredients | | | |
| Extra virgin olive oil, g | 7.7 | 1.3 | 3.5 |
| Onion, raw, g | 3.1 | 2.4 | 2.9 |
| Premium beef, g | 13.7 | 24.6 | 2.9 |
| Pasta sauce, g | 19.9 | 24.6 | 29 |
| Mixed herbs, g | <1 | | |
| Pasta, g | 18.3 | 15.2 | 32.5 |
| Pure cream, g | 7.7 | 1.7 | 3.8 |
| Whey protein powder, g | 2.5 | 14.7 | |
| Raw sugar, g | | | 0.9 |
| Corn flour, g | | | 2.6 |
| Skim milk, g | | | 2.9 |
| Full-fat vanilla yoghurt, g | 15.3 | 12.3 | 17.4 |
| Energy content, kJ | 940 | 888 | 892 |
| Macronutrient content | | | |
| Protein, g | 9.8 | 23.3 | 6.2 |
| Fat, g | 15.8 | 7.3 | 6.9 |
| Carbohydrate, g | 18.7 | 13.3 | 32.0 |

## 2.4 Single fibre recording

### 2.4.1 *In vitro* mouse gastro-oesophageal vagal afferent preparation

The *in vitro* mouse gastro-oesophageal vagal afferent preparation has been described in detail previously [166, 281], as illustrated in Figure 2.1. Mice were humanly killed by placing in a carbon dioxide-filled chamber. The thoracic and abdominal cavity was opened by midline incision. The stomach and oesophagus with attached vagal nerves were removed from the mouse and placed in a modified Krebs' buffer solution containing: 118.1 mmol/l NaCl, 4.7 mmol/l KCl, 25.1 mmol/l $NaHCO_3$, 1.3 mmol/l $NaH_2PO_4$, 1.2 mmol/l $MgSO_4.7H_2O$, 1.5 mmol/l $CaCl_2$, 1.0 mmol/l citric acid, and 11.1 mmol/l D-glucose, bubbled with 95% $O_2$ / 5% $CO_2$. Nifedipine (1 μmol/l) was also added to the Krebs to prevent smooth muscle contraction. It has previously been demonstrated that nifedipine does not affect the mechanical sensitivity of gastro-oesophageal vagal afferents [282]. The temperature of the Krebs' buffer was maintained at 4 ℃ during dissection to prevent metabolic degradation.

After further dissection, involving removal of adjacent tissue, the stomach, oesophagus and attached vagus nerves were left intact. The preparation was opened out longitudinally along the oesophagus and greater curvature of the stomach. The dorsal part of the stomach was completely removed to enable the tissue to be pinned flat in the organ bath with a straight edge. The ventral part of the stomach and oesophagus was pinned mucosal side up in a perspex chamber (dimensions: 6 x 2.5 x 1.2 cm; Danz Instruments) with Krebs' buffer flowing through at a rate of 12-13 ml/min at 34 ℃. The vagal nerves (< 1.5 cm) were extended into a second round chamber (dimensions: 3.7 cm diameter, 1.2 cm deep) filled with liquid paraffin oil through a sliding wall via a small hole for the vagus nerves to pass through. Laid on a mirror, the nerve sheath was

gently peeled back to expose the nerve trunk under a dissecting microscope. Nerve fibres were teased apart into 8-14 bundles using fine forceps. The small nerve bundles were placed one by one onto a platinum recording electrode for electrical signal recording. A reference electrode rested on the glass plate in a small pool of Krebs' solution.

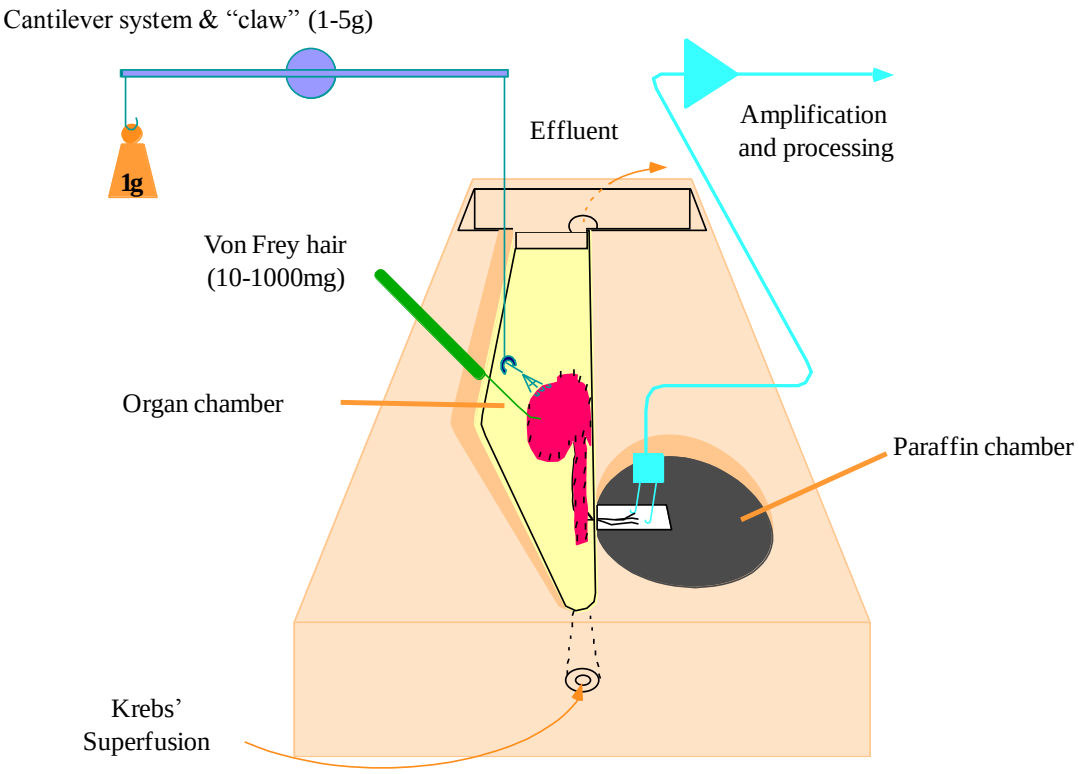


**Figure 2.1 In vitro mouse gastro-oesophageal vagal afferent preparation [166]**

The ventral part of the stomach and oesophagus of the mouse are pinned out flat mucosa uppermost and superfused with Krebs’ solution. The vagus nerve was drawn into a second chamber where teased fibres are placed onto a recording electrode. Mucosal stimuli are applied by stroking over the receptive field with calibrated von Frey hairs (10-1000 mg). Tension is applied by placing a hook adjacent to the receptive field. This hook is connected via a pulley to a cantilever system upon which weights (1-5 g) can be applied.

### 2.4.2 Characterisation of gastro-oesophageal vagal afferent properties

Two types of mechanosensitive vagal afferents in mouse stomach were studied: mucosal receptors which respond to mucosal stroking but not circular tension, and tension receptors which respond to both mucosal stroking and circular tension, as previously reported [166].

The location of receptive fields for both types of vagal afferent fibre was determined by mechanical stimulation throughout the preparation with a brush. Then subtypes of mechanosensitive vagal afferents were distinguished by a combination of mechanical stimuli involving mucosal stroking with calibrated von Frey hairs and circular tension using a cantilever claw system. Qualification of mechanical responses was performed differently according to the primary adequate stimulus for the type of fibre.

Mechanical thresholds of mucosal receptors were determined using calibrated von Frey hairs. The most reproducible, stimulus-dependent responses of these afferents to mucosal stroking was evoked when the von Frey hairs was moved at a rate of 5mm/s across the receptive field rather than being static. Different intensity of mucosal stroking including 10 mg, 50 mg, 200 mg and 1000 mg was placed on the receptive field by using different sized von Frey Hair, with 10 times strokes at each intensity and 1second intervals between each stroke. The von Frey hair was bent throughout the stroking stimulus to provide an even force. Due to the fact that the receptive fields were small (< 1 $mm^2$), a single test at each intensity is prone to missing the centre of the receptive field on some occasions. Therefore, we minimised experimental error by measuring the mean response to the middle eight of ten standard strokes given at 1 second intervals. The response was calculated as mean discharge per stroke.

Tension stimuli were applied via a fine suture silk attached via a hook to an unpinned point adjacent to the mechanoreceptive field. The thread was attached to a cantilever via a pulley close to the preparation. Reference standard weights were then placed on the opposite end of the cantilever. Each weight was applied in a step wise manner and maintained for 1 minute and the response was measured as the mean discharge evoked over this period. Due to the fact that all responses to tension adapted slowly, this method of assessment was considered representative of physiological responsiveness. The tension-response curves were produced by applying weights to the cantilever system in the range of 1-5 g. A recovery period of at least 1 minute was allowed between each tension stimulus.

### 2.4.3 Effects of NPW on mechanosensitivity of vagal afferents

After mechanical sensitivity of the gastric vagal afferents had been established, the effect of NPW on mechanical sensitivity was determined. NPW (1 nM) was added to the superfusing Krebs solution and allowed to equilibrate for 20 minutes after which time the tension-response and stroke-response curves were re-determined. This equilibration period ensured penetration of the peptide into all layers of the tissue. This procedure was repeated for NPW at increasingly higher doses (3-10 nM), consistent with other reports [206, 251] and with plasma NPW levels in mice [283]. We have previously established that there is no effect of time on gastric vagal afferent mechanosensitivity [284], asillustrated in Figure 2.2.

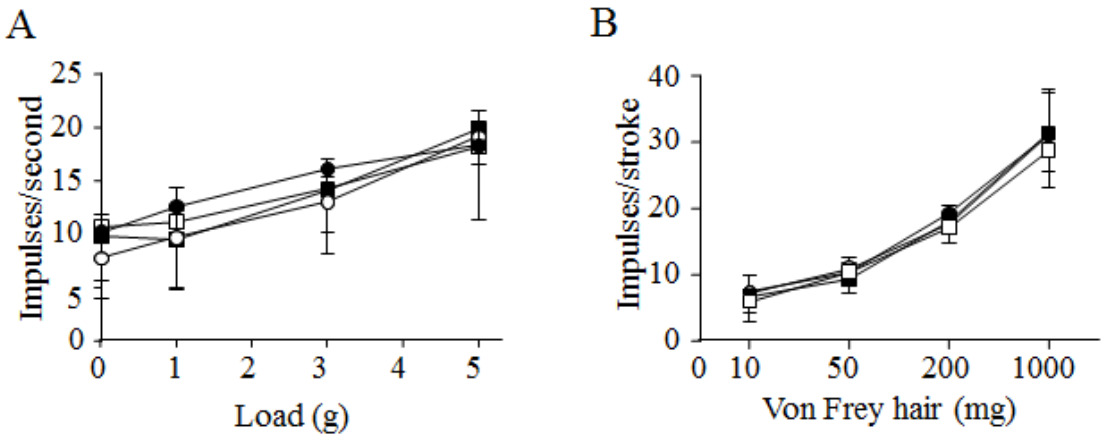


**Figure 2.2 The effect of time on the mechanosensitivity of mouse gastric vagal afferents**

A. Stimulus-response of gastric tension receptors to circular tension before (●) and after incubation for 20 (○), 40 (■) and 60 (□) minutes (*N* = 5). B. Stimulus-response of gastric mucosal receptors to mucosal stroking before (●) and after incubation for 20 (○), 40 (■) and 60 (□) minutes (*N* = 4). Time has no effect on the response of gastric tension receptors and mucosal receptors.

### 2.4.4 Data recording and analysis

Electrical signals were amplified with a biological amplifier (DAM 50; World Precision Instrumetns) and scaling amplifier (JRAK), filtered (band-pass filter-932; CWE Inc.) and recorded using an oscilloscope (DL 1200A; Yakogawa). Single units were discriminated on the basis of action potential shape, duration and amplitude off-line using Spike II software (Cambridge Electronic Design). Discharge traces were produced from Spike 2 software. All data are represented as the mean ± SEM with n= number of individual afferents. The difference in the overall pattern of mechanosensitivity after different doses of NPW exposure was evaluated using two-way ANOVA (Prism 5.02, Graphpad). Multiple comparisons by Bonferroni's correction were performed when ANOVAs revealed significance.

### 2.4.5 Drug

Stock solutions of the peptide NPW (0.1 mM; Tocris biosciences) was kept frozen (-80°C) and diluted to its final concentration in Krebs solution on the day of the experiment.

## 2.5 Tracing studies

### 2.5.1 Anterograde tracing

Vagal afferents endings in the mouse stomach were identified following anterograde tracing from their cell bodies located in the nodose ganglia, using a previously documented procedure [174, 285]. After an overnight fast (1600 hours to 0900 hours), mice were anaesthetised with isoflurane (1-1.5% in 15% oxygen). The left nodose was exposed and 0.5 µl of wheat-germ agglutinin-conjugated horse-radish peroxidise

(WGA-HRP; 4 mg/ml; PL-1026; Vector Laboratories) was pressure injected into the nodose ganglion via a glass micropipette (internal diameter = 25 μm; 1B100-3, WPI) prepared with an electrode puller. Multiple injections of 0.5-1 μl of tracer were injected into the nodose ganglion from three directions which ensure the tracer can be absorbed by the maximum number of possible nodose cells. The injection site was then dried, the skin incision closed and 0.05 ml antibiotics (terramycin; 10 mg/kg) and 0.05 ml analgesic (butorphanol; 5 mg/kg) were administered subcutaneously as mice regained consciousness. Mice were then individually housed and routinely monitored. After surgery mice recovered for two days allowing the tracer to anterogradely fill vagal afferent neurons to their peripheral nerve endings within the stomach.

### 2.5.2 Retrograde tracing

Cell bodies of gastric vagal afferents innervating specific stomach layers were identified using differential tracing from mouse stomach as previously documented [285, 286].

***Gastric muscle:*** C57/BL6 mice were fasted for 2 hours (0700 hours to 0900 hours) and anesthetized with isoflurane (1-1.5% in 15% oxygen), a laparotomy performed, and an Alexa Fluor® 555 conjugate of cholera toxin β-subunit (CTB-AF555; 500 mg in 100 μl 0.1M PB; C22843, Invitrogen) was injected subserosally into the muscularis externa of the whole stomach using a 30-gauge Hamilton syringe (Hamilton). Multiple equally spaced injections of 1-2 μl were made parallel to and 1-2 mm from the lesser curvature on both dorsal and ventral surfaces (total volume 10 μl), which ensures a greater area of the stomach is exposed to the tracer. The injection sites were then dried, the laparotomy incision closed, and antibiotic and analgesic administered as above.

***Gastric mucosa:*** C57/BL6 mice were fasted overnight (1600 hours to 0900 hours) to ensure an empty stomach during tracing surgery. Mice were anesthetized with isoflurane (1-1.5% in 15% oxygen), a laparotomy performed and a mucolytic (10% N-acetylcysteine; 200 µl; Bristol-Myers Squibb) was injected into the stomach lumen. The mucolytic stayed in the stomach for 5 min, which broke the lumen mucus allowing the tracer injected into the lumen to reach the mucosa more easily. Then the mucolytic was removed and the stomach lumen was rinsed twice with 200 µl saline. Subsequently, 10 µl of 0.5% CTB-AF555 was injected into the proximal gastric lumen via a 30-gauge Hamilton syringe and the stomach walls gently opposed to expose the dorsal and ventral surfaces to tracer. The laparotomy incision was then closed and antibiotic and analgesic administered as mentioned above. Food and water were withheld for 2 hours postoperatively to maximize mucosal exposure of tracer.

Mice, after both muscular and mucosal tracing surgeries, were individually housed and routinely monitored. Mice recovered for two days following surgery, allowing tracer to proceed from the stomach to cell bodies in the nodose ganglia. Mice were then processed for nodose cell culture and laser capture microdissection.

## 2.6 Immunohistochemistry

### 2.6.1 Perfusion fixation

Mice were anesthetized with intraperitoneal administration of sodium pentobarbitone (0.2 ml, 60 mg/ml; Troy laboratories). Their response to painful stimulus was checked by tail pinch. When mice were properly sedated, the rib cage was opened up to expose the heart and lungs. 0.5 ml of heparin (Pfizer) was directly injected into the left ventricle, and then the inferior vena cava was cut immediately. Warm heparinised saline

(3 ml heparin in 100 ml 0.85% sterile saline) was pumped into the left ventricle through a 21G blunt needle at a speed of 17 ml/min. Saline perfusion was continued until the blood start to run clear, and then switched to ice-cold fixative 4% paraformaldehyde in 0.1 M phosphate buffer (PFA-PB) at 4 ℃. Perfusion was stopped when the abdomen, head and tail were rigid. The stomach was then removed and cut along the greater curvature into ventral and dorsal sides. Tissue was post-fixed for 2 hours in 4% PFA-PB at room temperature and then was cryo-protected in 30% sucrose solution (30 g sucrose in 100 ml 0.1M PB) at 4℃ for a minimum of 18 hours. The tissue was then embedded in optimal cutting temperature compound (OCT; Tissue-Tek) and snap frozen in liquid nitrogen. Samples were kept in 80℃ until ready for cutting. 10um frozen section was cut and mounted onto gelatine coated slides. Slides were stored at -20℃ for less than a week before being processed for HRP detection and/or immunofluorescent staining.

### 2.6.2 WGA-HRP detection

Permanent visualization of WGA-HRP was achieved using a tyramide signal amplification system (TSA biotin system; NEL700A001KT; PerkinElmer, VIC, Australia). The stomach sections were air dried for 40 minutes and washed twice for 5 minutes in TNT wash buffer (0.05% Tween 20 (sigma), 0.15M NaCl, and 0.1M Tris-HCl, Ph 7.5). Then the sections were blocked with TNB (0.1M Tris-HCl, Ph 7.5, 0.15M NaCl; 0.5% blocking reagent provided with TSA biotin kit) for 60 minutes, and then reacted with tyramide-biotin (provided with TSA biotin kit) for 30 minutes at room temperature. The sections were then rinsed three times for 5 minutes with TNT buffer and reacted with streptavidin conjugated to Alexa Fluor® 647 (1:200 diluted in TNT

buffer; Invitrogen) for 2 hours at room temperature. After the final 3 × 5 minutes washes with TNT, the sections were processed for immunofluorescent staining.

### 2.6.3 Immunofluorescent staining

Frozen sections were air dried at room temperature for 40 minutes and rinsed three times for 5 minutes in PBS + 0.2% Triton X-100 (PBS-TX, pH 7.4; Sigma-Aldrich) to facilitate antibody penetration. Then all sections (including sections processed from HRP detection) were blocked with 10% normal chicken serum (for single-labelling) or 10% normal donkey serum (for double-labelling) in PBS-TX for 40 minutes at room temperature, and then incubated with rabbit anti-NPW polyclonal antibody (PH-005-61; Phoenix Pharmaceuticals) at a 1:800 dilution in PBS-TX at 4℃ for 20 hours. After three washes in PBS for 5 minutes, primary antibody was visualised using chicken anti rabbit secondary antibody conjugated to Alexa 488 (for single-labelling; Invitrogen) or donkey anti rabbit secondary antibody conjugasted to Alexa 568 (for double-labelling; Invitrogen) at a 1:200 dilution in PBS-TX for 1 hour at room temperature. For single-labelling, the sections were then given a final three times for 5 minutes PBS washes, mounted on slides and cover slipped using ProLong antifade (Invitrogen). For double-labelling, after three wash in PBS for 5 minutes, sections were then blocked in 10% goat anti mouse IgG (Invitrogen) in PBS-TX for 1 hour at room temperature, and then incubated in mouse anti-calcium sensing receptor monoclonal antibody (1:100 diluted in PBS-TX; MA1-934, ABR Affinity BioReagents) for 20 hours at room temperature. Unbound antibody was then removed with PBS washes and sections incubated for 1 hour at room temperature with donkey anti mouse secondary antibody conjugasted to Alexa 488 (1:200 in PBS-TX). The sections were then given final PBS-TX washes, mounted on slides and cover slipped using ProLong antifade.

### 2.6.4 Visualization

Slide sections were visualized using an epifluorescence microscope (BX-51, Olympus, Australia) equipped with filters for Alexa Fluor® 488, 568 and 647, with images acquired by a CoolSnapfx monochrome digital camera (Roper Scientific). Slides where primary antibody was omitted showed no labelling and served as negative controls. Typical NPW immunoreactive cells and negative control are shown in Figure 2.3. Typical colocalization of NPW and CaSR immunoreactive cells and negative control are shown in Figure 2.4.

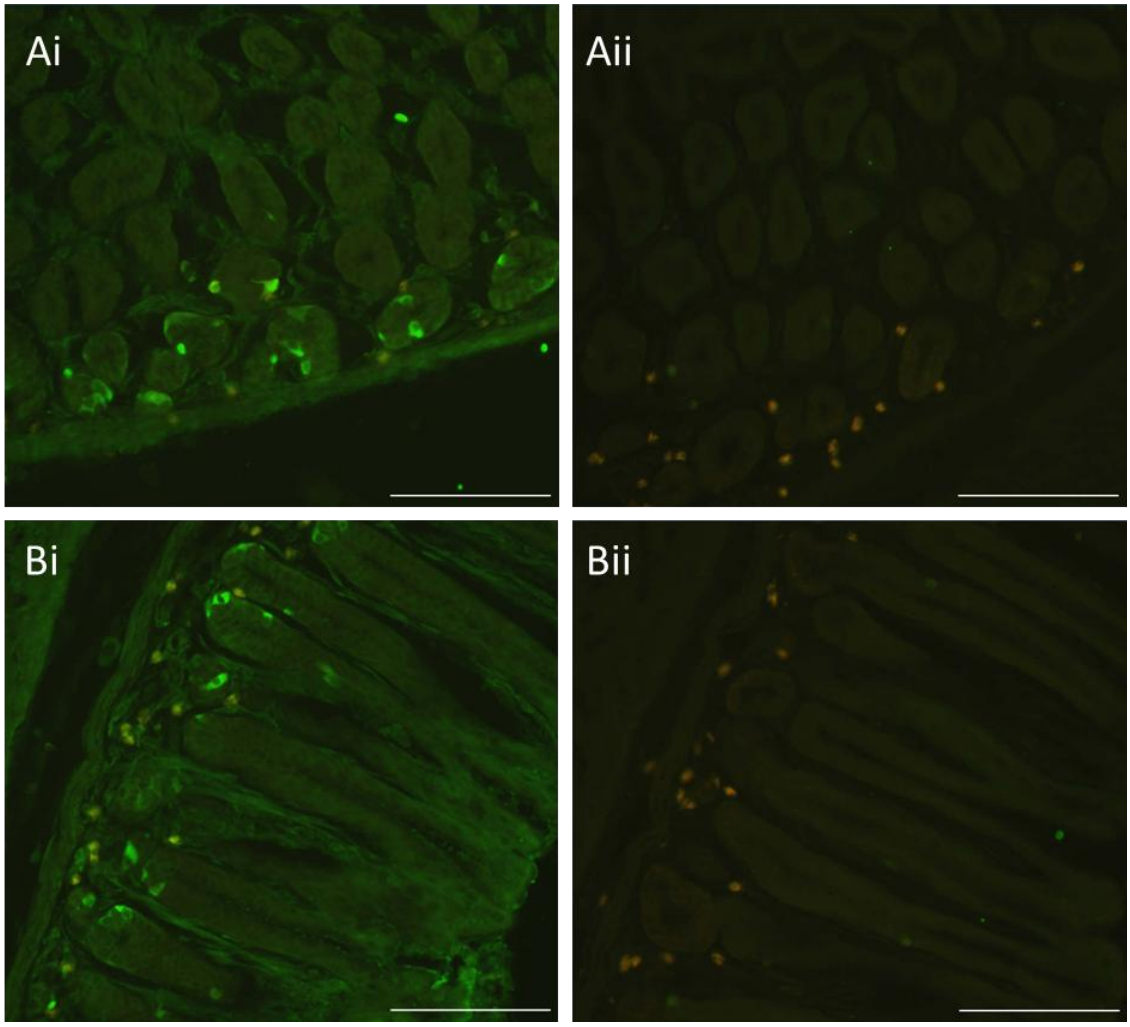


**Figure 2.3 Neuropeptide W immunoreactive cells in mouse gastric mucosa**

Ai, Bi. NPW immunoreactive cells in mouse gastric antrum. Aii, Bii. Negative controls with primary antibody omitted. Scale bar, 100 μm.

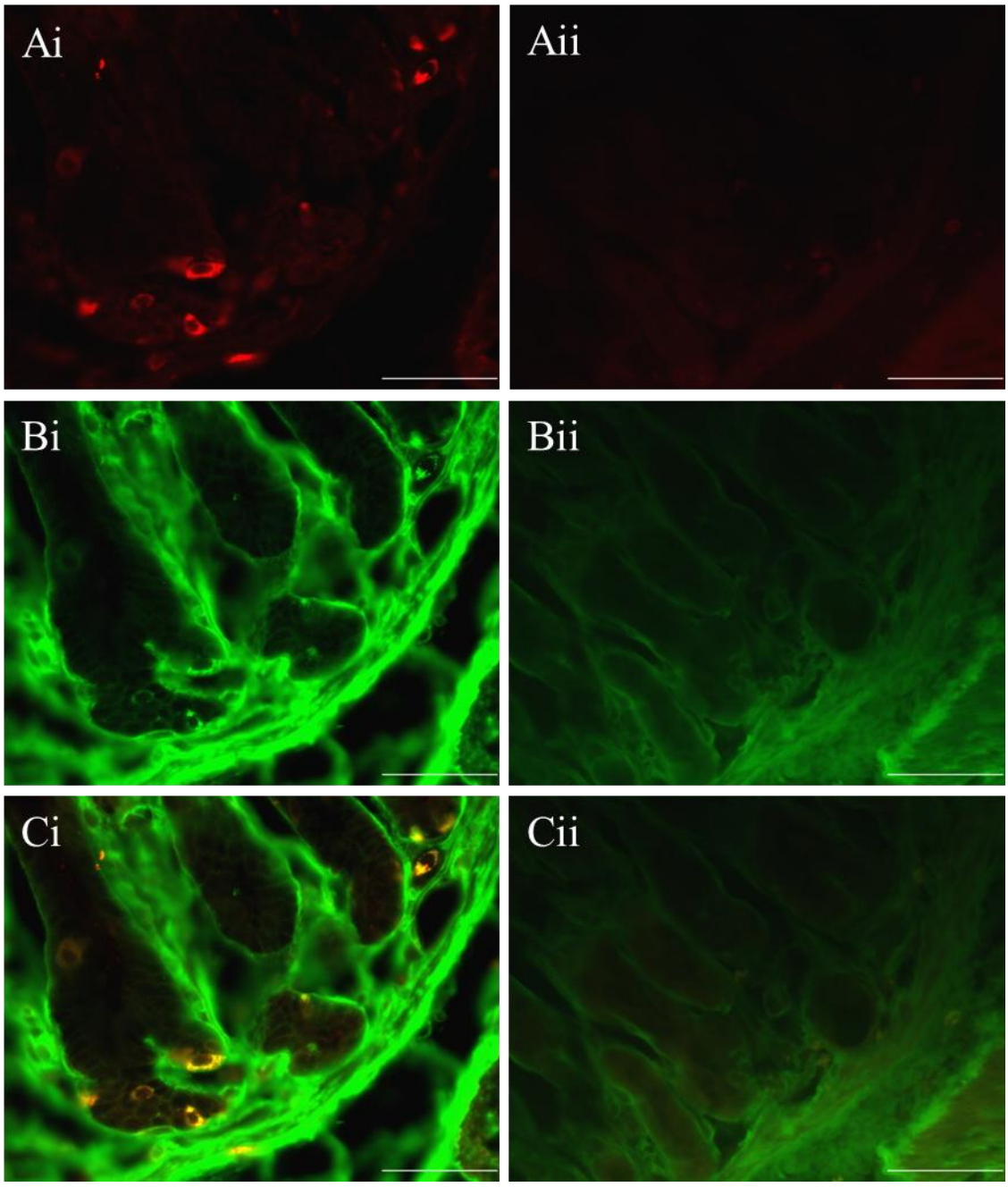


**Figure 2.4 Colocalization of neuropeptide W and calcium sensing receptor immunoreactive cells in mouse gastric antrum**

Ai, Bi and Ci. Typical colocalization of NPW and CaSR immunoreactive cells in mouse antrum. Ai, NPW immunoreactive cells. Bi, CaSR immunoreactive cells. Ci, overlay of Ai and Bi. Aii, Bii and Cii. Negative control with antibody omitted. Aii, NPW negative control; Bii, CaSR negative control; Cii, overlay of Aii and Bii. Scale bar, 100 μm.

### 2.6.5 Cell quantification

For quantification of the numbers of NPW immunoreactive cells, serial sections of gastric antrum were viewed at x 20 magnification and NPW immunoreactive cells were counted manually on 5 consecutive glands per section from antral glandular regions where strongly immunoreactive cells resided. The number of NPW positive cells per gland from each mouse was averaged from 10 non-overlapping sections. The NPW positive cells per gland were compared between feeding groups. Immunohistochemistry procedures were always performed on tissue from different groups at the same time. The number of NPW immunoreactive cells per gland was expressed as mean ± SEM.

## 2.7 Cell culture

### 2.7.1 Primary cell culture of mouse nodose ganglion

Retrogradely traced mice were anaesthetized with sodium pentobarbitone (0.2 ml, 60 mg/ml; Troy laboratories; i.p.), decapitated and both nodose ganglia from each animal were rapidly removed and placed into 10 ml F12 culture medium (11765, Invitrogen) at 4˚C. To obtain optimal cell density, nodose ganglia from four mice were combined into a tube containing F12 complete nutrient medium (10% foetal calf serum and 1% penicillin/streptomycin) and stored on ice. After all nodose ganglion were collected, F12 culture medium was removed and nodose ganglia were dissociated by incubating at 37℃ in a 4 mg/ml solution of dispase (17105; Invitrogen) and collagenase II (17101; Invitrogen) made up in Hank's Balanced Salt Solution (HBSS; 14170, Invitrogen) with agitation at 5 minutes intervals. After 30 minutes the dispase/collagenase solution was removed, and after 10 minutes incubation in a 4 mg/ml solution of just collagenase II in HBSS, the cells were rinsed twice in 2 ml cold HBSS and then incubated in 2 ml F12

culture medium. The ganglia were allowed to settle to the bottom of tube, and 1 ml F12 culture medium was removed. The nodose ganglia were mechanically dissociated into single cells by passing through a fire polished pasteur pipette until no cell clumps were visible. Generally 10 times trituration would be enough to obtain a cloudy suspension with no obvious friction observed. Cells in suspension were collected by centrifugation at 50 g for 1 minute (ramp up 1 minute, spin 1minute and break 1minute). Then the cells were washed twice in 1ml ice-cold HBSS, and resuspended in 120 µl HBSS. A cell count was performed using Trypan blue (0.4%; T10282, Invitrogen) exclusion, and then cells were diluted to 500 - 1000 cells per 10 µl suspension and seeded to 50 mm duplex dishes (415190-9111-000; Carl Zeiss). The dishes were placed in a humidified 95% air and 5% $CO_2$ incubator at 37℃ for 2 hours to allow cell adherence. After incubation, cells were then subject to laser-capture microdissection. Sterile techniques were applied throughout the cell separation and the culture hood and equipment were sterilized using 70% ethanol.

### 2.7.2 Primary cell culture of mouse gastric antral mucosal cells

#### 2.7.2.1 Isolation of mouse gastric antral mucosal cells

Mice were anaesthetised with sodium pentobarbitone (0.2 ml, 60 mg/ml; Troy laboratories; i.p.), and the gastric antrum was removed and placed in cold HBSS and washed vigorously three times. The mucosal layer was carefully striped off using forceps under the microscope and minced using razor blades into 2-3 $mm^2$ in cold HBSS medium and placed in 2 ml DMEM/F-12 (Invitrogen). Mucosal tissue was incubated in a 1mg/ml digestion solution containing collagenase II and dispase made up in HBSS in a 37℃ water bath for 30 minutes with a gentle agitation at 5 minutes intervals, and then in 1mg/ml collagenase solution for 30 minutes. The cells suspension

were centrifuged at 600 rpm for 2 minutes, washed twice in 2 ml cold HBSS, and triturated using a fire polished Pasteur pipette in 2 ml culture medium (DMEM/F-12 supplimented with 10% fetal calf serum and 1% penicillin/streptomycin) and filtered through a 100 µm sterile nylon mesh (Cell strainer; 352360, BD Falcon). The sediment was washed twice with 2 ml cold HBSS and resuspended in 2 ml culture medium. Tissue was triturated using a fire polished Pasteur pipette gently and the cell suspension was filtered through a 100 µm sterile nylon mesh. The isolated gastric mucosal cells in suspension and sediment were then combined. A cell count was performed using Trypan blue exclusion. On average, 6.4 x $10^5$ cells were isolated from each stomach. Cells from 3 stomachs were combined together and diluted to a final concentration of 1 x $10^5$ cells/ml in culture medium.

#### 2.7.2.2 Peptide expression study in mucosal cell culture

24-well plates (BD) were coated with 120 µl MAP (0.25 mg/ml in PBS; USUN) at 37 ℃ for 10 minutes. Then MAP was removed and plates were air dried. The cells were placed into the pre-coated 24-well culture plates (1 x $10^5$ cells per well) and were incubated in humidified 95% air and 5% $CO_2$ incubator at 37 ℃ for 20 hours before each study. After overnight incubation, cells were washed twice with Hepes buffer to remove the culture medium and unadhesive cells, and exposed to various test reagents dissolved in 500 µl Hepes buffer for 60 minutes in humidified 95% air and 5% $CO^2$ incubator at 37 ℃. The Hepes buffer had the following composition: 140 mM NaCl, 4.5mM KCl, 20mM Hepes (15630-106, Invitrogen), 1.2 mM CaCl2, 1.2 mM MgCl2 and 0.1% BSA. The test reagents include L-Phenylalanine (5 mM or 50 mM; P5482, Sigma), D-glucose (5 mM or 50 mM; Merck Millipore) and Calcium sensing receptor antagonist NPS2143 (25 µM; sc-361280, Santa Cruz). After incubation, culture medium

was completely aspirated off, and 120 µl buffer RLT (provided with the RNeasy micro kit) was added into each well followed by gentle pipetting and mixing. The lysate was then snap-frozen in liquid nitrogen until it was processed to total RNA isolation.

## 2.8 Laser capture microdissection

Laser capture microdissection was performed on a PALM microlaser technologies microbeam microdissection system (Carl Zeiss). Cell spot was dehydrated by a UV processed teri wiper. As illustrated in Figure 2.5, fluorescent labelled nodose neurons were identified and selected under the microscope. Then cells were UV laser micro-dissected and catapulted directly into the cap of a 0.5 ml sterile centrifuge tube, filled with 40 µl extraction buffer provided in the Picopure RNA Isolation Kit (KIT0202, Arcturus). After collection (usually take around 1 hour), the cap with collected cells was placed onto the attached tube, immediately spun down, snap-frozen in liquid nitrogen, and stored at -80 ℃ until it was processed for total RNA extraction.

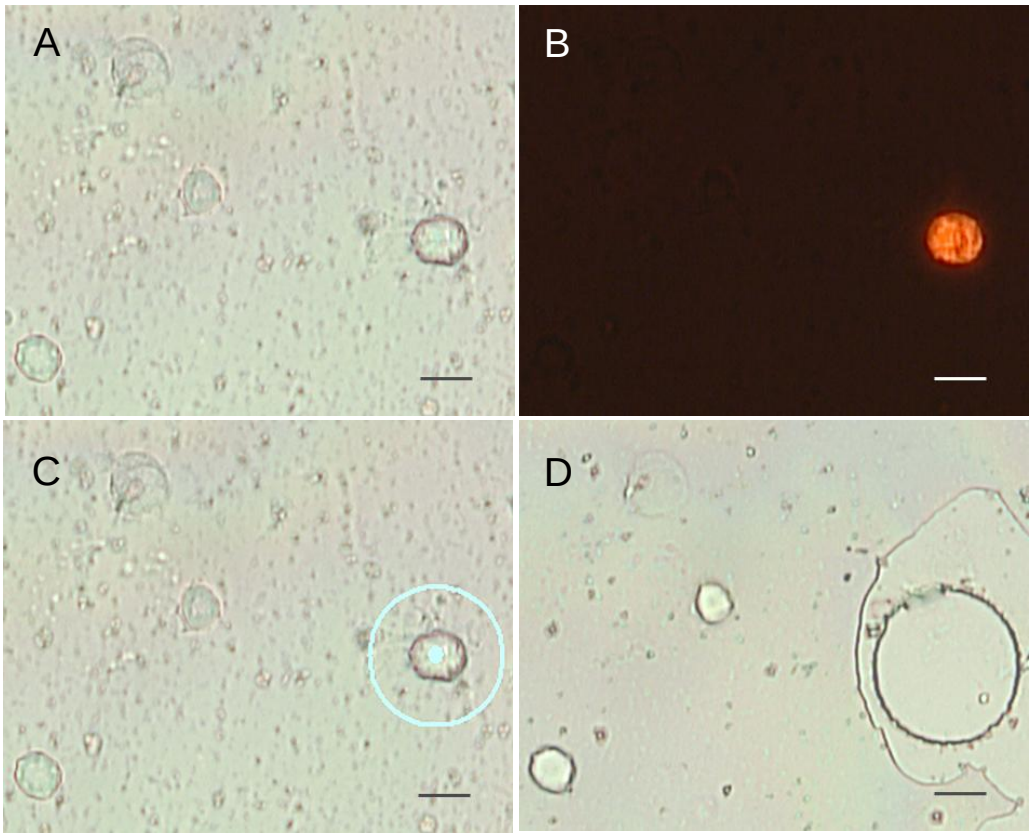


**Figure 2.5 Typical process of lasercapture microdissection of fluorescent labelled nodose neurons**

A. Cultured nodose neurons were observed under microscope. B. Fluorescent labelled nodose neuron was identified and distinguished from untraced cells. C. Labelled nodose neuron was circled as targets for UV laser cutting. D. Cells after UV laser cutting.

## 2.9 Total RNA isolation

Total RNA from gastric mucosa was extracted using an RNeasy Mini Kit (74104; Qiagen). Total RNA from nodose ganglia or cell culture was extracted using a RNeasy Micro Kit (74004; Qiagen). QIAshredder (79654; Qiagen) and RNase-free Dnase set (79254; Qiagen) were used in combination with RNeasy Mini Kit and RNeasy Micro Kit. Total RNA from laser captured cells were extracted using a PicoPure™ RNA Isolation Kit (Arcturus). The extraction process was in accordance with the manufacturer's instructions. Total RNA was quantified by measuring the absorbance at 260nm (A260) using a NanoDrop™ ND 1000 spectrophotometer (Thermo Scientific) and RNA purity was estimated via the 260/280 absorbance ratio.

## 2.10 Quantitative real-time PCR

Quantitative real-time PCR reactions (QRT-PCR) were performed as described in detail previously [287]. In short, reactions were performed using a Chromo4 (MJ Research) real-time instrument attached to a PTC-200 Peltier thermal cycler (MJ Research) and analysed with Opticon Monitor Software (MJ research). QRT- PCR reactions were performed using a QuantiTect SYBRgreen RT-PCR one-step RT-PCR kit (204245; Qiagen) according to the manufacturer's instructions. All primers used were pre-designed validated QuantiTect Primer assays (Qiagen) and targeted GPR7 (QT00299040) and $\beta$-tubulin (QT00124733) in the nodose ganglia, and NPW (QT00295400), gastrin (QT00107702) and $\beta$-actin (QT01136772) in the stomach. QRT-PCR reactions were carried out under the following conditions: reverse transcription, 50 ℃ for 30 minutes; initial PCR activation, 95 ℃ for 15 minutes; PCR cycles 94 ℃ for 15 seconds, 55 ℃ for 30 seconds and 72 ℃ for 30 seconds repeated for 50 cycles. A melt curve was obtained to confirm the specificity of the products

produced and products were visualised using 3% agarose gel electrophoresis with ethidium bromide staining. Each assay was run in triplicate and repeated in a duplicate PCR experiment. Control PCRs were carried out substituting RNase-free water for template RNA. Relative RNA levels were calculated using the comparative CT method as described previously [288], or normalized to corresponding control by using the delta-delta CT method as described previously [289].

### 2.11 Breath test analysis

The effect of NPW on gastric half emptying time (t ½) was determined using breath test analysis as previously described [290-292]. Mice underwent repeated breath tests to examine different doses of NPW and test meals. Briefly, after an overnight fast (1600 to 0900 hours), the mice were placed into a gas-tight rubber-sealed 100 ml glass containers, with a two-way valve inserted into the lid as an outlet for sampling with a 10 ml plastic syringe. Following the baseline breath sample collection, mice were intraperitoneally injected with 200 µl 0.9% saline or 200 µl saline containing 1 or 3 nmol NPW /100 g body weight, doses shown to exert other short-term physiological functions peripherally [216, 222]. After 30 minutes, mice were gavaged intragastrically with 100 µl of water containing 1 µl/ml 13C-acetic acid (99% enrichment; Cambridge Isotope Laboratories) or given 0.1 g of baked egg yolk containing 1 µg/ml of 13C-labelled octanoic acid (99% enrichment, Cambridge Isotope Laboratories) to consume within 1 minute. Breath samples were collected following liquid feeding at 2.5, 5, 7.5, 10, 15, 20, 25, 30, 45, 60, 75, 90, 105, 120 minutes, and was collected following solid meal consumption at 5, 10, 15, 20, 25, 30, 45, 60, 75, 90, 105, 120, 135, 150 minutes. Breath air was accumulated for 2 minutes before each breath sampling. During the sampling intervals, the lid was replaced with a wire mesh to allow fresh air flow into

the chamber. Breath samples were analyzed for $^{13}CO_2$ content with an isotope ratio mass spectrometer (ABCA 20/20; Europa Scientific). There was a minimum of 3 days between subsequent breath tests to allow for full recovery. The $^{13}CO_2$ excretion data were analyzed by nonlinear regression analysis for curve fitting and for calculation of gastric half-emptying time (t ½). Data are expressed as mean ± SEM.

### 2.12 Enzyme immunoassay and radioimmunoassay

#### 2.12.1 Plasma extraction

Mice blood samples were collected into pre-chilled $K_2$EDTA coated tubes and plasma was separated by centrifugation at 3000 rpm for 15 minutes at 4 ℃ and stored at -80 ℃ until assayed. Human blood samples were collected into ice-chilled EDTA-treated tubes containing 400 kIU aproptinin (Trayslol) per milliliter blood. Plasma was separated by centrifugation at 3200 rpm for 15 minutes at 4 ℃ within 30 minutes of collection and stored at -80 ℃ until assayed.

#### 2.12.2 Enzyme immunoassay

Enzyme immunoassay (EIA) was used to test NPW concentrations in both human and mouse samples. Human plasma NPW concentration was determined using a Human NPW-23 EIA kit (EK-005-60; Phoenix Pharmaceuticals) according to manufacturer's instructions. All samples were tested in duplicate. The NPW assay recognises both human NPW-23 and NPW-30. The minimum detectable concentration was 0.1 ng/ml. A typical standard curve of this assay is shown in Figure 2.6. The linear range is between 0.1-1.72 ng/ml, and all samples were diluted in 1:10 to lie within this range. The intra-assay and inter-assay coefficient variation was <10%. NPW concentration in mouse plasma was determined using a mouse NPW-23 EIA kit (EK-005-61; Phoenix

Pharmaceuticals) according to manufacturer's instructions. The NPW assay recognises mouse NPW-23. A typical standard curve of this assay is shown in Figure 2.6. The linear range is between 0.2-5.07 ng/ml, and all samples were diluted in 1:10 to lie within this range. The minimum detectable concentration was 0.2 ng/ml, and the intra-assay variation was 1.7%.

### 2.12.3 Radioimmunoassay

Radioimmunoassay (RIA) was used to test gastrin concentrations in both human and mouse samples. Human plasma gastrin concentration was determined by gastrin RIA kit (06B255017; MP Biomedicals) according to manufacturer's instructions. All samples were tested in duplicate. The cross-reactivity to gastrin-17-I, gastrin-17-II, gastrin 34-I and gastrin-(5-17) was 100%, 77%, 42% and 54% respectively. A typical standard curve of this assay is shown in Figure 2.7. The minimum detectable concentration was 3.3 pg/ml, and the intra-assay and inter-assay coefficient variation was < 15%. Gastrin concentration in mouse plasma was determined using gastrin EURIA RIA kit (MD 302; Euro-Diagnostica) according to manufacturer's instructions. The cross-reactivity to gastrin-17, sulphated gastrin-17 and gastrin-34 was 100%, 83% and 61% respectively. The minimum detectable concentration was 5 pmol/l, and the intra-assay variation was 3.0%. A typical standard curve of this assay is shown in Figure 2.7.

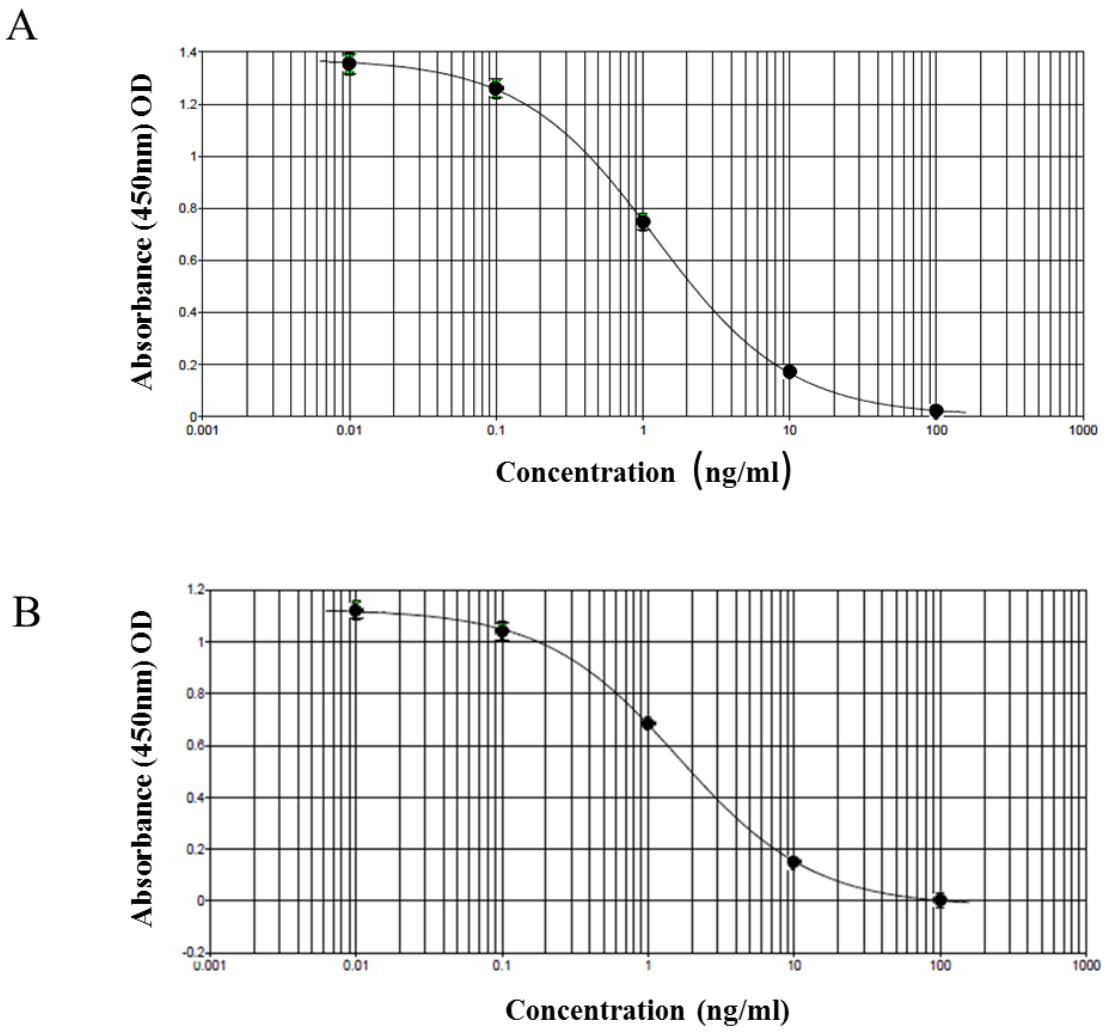


**Figure 2.6 Typical standard curve of human or mouse NPW-23 enzyme immunoassay kit**

A. Typical standard curve of Human NPW-23 EIA kit with logit log transformation. B. Typical standard curve of mouse NPW-23 EIA kit with logit log transformation.

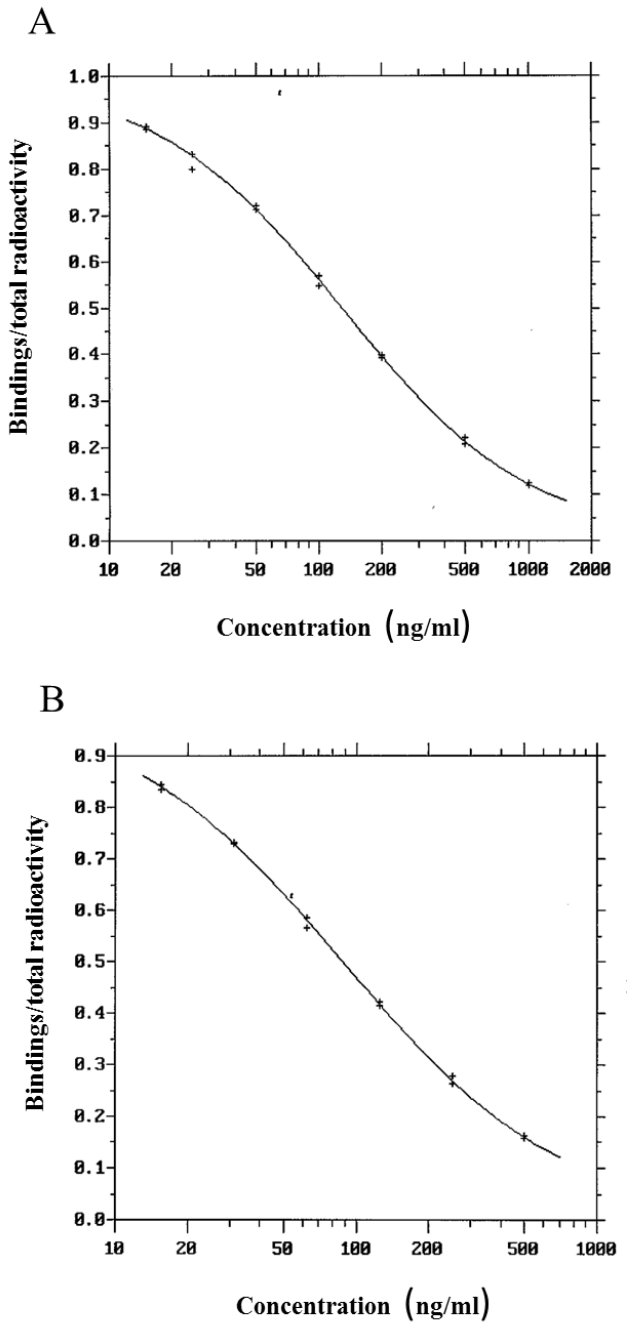


**Figure 2.7 Typical standard curve of gastrin radioimmunoassay kit**

A. Typical standard curve of gastrin radioimmunoassay kit with logit log transformation for human samples. B. Typical standard curve of gastrin EURIA radioimmunoassay kit with logit log transformation for mice samples.

## 2.13 Statistical analysis

Two-way ANOVA was used to determine the difference in the overall pattern of mechanosensitivity after different doses of NPW exposure. Un-paired *t*-test was used to compare mean values between two groups. In human study, repeated-measures one-way ANOVA was used in the human studies to compare baseline peptides concentrations between study days. Repeated-measures two-way ANOVA was used to evaluate peptides concentrations with time and treatment as factors. Areas under the curves (AUCs; $t$ = -30 - 180 min) were calculated using the trapezoidal rule for human plasma peptides levels after test meal intake, and they were compared among treatment groups using repeated-measures one-way ANOVA. Post hoc paired comparisons adjusted for multiple comparisons by Bonferroni's correction were performed when ANOVAs revealed significance. Gastric emptying rates between different doses of NPW and saline injected groups were compared by one-way ANOVA with a Tukey post hoc test. All comparisons were performed by using Graphpad Prism 5.02 (Graphpad). Statistical significance was set at $P < 0.05$.

## 2.14 Media and solutions

Values in parentheses are final concentrations.

**Modified Krebs' buffer solution (10000 ml)**

| | | |
|---|---|---|
| NaCl | 69.0 g | (118.1 mmol/l) |
| KCl | 3.54 g | (4.7 mmol/l) |
| $NaHCO_3$ | 21.0 g | (25.1 mmol/l) |
| $NaH_2PO_4$ | 1.56 g | (1.3 mmol/l) |
| $MgSO_4.7H_2O$ | 2.96 g | (1.2 mmol/l) |

| | | |
|---|---|---|
| $CaCl_2$ | 25 ml | (1.5 mmol/l) |
| citric acid | 2.0 g | (1.0 mmol/l) |
| D-Glucose | 20.0 g | (11.1 mmol/l) |
| Nifedipine | 1 ml | (1 µmol/l) |
| Water | Top up to 10000ml | |

bubbled with 95% $O_2$ / 5% $CO_2$.

**0.1 M Phosphate buffer, pH 7.4 (1000 ml)**

| | |
|---|---|
| $NaH_2PH_4$ | 2.6 g |
| $NaHPO_4$ | 11.5g |
| Distilled water | 800ml |
| pH to 7.4 using 1M HCL/1M NaOH | |
| Distilled water | top up to 1L |

**PFA-PB (1000 ml)**

| | |
|---|---|
| Paraformaldeyhe | 40 g |
| 0.1M Phosphate Buffer, pH 7.4 | 800 ml |
| pH to 7.4 using 1M HCL/1M NaOH | |
| 0.1M Phosphate Buffer, pH 7.4 | top up to 1L |

**Phosphate buffered saline, pH 7.4 (1000 ml)**

| | |
|---|---|
| NaCl | 80 g |
| KCl | 2 g |
| $NaH_2PO_4$ | 14.4 g |
| $KH_2PO_4$ | 2.4 g |

| | |
|---|---|
| Distilled water | 800 ml |
| pH to 7.4 using 1M HCL/1M NaOH | |
| Distilled water | top up to 1L |

**PBS-TX (100 ml)**

| | |
|---|---|
| Phosphate buffered saline, pH 7.4 | 100 ml |
| Triton X-100 | 200 µl |

**0.1M Tris-HCl, pH 7.5 (1000 ml)**

| | |
|---|---|
| Tris | 12.1 g |
| Distilled water | 900ml |
| pH to 7.4 using 1M HCL | |
| Distilled water | top up to 1L |

**TNT buffer (1000 ml)**

| | |
|---|---|
| NaCl | 8.76 g |
| Tween20 | 500 µl |
| 0.1M Tris-HCl, pH 7.5 | top up to 1L |

**TNB buffer (1000 ml)**

| | |
|---|---|
| NaCl | 8.76 g |
| Blocking reagent (TSA biotin kit) | 5 ml |
| 0.1M Tris-HCl, Ph 7.5 | top up to 1L |

# CHAPTER 3. MODULATION OF MURINE GASTRIC VAGAL AFFERENT MECHANOSENSITIVITY BY NEUROPEPTIDE W

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# Statement of Authorship

## Modulation of Murine Gastric Vagal Afferent Mechanosensitivity by Neuropeptide W

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***By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.***

**Hui Li** (Candidate)

Performed most electrophysiology experiments, retrograde tracing surgery, cell culture, laser capture microdissection, QRT-PCR, breath test experiments, husbandry of the mice, data analysis and interpretations and wrote the manuscript.

Signed Date 26/9/2013

**Stephen Kentish**

Aided in the husbandry of animal and construction of manuscript.

Signed Date 26/9/2013

**Stamatiki Kritas**

Analysed the breath test results.

Signed Date 26/9/2013

**Richard L. Young**

Performed the anterograde tracing surgery and manuscript construction.

Signed Date 26/9/2013

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Performed the retrograde tracing surgery and immunohistochemistry experiments.

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**Tracey A. O'Donnell**

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Signed Date 26/9/2013

**L. Ashley Blackshaw**

Assisted in the conceptualisation of experiments and construction of manuscript.

Signed Date 27/9/2013

**Gary A. Wittert**

Assisted in the interpretation of data and construction of manuscript.

Signed Date 3/9/2013

**Amanda J. Page**

Assisted in the conceptualisation of experiments, performed some electrophysiology experiments, data analysis and interpretation and manuscript construction.

Signed Date 27/9/2013

## 3.1 Abstract

**Aim:** Neuropeptide W (NPW) is an endogenous ligand for the receptors GPR7 and GPR8 and is involved in central regulation of energy homeostasis. NPW in the periphery is found in gastric gastrin (G)-cells. In the stomach, energy intake is influenced by vagal afferent signals so we aimed to determine the effect of NPW on mechanosensitive gastric vagal afferents under different feeding conditions.

**Methods:** Female C57BL/6 mice (N >10/group) were fed a standard laboratory diet (SLD), high fat diet (HFD), or were food restricted. The relationship between NPW immunopositive cells and gastric vagal afferent endings was determined by anterograde tracing and NPW immunohistochemistry. An *in vitro* gastro-oesophageal preparation was used to determine the functional effects of NPW on gastric vagal afferents. Expression of NPW in the gastric mucosa and GPR7 in whole nodose ganglia was determined by quantitative RT-PCR (QRT-PCR). The expression of GPR7 in gastric vagal afferent neurons was determined by retrograde tracing and QRT-PCR.

**Results:** NPW immunoreactive cells were found in close proximity to traced vagal afferents. NPW selectively inhibited responses of gastric vagal tension receptors to stretch in SLD but not HFD or fasted mice. In the nodose ganglia, GPR7 mRNA was specifically expressed in gastric vagal afferent neurons. In fasted mice gastric mucosal NPW and nodose GPR7 mRNA was reduced compared to SLD. A HFD had no effect on gastric NPW mRNA, but down-regulated nodose GPR7 expression.

**Conclusion:** NPW modulates gastric vagal afferent activity, but the effect is dynamic and related to feeding status.

## 3.2 Introduction

Neuropeptide W (NPW) is a peptide originally identified in the hypothalamus as an endogenous ligand for G-protein-coupled receptors (GPCRs), GPR7 and GPR8 [206-208]. It is widely expressed in the central nervous system (CNS) as well as peripheral tissues of rats [209, 215, 220], mice [208] and humans [207, 226]. Although GPR7 and GPR8 are also widely distributed, to date GPR8 has only been found in humans and is absent in rats and mice [205]. NPW has been found to play a role in many physiological functions including stress, inflammatory pain, cardiovascular function as well as energy balance [248, 258]. Within the hypothalamus, NPW is involved in the regulation of feeding behaviour, but its role is complicated and controversial. Some studies suggest NPW inhibits food intake [211, 219], via activation of pro-opiomelanocortin/cocaine- and amphetamine-regulated transcript neurons and inhibition of neuropeptide Y/agouti-related peptide neuron [219]. Other studies indicate an orexigenic role [206, 251, 252], showing that NPW activates orexigenic neurons and transiently reduces plasma leptin levels [222, 252].

NPW is abundantly expressed within gastrin cells (G-cells) of the gastric antrum in rats, mice and humans [226]. Like many enteroendocrine cells, G-cells are open-type flask shaped cells which are typically responsive to luminal nutrients [293]. Plasma NPW levels in the rat gastric antral vein decrease upon fasting and increase again after refeeding [226], and a similar pattern of NPW mRNA and protein expression has been observed in the gastric mucosa of rats [229]. Gastrointestinal peptides regulated in response to food ingestion are generally thought to be involved in the short-term regulation of gastric function and food intake [78, 294] but the physiology of gastric NPW remains unclear.

Gastric vagal afferents play a major role in the regulation of food intake and gastric function [159, 163]. There are two functional classes of vagal afferents which detect mechanical stimuli within different layers of the mouse stomach [165, 166]. In the muscular layer tension receptors detect stomach fullness by responding to distension of the stomach wall [171, 295]. In contrast, mucosal receptors, located within the mucosal layer of the stomach, are activated by mechanical contact with large food particles and may contribute to the discrimination of particle size, acting to slow gastric emptying and facilitate mechanical digestion in the stomach [168]. We have shown that a number of peptides, known to be involved in energy homeostasis, modify the sensitivity of gastric vagal endings to mechanical stimuli [174, 176]. In addition, the effect of ghrelin and leptin on gastric vagal afferent mechanosensitivity is dependent on feeding status [174, 180]. NPW is an ideal candidate for modulation of gastric vagal afferent satiety signals, as NPW containing G-cells are abundant in the gastric antrum, and thus NPW released from these cells may modulate the activity of adjacent gastric vagal afferents. However, it remains unknown whether NPW elicits such a modulatory role and if so, whether this is dependent on the state of nutrition.

We therefore determined the effect of NPW on the mechanosensitivity of gastric vagal afferents in mice on standard laboratory diet (SLD), following a short-term restriction in food intake or following long-term consumption of a high-fat diet (HFD). We also established the anatomical relationship between NPW containing cells in the mucosa and vagal afferent endings. In addition, we investigated expression of GPR7 in vagal afferent cell bodies, and changes in the expression of NPW and GPR7 in gastric mucosa and nodose ganglia respectively.

## 3.3 Materials and methods

This study conforms to Good Publishing Practice in Physiology [296].

### Ethical approval

All studies were performed with the approval of the Animal Ethics Committees of the Institute for Medical and Veterinary Science and University of Adelaide, Adelaide, Australia, and in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The experiments also conform to the principles of UK regulations, as described in Drummond (2009).

### Mice

Female C57BL/6 mice were group housed with free access to water and food. At 8 weeks of age, mice were randomly assigned to one of three feeding groups. The first two groups were fed standard laboratory diet (SLD (14MJ/Kg); energy from fat-12%, protein-23%, carbohydrate-65%) for 12 weeks. In the SLD the fat is comprised of mixed vegetable and canola oils and the sugar from wheat and barley (Specialty Feeds, Glen Forrest, WA, Australia). Prior to experimentation one group was fed SLD ad libitum (SLD group) while the other group was fasted overnight for 17 h (SLD fasted group). During the fasting period, mice were housed individually, with free access to water, in a cage where bedding and nesting material were withdrawn. Coprophagia was restricted using a raised wire mesh inserted into the bottom of the mouse cages that allowed faecal pellets to drop to the cage floor. Over the duration of the fasting period the body weight dropped by ~2g (9.5%). The third group of mice (HFD group) were fed a HFD (21.4MJ/Kg; energy from fat – 61%, protein – 16%, carbohydrate – 23%) ad libitum for 12 weeks. In the HFD the fat is predominantly cocoa butter and the sugar is

a mixture of sucrose, wheat starch and dextrinised starch (Specialty Feeds). We have previously determined that female C57BL/6 mice fed a HFD have increased total body weight and fat mass over a 12 week feeding period compared to SLD mice [174]. The weights of the mice were monitored weekly.

### *In vitro* mouse gastro-oesophageal vagal afferent preparation

This preparation has been described in detail previously [166, 281]. Briefly, C57BL/6 mice ($N \geq 3$/receptor) were killed via $CO_2$ inhalation and the thorax opened by midline incision. The stomach and oesophagus with attached vagal nerves were removed and placed into a modified Krebs solution containing (mM): NaCl 118.1, KCl 4.7, $NaHCO_3$ 25.1, $NaH_2PO_4$ 1.3, $MgSO_4.7H_2O$ 1.2, $CaCl_2$ 1.5, citric acid 1.0 and glucose 11.1, bubbled with 95% $O_2$-5% $CO_2$ at 4℃ during dissection to prevent metabolic degradation. Nifedipine (1 μM) was also added to the Krebs superfusate to prevent smooth muscle contraction. In a previous preliminary study we have shown that nifedipine has no effect on the mechanical sensitivity of gastro-oesophageal vagal afferents [282]. After further dissection, the preparation was opened out longitudinally along the oesophagus and the greater curvature of the stomach. The preparation was then placed mucosal side up in an organ bath (Fig. 3.1). The vagus nerves (< 1.5 cm long) were extended into a second chamber where they were rested on a glass plate and bathed in paraffin oil. Under a dissecting microscope the nerve sheath was gently peeled back to expose the nerve trunk. Using fine forceps, nerve fibres were teased apart into 8-14 bundles then, one by one, the small bundles were placed onto a platinum recording electrode. A reference electrode rested on the glass plate in a small pool of Krebs' solution.

### Characterization of gastric vagal afferent properties

Two types of mechanosensitive afferent fibre were studied, those responding to mucosal stroking but not circular tension (mucosal receptors) and those responding to mucosal stroking and circular tension (tension receptors) as reported previously [166].

Location of receptive fields for both types of afferent fibres was determined by mechanical stimulation throughout the preparation with a brush. In this study, the receptive fields tested were located in the body and antrum of the stomach. Accurate quantification of mechanical responses was performed differently according to the primary adequate stimulus for the type of fibre. Mechanical thresholds of mucosal receptors were determined using calibrated von Frey hairs. The most reproducible, stimulus-dependent responses of these afferents to mucosal stroking were evoked when the probe was moved at a rate of 5 $mm.s^{-1}$ across the receptive field rather than being kept static. Due to the fact that the receptive fields were small (< 1 $mm^2$), a single test at each intensity is prone to missing the centre of the receptive field on some occasions. Therefore, we minimised experimental error by measuring the mean response to the middle eight of ten standard strokes given at 1 s intervals. Tension-response curves were also obtained and used in combination with von Frey thresholds to determine whether the receptive field of fibres were located in the mucosal or the muscular layer. Tension stimuli were applied via fine suture silk attached via a hook to an unpinned point adjacent to the mechanoreceptive field. The thread was attached to a cantilever via a pulley close to the preparation. Reference standard weights were then placed on the opposite end of the cantilever. Each weight was applied as a step and maintained for 1 min, and the response was measured as the mean discharge frequency evoked over this entire period. Due to the fact that all responses to tension adapted slowly, this method of assessment was considered representative of physiological responsiveness. The tension-response curves were produced by applying weights to the cantilever system in

the range of 1-5 g. A recovery period of at least 1 min was allowed between each tension stimulus.

**Effect of neuropeptide W on gastric vagal afferent mechanosensitivity**

After mechanical sensitivity of the gastric vagal afferents had been established, the effect of NPW on mechanical sensitivity was determined. NPW (1 nM) was added to the superfusing Krebs solution and allowed to equilibrate for 20 min after which time the tension-response and stroke-response curves were re-determined. This equilibration period ensured penetration of the peptide into all layers of the tissue. This procedure was repeated for NPW at increasingly higher doses (3-10 nM), consistent with other reports [206, 251]. In a separate cohort of mice on the same diet regime plasma NPW levels in female SLD and HFD mice were 9.7 ± 0.7 ng/ml (3.5 nM) and 5.9 ± 0.5 ng/ml (2.1 nM) respectively [297]. Unpublished data from female 8 week old fasted SLD mice reveal plasma NPW levels are 7.9 ± 0.9 ng/ml (2.8 nM; $N$ = 10). Therefore the concentrations of NPW used in the in vitro preparation are consistent with plasma levels in the SLD, fasted SLD and HFD mice. There was no apparent difference in the effect of NPW on afferents located in the corpus or antrum of the stomach and therefore the data from these afferents were pooled.

**Data recording and analysis**

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

computer (Dell Latitude). Trace images were produced from Spike 2 software. Data are expressed as mean ± SEM with *n* = number of individual afferents and *N* = number of mice in all cases. The difference in the overall pattern of mechanosensitivity of the individual afferents (n) after different doses of NPW exposure was evaluated using two-way ANOVA (Prism 5.02, Graphpad, CA, USA).

**Drugs**

Stock solutions of the peptide NPW (0.1 mM; Tocris Bioscience, Bristol, UK) were kept frozen (-80°C) and diluted to final concentration in Krebs solution on the day of the experiment. We have previously established that there is no effect of time on gastric vagal afferent mechanosensitivity [284].

**Anterograde tracing**

Vagal afferents endings in the mouse stomach were identified following anterograde tracing from their cell bodies located in the nodose ganglia, using a previously documented procedure [285]. Following an overnight fast, mice (*N* = 4) were anaesthetized with isoflurane (1-1.5% in oxygen). The left nodose ganglia was exposed and 0.5 µl of wheat-germ agglutinin-conjugated horseradish peroxidase (WGA-HRP; 4 mg $ml^{-1}$; Vector Laboratories, QLD, Australia) pressure injected into the nodose ganglion via a glass micropipette (internal diameter = 25 µm). The injection site was then dried and the skin incision closed. Antibiotics (terramycin; 10 mg $kg^{-1}$) and analgesic (butorphanol; 5 mg $kg^{-1}$) were administered subcutaneously. After surgery mice recovered for two days allowing the tracer to anterogradely fill vagal afferent neurons to their peripheral nerve endings within the stomach.

**Horseradish peroxidase detection and immunohistochemistry**

Anterogradely traced mice were anaesthetized with pentobarbitone (0.2 ml i.p., 60mg $ml^{-1}$) and transcardially perfused with heparinised saline at 40 ℃, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PFA-PB) at 4 ℃. The ventral side of the stomach was removed and placed in PFA-PB at 4 ℃ for 2 h. After post fixation the stomach was cryoprotected in 30% sucrose-PB for a minimum of 18 h. The stomach was then frozen in optimal cutting temperature compound (O.C.T.; Tissue-Tek, Sakura Finetek, Alphen aan den Rijn, Netherlands) and 10 μm transverse sections cut. Permanent visualisation of WGA-HRP was achieved using tyramide signal amplification (TSA; TSA biotin reagent kit; PerkinElmer, VIC, Australia). Briefly, the tissue sections were rinsed in TNT buffer (0.05% Tween 20, 0.15 M NaCl and 0.1 M Tris-HCl, pH 7.5), blocked for 30 min in TNB (0.1 M Tris-HCl, pH 7.5; 0.15 M NaCl; 0.5% blocking agent (provided with the TSA biotin reagent kit)), and then incubated for 20 min with tyramide-biotin diluted 1:50 in amplification diluent. The tissue was then rinsed in TNT buffer and reacted with streptavidin conjugated to Alexa Fluor® 647 (Life technologies (Invitrogen), VIC, Australia) for 2 h at room temperature. Sections were subsequently processed for NPW immunohistochemistry.

Immunoreactivity for NPW was detected using a rabbit polyclonal antibody to NPW (H-005-61; Phoenix Pharmaceuticals, QLD, Australia). Primary antibody was visualised using chicken anti rabbit secondary antibody conjugated to Alexa Fluor® 488 (Invitrogen). Briefly, sections were air dried at room temperature and rinsed in PBS + 0.2% Triton X-100 (PBS-TX, pH 7.4; Sigma-Aldrich, NSW, Australia) to facilitate antibody penetration. Primary antibody diluted 1:200 in PBS-TX was incubated at 4 ℃ for 20 h. Unbound antibody was then removed with PBS-TX washes and sections incubated for 1 h at room temperature with secondary antibody (1:200 in PBS-TX). The sections were then given final PBS-TX washes, mounted on slides and cover slipped

using ProLong antifade (Invitrogen). Slides where primary antibody was omitted showed no labelling and served as negative controls.

Slide sections and whole mounts were visualized using an epifluorescence microscope (BX-51, Olympus, Australia) equipped with filters for Alexa Fluor® 488 and 647, with images acquired by a CoolSnapfx monochrome digital camera (Roper Scientific, Tuscon, AZ, USA). Pseudocoloured fluorescence images were overlaid and luminance of images was not adjusted.

**Retrograde tracing**

Cell bodies of gastric vagal afferents innervating specific stomach layers were identified using differential tracing from the stomach as previously documented [285]. These studies were only performed on SLD and HFD mice due to the ethical consideration of restricting food intake in mice a few days after surgery.

*Gastric muscle:* Mice ($N$ = 4) were anaesthetized with isoflurane (1-1.5% in oxygen), a laparotomy performed, and an Alexa Fluor® 555 conjugate of cholera toxin β-subunit (CTB-AF555; 0.5%; Invitrogen) injected subserosally into the muscularis externa of the whole stomach using a 30 gauge Hamilton syringe. Multiple injections (5 µl tracer per surface) were made on both dorsal and ventral surfaces. The injection sites were dried, the laparotomy incision closed, and antibiotic and analgesic administered as above.

*Gastric mucosa:* Mice ($N$ = 4) were fasted overnight and anaesthetized with isoflurane, a laparotomy performed and a mucolytic (10% N-acetylcysteine; 200 µl; Bristol-Myers Squibb, VIC, Australia) injected into the stomach lumen. After 5 min the mucolytic was removed and followed by two saline rinses (200 µl each). Subsequently, 10 µl of 0.5% CTB-AF555 was injected into the proximal gastric lumen via a 30 gauge Hamilton

syringe and the proximal stomach walls gently opposed to expose the dorsal and ventral surfaces to tracer. The laparotomy incision was then closed and antibiotic and analgesic administered as above. Food and water were withheld for 2 h postoperatively to maximize exposure of tracer. Mice recovered for two days following surgery, allowing tracer to proceed from the stomach to cell bodies in the nodose ganglia. The mice were then processed for nodose cell culture and laser capture microdissection.

**Cell culture and laser capture microdissection**

Retrogradely traced mice were anaesthetized with pentobarbitone (0.2 ml, 60 mg $ml^{-1}$; i.p.), decapitated and both nodose ganglia from each animal were harvested. To obtain optimal cell density, nodose ganglia from four mice were combined into a tube containing F12 (Invitrogen) complete nutrient medium (10% foetal calf serum FCS and 1% penicillin/streptomycin) and stored on ice. Nodose ganglia were dissociated by incubating at 37 ℃ in a 4 mg $ml^{-1}$ solution of dispase and collagenase II (Invitrogen) made up in Hank's Balanced Salt Solution (HBSS; Invitrogen) with agitation at 5 min intervals. After 30 min the dispase/collagenase solution was removed, and after 10 min incubation in a 4 mg $ml^{-1}$ solution of just collagenase II in HBSS, the cells were rinsed in cold HBSS and F12 and then further dissociated by passing them through a fire polished pasteur pipette until no cell clumps were visible. The cells were then pelleted, rinsed in HBSS, and resuspended in HBSS. A cell count was performed using Trypan blue exclusion, and then cells were diluted to 500-1000 cells per 10 µl suspension and seeded to 50 mm duplex dishes (Carl Zeiss, Jena, Germany). The dishes were placed in a 5% $CO_2$ incubator set at 37 ℃ for 2 h to allow cell adherence.

Cells were then subject to laser-capture microdissection, performed on a P.A.L.M.® microbeam microdissection system (Carl Zeiss). Fluorescent labelled nodose neurons

were micro-dissected and catapulted directly into an extraction buffer provided by PicoPure™ RNA Isolation Kit (Arcturus, Fostercity, CA, USA). RNA was extracted from these cells using a PicoPure™ RNA Isolation Kit (Arcturus) in accordance with manufacturer's instructions.

**Quantitative RT-PCR**

Total RNA extracted from laser captured gastric vagal neurons was processed for quantitative RT-PCR (QRT-PCR) to determine GPR7 expression. To enable comparison of expression of NPW in gastric mucosa and GPR7 in nodose ganglia between different groups, gastric mucosal scrapings and nodose ganglia were collected from 20 week old female C57BL/6 mice ($N \geq 5$/group). Total RNA was extracted using an RNeasy Mini Kit (Qiagen, VIC, Australia) and RNeasy Micro Kit (Qiagen) respectively in accordance with the manufacturer's instructions. RNA was quantified by measuring the absorbance at 260 nm (A260) using a NanoDrop™ ND 1000 spectrophotometer (Thermo Fisher Scientific, Vic, Australia) and RNA purity was estimated via the 260/280 absorbance ratio.

QRT-PCR reactions were performed as described in detail previously [287]. In short, reactions were performed using a Chromo4 (MJ Research, Bio-Rad, NSW, Australia) real-time instrument attached to a PTC-200 Peltier thermal cycler (MJ Research) and analyzed with Opticon Monitor Software (MJ research). QRT-PCR reactions were performed with a QuantiTect SYBRgreen RT-PCR one-step RT-PCR kit (Qiagen) according to the manufacturer's specifications with predesigned primer assays (Qiagen) optimized for the detection of the known sequence of mouse GPR7 (QT00299040), β-tubulin (QT00124733), NPW (QT00295400) and β-actin (QT01136772) transcripts contained in the NCBI reference sequence database (www.ncbi.nlm.nih.gov/RefSeq).

QRT-PCR reactions were carried out under the following conditions: reverse transcription, 50 ℃ for 30 min; initial PCR activation, 95 ℃ for 15 min; PCR cycles 94 ℃ for 15 s, 55 ℃ for 30 s and 72 ℃ for 30 s repeated for 50 cycles. A melt curve was obtained to confirm the specificity of the products produced and products were visualised using 3% agarose gel electrophoresis with ethidium bromide staining. Each assay was run in triplicate and repeated in a duplicate PCR experiment. Control PCRs were carried out substituting RNase-free water for template RNA. Relative RNA levels were calculated using the comparative CT method as described previously [288]. Quantitative data are expressed as mean ± SEM. Transcript expression between groups was compared using Student's unpaired *t*-test (Prism 5.02, Graphpad, CA, USA). Statistical significance was set at $P < 0.05$.

**Breath test analysis to determine gastric emptying rate**

The effect of NPW on gastric half emptying time (t ½) was determined using breath test analysis as previously described [290, 291]. SLD mice ($N = 10$) underwent repeated breath tests examining different doses of NPW and test meals. Briefly, after an overnight fast and following the baseline breath sample collection, mice were intraperitoneally injected with 200 µl 0.9% saline or 200 µl saline containing 1 or 3 nmol 100 $g^{-1}$ NPW, doses shown to exert other short-term physiological functions peripherally [216, 222]. After 30 min, mice were gavaged intragastrically with 100 µl of water containing 1 µl $ml^{-1}$ 13C-acetic acid (99% enrichment; Cambridge Isotope Laboratories, Andover, MA, USA) or given 0.1 g of baked egg yolk containing 1 µg $ml^{-1}$ of 13C-labelled octanoic acid (99% enrichment, Cambridge Isotope Laboratories) to consume within 1 min. Breath samples were collected at regular intervals for liquid (0-120 min) and solid meal emptying (0-150 min) and analyzed for $^{13}CO_2$ content with an

isotope ratio mass spectrometer (ABCA 20/20 Europa Scientific, Crewe, UK). There was a minimum of 3 days between subsequent breath tests to allow for full recovery. The $^{13}CO_2$ excretion data were analyzed by nonlinear regression analysis for curve fitting and for calculation of gastric half-emptying time (t ½). Gastric emptying rates between different doses of NPW and saline injected groups were compared by one-way ANOVA with a Tukey post hoc test. Data are expressed as mean ± SEM. $P < 0.05$ was considered significant.

## 3.4 Results

### Diet induced changes in mouse body weight and blood glucose

HFD mice gained significantly more body weight over the 12 week period than mice fed a SLD (6.9 ± 0.8 g ($N$ = 13) and 5.0 ± 0.4 g ($N$ = 16) respectively; $P < 0.05$; unpaired *t*-test), in accordance with previous observations [174]. In a separate cohort of animals on the same diet regime the blood glucose level of SLD, fasted SLD and HFD mice at the time of sacrifice was 9.4 ± 0.4 mmol/L ($N$ = 20), 5.6 ± 0.3 mmol/L ($N$ = 10) and 12.07 ± 0.4mmol/L ($N$ = 24) respectively ($P < 0.001$ HFD v SLD, fasted SLD; $P < 0.001$ SLD v fasted SLD; data not shown).

### Anatomy of vagal afferent endings and neuropeptide W-containing cells in the gastric mucosa

NPW containing cells in the gastric mucosa were identified in close apposition with anterogradely traced vagal afferent fibres. NPW immunopositive cells were located basolaterally within mucosal glands of the gastric antrum (Fig. 3.2a). Anterograde traced vagal afferents from the nodose ganglion revealed intraepithelial fibres within the mucosal layer of the stomach (Fig. 3.2b). Vagal afferents were located in close proximity (within a few micrometres) to NPW-containing cells (Fig. 3.2c).

### The effect of neuropeptide W on gastric vagal afferent mechanosensitivity

The effect of NPW on the mechanosensitivity of gastric vagal afferent tension receptors is illustrated in Fig. 3.3. In SLD mice fed *ad libitum*, NPW (3-10 nM) significantly and dose dependently reduced the response of gastric tension receptors to circular tension (1-5 g; $P < 0.05$, two-way ANOVA; Fig. 3.3a and Fig. 3.3d). However, NPW (1-10

nM) had no effect on gastric tension receptors in SLD fasted mice and HFD mice (Fig. 3.3b and Fig. 3.3c).

The effect of NPW on the mechanosensitivity of gastric vagal afferent mucosal receptors is illustrated in Fig.4. NPW (1-10 nM) did not significantly alter the response of gastric mucosal receptors to mucosal stroking in SLD mice (Fig. 3.4a and Fig. 3.4d), SLD fasted mice (Fig. 3.4b) or HFD mice (Fig. 3.4c).

There was no significant difference in the spontaneous activity of gastric tension and mucosal receptors in the absence and presence of NPW (1-10 nM; data not shown) in the different groups.

**Identification of neuropeptide W receptor GPR7 in gastric vagal afferent pathway**

The expression of GPR7 mRNA was identified in whole nodose ganglia using QRT-PCR. Agarose gel electrophoresis confirmed amplification of GPR7 and housekeeper (β-tubulin) with expected product sizes of 84 bp and 107 bp, respectively (Fig. 3.5a). The neurons innervating the stomach represent only a small proportion of the neurons within the whole nodose ganglia [285]. Following differential retrograde tracing, to identify vagal afferent cell bodies innervating either muscular or mucosal layers of the stomach [285], it was determined that GPR7 mRNA was expressed at levels approximately 30-fold higher in gastric muscle and 10-fold higher levels in neurons innervating the gastric mucosa, in comparison to expression in the whole nodose ganglia (Fig. 3.5b, $P < 0.001$; unpaired *t*-test).

**The effect of food intake on neuropeptide W and GPR7 expression in the gastric mucosa and whole nodose ganglia**

QRT-PCR data revealed that NPW mRNA expression in the gastric mucosa was reduced in SLD fasted mice in comparison to *ad libitum* fed SLD mice ($P < 0.05$; unpaired *t*-test), however, no difference was observed between HFD and SLD mice (Fig. 3.6a). Similarly, GPR7 mRNA expression in the whole nodose ganglia was lower in SLD fasted mice compared to SLD mice ($P < 0.05$; unpaired *t*-test), with no difference evident between HFD and SLD mice (Fig. 3.6b). Due to the selectivity of NPW effect on gastric tension receptors, we determined expression of GPR7 mRNA specifically in the cell bodies of muscular gastric vagal afferents in HFD mice and SLD mice. Here, expression of GPR7 mRNA was significantly reduced in HFD mice compared to SLD mice ($P < 0.01$, unpaired *t*-test; Fig. 3.6c).

**The effect of neuropeptide W on gastric emptying rate**

Non-invasive breath testing was undertaken to determine the gastric half emptying time of liquid and solid test meals after intraperitoneal injection with saline or NPW in mice. The gastric emptying of water was not changed in mice injected NPW (1 or 3 nmol $100g^{-1}$; t ½ = 15.11 ± 1.39 and 14.27 ± 1.21 min, respectively) compared to mice injected saline (t ½ = 14.23 ± 1.09 min). Similarly, the rate of gastric emptying after the solid test meal was unchanged between mice injected with NPW doses (t ½ = 103.1 ± 10.14 and 98.89 ± 10.64 min, respectively) or saline (t ½ = 96.55 ± 9.84 min).

## 3.5 Discussion

The present study provides the first evidence that NPW modulates gastric vagal afferent signalling pathways. Our data shows that anterogradely labelled gastric vagal afferents innervating the mucosal layer of the stomach were closely associated with NPW immunopositive cells suggesting that vagal afferent endings are appropriately located to respond to local release of NPW. In addition, we found that NPW receptor GPR7 was expressed in the nodose ganglia, where the cell bodies of gastric vagal afferents are located. It is known that receptors expressed in whole nodose ganglia can be transported to vagal afferent terminals in the stomach [153], therefore NPW released within the stomach may act on GPR7 receptors at these peripheral afferent endings. In addition to direct effects, NPW carried in blood capillaries may also act on vagal afferents terminals in different regions and layers of the stomach. There are two types of vagal afferents innervating the stomach, mucosal and tension receptors, which represent around 7% and 9% of the total nodose neuron population respectively [285]. When we investigated the expression of GPR7 in these subsets of neurons, it was evident that GPR7 mRNA expression was significantly higher in gastric neurons than whole nodose ganglia, suggesting a gastric specific role for GPR7 in vagal afferent signalling.

Tension sensitive vagal afferents signal the extent of gastric distension and contraction to the CNS, which is a critical input to generate satiety and regulate food intake [171, 295]. Indeed, vagal electrical stimulation has been shown to effectively reduce food intake and body weight in both rats and humans [163, 164]. The observation that NPW reduced the mechanosensitivity of tension sensitive gastric vagal afferents in lean mice fed *ad libitum* would be consistent with a role to abrogate the signalling of satiety by these afferents generated in response to gastric distension; an effect dependent on the

state of nutrition. This observation stands in contrast to what is generally considered to occur in response to food intake whereby gastrointestinal hormones secreted in response to food intake provide negative feedback, ultimately terminating a meal [298]. Under certain circumstances, however, inhibitory responses have a physiological role. For example, after detecting food the proximal stomach relaxes to provide an increased reservoir for food [299] . Therefore, one hypothesis is that the inhibitory effect of NPW on gastric tension receptor satiety signalling may be part of an early physiological response to meal initiation permitting the stomach to tolerate the accommodation of larger volumes of food with any residual effect overcome by more potent meal termination signals. Although NPW may facilitate gastric accommodation no change in gastric emptying was observed in the solid meal gastric emptying studies. This is consistent with a study in humans with impaired postprandial accommodation where proximal stomach emptying was reduced but overall gastric emptying remained normal [300].

The inhibitory effect of NPW on gastric vagal tension receptors is abolished after an overnight fast. If the aforementioned hypothesis is correct then one might expect a reduction in the amount of food consumed during the initial eating episodes after short term food restriction, and this has been observed in rats [301]. The reduced sensitivity of gastric vagal tension receptors to NPW after an overnight fast is consistent with the reduced expression of NPW mRNA in the gastric mucosa and reduced expression of GPR7 in the cell bodies of vagal afferents. This is not the first report of diet induced changes in receptor expression in vagal afferents. It is well established that the expression of other G-protein coupled receptors in the nodose ganglion, including cannabinoid receptor type 1, Y2 receptor, growth hormone secretagogue receptor and melanin-concentrating hormone-1 receptor, vary in response to feeding state [174, 191].

The mechanism leading to the diet induced change in GPR7 levels within the vagal afferent cell bodies is unknown and requires further investigation. It should be noted that decreased receptor expression observed in the whole nodose ganglia may not reflect receptor expression in specific gastric vagal afferent populations, as evidenced by our expression data in HFD mice.

The inhibitory effect of NPW on satiety signals from tension sensitive vagal afferents was also abolished after a HFD. The absence of any effect of NPW, despite the finding that NPW expression in the gastric mucosa is unaltered in HFD conditions, is consistent with the reduction in expression of GPR7 mRNA in specific gastric tension sensitive vagal afferent cell bodies. A chronic HFD has been reported to reduce the response of gastric vagal afferents to stretch [174]. The results of the current study suggest that NPW is unlikely to impact any further on the physiological consequences of tension receptors that are already poorly responsive to stretch.

Gastric mucosal receptors were not regulated by NPW in SLD mice fed *ad libitum*, despite close proximity of NPW containing cells and vagal afferent fibres in the stomach. Gastric mucosal receptors are known to be responsible for discriminating food particle size that may ultimately provide negative feedback onto control of gastric motor patterns that promote gastric emptying [168]. We therefore predicted that NPW would not be involved in gastric motility via vagal afferents, and the absence of an effect of NPW on gastric empting of either liquid or solid meal confirmed this.

In the current study, the modulatory role of NPW on gastric vagal afferent mechanosensitivity was only studied in female mice. However, in the hypothalamus, NPW immunoreactive cells tend to be more abundant in male compared to female rats [215]. In addition, unlike female mice, male GPR7-/- mice are hyperphagic and develop

adult-onset obesity [255]. Therefore, it is possible that modulation of gastric vagal afferents by NPW is gender specific. This along with the effect of the estrous cycle on the modulatory role of NPW requires further investigation.

In conclusion, our anatomical, molecular and functional data suggest that NPW selectively modulates the mechanosensitivity of gastric vagal afferents. NPW selectively inhibits gastric tension receptors but has no effect on mucosal receptors. The inhibition on tension sensitive vagal afferents was only observed in fed conditions, consistent with NPW and GPR7 expression in this pathway, suggesting a more important role of NPW following food intake. However, this effect was abolished after fasting and chronic HFD feeding, highlighting the plasticity in the action of NPW under different feeding conditions.

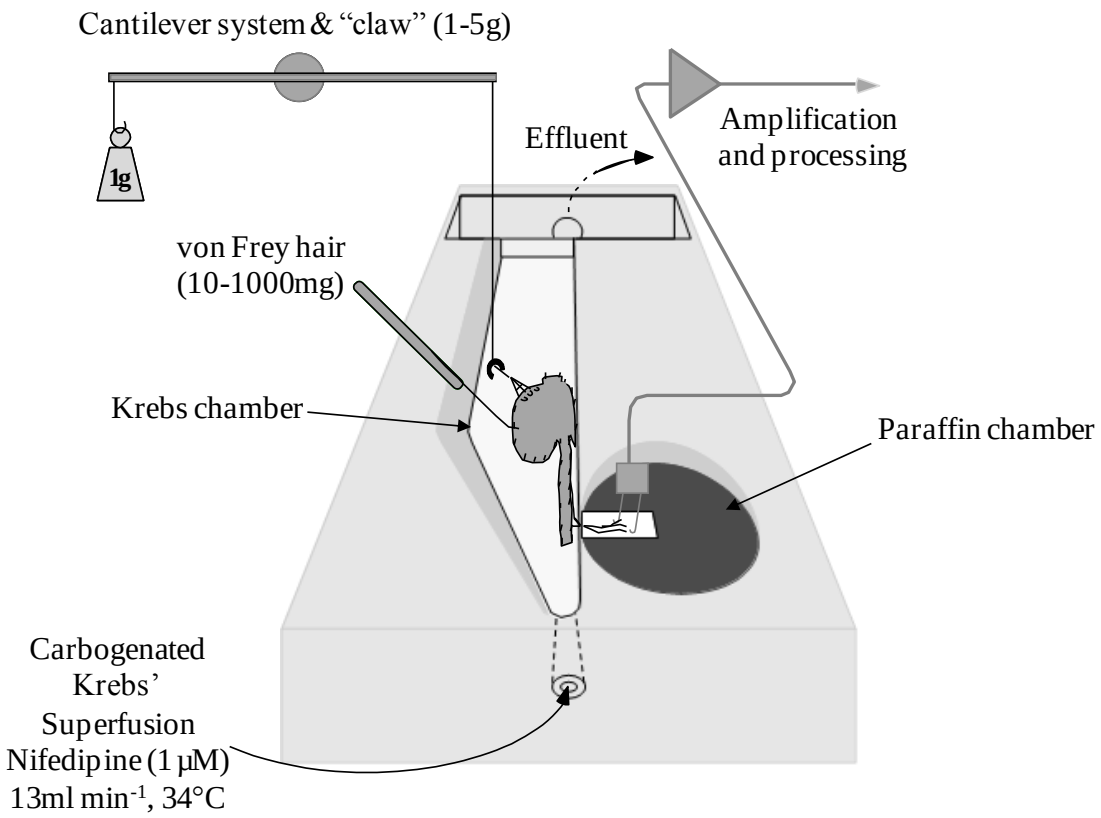



**Figure 3.1 Schematic diagram of the apparatus used for recording single gastric vagal afferent fibres *in vitro***

The oesophagus and stomach are pinned out flat mucosa uppermost and superfused with Krebs' solution. The vagus nerve was drawn into a second chamber where teased fibres are placed onto a recording electrode. Mucosal stimuli are applied by stroking over the receptive field with calibrated von Frey hairs. Tension is applied by placing a hook adjacent to the receptive field. This hook is connected via a pulley to a cantilever system upon which weights can be applied.

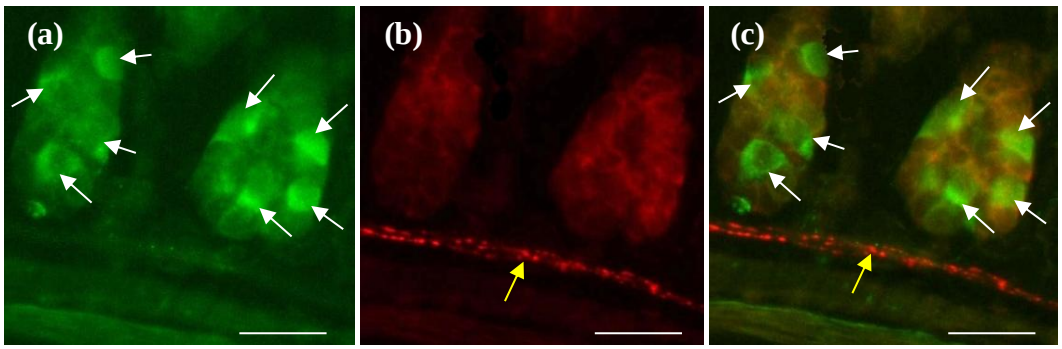



**Figure 3.2 Relationship between vagal afferents and neuropeptide W-containing cells in the gastric mucosa**

Combined immunohistochemistry and anterograde traced vagal afferent fibres in the mouse stomach (*N* = 4). Images show a cross section through the base of a gastric villus. (a) epithelial cells immunopositive for NPW (white arrows). (b) anterogradely traced (WGA-HRP) vagal afferent fibre (yellow arrow). (c) overlay of a and b showing close proximity of NPW containing epithelial cells and anterogradely labeled vagal afferent fibres. *Scale Bar = 25 µm.*

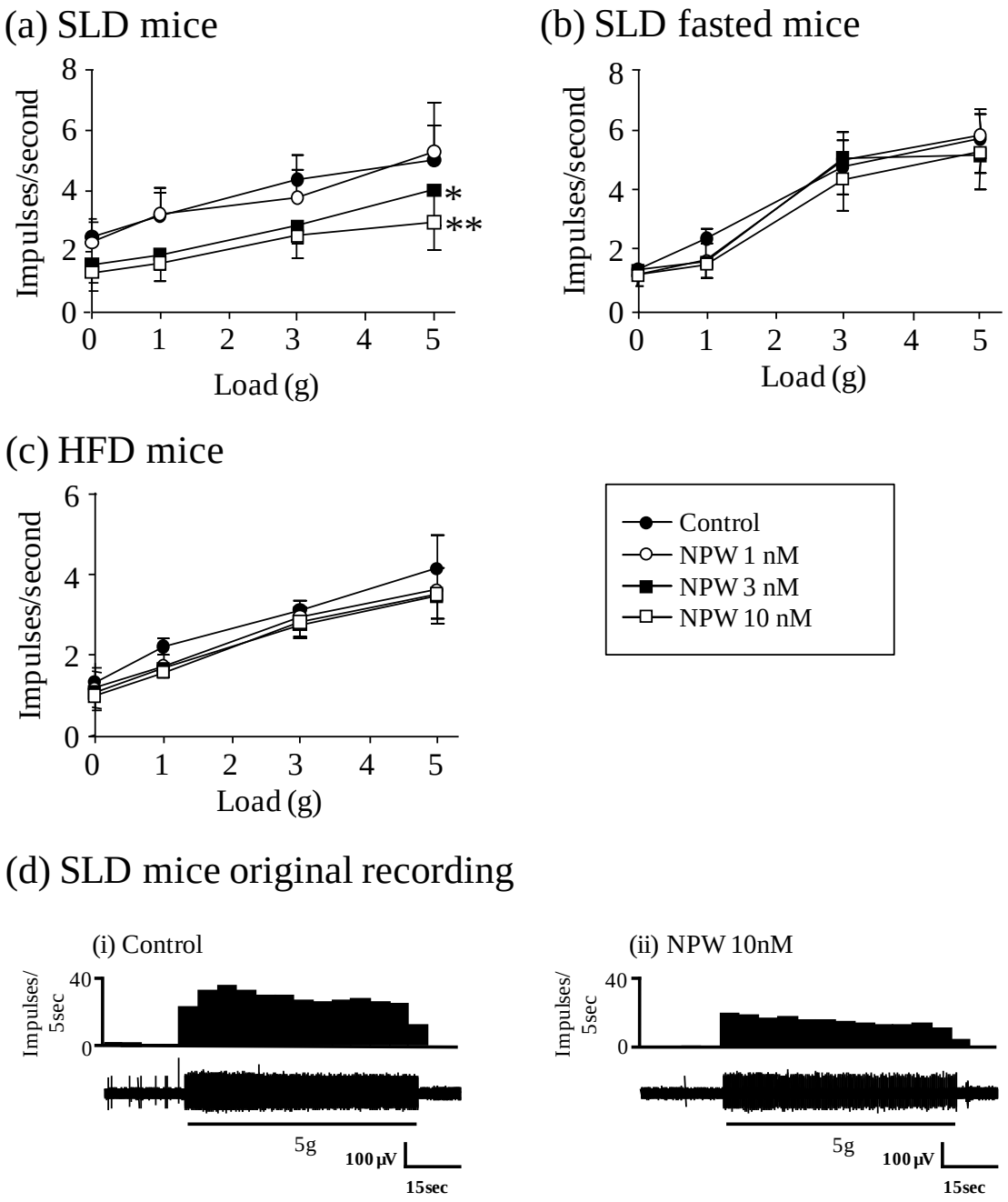



**Figure 3.3 The effect of neuropeptide W on the response of mouse gastric tension receptors**

Stimulus-response of gastric tension receptors to circular tension before and after exposure to NPW (1, 3 or 10 nM) in SLD mice (a: $n = 8$, $N = 5$), SLD fasted mice (b: $n = 5$, $N = 5$), and HFD mice (c: $n = 4$, $N = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control. (d) Typical recording of gastric tension receptors with a circumferential stretch of 5 g in SLD mice before and after 10 nM NPW application.

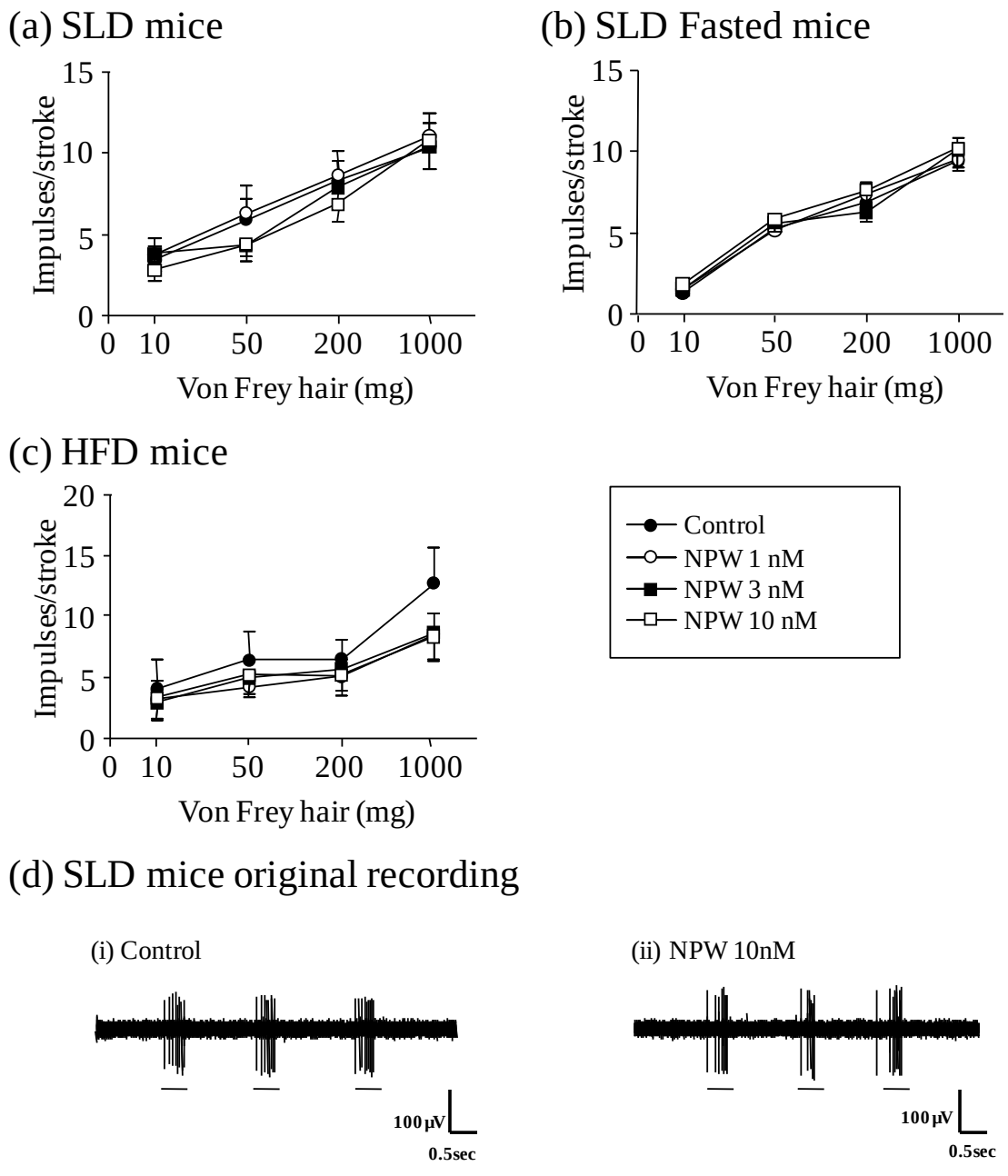






**Figure 3.4 The effect of neuropeptide W on the response of mouse gastric mucosal receptors**

Stimulus-response functions of gastric mucosal receptors to mucosal stroking before and after exposure to NPW (1, 3 or 10 nM) in SLD mice (a: $n$ = 9, $N$ = 6), SLD fasted mice (b: $n$ = 6, $N$ = 4), and HFD mice (c: $n$ = 5, $N$ = 4). (d) Typical recording of gastric mucosal receptors with a 50 mg von Frey hair in SLD mice before and after 10 nM NPW application.

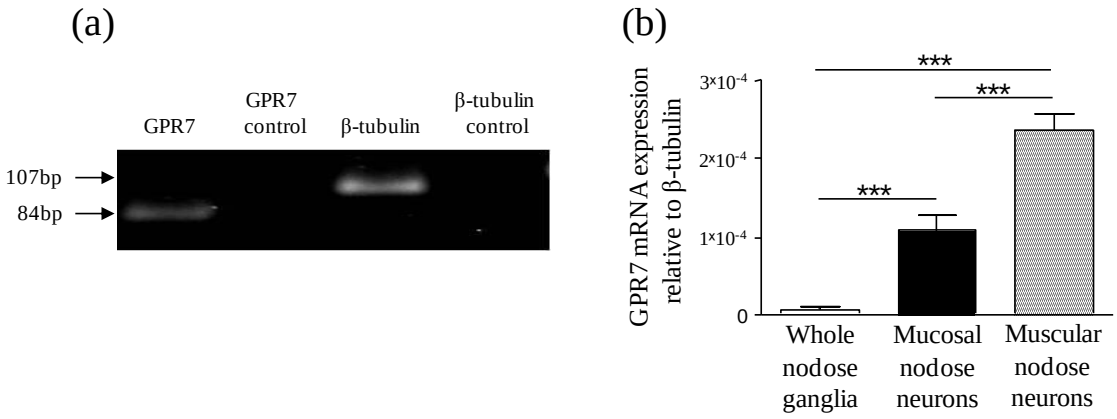
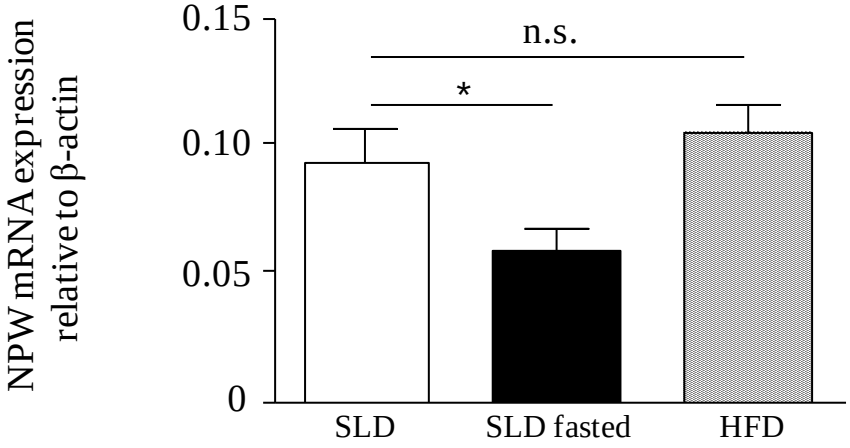


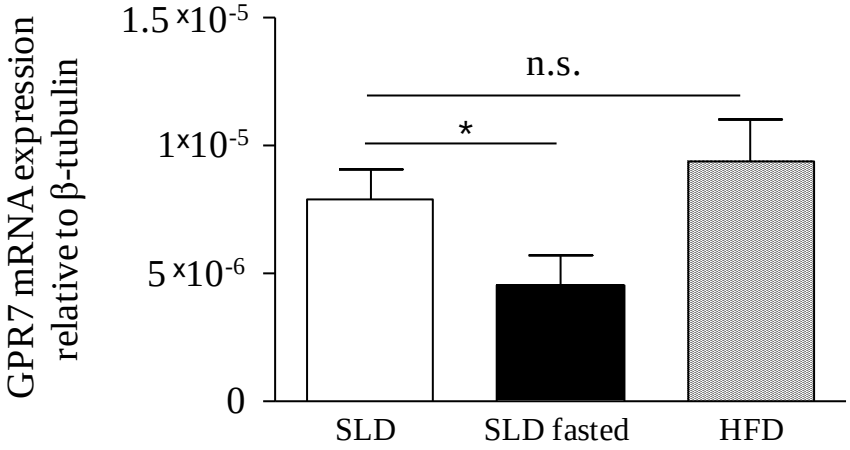

**Figure 3.5 Expression of GPR7 mRNA in vagal afferent cell bodies**

(a) Agarose gel electrophoresis of amplified QRT-PCR products from whole nodose ganglia using specific primers for GPR7 and β-tubulin. The controls are PCR amplification products from reactions with no template added (water controls) (b) Relative expression of GPR7 mRNA in whole nodose ganglia ($N$ = 5), gastric mucosal nodose neurons ($N$ = 4) and gastric muscular nodose neurons ($N$ = 4) in 20 week old mice fed *ad libitum*. ***$P < 0.001$.

(a) Gastric mucosa




(b) Whole nodose ganglia




(c) Gastric muscular nodose neurons

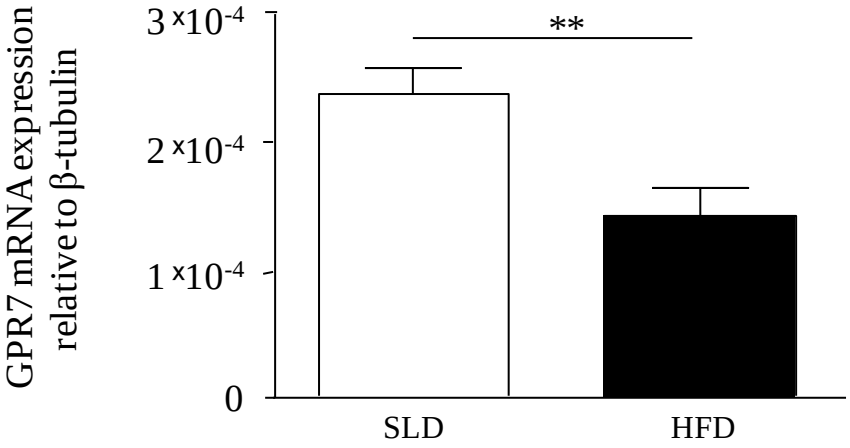


**Figure 3.6 Expression of neuropeptide W and GPR7 mRNA in the gastric mucosa and whole nodose ganglia respectively**

(a) Expression of NPW mRNA relative to β-actin in gastric mucosa of SLD mice ($N$ = 5), SLD fasted mice ($N$ = 6) and HFD mice ($N$ = 5) *$P$ < 0.05 compared with SLD mice. (b) Expression of GPR7 mRNA relative to β-tubulin in whole nodose ganglia in SLD mice ($N$ = 5), SLD fasted mice ($N$ = 6) and HFD mice ($N$ = 5). *$P$ < 0.05 compared with SLD mice. (c) Expression of GPR7 mRNA relative to β-tubulin in nodose neurons innervating the gastric muscular layer in SLD mice and HFD mice ($N$ = 4/group). **$P$ < 0.01 compared with SLD mice.

# CHAPTER 4. NEUROPEPTIDE W MODULATION OF GASTRIC VAGAL AFFERENT MECHANOSENSITIVITY: IMPACT OF AGE AND GENDER

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# Statement of Authorship

## Neuropeptide W Modulation of Gastric Vagal Afferent Mechanosensitivity: Impact of Age and Gender

Submitted to: Acta Physiologica

***By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the manuscript to be included in the candidate's thesis.***

**Hui Li (Candidate)**

Performed most electrophysiology experiments, retrograde tracing surgery, cell culture, laser capture microdissection, QRT-PCR, data analysis and interpretations and wrote the manuscript.

Signed Date 26/9/2013

**Claudine Frisby**

Performed RNA extraction and QRT-PCR and manuscript editing.

Signed Date 26/9/2013

**Tracey A. O'Donnell**

Performed some electrophysiology experiments, husbandry of animal and manuscript editing.

Signed Date 26/9/2013

**Stephen Kentish**

Aided in the husbandry of animal and manuscript construction.

Signed Date 26/9/2013

**Gary A. Wittert**

Assisted in the conceptualisation of experiments, interpretation of data and construction of manuscript.

Signed Date 3/9/2013

**Amanda J. Page**

Assisted in the conceptualisation of experiments, performed some electrophysiology experiments, data analysis and interpretation and manuscript construction.

Signed Date 27/9/2013

## 4.1 Abstract

**Aim**: Gastric vagal afferents can regulate food intake and gastric function in response to mechanical stimulation. Neuropeptide W (NPW) reduces the response of gastric vagal afferents to circular tension in 20-week-old female mice, suggesting a possible role of NPW in inhibition of vagal afferent satiety signalling after food intake. However, food intake varies with age and gender, thus in this study we aimed to determine the role of NPW on vagal afferent mechanosensitivity in different aged male and female mice.

**Methods**: An *in vitro* gastro-oesophageal preparation was used to determine the effect of NPW on gastric vagal afferent mechanosensitivity from male and female C57BL/6 mice aged 8 and 20 week. Retrograde tracing and laser capture microdissection were used to selectively collect gastric vagal afferent neurons innervating sub-layers of the stomach. Expression of NPW in the gastric mucosa and GPR7 in gastric vagal afferent neurons was determined using quantitative RT-PCR.

**Results**: We found that there were no gender differences in the inhibitory effect of NPW on gastric tension sensitive vagal afferents. However, the inhibitory effect was only observed in the 20 week, and not 8 week old mice. NPW inhibits the mechanosensitivity of gastric mucosal vagal afferents in 8-week-old female mice, but not in 20-week-old female mice or age-matched male mice. NPW mRNA expression in gastric mucosa and GPR7 mRNA expression in vagal afferent neurons innervating gastric muscular layers are higher in 20-week-old mice compared to 8-week-old mice in both genders.

**Conclusion**: The inhibitory effect of NPW on gastric tension sensitive vagal afferents is not gender-specific, but is age-dependent, and NPW inhibits the mechanosensitivity of

mucosal vagal afferents in young female mice. Thus, NPW's role in the stomach varies depending on the age of the mouse.

## 4.2 Introduction

Vagal afferents play an important role in the regulation of short-term food intake and gastrointestinal function [134, 159]. In the stomach, mechanical stimuli exerted by the presence of food are sensed by vagal afferents and transmitted to the central nervous system (CNS). In mice there are two types of gastric mechanosensitive vagal afferent, mucosal receptors and tension receptors, which are classified according to their responses to mechanical stimuli [165, 166]. Tension receptors, located in the muscular layer, sense the distension and contraction of the stomach [166], and transfer signals to the CNS to induce satiety [295]. Mucosal receptors, located in the mucosal layer, are sensitive to mucosal stroking but not circular tension [166]. The role of mucosal receptors is less obvious, but it is thought they might act to selectively distinguish food particle size and thus determine when gastric emptying occurs [168]. Vagal afferent endings innervating the stomach are closely located to endocrine cells, and the responses of vagal afferents to mechanical stimuli are modulated by locally released hormones, such as leptin and ghrelin [174, 176, 180].

Neuropeptide W (NPW) is an endogenous ligand for the G-protein-coupled receptors GPR7 and GPR8 [206-208]. It has multiple functions both centrally and peripherally, including a role in the regulation of short-term food intake and long-term energy homeostasis [247-250]. In the CNS, NPW has been demonstrated to have both food intake stimulatory and inhibitory effects [206, 211, 219, 251, 252]. Chronic administration of NPW into the CNS of rats reduces body weight, food intake and increases energy expenditure [211], with the opposite being observed in GPR7 knockout mice [255]. In the stomach, NPW is abundantly expressed in gastric antral G cells [226]. Previously, we have shown that in 20-week-old female mice NPW inhibits

the response of tension-sensitive gastric vagal afferents to stretch [302], suggesting a potential role of NPW in delaying gastric vagal afferent satiety signals.

Evidence suggests that satiety signalling via hormones could be different between the period of early development and adulthood. Developing rats and mice are less sensitive to the feeding-inhibitory effect of CCK and leptin in comparison to adult animals [303-305]. The reduced sensitivity to satiety signalling could be associated with rapid growth rates, as supported by the findings that rapid-growth chickens are less sensitive to the anorectic effect of leptin compared to chickens with slower growth rate at the same age [306]. Based on this evidence, we sought to determine whether NPW has a differential effect on gastric vagal afferent mechanosensitivity in mice at different ages.

In addition to age, gender appears to have a large effect on the regulation of body weight with female humans being more prone to obesity [307]. NPW receptor, GPR7, knockout male, but not female mice were hyperphagic and developed adult-onset obesity [255]. Moreover, NPW containing cells in hypothalamus tend to be more abundant in male rats compared to female rats [215]. Therefore, NPW may have gender specific effects.

In this study, we aimed to determine the role of NPW on the mechanosensitivity of individual subtypes of gastric vagal afferent in male and female mice at 8 weeks of age (a time of rapid growth), and 20 weeks of age (a time of slower growth). We then determined the expression of NPW in the gastric mucosa and also the expression of its receptor, GPR7, in gastric vagal afferent cell bodies.

## 4.3 Materials and methods

### Mice

Male and female C57BL/6 mice were group housed under a 12:12-h light-dark cycle with *ad libitum* access to water and a standard laboratory diet (14MJ/Kg; energy from fat-12%, protein-23%, carbohydrate-65%). The weight of the mice was monitored weekly. The body weight gain of the mice during the week they were 8 and 20 weeks old was calculated as percentage difference in the body weight. All experimental studies were performed with the approval of the animal ethics committee of the Institute of Medical and Veterinary Science and the University of Adelaide. The experiments also conform to the principles of UK regulations, as described in Drummond [279].

### *In vitro* mouse gastro-oesophageal afferent preparation

This preparation has been described in detail previously [166, 281, 302]. Briefly, 8 or 20 week old C57BL/6 mice were killed via $CO_2$ inhalation and the thorax was opened by midline incision. The stomach and oesophagus with attached vagal nerves were removed and placed into a modified Krebs solution containing (mM): NaCl 118.1, KCl 4.7, $NaHCO_3$ 25.1, $NaH_2PO_4$ 1.3, $MgSO_4.7H_2O$ 1.2, $CaCl_2$ 1.5, citric acid 1.0 and glucose 11.1, bubbled with 95% $O_2$-5% $CO_2$ at 4°C during dissection to prevent metabolic degradation. Nifedipine (1μM) was also added to the Krebs solution to prevent smooth muscle contraction. After further dissection, the preparation was opened out longitudinally along the oesophagus and the greater curvature of the stomach. The preparation was then placed mucosal side up in the organ bath.

### Characterisation of gastric vagal afferent properties

Two types of mechanosensitive gastric vagal afferent fibre were studied, those responding to mucosal stroking and circular tension (tension receptors), and those responding to mucosal stroking but not circular tension (mucosal receptors), as reported previously [166].

Location of receptive fields for vagal afferent endings in the stomach was determined by mechanical stimulation throughout the preparation with a brush. In this study, the receptive fields tested were in the body and antrum of the stomach. Tension and mucosal receptors were distinguished by their responses to circular stretch and mucosal stroking. Mucosal stroking were performed using calibrated von Frey hairs (10- 1000 mg) moving across the receptive field. To minimise the experimental error, ten standard strokes were given at 1 s intervals for each weight, and the response was measured as the mean discharge of the middle eight strokes. Tension stimuli were applied using a cantilever system with a fine suture silk attached via a hook adjacent to the mechanoreceptive field. The tension-response curves were produced by applying weights to the cantilever system in the range of 1 - 5 g. Each weight was applied for 1 min, and the response was measured as the mean discharge frequency evoked over this period. A recovery period of at least 1 min was allowed between each tension stimulus.

**Effect of neuropeptide W on gastric vagal afferent mechanosensitivity**

After mechanical sensitivity of the gastric vagal afferents had been established, NPW (1 nM) was added to the superfusing Krebs solution and allowed to equilibrate for 20 min after which time the tension-response or stroke-response curves were re-determined. This equilibration period ensured penetration of the peptide into all layers of the tissue. This procedure was repeated for NPW at increasingly higher doses (3 - 10 nM), consistent with other reports [206, 251] and with plasma NPW levels in mice [308].

**Data recording and analysis**

Afferent impulses were amplified with a biological amplifier (DAM 50, World Precision Instruments, Sarasota, FL, USA), and filtered (band-pass filter 932, CWE, Ardmore, PA, USA). Single units were discriminated on the basis of action potential shape, duration and amplitude by use of Spike 2 software (Cambridge Electronic Design, Cambridge, UK). Data are expressed as mean ± SEM with *n* = number of individual afferents and *N* = number of mice in all cases. The difference in the overall pattern of mechanosensitivity after different doses of NPW exposure was evaluated using a two-way ANOVA (Prism 5.02, Graphpad).

**Drugs**

Stock solutions of the peptide NPW (0.1 mM; Tocris Bioscience, Bristol, UK) were kept frozen (-80°C) and diluted to final concentration in Krebs solution on the day of the experiment.

**Retrograde tracing**

Cell bodies of gastric vagal afferents innervating specific stomach layers were identified using differential tracing from the stomach as previously documented [285].

*Gastric muscle:* Male and female mice (8 or 20 weeks old) were anaesthetized with isoflurane (1-1.5% in oxygen), a laparotomy performed, and an Alexa Fluor® 555 conjugate of cholera toxin β-subunit (CTB-AF555; 0.5%; Invitrogen) injected into the muscularis externa of the whole stomach using a 30 gauge Hamilton syringe (Hamilton company, Reno, NV, USA). Multiple injections (5 µl tracer per surface) were made on both dorsal and ventral surfaces. The injection sites were dried, and the laparotomy

incision closed. Antibiotic (terramycin; 10 mg $kg^{-1}$) and analgesic (butorphanol; 5 mg $kg^{-1}$) were administered subcutaneously.

*Gastric mucosa:* Male and female mice (8 or 20 weeks old) were fasted overnight and anaesthetized with isoflurane, a laparotomy performed and a mucolytic (10% N-acetylcysteine; 200 µl; Bristol-Myers Squibb, VIC, Australia) injected into the stomach lumen. After 5 min the mucolytic was removed and followed by two saline rinses (200 µl each). Subsequently, 10 µl of 0.5% CTB-AF555 was injected into the proximal gastric lumen via a 30 gauge Hamilton syringe and the proximal stomach walls gently opposed to expose the dorsal and ventral surfaces to tracer. The laparotomy incision was then closed and antibiotic and analgesic administered as above. Food and water were withheld for 2 h postoperatively to maximize exposure of tracer.

Mice recovered for two days following each surgery, allowing tracer to proceed from the stomach to the cell bodies in the nodose ganglia. The mice were then processed for nodose cell culture and laser capture microdissection.

**Cell culture and laser capture microdissection**

Retrogradely traced mice were anaesthetized with pentobarbitone (0.2 ml, 60 mg $ml^{-1}$; i.p.), decapitated and both nodose ganglia from each animal were harvested. To obtain optimal cell density, nodose ganglia from four mice were combined into a tube containing F12 (Invitrogen) complete nutrient medium + 10% foetal calf serum and 1% penicillin/streptomycin and stored on ice. Nodose ganglia were dissociated by incubating at 37 ℃ in a 4 mg $ml^{-1}$ solution of dispase and collagenase II (Invitrogen) made up in Hank's Balanced Salt Solution (HBSS; Invitrogen) with agitation at 5 min intervals. After 30 min the dispase/collagenase solution was removed, and nodose

ganglia were further incubated in a 4 mg $ml^{-1}$ solution of collagenase II in HBSS for 10 min. Then the cells were rinsed in cold HBSS and F12 and further dissociated by passing them through a fire polished pasteur pipette until no cell clumps were visible. The cells were then pelleted, rinsed in HBSS, and resuspended in HBSS. A cell count was performed using Trypan blue exclusion, and then cells were diluted to 500-1000 cells per 10 µl suspension and seeded to 50 mm duplex dishes (Carl Zeiss, Jena, Germany). The dishes were placed in a 5% $CO_2$ incubator set at 37 ℃ for 2 h to allow cell adherence.

Cells were then subject to laser-capture microdissection, performed on a P.A.L.M.® microbeam microdissection system (Carl Zeiss). Fluorescent labelled nodose neurons were micro-dissected and catapulted directly into an extraction buffer provided in the PicoPure™ RNA isolation kit (Arcturus, Fostercity, CA, USA). Neurons were snap frozen in liquid nitrogen and stored at -80 ℃ until processed for total RNA extraction.

**RNA extraction and quantitative real-time PCR**

Gastric mucosal scrapings were collected from 8 and 20-week-old male and female mice. Total RNA was extracted using an RNeasy mini kit (Qiagen, VIC, Australia) in accordance with the manufacturer's instructions. Total RNA was extracted from laser captured cells using a PicoPure™ RNA isolation kit (Arcturus) in accordance with the manufacturer's instructions. RNA was quantified by measuring the absorbance at 260 nm (A260) using a NanoDrop™ ND 1000 spectrophotometer (Thermo Fisher Scientific, Vic, Australia) and RNA purity was estimated via the 260/280 absorbance ratio.

Quantitative real-time PCR (QRT-PCR) reactions were performed as described in detail previously [287] to determine NPW or GPR7 expression in gastric mucosa and laser captured gastric vagal neurons respectively. In short, reactions were performed using a Chromo4 (MJ Research, Bio-Rad, NSW, Australia) real-time instrument attached to a PTC-200 Peltier thermal cycler (MJ Research) and analyzed with Opticon Monitor Software (MJ research). QRT-PCR reactions were performed using a QuantiTect SYBR green one-step RT-PCR kit (Qiagen) according to the manufacturer's instructions. All primers used were pre-designed validated QuantiTect Primer assays (Qiagen) and targeted NPW and β-actin in the stomach mucosa, and GPR7 and β-tubulin in the gastric vagal neurons. QRT-PCR reactions were carried out under the following conditions: reverse transcription, 50 ℃ for 30 min; initial PCR activation, 95 ℃ for 15 min; PCR cycles 94 ℃ for 15 s, 55 ℃ for 30 s and 72 ℃ for 30 s repeated for 50 cycles. A melt curve was obtained to confirm the specificity of the products produced and products were visualised using 3% agarose gel electrophoresis with ethidium bromide staining. Each assay was run in triplicate and repeated in a duplicate PCR experiment. Control PCRs were carried out substituting RNase-free water for template RNA. Relative RNA levels were calculated using the comparative CT method as described previously [288]. Quantitative data are expressed as mean ± SEM. Transcript expression between groups was compared using Student's unpaired *t*-test (Prism 5.02, Graphpad, CA, USA). Statistical significance was set at $P < 0.05$.

## 4.4 Results

### Body weight gain in mice at different age and gender

The rate of body weight gain in male and female mice at different ages and gender are illustrated in Fig. 4.1. In male mice, the body weight gain per week was significantly higher in 8-week-old mice (6.43% ± 1.04%) in comparison to 20-week-old mice (1.35 ± 0.47%; $P < 0.01$). In female mice, the body weight gain per week was significantly higher in 8-week-old mice (4.71% ± 0.61%) in comparison to 20-week-old mice (0.49% ± 0.75%; $P < 0.001$). There is no difference in the rate of body weight gain between male and female mice at the same age. Therefore, 8-week-old mice were gaining weight at a significantly higher rate than 20-week-old mice in both male and female mice.

### The effect of neuropeptide W on mechanosensitivity of gastric tension receptors

There is no age or gender difference in the baseline mechanosensitivity of gastric vagal afferent tension receptors ($P = 0.317$; data not shown). The effect of NPW on the mechanosensitivity of gastric vagal afferent tension receptors in 8 and 20-week-old male and female mice is illustrated in Fig. 4.2. In 8-week-old male mice NPW (1-10 nM) had no effect on the mechanosensitivity of gastric tension receptors (Fig. 4.2ai; $P > 0.05$). However, in 20-week-old male mice 1-10 nM NPW significantly reduced the response of gastric tension receptors to circular tension ($P < 0.001$ vs. Control; 1-5 g; Fig. 4.2aii). Similarly, in 8-week-old female mice NPW had no effect on the mechanosensitivity of gastric tension receptors (Fig. 4.2bi and 4.2ci, $P > 0.05$), but in 20-week-old female mice NPW (3-10 nM) reduced the response of gastric tension receptors to circular tension (3 nM $P < 0.05$, 10 nM $P < 0.01$ vs. Control; 1-5 g; Fig.

4.2bii and 4.2cii). Therefore gastric tension receptor sensitivity to NPW was significantly increased with age, but was not affected by gender.

**The effect of neuropeptide W on mechanosensitivity of gastric mucosal receptors**

There are no age or gender differences in the baseline mechanosensitivity of gastric vagal afferent mucosal receptors ($P = 0.443$; data not shown). The effect of NPW on the mechanosensitivity of gastric vagal afferent mucosal receptors in 8 and 20-week-old male and female mice is illustrated in Fig. 4.3. In male mice, at 8 weeks (Fig. 4.3ai) and 20 weeks of age (Fig. 4.3aii), NPW (1-10 nM) had no effect on the mechanosensitivity of gastric mucosal receptors to mucosal stroking (10-1000 mg; $P > 0.05$). In 8-week-old female mice, NPW (3-10 nM) significantly reduced the response of mucosal receptors to mucosal stroking with calibrated von Frey hairs (3 nM $P < 0.05$, 10 nM $P < 0.001$ vs. Control; 10-1000 mg; Fig. 4.3bi and 4.3ci). However, this inhibitory effect of NPW was not observed in 20-week-old female mice ($P > 0.05$ vs. Control; Fig. 4.3bii and 4.3cii). Therefore, NPW had no effect on gastric mucosal afferent mechanosensitivity in male mice, but inhibited the response in 8-week-old female mice; an effect no longer observed in the older 20-week-old female mice.

**Neuropeptide W mRNA expression in gastric mucosa**

To further illustrate the role of NPW in the stomach, expression of NPW in the gastric mucosa was determined in different age and gender groups, as shown in Fig. 4.4. There was no significant difference in the expression of NPW mRNA levels between genders at age 8 or 20 weeks old. However, in male mice, NPW mRNA content in the gastric mucosal was significantly higher in 20-week-old mice compared to 8-week-old mice (Fig. 4.4a, $P < 0.001$). In female mice, the NPW mRNA levels in 8-week-old and 20-

week-old mice was not significantly different ($P$ = 0.08), but showed the same trend as male mice. Amplification products of NPW and housekeeper (β-actin) were confirmed by agarose gel electrophoresis (Fig. 4.4b). Therefore, NPW mRNA expression in mouse stomach is not different between genders, but increases with age.

**Neuropeptide W receptor GPR7 mRNA expression in gastric vagal afferent cell bodies**

Previously we have shown that GPR7 expression in whole nodose ganglia does not represents its expression in specific gastric neurons [302]. Therefore, the expression of GPR7 mRNA in nodose neurons specifically innervating the gastric mucosal layer and muscular layer was determined in different age and gender groups, as illustrated in Fig. 4.5. In muscular traced neurons, GPR7 mRNA levels were significantly higher in 20 week old mice compared to 8 week old mice in both genders (Fig. 4.5a; male $P < 0.05$, female $P < 0.001$). In mucosal traced neurons, expression of GPR7 mRNA was not different between 8-week-old and 20-week-old mice in both genders (Fig. 4.5b). However, there was a trend for a decrease in expression in 20-week-old female mice ($P$ = 0.06 vs. 8-week-old female mice). There was no difference in GPR7 expression in gastric tension or mucosal neurons between male and female at the same age (8 weeks or 20 weeks old). Amplification products of GPR7 and housekeeper (β-tubulin) were confirmed by agarose gel electrophoresis (Fig. 4.5c).

## 4.5 Discussion

The current study demonstrates that the previously identified inhibitory effect of NPW on gastric tension sensitive vagal afferents [302] is age but not gender specific. The levels of NPW and GPR7 transcript in the gastric mucosa and tension sensitive vagal afferent neurons respectively increase with age, which may account for the increased inhibitory effect of NPW on gastric tension sensitive vagal afferents in the older mice.

It is well known that gastric tension receptors signal the extent of gastric distension and contraction to the CNS, which is critical in generating satiety and fullness to regulate food intake [171, 295]. Our previous [302] and current study show that NPW inhibits the response of tension sensitive gastric vagal afferents to circular stretch, an effect independent of gender, indicating that the role of NPW on vagal afferents should be important physiologically in both genders. However, GPR7 deleted mice are sexually dimorphic in food intake and body weight [255] with hyperphagia and obesity observed only in male mice. This suggests that NPW modulation of gastric vagal afferent pathways are not responsible for the GPR7 deletion induced hyperphagia and obesity in male mice. Alternatively, the disrupted energy homeostasis by GPR7 deletion could be due to central effects of NPW, as chronic central administration of NPW inhibits food intake [211].

The inhibitory effect of NPW on tension sensitive vagal afferents is found in 20-week-old mice but not in 8-week-old mice in both genders, suggesting this effect is age dependent. Eight-week-old mice are in the stage of early development with a higher growth rate compared to the 20-week-old mice. During this rapid growth rate period the level of food intake is also higher compared to the 20 week old mice [309, 310]. The hyperphagia phenotype during the early adulthood period could be associated with the

ineffectiveness of satiety hormones on food intake, as shown with CCK [303 ]. However, the regulation of NPW on tension sensitive vagal afferents is not observed in young mice, suggesting the role of NPW via vagal afferents is not responsible for the hyperphagia during this period. The decreased sensitivity of gastric vagal afferents to NPW in 8-week-old mice could be a negative feedback of the positive energy balance associated with high calorie intake and body fat accumulation during the rapid growth period, as a similar lack of effectiveness of NPW was also observed in mice fed a chronic high-fat diet [302]. Alternatively, it is possible that the insensitivity of younger mice to NPW is because the effect of NPW is triggered later in the development of the mice. Gastric distension via vagal afferents is the major satiety factor controlling food intake in postnatal rats, and other signals such as hormones become involved later in development [311], which is consistent with the effect of NPW on tension receptors not being seen in the 8 week old mice.

Consistent with the increased neuromodulatory action of NPW on gastric tension receptors during growth, the expression of NPW and GPR7 mRNA in this pathway were also increased with age. It has been reported that both NPW transcript and protein level in the stomach gradually increase before reaching adulthood [228] and we now demonstrate an increase of NPW transcript levels in the gastric mucosa during early adulthood, although in female mice it did not reach significance. Changes in expression of gastrointestinal hormones during development have previously been reported. Compared to 3 month old mice, younger (1 month old) mice have lower protein levels of somatostatin, peptide YY, galanin, and higher levels of neurotensin, secretin and gastric inhibitory polypeptide in the gastrointestinal tract [312]. However, changes in hormone expression may occur in a tissue-specific manner. For example, during early development in rodents neuropeptide Y levels are decreased in the stomach and some

brain areas including the striatum and hypocampus, increased in the duodenum and adrenal gland and are not changed in the colon, hypothalamus and circulation [312, 313]. Therefore, the increased NPW mRNA content in the stomach during growth may only cause an age-specific effect in regards to the effect of NPW in the stomach; this requires further investigation.

Similarly, the expression of the NPW receptor GPR7 in vagal afferent neurons innervating the muscular layers is also increased with age, which may account for the increased sensitivity of tension vagal afferents to NPW. Such a phenomenon has previously been observed with the acquisition of the anorectic effect of leptin in chickens being accompanied by increased expression of the leptin receptor in the brain [306]. The mechanisms leading to the changes in NPW and GPR7 expression with age are unknown. However, considerable evidence suggests there are substantial interactions between various gastrointestinal hormones and their receptors [191, 308], whether the changes in NPW and GPR7 expression were due to such interactions requires further investigation.

In 8-week-old female mice, NPW significantly inhibits the response of gastric mucosal vagal afferents to stroking. This modulation of NPW on gastric mucosal vagal afferents in 8-week-old female mice was not found in age-matched male mice or older female mice, suggesting a possible physiological role of NPW during early adulthood in female mice, which requires further investigation to determine the physiological significance.

In conclusion, the current study shows that the inhibitory role of NPW on gastric tension sensitive vagal afferents is age but not gender specific. The effective modulation of NPW on tension sensitive vagal afferents in adult mice is associated with the increased NPW and GPR7 expression in this pathway. Moreover, in young female

mice NPW inhibits the mechanosensitivity of mucosal vagal afferents. These findings suggest that NPW's role varies depending on the age of the mice.

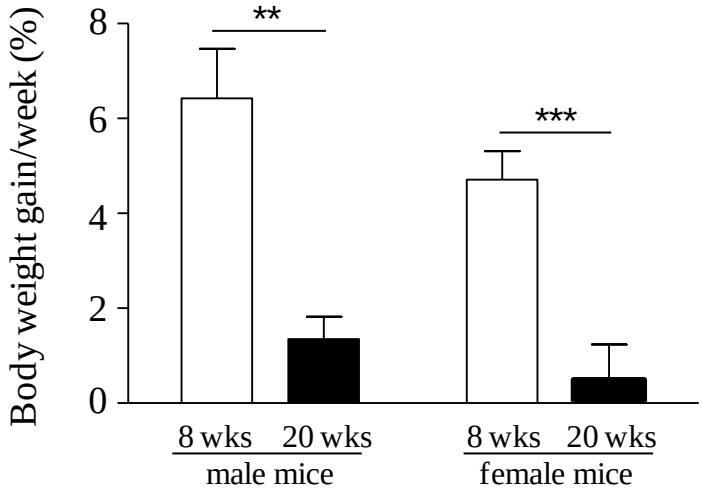
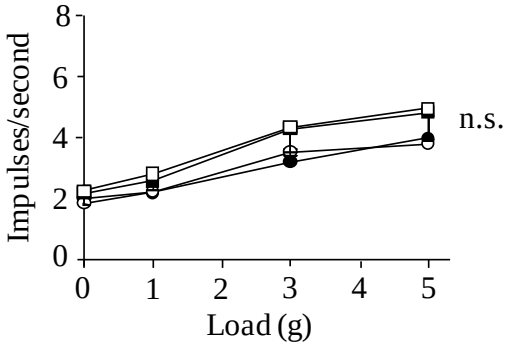



**Figure 4.1 The weekly body weight gain of mice**

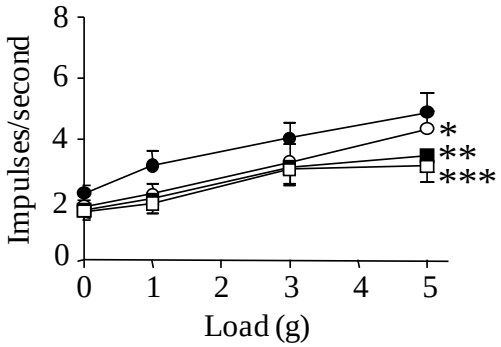
The weekly body weight gain of male ($N$ = 12) and female mice ($N$ = 32) at 8 and 20 weeks old. **$P$ < 0.01, ***$P$ < 0.001.

(a) Male mice

(i) 8 weeks old

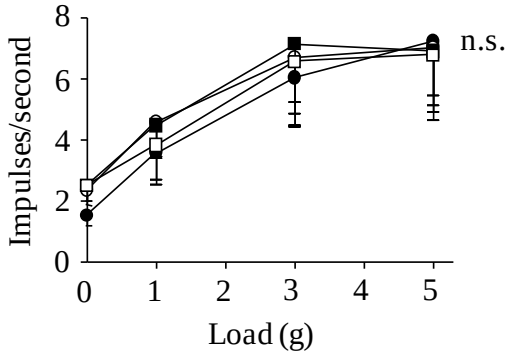



(ii) 20 weeks old

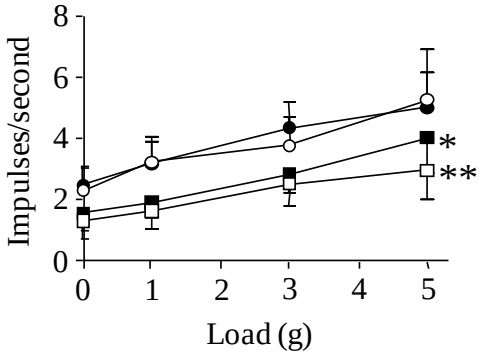



(b) Female mice

(i) 8 weeks old

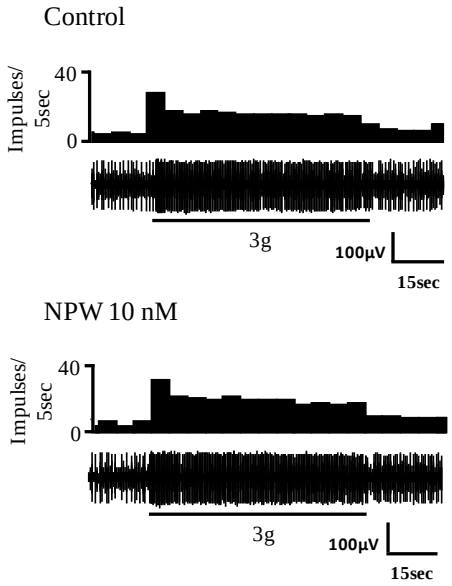



(ii) 20 weeks old




(c) Typical original recording

(i) 8 weeks old female




(ii) 20 weeks old female

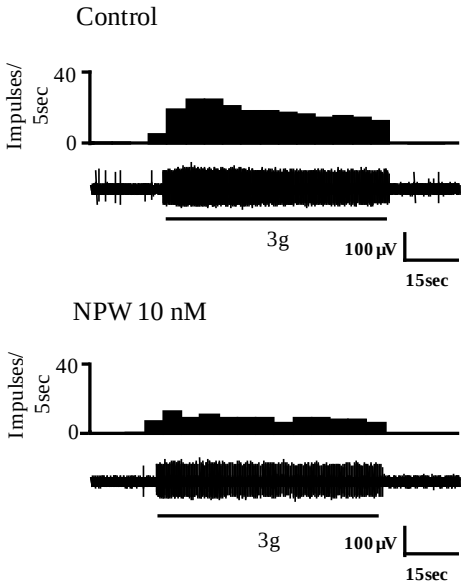
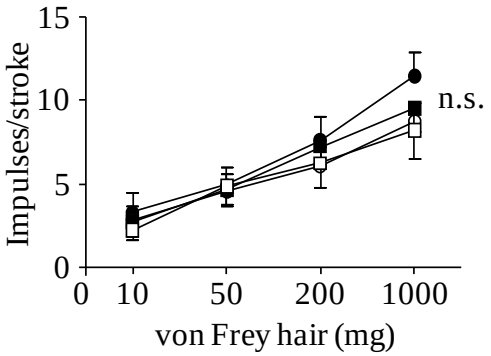


**Figure 4.2 The effect of neuropeptide W on the response of mouse gastric tension receptors**

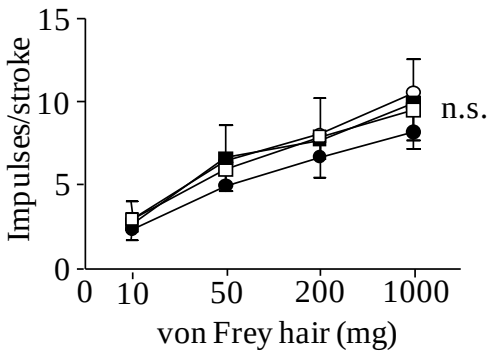
(a.b) Stimulus-response of gastric tension receptors to circular tension before (●) and after exposure to NPW 1 nM (○), 3 nM (■) and 10 nM (□) in male mice at 8 weeks old (ai: n = 5 , N = 5) or 20 weeks old (aii: n = 6, N = 5) , and female mice at 8 weeks old (bi: n = 10, N = 10) or 20 weeks old (bii: n = 8 , N = 5). *P < 0.05, **P < 0.01, ***P < 0.001 compared with control (two-way ANOVA). (c) Typical recording of gastric tension receptors to 3 g circular tension in 8 weeks old (i) and 20 weeks old (ii) female mice before and after 10 nM NPW application.

(a) Male mice

(i) 8 weeks old

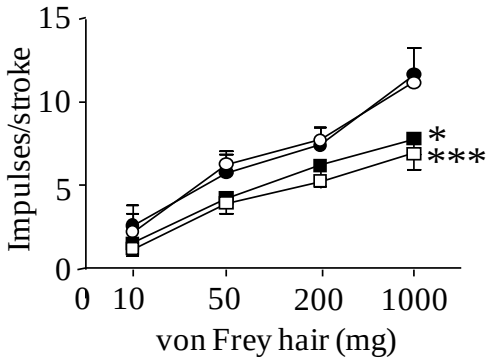



(ii) 20 weeks old

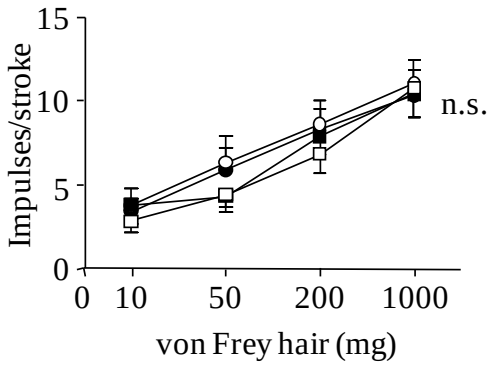



(b) Female mice

(i) 8 weeks old

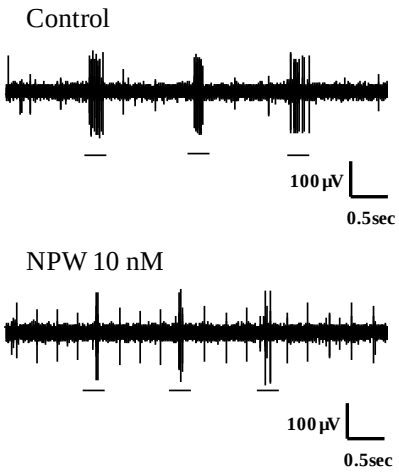



(ii) 20 weeks old




(c) Typical original recording

(i) 8 weeks old female




(ii) 20 weeks old female

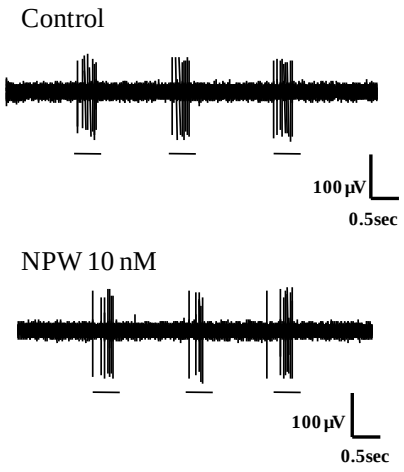


**Figure 4.3 The effect of neuropeptide W on the response of mouse gastric mucosal receptors**

Stimulus-response of gastric mucosal receptors to mucosal stroking before (●) and after exposure to NPW 1 nM (○), 3 nM (■) and 10 nM (□) in male mice at 8 weeks old (ai: $n$ = 10 , $N$ = 8) or 20 weeks old (aii: $n$ = 5, $N$ = 4) , and female mice at 8 weeks old (bi: $n$ = 6, $N$ = 6) or 20 weeks old (bii: $n$ = 9 , $N$ = 6). *$P$ < 0.05, ***$P$ < 0.001 compared with control. (c) Typical recording of gastric mucosal receptors with a 50 mg von Frey hair from 8 weeks old (i) and 20 weeks old (ii) female mice before and after 10 nM NPW application.

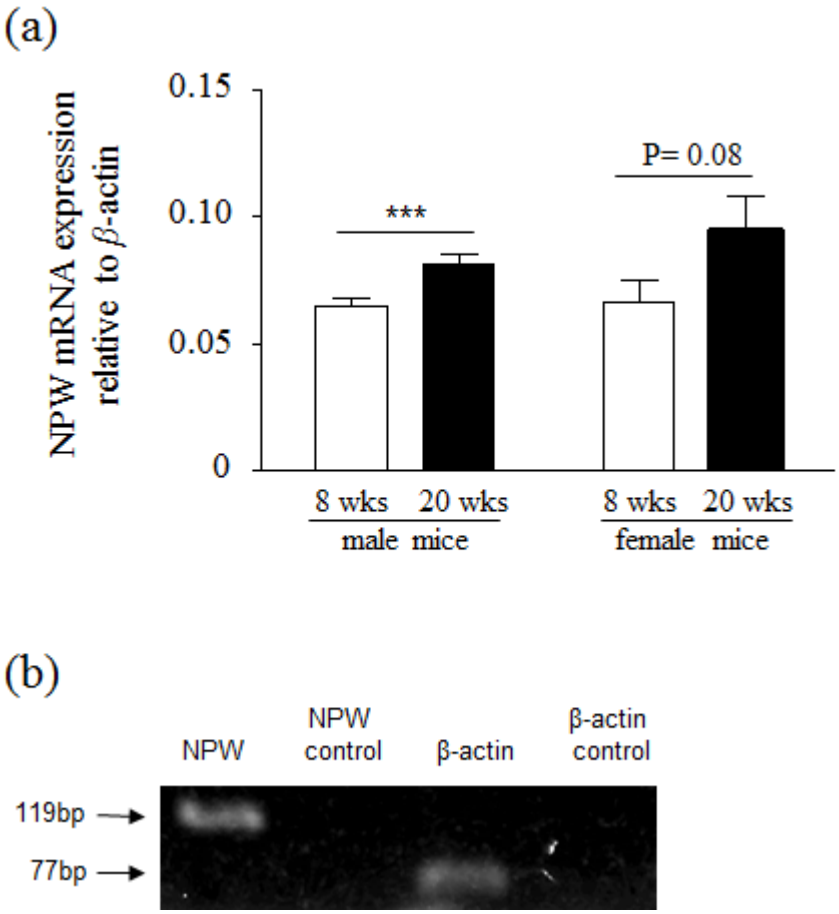
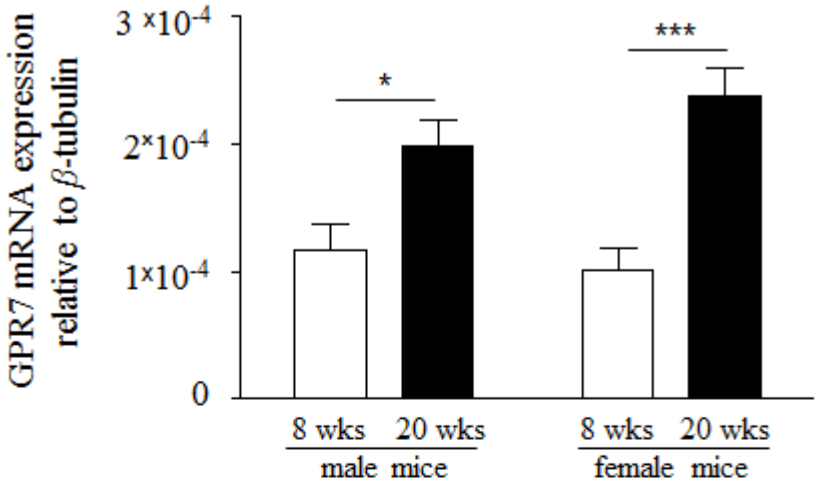


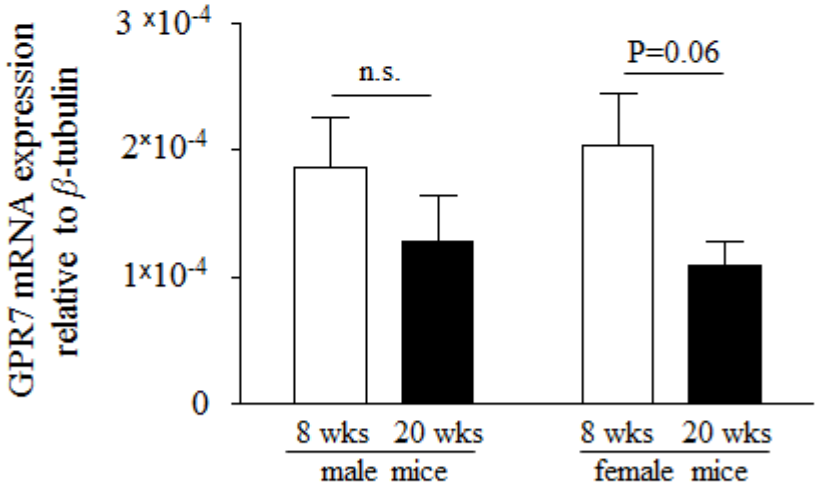

**Figure 4.4 Expression of NPW mRNA in gastric mucosa**

(a) Expression of NPW mRNA relative to β-actin in gastric mucosa of male mice at 8 weeks old and 20 weeks old, and female mice at 8 weeks old and 20 weeks old. $N$ = 5/group. ***$P$ < 0.001. (b) Agarose gel electrophoresis of amplified QRT-PCR products from gastric mucosa using specific primers for NPW and β-actin. The controls are PCR amplification products from reactions with no template added (water controls)

(a) Gastric muscular nodose neurons




(b) Gastric mucosal nodose neurons




(c)

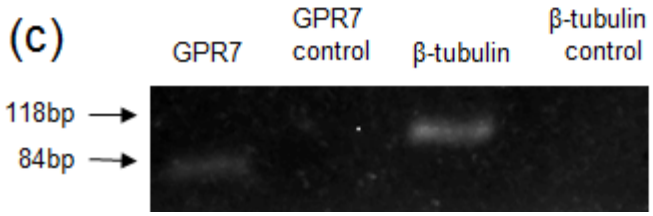


**Figure 4.5 Expression of GPR7 mRNA in nodose neurons innervating the stomach**

Expression of GPR7 mRNA relative to β-tubulin in nodose neurons innervating the gastric muscular layer (a) or gastric mucosal layers (b) in male mice at 8 weeks old and 20 weeks old, and female mice at 8 weeks old and 20 weeks old. $N$ = 4/group. *$P$ < 0.05, ***$P$ < 0.001. (c) Agarose gel electrophoresis of amplified QRT-PCR products from nodose neurons using specific primers for GPR7 and β-tubulin. The controls are PCR amplification products from reactions with no template added (water controls)

# CHAPTER 5. NUTRITIONAL REGULATION OF GASTRIC NEUROPEPTIDE W

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Submitted to: Peptides

# Statement of Authorship

## Nutritional Regulation of Gastric Neuropeptide W

Submitted to: Peptides

***By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the manuscript to be included in the candidate's thesis.***

**Hui Li (Candidate)**

Performed peptides quantification, mice study, QRT-PCR, immunohistochemistry and cell quantification, cell culture study, data analysis and interpretations and wrote the manuscript.

Signed Date 26/9/2013

**Christine Feinle-Bisset**

Provide human samples.

Signed Date 26/9/2013

**Claudine Frisby**

Assisted in the organizing radioimmuoassay performance.

Signed Date 26/9/2013

**Tracey A. O'Donnell**

Assisted in the manuscript editing.

Signed Date 26/9/2013

**Stephen Kentish**

Assisted in the manuscript construction.

Signed Date 26/9/2013

**Gary A. Wittert**

Assisted in the interpretation of data and construction of manuscript.

Signed Date 3/9/2013

**Amanda J. Page**

Assisted in the conceptualisation of experiments, interpretation of data and manuscript construction.

Signed Date 27/9/2013

## 5.1 Abstract

The peptide, neuropeptide W (NPW), is secreted from gastrin (G) cells in the stomach in response to food intake. The mechanisms underlying food intake-induced regulation of gastric NPW is largely unknown. We hypothesised that specific macronutrients were responsible for food-induced NPW secretion. We evaluated the acute effects of fat, carbohydrate and protein on plasma NPW concentrations in human and mice. The effect of different nutrients on expression of NPW in the antral stomach was also determined in mice. Primary cell cultures of mouse gastric antral mucosal cells were used to investigate the signalling pathway of NPW expression. Plasma NPW concentrations did not change after nutrients intake in either humans or mice. NPW mRNA expression and the number of NPW immunoreactive cells in the mouse antrum were increased in mice gavage fed with protein or glucose, but not lipid. In primary antral mucosal cell culture, NPW mRNA expression was stimulated by L-phenylalanine, but not influenced by glucose. Calcium-sensing receptor (CaSR) immunoreactive cells were largely co-localized with NPW containing cells in the mouse gastric antral glands, and NPW mRNA expression was inhibited by a selective antagonist of CaSR NPS2143; however, L-phenylalanine-induced NPW increase is independent of NPS2143. In conclusion, these studies indicated an inconsistency between plasma and gastric NPW levels in response to food intake, suggesting gastric released NPW may play a more important role locally. Moreover, meal-related nutrients, glucose and especially protein are potent regulators of gastric NPW, via distinct mechanisms. Overall, the current studies demonstrated that specific nutrients are responsible for the regulation of NPW in the stomach.

## 5.2 Introduction

Neuropeptide W (NPW), the endogenous ligand for G protein-coupled receptors GPR7 and GPR8, is widely expressed both centrally and peripherally and plays a pivotal role in many physiological functions including stress, inflammatory pain, neuroendocrine function and energy balance [206, 208, 248, 249]. There are two forms of NPW, NPW23 and NPW30, sharing identical N-terminal 23 residues and binding with similar affinity to GPR7 and GPR8 [206, 207]. In the stomach, NPW is produced by gastrin (G) cells in antral glands of both rodents and human [226]. Both NPW23 and NPW30 are found in the stomach, with expression of NPW30 higher than that of NPW23 [226]. The role of gastric NPW is not well defined. Evidence suggests NPW may be involved in the regulation of food intake and digestion by modulating gastric vagal afferents [302] and inducing gastric acid secretion [226].

In the stomach, NPW is secreted in response to acute intake of a standard laboratory diet in rats [226, 229]. Gastric mucosal NPW mRNA and peptide levels are low after an overnight fast in rats and increase after refeeding with standard laboratory diet [229]. Similarly, fasting plasma NPW concentrations in the rat gastric antral vein are low and increase in response to feeding with standard laboratory diet [226]. Dietary nutrients are generally potent stimulants of gastrointestinal hormones, including CCK and PYY [231-233], however, the role of macronutrients in the regulation of gastric NPW is unclear. Two studies reported that there were no differences in plasma NPW concentrations in rats chronically fed with high-fat, -carbohydrate or -protein diets [226, 230]. However, these studies did not evaluate the acute effect of specific macronutrients on gastric NPW expression and plasma concentrations. Therefore, in this study we sought to determine the acute effect of macronutrients on gastric NPW secretion.

G-cells are open-type, flask-shaped, endocrine cells that are in direct contact with the gastric lumen and potentially sense luminal nutrients [235]. Gastrin secretion is induced by chemical and mechanical stimulants sensed by receptors expressed in G-cells [238, 243, 314]. More than 80% of gastrin cells in the stomach co-express NPW [226], thus, it is possible that NPW secretion in the stomach may be stimulated via the same mechanism(s) as gastrin. Amino acids, especially the aromatic amino acids, L-phenylalanine and L-tryptophan, have been recognized as major stimuli for gastrin secretion [240]. Glucose and lipid from the lumen or systemic circulation also induce gastrin release [238, 242, 243]. The nutrient-sensing pathway of gastrin secretion is not fully understood, but many studies have demonstrated that calcium sensing receptors (CaSR) are involved in amino acid-induced gastrin secretion. Amino acids, especially aromatic amino acids, sensitize the CaSR to $Ca^{2+}$, and the subsequent $Ca^{2+}$ influx evokes gastrin secretion [280, 315, 316].

The overall aim of this series of studies was, therefore, to determine the nutritional regulation of gastric NPW. Firstly, we determined plasma NPW concentrations in humans and mice in response to acute ingestion of meals differing in their macronutrient composition. Then, the expression of NPW in gastric antral mucosa was quantified in mice after ingestion of different macronutrients. Primary antral mucosal cell culture studies were used to determine the mechanisms of increased NPW expression. Gastrin levels in response to macronutrients were also investigated in these studies. .

## 5.3 Materials and methods

### Ethics

The human study protocol was approved by the Royal Adelaide Hospital Human Research Ethics Committee. Subjects provided written, informed consent prior to their inclusion to the study. The studies on mice were performed with the approval of the Animal Ethics Committees of the Institute for Medical and Veterinary Science and University of Adelaide, Adelaide, Australia, and in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The experiments conform to the principles of UK regulations, as described in Drummond [279].

### Human study

Human blood samples were collected from a previously reported study [233]. Samples from healthy lean male subjects ($N$ = 8; BMI = 23.54 ± 0.79) were included in the current study. Briefly, subjects attended the study on three occasions, separated by 3-7 days. They were provided with a standard meal at 1900 h on the evening prior to each study day and requested to fast after the meal. On the morning of the study day, an i.v. cannula was positioned in a forearm vein, and the subject was provided with a standard breakfast at 0845 h. At 1130 h ($t$ = -30 min) a 10 ml blood sample was taken. At $t$ = -20 min subjects ingested, in randomized order, either a *1*) high-fat (% energy from fat/protein/carbohydrate 55:15:30; protein content (g/kg body wt): 0.4), *2*) high-carbohydrate (30:10:60; 0.2) or *3*) high-protein (25:45:30; 1.35) test meal within 20 min. The energy content of each test meal represented ~30% of each subject's estimated daily energy requirement. Whey protein isolate, pure cream, or a combination of corn

flour and raw sugar was added, as required, to achieve the described protein, fat or carbohydrate composition. Further blood samples were collected immediately after the preload and at *t* = 15, 30, 45, 60, 75, 90, 120, and 180 min.

**Quantification of peptides concentrations in human plasma**

Human blood samples were collected into ice-chilled EDTA-treated tubes containing 400 kIU aproptinin per millilitre blood (Trayslol; Bayer Australia, NSW, Australia). Plasma was separated by centrifugation at 3200 rpm for 15 min at 4 ℃ within 30 min of collection and stored at -80 ℃ until assayed. Human plasma NPW and gastrin concentrations were determined using a Human NPW-23 EIA Kit (EK-005-60; Phoenix Pharmaceuticals, QLD, Australia) and gastrin RIA kit (06B255017; MP Biomedicals, NSW, Australia), respectively. All samples were tested in duplicate. The NPW assay recognises both human NPW-23 and NPW-30. For the gastrin assay, the cross-reactivity with gastrin-17-I, gastrin-17-II, gastrin 34-I and gastrin-(5-17) was 100%, 77%, 42% and 54% respectively. The minimum detectable concentrations were 0.46 ng/ml and 3.3pg/ml for NPW and gastrin, respectively. The intra-assay and inter-assay coefficient variations for both assays were <10% and <15%, respectively.

**Mouse study**

Eight week old female C57BL/6 mice were group-housed under a 12 h dark/light cycle with *ad libitum* access to water and standard laboratory diet. Mice were fasted at 1600 h on the day before the experiment. On the following morning, between 0900-1100 h, mice were intragastric gavage-fed with a bolus (200 µl) of water or nutrient solution of either whey protein (dissolved in water; Bulk Nutrition, VIC, Australia), D-glucose (dissolved in water; Merck Millipore, VIC, Australia) or 10% Intralipid (Pharmatel

Fresenius Kabi, NSW, Australia). Each nutrient solution contained 0.22 kcal. Thirty min after gavage feeding, mice were either anaesthetised (isoflurane, 1-1.5% in oxygen) for collection of blood from the abdominal aorta or perfuse-fixed for immunohistochemistry.

**Quantification of peptides concentrations in mouse plasma**

Mouse ($N$ = 5/group) blood samples were collected into ice-chilled $K_2$EDTA coated tubes, and plasma was separated by centrifugation at 3000 rpm for 15 min at 4 ℃ and stored at -80 ℃ until assayed. NPW and gastrin concentrations in mouse plasma were determined using a mouse NPW-23 EIA kit (EK-005-61; Phoenix Pharmaceuticals) and gastrin EURIA RIA kit (MD 302; Euro-Diagnostica), respectively. The NPW assay recognises mouse NPW-23. For the gastrin assay, the cross-reactivity with gastrin-17, sulphated gastrin-17 and gastrin-34 was 100%, 83% and 61% respectively. The minimum detectable concentrations were 0.2 ng/ml and 10.5 pg/ml for NPW and gastrin, respectively. Intra-assay variations were 1.7% (NPW) and 3.0% (gastrin).

**Quantitative RT-PCR**

Mouse gastric mucosal scrapings ($N$ = 5/group) were collected, and total RNA was extracted using an RNeasy Mini Kit (Qiagen, VIC, Australia). RNA was quantified by measuring the absorbance at 260 nm (A260) using a NanoDrop™ ND 1000 spectrophotometer (Thermo Scientific, VIC, Australia), and RNA purity was estimated via the 260/280 absorbance ratio. QRT-PCR reactions were performed as described in detail previously [302]. In short, reactions were performed using a Chromo4 (MJ Research, Bio-Rad, NSW, Australia) real-time instrument attached to a PTC-200 Peltier thermal cycler (MJ Research) and analysed with Opticon Monitor Software (MJ

research). QRT-PCR reactions were performed using a QuantiTect SYBR green one-step RT-PCR kit (Qiagen). Primers targeting NPW and β-actin were pre-designed validated QuantiTect Primer assays (Qiagen). QRT-PCR reactions were carried out under the following conditions: reverse transcription, 50 ℃ for 30 min; initial PCR activation, 95 ℃ for 15 min; PCR cycles 94 ℃ for 15 s, 55 ℃ for 30 s and 72 ℃ for 30 s repeated for 50 cycles. A melt curve was obtained to confirm the specificity of the products produced, and products were visualised using 3% agarose gel electrophoresis with ethidium bromide staining. Each assay was run in triplicate and repeated in a duplicate PCR experiment. Control PCRs were carried out substituting RNase-free water for template RNA. Relative RNA levels were normalized to corresponding control by using the delta-delta CT method as described previously [289].

**Immunohistochemistry**

Mice were anaesthetised with pentobarbitone (0.2 ml i.p., 60 mg/ml) and then transcardially perfused with warm heparinised saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PFA-PB) at 4 ℃. The ventral side of the stomach was removed and placed in ice cold PFA-PB for 2 h. After fixation the stomach was cryoprotected with 30% sucrose for a minimum of 18 h, then embedded in optimal cutting temperature compound (OCT; Tissue-Tek, Sakura Finetek, Alphen aan den Rijn, Netherlands) and snap frozen in liquid nitrogen.

For single- and double-labelling experiments, frozen sections (10 μm) were cut, air dried at room temperature and rinsed in PBS with 0.2% Triton X-100 (PBS-TX, pH 7.4; Sigma-Aldrich, MO, USA) to facilitate antibody penetration. Sections were blocked with 10% normal goat serum (for single-labelling; Sigma-Aldrich) or 10% normal donkey serum (for double-labelling; Sigma-Aldrich) in PBS-TX for 40 min at room

temperature, and then incubated with rabbit anti-NPW polyclonal antibody (PH-005-61; Phoenix Pharmaceuticals) at a 1:800 dilution in PBS-TX at 4 ℃ for 20 h. After three washes in PBS for 5 min, primary antibody was visualised using chicken anti rabbit secondary antibody conjugated to Alexa 488 (for single-labelling; Invitrogen, VIC, Australia) or donkey anti rabbit secondary antibody conjugated to Alexa 568 (for double-labelling; Invitrogen) at a 1:200 dilution in PBS-TX for 1 h at room temperature. For single-labelling, the sections were then given final PBS washes, mounted on slides and cover slipped using ProLong antifade (Invitrogen). For double-labelling, after three washes in PBS for 5 min, sections were blocked in 10% goat anti mouse IgG (Invitrogen) in PBS-TX for 1 h at room temperature, and then incubated in mouse anti-CaR monoclonal antibody (1:100 diluted in PBS-TX; MA1-934, ABR Affinity BioReagents, CO, USA) for 20 h at room temperature. Unbound antibody was then removed with PBS washes and sections incubated for 1 h at room temperature with donkey anti mouse secondary antibody conjugated to Alexa 488 (Invitrogen) at a 1:200 dilution in PBS-TX. The sections were then given final PBS washes, mounted on slides and cover slipped using ProLong antifade.

Slide sections and whole mounts were visualized using an epifluorescence microscope (BX-51, Olympus, NSW, Australia) equipped with filters for Alexa Fluor® 488 and 568, with images acquired by a CoolSnapfx monochrome digital camera (Roper Scientific, AZ, USA). Pseudocoloured fluorescence images were overlaid. Slides where primary antibody was omitted showed no labelling and served as negative controls.

**Cell quantification**

For quantification of the number of NPW immunoreactive cells, serial sections of gastric antrum were viewed at 20x magnification. NPW immunoreactive cells were

counted manually, within the antral glandular regions where strongly immunoreactive cells were located, on 5 consecutive glands per section. The number of NPW-positive cells per gland from each mouse was averaged from 10 sections. The NPW-positive cells per gland were compared between feeding groups.

**Primary cell culture of mice gastric antral mucosal cells**

Eight week old female C57BL/6 mice fed *ad libitum* ($N$ = 3) were anaesthetised with pentobarbitone (0.2 ml i.p., 60 mg/ml), gastric antrums were removed and placed in cold Hanks' balanced salts solution (HBSS; Invitrogen) and washed vigorously three times. The mucosal layer was bluntly separated from the submucosal layer, cut into 2-3mm$^2$ sections in cold HBSS medium and placed in 2 ml DMEM/F-12 (Invitrogen). Mucosal tissue was incubated at 37℃ in a 1 mg/ml digestion solution containing collagenase II (Invitrogen) and dispase (Invitrogen) for 30 min, and then in 1 mg/ml collagenase solution for 30 min. The cell suspension was centrifuged at 600 rpm for 2 min, washed twice in 2 ml cold HBSS, and triturated using a fire polished Pasteur pipette in 2 ml culture medium (DMEM/F-12 supplemented with 10% fetal calf serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen)) and filtered through a 100 μm sterile nylon mesh (Cell strainer; BD Falcon, NSW, Australia). The sediment was washed twice with 2 ml cold HBSS and resuspended in 2 ml culture medium. Tissue was gently triturated using a fire polished Pasteur pipette and the cell suspension was filtered through a 100 μm sterile nylon mesh. The isolated gastric mucosal cells from the suspension and sediment were then combined. A cell count was performed using Trypan blue exclusion. On average, 6.4 $\times$ 10$^5$ cells were isolated from each stomach. Cells from 3 stomachs were pooled together and diluted to a final concentration of 1 $\times$ 10$^5$ cells/ml in culture medium. 24-well culture plates were pre-coated with mussel

adhesive protein (0.25 mg/ml in PBS; USUN, P.R.China) according to a previous report [317]. The cells were plated at a density of $1 \times 10^5$ cells per well and incubated in humidified 95% air and 5% $CO_2$ at 37 ℃ for 20 h.

**Peptide expression studies in cell culture**

After overnight incubation, cells were washed twice with Hepes buffer (Invitrogen) to remove the culture medium and non-adhesive cells, and exposed to various test reagents dissolved in 500 µl Hepes buffer for 60 min in humidified 95% air and 5% $CO^2$ at 37 ℃. The Hepes buffer included the following composition: 140 mM NaCl, 4.5 mM KCl, 20 mM Hepes, 1.2 mM $CaCl_2$, 1.2mM $MgCl_2$ and 0.1% BSA. The test reagents include L-phenylalanine (5 mM or 50 mM; P5482, Sigma), D-glucose (5 mM or 50 mM; Merck Millipore) and calcium sensing receptor antagonist NPS2143 (25 µM; sc-361280, Santa Cruz, CA, USA) at a concentration previously reported [318]. After incubation, culture medium was completely aspirated off and 120 µl lysis buffer (provided with the RNeasy micro kit; Qiagen) was added to each well followed by gentle pipetting and mixing. The lysate was then snap-frozen in liquid nitrogen prior to processing for RNA isolation. RNA was extracted from these cells using an RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions prior to QRT-PCR.

**Statistics**

Results were expressed as mean ± SEM. Repeated-measures one-way ANOVA was used in the human studies to compare baseline peptide concentrations between study days. Repeated-measures two-way ANOVA was used to evaluate peptide concentrations with time and treatment as factors. Area under the curves (AUCs; $t$ = -30 - 180 min) were calculated using the trapezoidal rule for human plasma peptides levels after test meal intake, and they were compared among treatment groups using repeated-

measures one-way ANOVA. Post hoc paired comparisons, adjusted for multiple comparisons by Bonferroni's correction, were performed when ANOVAs revealed significance. The differences in mean values for variables within mice studies and cell culture studies were compared using un-paired *t*-tests. All comparisons were performed using Prism 5.02 (Graphpad, CA, USA). Statistical significance was accepted at $p < 0.05$.

## 5.4 Results

### Plasma neurpeptide W and gastrin concentrations in response to nutrients in humans

To determine the effect of macronutrients on plasma NPW levels, plasma NPW and gastrin concentrations were tested in healthy lean subjects after intake of high-protein, high-fat or high-carbohydrate meal. There were no differences in baseline NPW or gastrin concentrations in human plasma on the different study days. Plasma NPW levels were not altered by time and did not differ between test meals (Fig. 1Ai). For gastrin, plasma concentrations increased in response to all test meals (time effect, $p < 0.001$; Fig. 1Aii), and there was a significant difference among test meals (treatment effect, $p < 0.001$; Fig. 1Aii). The magnitude of the rise in gastrin concentration was greater following high-protein meal (AUC: 19034 ± 2914 pg/ml $^{x}$ min) compared with high-carbohydrate meal (AUC: 13211 ± 1786 pg/ml $^{x}$ min; $p < 0.01$), and there was no difference between high-protein meal and high-fat meal (AUC: 15767 ± 2090 pg/ml $^{x}$ min), or high-carbohydrate meal and high-fat meal. These results indicate that human plasma NPW levels were not affected by intake of different nutrients, but plasma gastrin levels were increased after a high-protein meal.

### Plasma neurpeptide W and gastrin concentrations in response to nutrients in mice

To confirm the findings in the human study, we determined the plasma NPW levels in mice after intake of whey protein, lipid or glucose. These nutrients were chosen as their effects would be more specific and profound compared to complex undigested macronutrients. Plasma NPW levels were determined at 30 min after nutrient feeding, the previously determined peak gastrin response for nutrients [242, 280]. Mouse plasma

NPW concentrations following lipid, glucose or protein feeding were not significantly different compared with the water control (Fig. 1Bi). Plasma gastrin levels were significantly higher following a protein feed compared with the water control ($p < 0.05$), but was not affected by lipid or glucose (Fig. 1Bii). Therefore, nutrients intake has no effect on systemic plasma NPW levels in mice.

**Gastric neuropeptide W content in response to nutrient feeding in mice**

We also determined NPW mRNA expression in mouse gastric mucosa 30 min after lipid, protein or glucose gavage feeding. QRT-PCR results indicate that NPW mRNA expression in mouse gastric mucosa 30 min following glucose or protein feeding was 1 and 2 fold higher respectively compared with the water control mice ($p < 0.01$ and $p < 0.001$ respectively; Fig. 2A). Lipid feeding had no effect on gastric NPW expression compared to the water control (Fig. 2A). Protein induced a significantly higher level of NPW mRNA expression compared to glucose ($p < 0.05$; Fig. 2A). A qualitatively similar but more pronounced increase was observed for gastrin mRNA. The expression of gastrin mRNA in mouse gastric mucosa was significantly increased following intake of either lipid (1 fold; $p < 0.01$), glucose (6 fold; $p < 0.001$) or protein (10 fold; $p < 0.001$) compared with the water control (Fig. 2B). The effect of protein was significantly higher than that of glucose and lipid ($p < 0.05$; Fig. 2B).

To further confirm whether protein and glucose induced increases in NPW mRNA expression was reflected in changes in protein level, the number of NPW immunoreactive cells in gastric antrum was determined. Similar to NPW mRNA expression, the number of NPW immunoreactive cells in the gastric antrum were significantly higher in protein ($2.31 \pm 0.12$ cells/gland), and glucose gavage fed mice ($1.71 \pm 0.10$ cells/gland) compared to water control mice ($1.28 \pm 0.07$ cells/gland; $p <$

0.01 and $p < 0.05$; Fig. 2C). Moreover, the number of NPW immunoreactive cells was significantly higher in protein gavage fed mice compared to glucose gavage fed mice ($p < 0.01$; Fig. 2C).

**NPW mRNA levels in mouse primary antral mucosal cells in response to L-phenylalanine and calcium sensing receptor antagonist**

To determine whether protein directly modulates NPW expression in the gastric mucosa, primary gastric antral mucosal cells were exposed to control buffer or buffer with L-phenylalanine (5 or 50 mM). Exposure to 50 mM L-phenylalanine, but not 5 mM L-phenylalanine, induced higher NPW mRNA expression than in the absence of L-phenylalanine ($p < 0.05$; Fig. 3A). Similarly, gastrin mRNA expression was stimulated by exposure to both 5 mM and 50 mM L-phenylalanine ($p < 0.05$; Fig. 3B). We further tested whether protein induced NPW expression in G-cells was mediated via CaSR, sharing the same mechanism as gastrin. Firstly, we confirmed a large degree of co-localization of NPW and CaSR immunoreactive cells in the gastric antrum (Fig. 3C). The selective CaSR antagonist NPS2143 was used to treat antral mucosal cells. 25 μM NPS2143 significantly inhibited NPW mRNA and gastrin mRNA expression in cells with or without exposure to L-phenylalanine ($p < 0.01$; Fig. 3A and Fig. 3B). L-phenylalanine (50 mM) significantly increased mRNA expression of NPW in cells incubated with or without NPS2143. There was no interaction between the effect of the antagonist and the effect of L-phenylalanine and thus the effects of L-phenylalanine are independent of CaSR. L-phenylalanine (5 and 50 mM) increased mRNA expression of gastrin, but the increase was not observed in cells incubated with L-phenylalanine (5 mM) and NPS2143. There was a significant interaction between the effect of the

antagonist and the effect of L-phenylalanine, therefore the effect of L-phenylalanine is dependent on CaSR.

**NPW mRNA levels in mouse primary antral mucosal cells in response to glucose**

To determine whether glucose directly modulates NPW expression in gastric mucosa, primary gastric antral mucosa cells were exposed to buffer with or without glucose (5 or 50 mM). NPW mRNA expression in antral mucosal cells was not affected by 5 mM or 50 mM glucose (Fig. 4A). In contrast, gastrin mRNA expression in antral mucosal cells was significantly stimulated by both 5 mM and 50 mM glucose ($p < 0.05$; Fig. 4B).

## 5.5 Discussion

In the current study, the acute effect of macronutrients on gastric NPW secretion was determined at different levels. Firstly, we did not observe any variation in NPW levels in the systemic circulation after food intake in both human and mouse, independent of nutrients composition. However, more specifically in the mouse stomach, NPW expression increased in response to specific nutrients intake, consistent to previous findings demonstrating that gastric NPW was up-regulated by food intake [226, 229]. These findings suggest a discrepancy in NPW levels in the blood circulation and the stomach after acute food intake. Although *in vivo* mouse studies indicate that protein and glucose are stimulators of gastric NPW, our *in vitro* cell culture study suggest protein, but not glucose directly induces NPW expression within the gastric mucosa, via a mechanism independent of CaSR.

Gastrointestinal hormones released in response to food intake may lead to the changes in circulating levels, including PYY and GLP-1 [233, 319]. In the current studies, we observed an increase in plasma gastrin levels after food intake, more specifically protein intake, consistent with previous reports [242]. However, our results indicate that plasma NPW levels were not changed after food intake in both human and mouse, independent of nutrients composition. Similar to this findings, Beck *et al*. [230] also failed to detect any changes in circulating NPW levels following an overnight fast in either obese leptin receptor deficient rats (fa/fa) or their lean littermates. It is reported that NPW levels more locally in the gastric antral vein are decreased after fasting and increased in response to food intake in rats [226]. Taken together, this evidence suggests that NPW is released from the stomach upon food intake, but may not affect NPW levels in the systemic circulation.

In the stomach, gastric NPW was up-regulated at both mRNA and protein levels upon food intake [229, 302]. The current study revealed that glucose and protein but not lipid, induced an increase in NPW mRNA expression in mouse stomach. Consistently, the number of NPW immunoreactive cells was also increased after protein and glucose intake. These nutrients were given at the same energy level, suggesting specific nutrients rather than energy content are responsible for the food induced NPW secretion. A similar expression pattern was observed for gastrin, with protein being the major stimulant followed by glucose. However, the magnitude of NPW mRNA expression was lower compared to gastrin and unlike gastrin was not affected by lipid intake. In the current study expression of NPW was only tested at the previously determined peak time of gastrin secretion and it did not reflect the release of NPW from the stomach. Therefore, an overall dynamic secretion of NPW following nutrients intake remains to be determined.

The discrepancy found in NPW levels in the blood circulation and the stomach has also been reported with other peptides such as neuropeptide Y [313]. The mechanism is not clear. It is possible that upon release from the stomach NPW may be rapidly degraded in the liver, given that reported plasma NPW concentrations in the rat gastric antral vein are much higher (around 28 times) than that in the systemic circulation [226], and thus changes in circulating NPW levels are not detected. It is also possible that the stomach may not be the major source of circulating NPW, as NPW is also widely distributed in the CNS and peripheral tissues [207]. As circulating NPW levels were not regulated by food intake, food intake induced gastric NPW secretion may have limited, if any, physiological effects remotely via the circulation, and thus the function of NPW locally within the stomach after food intake could be more physiologically important. There are limited studies focused on the physiological role of NPW in the stomach. Evidence

suggests that NPW can modulate gastric vagal afferents and gastric acid secretion [226, 302], however, other roles of gastric NPW may also exist. For instance, other gastrointestinal hormones including gastrin have been found to be involved in the regulation of food intake, gastric acid secretion, gastric mucosal cell proliferation and gastric motility [298, 320, 321] .

The co-expression of NPW and gastrin in antral G-cells and the common sensitivity of their secretion to protein and glucose raise the possibility that the secretion of both peptides may share the same mechanisms. It is well known that the aromatic amino acids, L-phenylalanine and L-tryptophan, are major signals for gastrin release and expression [240, 322]. Our mouse antral mucosal cell culture studies demonstrate that L-phenylalanine, at high concentrations can induce an increase in NPW mRNA expression in gastric antral cells, suggesting amino acids may directly stimulate NPW expression in the stomach. However, again this effect was less significant than L-phenylalanine induced gastrin expression, as 5 mM L-phenylalanine was sufficient to increase gastrin expression in antral mucosal cells. The effective concentration of L-phenylalanine (50 mM) for NPW expression in the current study corresponds to L-phenylalanine concentrations found to stimulate CCK secretion in enteroendocrine STC-1 cell culture [318]. The current results only reflect changes in NPW mRNA level, whether there are similar changes in protein levels requires further investigation.

CaSR, expressed in G cells, is an amino acid sensor involved in gastrin secretion in response to protein intake [315, 316]. CaSR induced $Ca^{2+}$ mobilization was increased by L-amino acids [315], and peptone stimulated gastrin secretion was inhibited by the selective antagonist of CaSR NPS2143 [280]. In the current study, we confirmed that CaSR and NPW immunoreactive cells were largely co-localized in the mouse gastric

antrum. The cell culture studies demonstrated that NPS2143 inhibited gastrin expression in gastric mucosal cells, consistent to previous reports of reduced gastrin expression in CaSR null mice [280], and the phenylalanine-dependent stimulation on gastrin expression is dependent on NPS2143. Although NPW expression is also inhibited by NPS2143, the effect of L-phenylalanine is independent of NPS2143. Therefore, it is possible that CaSR is involved in inducing NPW expression, but may not be an important pathway of L-phenylalanine induced NPW expression. Other types of amino acid sensors may also be responsible for NPW expression, such as GPRC6A, a G-protein coupled receptor found mostly expressed in gastrin G cells [59]. Moreover, other hormones released in response to amino acids may also affect the NPW expression, as there are considerable interactions between gastrointestinal hormones [323, 324]. The role of different type of amino acids in NPW secretion and their possible pathways should be further investigated.

In line with another *in vitro* study [238], our results showed that gastrin mRNA expression in antral mucosal cells increased with glucose incubation. However, inconsistent to our *in vivo* findings, NPW mRNA expression in antral mucosal cells was not affected by exposure to glucose. This suggests that NPW secretion in G-cells may not be directly stimulated by glucose, which is at least partly different from the mechanism of glucose induced gastrin expression. Signals from the small intestine might account for glucose regulated gastric NPW secretion, given that glucose sensors are most densely distributed in the small intestine [325]. This requires further investigation.

In summary, this study showed an inconsistency between plasma and gastric NPW levels in response to food intake, suggesting gastric released NPW may play a more

significant role locally. Moreover, meal-related nutrients, glucose and especially protein, are stimulants of gastric NPW, and their underlying mechanisms are at least partly different from gastrin. Overall, our studies demonstrate that specific nutrients are responsible for the regulation of NPW in the stomach.

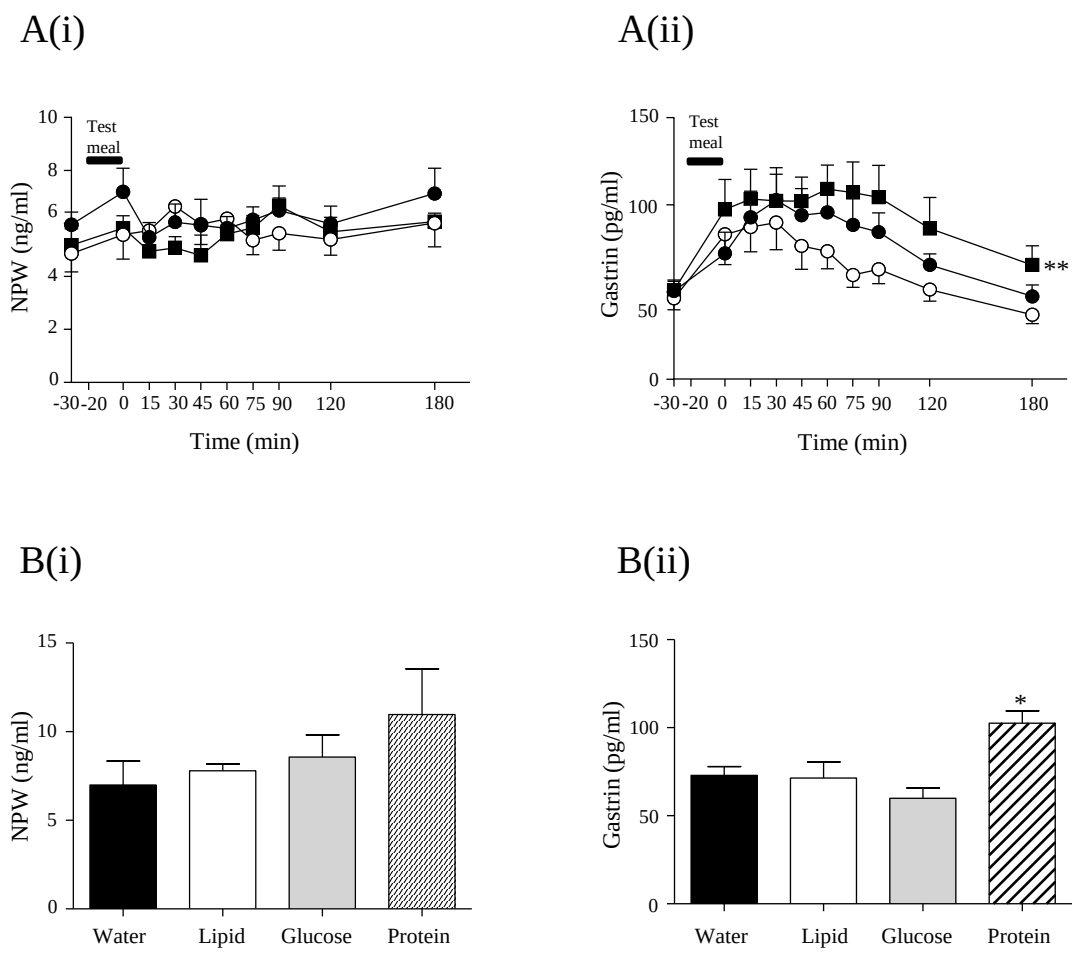



**Figure 5.1 Plasma NPW and gastrin levels in response to nutrient intake in humans and mice**

A, Plasma NPW (i) and gastrin (ii) levels after intake of food rich in different macronutrients (● High fat, ○ High carbohydrate, ■ High protein). $^{**}p < 0.01$ *vs.* gastrin levels after high carbohydrate ($t$ = -30 to 180 min). $N$ = 4. B, Plasma NPW (i) and gastrin (ii) levels ($t$ = 30 min) after water or specific nutrient feeding in mice. $^{*}p < 0.05$ *vs.* water feeding. $N$ = 5/group.

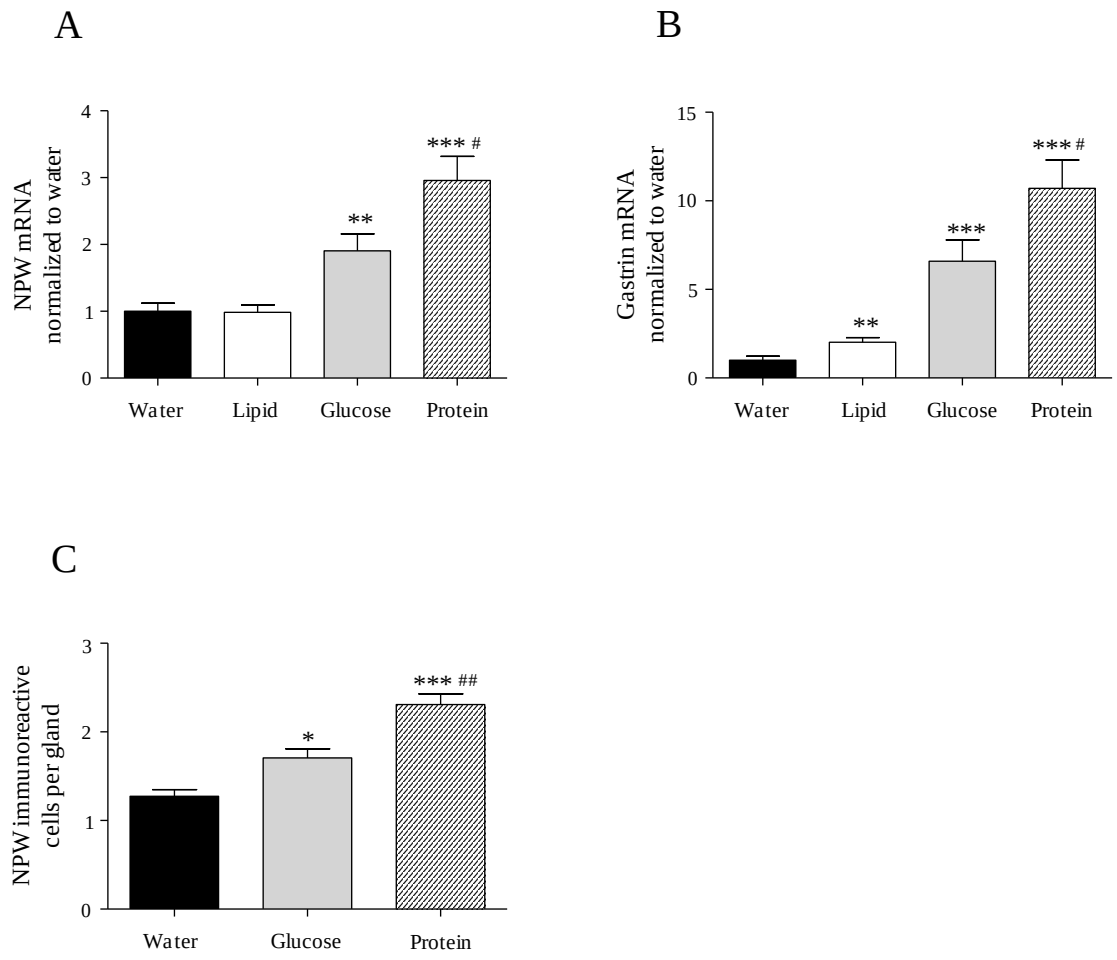



**Figure 5.2 NPW and gastrin levels in gastric mucosa in response to nutrient intake**

A, Expression of NPW mRNA in gastric mucosa after water or nutrient feeding in mice. $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* water feeding; $\#p < 0.05$ *vs.* glucose feeding. $N = 5$/group. B, Expression of gastrin mRNA in gastric mucosa after water or nutrient feeding in mice. $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* water feeding; $\# p < 0.05$ *vs.* glucose feeding. $N = 5$/group. C, Quantization of cells expressing NPW per gland in gastric antral mucosa after water, glucose or protein feeding in mice. $^{*}p < 0.05$, $^{***}p < 0.001$ *vs.* water feeding; $\#\# p < 0.01$ *vs.* glucose feeding. $N = 4$/group.

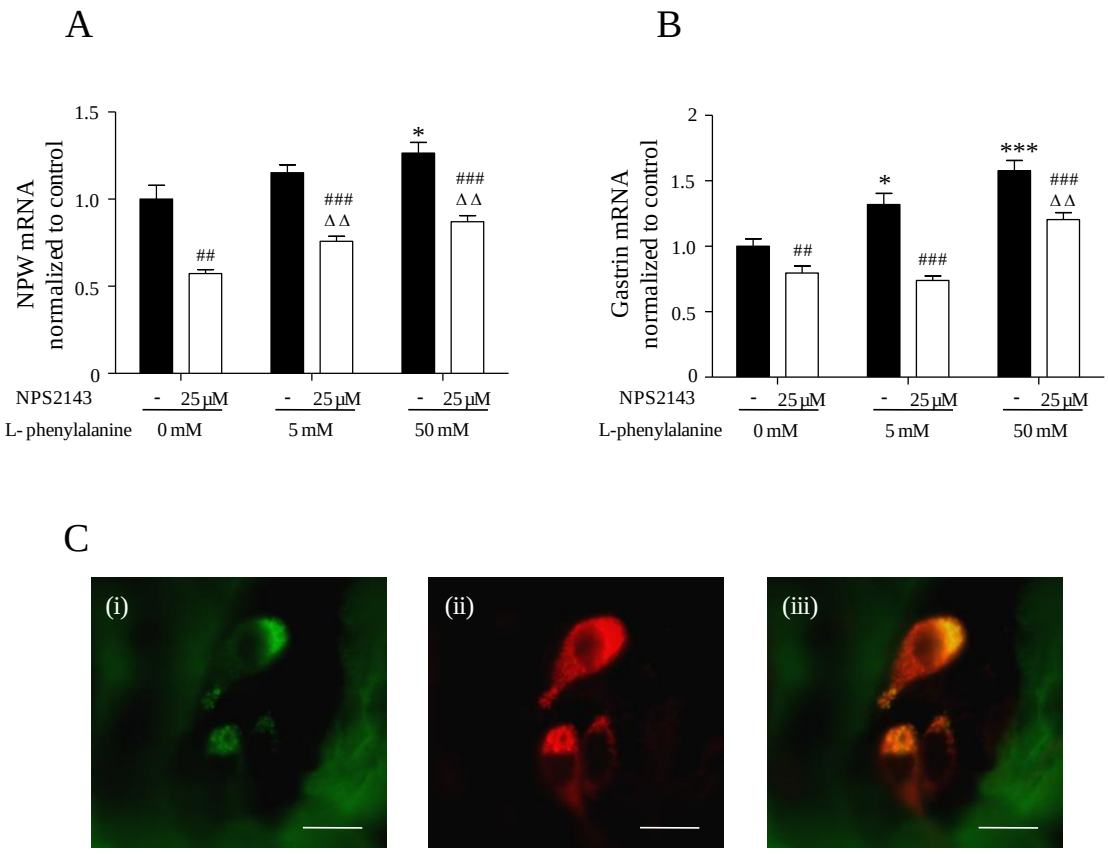



**Figure 5.3 Effect of phenylalanine and the CaSR antagonist NPS2143 on NPW and gastrin mRNA expression in mice antral mucosal cell culture**

A-B, Expression of NPW mRNA (A) and gastrin mRNA (B) in cells exposed to 0 mM, 5 mM or 50 mM L-phenylalanine with or without 25 µM NPS2143. *$p < 0.05$ and ***$p < 0.001$ vs. L-phenylalanine and NPS untreated cells; ##$p < 0.01$ and ###$p < 0.001$ vs. NPS untreated cells at the corresponding L-phenylalanine concentration; ΔΔ$p < 0.01$ vs. NPS2143 and L-phenylalanine untreated cells. C, Co-localization of CaSR and NPW immunoreactive cells in mouse gastric antral mucosa. (i) CaSR immunoreactive cells (green), (ii) NPW immunoreactive cells (red) and (iii) overlay (orange). Scale bars = 10 µm.

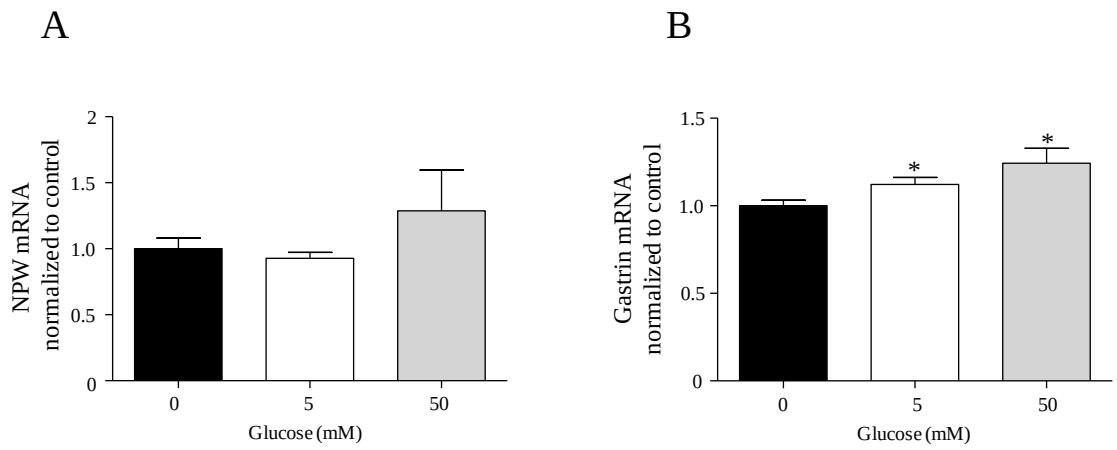



**Figure 5.4 Effect of glucose on NPW and gastrin mRNA expression in mouse antral mucosal cell culture**

Expression of NPW mRNA (A) and gastrin mRNA (B) in cells exposed to 0 mM, 5 mM or 50 mM glucose. *$p < 0.05$ *vs.* no glucose control.

# CHAPTER 6. GENERAL DISCUSSION AND CONCLUSION

This thesis provides the first evidence that NPW, the newly identified gut-brain peptide, modulates gastric vagal afferent signalling. This effect is dynamic and related to nutritional states, age and gender. It is known that vagal afferents innervating the proximal GI tract are responsive to a number of hormones, the current studies suggests NPW as a member of these hormones, which may regulate gastric vagal afferent function including satiety signalling.

**Molecular expression in vagal afferent pathways**

GI hormones modulate vagal afferents by activating their corresponding receptors secreted in the vagal afferent cell bodies and transmitted to the terminal endings [150, 153]. Numerous receptors for appetite-regulatory hormones are expressed in nodose ganglia, where the cell bodies of vagal afferents are located, such as GLP-1 receptor, leptin receptor and CCK-1 receptor [148, 151, 152]. In this thesis, NPW receptor GPR7 was identified in the nodose ganglia of mice. Furthermore, a higher level of GPR7 mRNA was found in the nodose neurons innervating the stomach compared to the whole nodose ganglia. This finding is consistent to the abundant expression of NPW in the stomach, where NPW containing cells are in close proximity to vagal afferent endings, suggesting that mucosal vagal afferents are in a good position to sense NPW upon its release. Moreover, vagal afferents innervating muscular layers could also sense NPW, as observed with other GI hormones [174, 176], which may probably be diffused locally or be carried in blood capillaries. Therefore, the expression of NPW and its receptor along the gastric vagal afferent pathway in the stomach indicates a potential role of NPW via gastric vagal afferents.

**Modulation of NPW on gastric vagal afferents**

Our *in vitro* electrophysiology study showed that in adult mice (20 weeks old) NPW inhibited the response of gastric vagal afferents to mechanical stretch. Mechanical distension of the stomach is an important satiety signal relayed from the periphery to the CNS after food intake. Classical studies using gastric balloon or non-nutrient gastric perfusion in human and rodents indicate that gastric load alone is enough to generate feeling of fullness [159, 160, 326]. Therefore, the inhibition of NPW on tension sensitive vagal afferents would reduce the satiety signalling in response to food accumulation in the stomach. Food intake stimulated hormones are usually satiety factors to terminate a meal [60, 298]. However, upon food intake several mechanisms are involved to facilitate food intake and processing, such as gastric acid secretion, and proximal stomach relaxation [299, 327]. The reduced sensitivity of vagal afferents by NPW may lead to delayed satiety signalling which would facilitate food intake. An observation of direct regulation of gastric NPW on food intake would be useful to confirm the current hypothesis of this finding, however, it is difficult to exclude bias factors in experimental design as exogenous administration of NPW may regulate food intake via other pathways, including regulation of appetite in the CNS via the blood circulation, and also regulating the endocrine system which would indirectly regulate food intake. Previous findings have shown that other gastric hormones including leptin and ghrelin are also involved in modulating gastric mechanosensitive vagal afferents. Leptin has no effect on gastric tension receptors in fed mice, but has an inhibitory effect in overnight fasted mice [180], whereas, ghrelin inhibits the response of tension sensitive vagal afferents in both fed and fasted states [174]. The current findings together with these evidences suggest that NPW is a member of the gastric hormones involved in modulating vagal afferents, but the role could be different in regulating food intake.

The peripheral effects of gut-brain peptides generally correspond to their role in the CNS. In the CNS, the chronic role of NPW is an anorectic and catabolic factor [211, 255], while acute effects of NPW show increase or decrease in food intake depending on administration time and sites [206, 211, 212, 251, 252]. These findings suggest that despite the chronic inhibitory effect of NPW on food intake, the acute role of NPW may have multiple ways to regulate food intake in the CNS. Although the current study cannot provide direct evidence on the regulation of NPW on food intake in the stomach, our findings suggest that the NPW gastric vagal afferent signalling pathway is not a mechanism of the anorectic role of NPW.

In the gastric mucosa, vagal afferent endings are in close proximity to NPW secreting cells, however, we did not observe any regulation of exogenous NPW on mechanosensitive mucosal vagal afferents in adult mice (20 weeks old), despite its role in younger mice (8 weeks old). This correlates well with the reduction in GPR7 mRNA expression in the older 20-week-old mice.

***Influence of acute food deprivation***

Our results showed that the modulation of NPW on gastric tension sensitive vagal afferents was only observed in mice fed *ad libitun* but not in fasted mice. This finding suggests the modulation of NPW on gastric vagal afferents is less effective in the fasted status, but is a response to food intake. This change is specifically observed in gastric vagal afferents, as the regulation of NPW on oesophageal tension sensitive vagal afferents was not affected by food intake (Supplementary Figure. S1). It is known that gastric vagal afferent function is adaptive in response to food intake. In the fasted state the response of gastric vagal afferents to gastric distension is lower compared to that in the fed state [174], therefore, NPW may not be necessary to inhibit the already reduced

mechanosensitivity of gastric vagal afferents in the fasted state. This role is related to the molecular expression of NPW and its receptor along the pathway. The increased sensitivity of vagal afferents to NPW is accompanied by increased gastric NPW mRNA and protein after food intake, which would enhance the NPW vagal afferent signalling pathway during food intake. Moreover, the up-regulated GPR7 expression in the nodose ganglia after food intake could be responsible for the increased sensitivity of vagal afferents to NPW. The mechanisms underlying the observed increase in GPR7 expression remains unclear, but evidence exclude the effects of hormones, as food intake associated hormones including NPW, leptin and ghrelin show the opposite effect on GPR7 expression [308]. Whether other food intake associated factors such as nutrients could modulate GPR7 remains to be investigated.

***Influence of chronic high-fat diet***

In the obese state, several signalling pathways involved in controlling food intake are disrupted, including central signalling circuits and peripheral appetite signals [16, 20]. The sensitivity of gastric vagal afferents to circular stretch is reduced in HFD-induced obesity [174], suggesting a reduced response to gastric load after food intake and a reduced satiety signalling to the CNS. This effect is consistent with the hyperphagia phenotype observed in obesity. The current study revealed that the inhibition of NPW on gastric tension sensitive vagal afferents is abolished in HFD-induced obesity and accompanied with a reduced NPW receptor expression in the vagal afferents neurons, despite the unchanged NPW mRNA expression in the stomach. These findings suggest that similar to the role of NPW in the fasted state, in HFD-induced obesity NPW is not effective in regulating gastric vagal afferents when their response is already reduced.

This role is specifically observed in vagal afferents innervating the stomach but not the oesophagus (Supplementary Figure. S1).

***Influence of age and gender***

The modulation of NPW on gastric tension sensitive vagal afferents is age-dependent. The physiological meaning of this effect is not clear. Eight-week-old mice are in a period of rapid growth, while in 20-week-old mice the growth rate has slowed down. In the rapid growth period, mice are gaining body weight more rapidly and having a higher food intake relative to body weight compared to older mice. The decreased sensitivity of gastric vagal afferents to NPW could be related to the positive energy balance associated with high calorie intake and body fat accumulation, as a similar lack of effectiveness of NPW was also observed in mice fed a chronic HFD [302]. Our results also showed that in female mice the inhibitory effect of NPW on gastric tension receptors was observed in 32-week-old mice, where the growth rates are not different from that in 20-week-old mice (Supplementary Figure. S2 and S3). On the other hand, it is also possible that the insensitivity of younger mice to NPW is due to developmental progression, as both NPW and GPR7 expression in the pathway are increased in 20 week-old mice compared to 8-week-old mice.

The modulation of NPW on gastric tension sensitive vagal afferents is consistently observed in both male and female mice, suggesting the role is not gender-specific. GPR7 deleted mice are sexually dimorphic in food intake and body weight [255], however, the current findings show a gender-consistent inhibition of NPW on gastric vagal afferents, suggesting that the NPW vagal afferent pathway may not be responsible for the GPR7 deletion induced hyperphagia and obesity in male mice.

In 8-week-old female mice, NPW inhibits the mucosal receptors to mucosal stroking. The role of mucosal mechanical sensitive vagal afferents is not clear, but evidence suggest it is involved in recognition of food particle size and respond to regulate gastric empty [168]. The inhibited response of mucosal receptor to stroking was supposed to lead to an increased gastric emptying, however, our *in vivo* gastric emptying study failed to observe this effect in 8-week-old mice (not shown data). The function of mucosal mechanical sensitive vagal afferents and the role of NPW involved in this pathway require further investigation.

**Regulation of gastric NPW expression**

This thesis also provides information about the regulation of gastric NPW mRNA levels by nutritional states, age, gender and nutrients. Firstly, we confirmed the previous findings that the expression of NPW in the stomach is reduced after an overnight fast, supporting the reports that gastric NPW is secreted in response to food intake [226, 229]. In the stomach, NPW is secreted from open-type flask-shaped gastrin-releasing G cells [226]. Open-type cells in the GI tract, such as K cells and L cells [328], typically respond to lumen nutrients and secrete hormones. We further found that protein and glucose are nutrients that induce NPW expression in the stomach, via different mechanisms. Although gastric NPW levels are regulated by acute food intake, we failed to detect any changes in gastric NPW mRNA levels in chronic HFD-induced obese mice, suggesting gastric NPW is not changed in the obese state. On the other hand, it is reported that gastric NPW expression was inhibited in chronically food restricted rats [229], indicating that chronic negative energy balance down-regulates gastric NPW expression, similar to the acute effect of energy restriction. The gastric NPW mRNA levels show no difference between male and female mice at different ages; however, its

levels are increased in mice from 8 week to 20 week of age. It is reported that gastric NPW mRNA levels in mice are increased in developing mice until adulthood [228], in the current study we found an increase of NPW mRNA levels during early adulthood. It is possible that the increase in NPW level from 8 week to 20 week is a developmental progression, as we did not observe different gastric NPW levels between 20 and 32-week-old female mice (Supplementary Figure. S3). The age-related changes in NPW levels are also observed in other GI hormones such as ghrelin [329], how they are relevant to the development of the GI tract or GI functions remains to be established.

In this thesis, we only quantify the mRNA levels but not the protein levels of NPW in the mouse stomach. Protein level would be a more meaningful indicator of functional NPW released from the stomach. However, multiple attempts to determine the NPW protein levels in the stomach using traditional techniques including western blot and enzyme immunoassay have been unsuccessful due to the low protein levels of NPW in the stomach. Evidence suggests that NPW protein and transcript levels may be consistently regulated by acute food intake. In response to fasting, both NPW mRNA and protein levels are reduced in rats, and returned back to normal after refeeding [229]. Our results also showed that using a semi-quantification method (immunohistochemistry), the number of NPW immunoreactive cells is increased in response to nutrients intake in mice, similar to the NPW mRNA levels in the gastric antrum. Therefore, food intake induced increase in NPW secretion is associated with increased NPW transcript levels, similar to what was found with other GI hormones such as CCK [330] . How NPW protein levels in the stomach are regulated in other situations and whether they are consistent to NPW mRNA levels remains to be illustrated.

NPW secretion from the stomach may not affect NPW concentrations in the blood circulation. Although gastric NPW is markedly increased by acute food intake, our human and mice studies failed to detect any changes in plasma NPW levels after food intake. Similarly, it is also reported that plasma NPW levels are not affected by fasting or re-feeding in obese Fa/Fa rats and its lean littermates [230]. Therefore, acute food intake may not be sufficient to change plasma NPW levels, despite its regulation on gastric NPW secretion. It is possible that the stomach is not the major source of circulating NPW, as NPW is also widely distributed in the CNS and other peripheral tissues [207]. It is also possible that gastric released NPW is degraded, although no reports are available on its degradation and excretion in the blood circulation. As food intake induced gastric NPW secretion does not affect circulating NPW levels, it is likely that gastric released NPW may have limited roles via the blood circulation. Many peripherally released hormones such as leptin and insulin could exert their functions partly via entering the CNS through blood circulation [20]. Although intense NPW binding sites was observed in subfornical organ where the blood brain barrier is lacking [214], there is limited information on the ability of blood-born NPW penetrating into the brain. It is reported that central administration but not intraperipheral administration of NPW exert anticonvulsant activity, suggesting peripheral NPW may not penetrate into the brain effectively [331]. Therefore, NPW released from the stomach in response to food intake may have limited central effect via the blood circulation, indicating the role of gastric released NPW is more important locally.

Plasma NPW levels could be regulated in a different way from gastric NPW levels. In HFD-induced obese mice, NPW expression in the stomach is not different from that of normal control mice, however, elevated plasma NPW levels were reported in obese fa/fa rats compared to their lean littermates [230]. The increased plasma NPW levels in

obesity could arise from the hypothalamus as elevated hypothalamic NPW levels are observed in different obese models including ob/ob mice, db/db mice, and diet induced obese rats [219]. The tissue-specific regulation of hormone secretion was also reported for NPY [313]. Under chronic food restriction gastric and plasma NPW levels are consistently reduced, suggesting a large weight loss associated with endocrine changes is sufficient to down-regulate plasma NPW levels. Therefore, plasma NPW levels may be more sensitive to endocrine changes associated with obesity or weight loss.

**Summary**

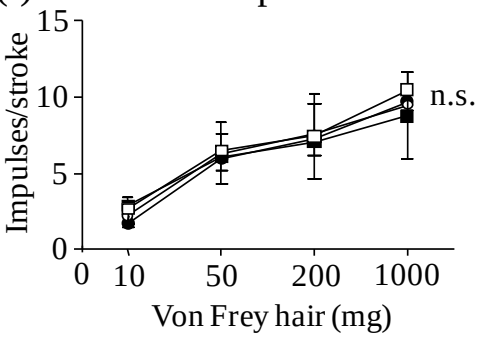
In summary, the evidence provided by this thesis demonstrates that NPW modulates gastric vagal afferent signalling pathway, an effect related to feeding status, age and gender. In response to food intake, especially protein and glucose ingestion, NPW is up-regulated in the stomach. Gastric released NPW could modulate the mechanosensitivity of gastric vagal afferents to stretch, which may delay the satiety signals generated from food load in the stomach. This effect together with other responses such as gastric secretion may facilitate food intake. This role is not gender-specific, but is not observed in young adult mice (8 weeks old). Food intake induced gastric NPW secretion may not be sufficient to affect plasma NPW levels, suggesting gastric NPW secretion may have more physiological importance locally within the stomach . GI hormones generally play multiple roles to regulate food intake and GI functions. The role of NPW in the GI tract is not fully understood but it is evident that NPW has numerous functions. Within vagal afferent cell bodies NPW has been shown to regulate the expression of receptors for ghrelin and leptin [308], suggesting that NPW modulates the effect of other appetite regulating hormones. Moreover, Mondal *et al* showed that NPW may induce gastric acid secretion [226]. Our preliminary finding suggests that GPR7 is also expressed in

enteric neurons of the myenteric plexus (Supplementary Figure. S4), indicating that NPW may also be involved in enteric neurons signalled functions. Other roles of gastric NPW within the stomach need to be investigated further.

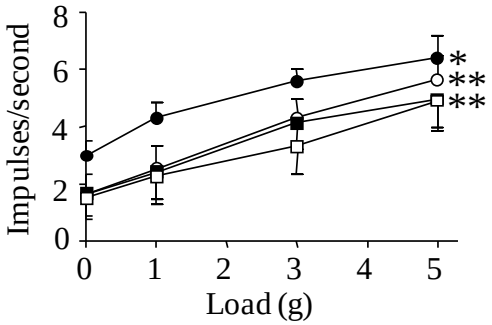
# APPENDIX

(a) SLD mice

(i) Mucosal receptor

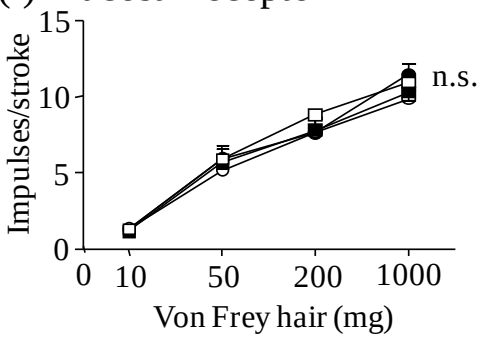



(ii) Tension receptor




(b) SLD fasted mice

(i) Mucosal receptor

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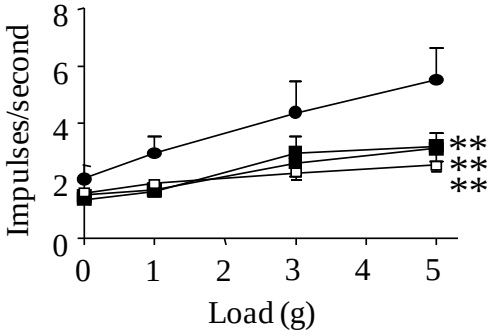
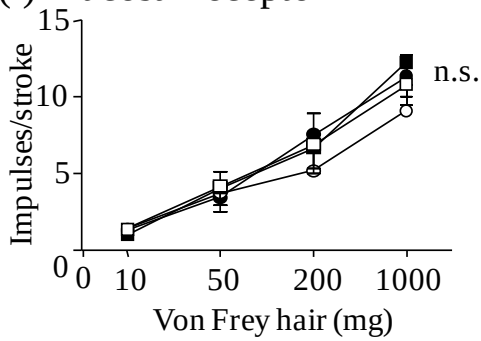



(c) HFD mice

(i) Mucosal receptor

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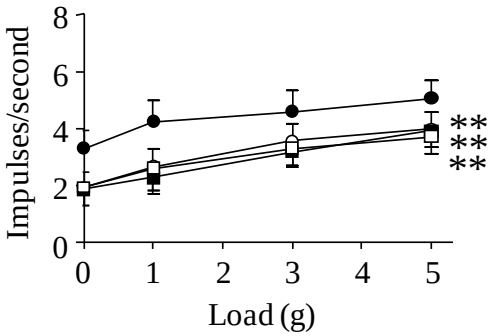
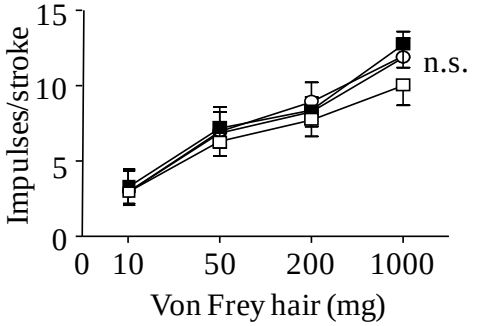


**Supplementary Figure S1 The effect of neuropeptide W on the response of oesophageal mechanosensitive vagal afferents in female SLD, SLD fasted and HFD mice**

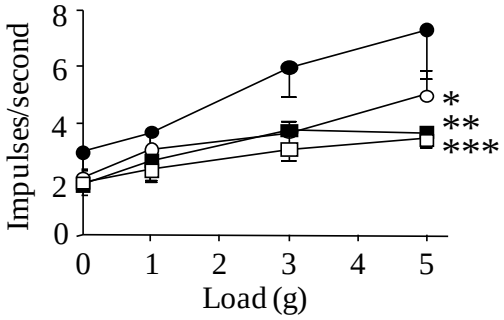
Stimulus-response functions of gastric mucosal receptors to mucosal stroking before (●) and after exposure to NPW (1 nM (○), 3 nM (■) or 10 nM (□)) in standard laboratory diet (SLD) mice (ai: $n = 5$, $N = 5$), SLD fasted mice (bi: $n = 5$, $N = 5$), and HFD mice (ci: $n = 5$, $N = 5$). Stimulus-response of oesophageal tension receptors to circular tension before (●) and after exposure to NPW (1 nM (○), 3 nM (■) or 10 nM (□)) in SLD mice (aii: $n = 5$, $N = 4$), SLD fasted mice (bii: $n = 5$, $N = 4$), and HFD mice (cii: $n = 6$, $N = 5$). *$P < 0.05$, **$P < 0.01$ compared with control.

(a)

(i) Mucosal receptor



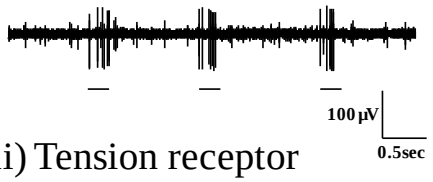

(ii) Tension receptor




(b) Typical original recording

(i) Mucosal receptor

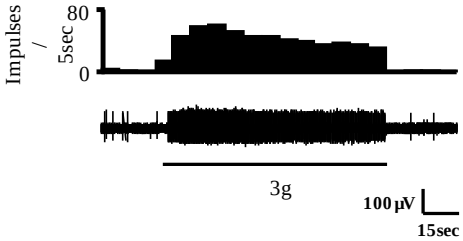
Control



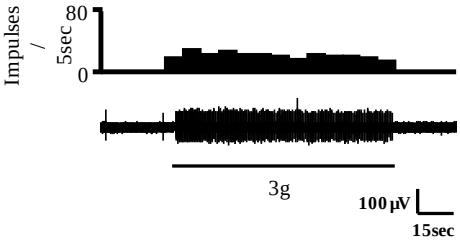

NPW (10nM)


(ii) Tension receptor

Control

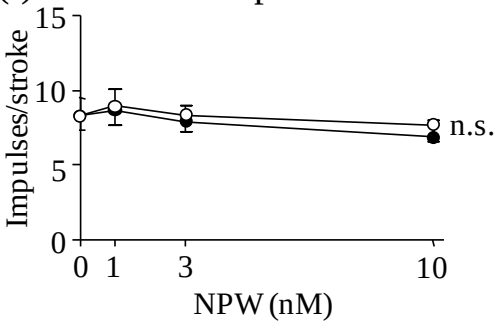



NPW (10nM)

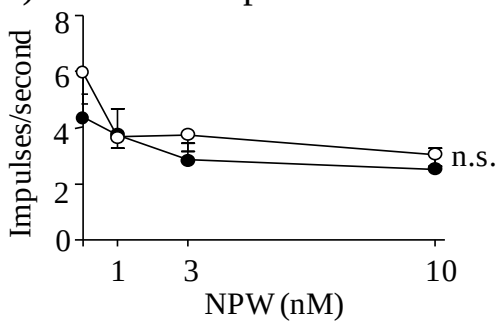



(c)

(i) Mucosal receptor



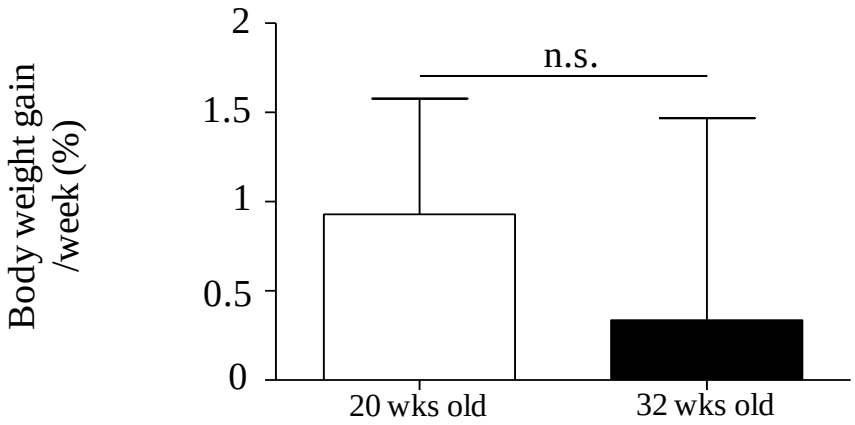

(ii) Tension receptor



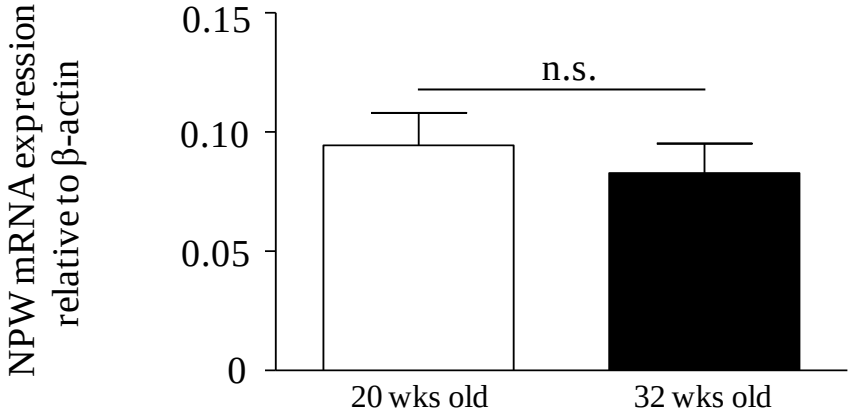
**Supplementary Figure S2 The effect of neuropeptide W on the response of gastric mechanosensitive vagal afferents in 32-week-old female mice**

(a) Stimulus-response functions of gastric mucosal receptors to mucosal stroking (ai: $n$ = 5, $N$ = 4), and gastric tension receptors to circular tension (aii: $n$ = 6, $N$ = 5) before (●) and after exposure to NPW (1 nM (○), 3 nM (■) or 10 nM (□)) in 32-week-old female mice. *$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.001 compared with control. (b) Typical recording of gastric mucosal receptors with a 200 mg von Frey hair (bi), and gastric tension receptors with a circumferential stretch of 3 g (bii), before and after 10 nM NPW application in 32-week-old female mice. (c) The effect of NPW on the response of mucosal receptors to 200 mg von Frey hair (ci) and tension receptors to 3 g circular tension (cii) in 20 (●) and 32 (○)-week-old mice.

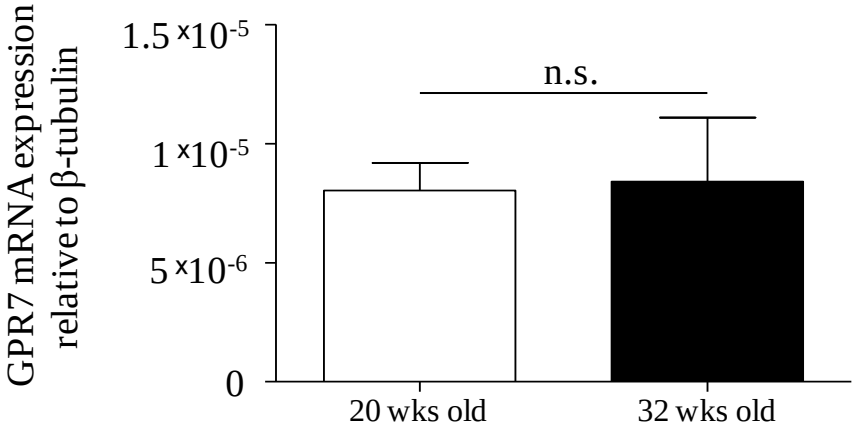
(a)




(b) Gastric mucosa

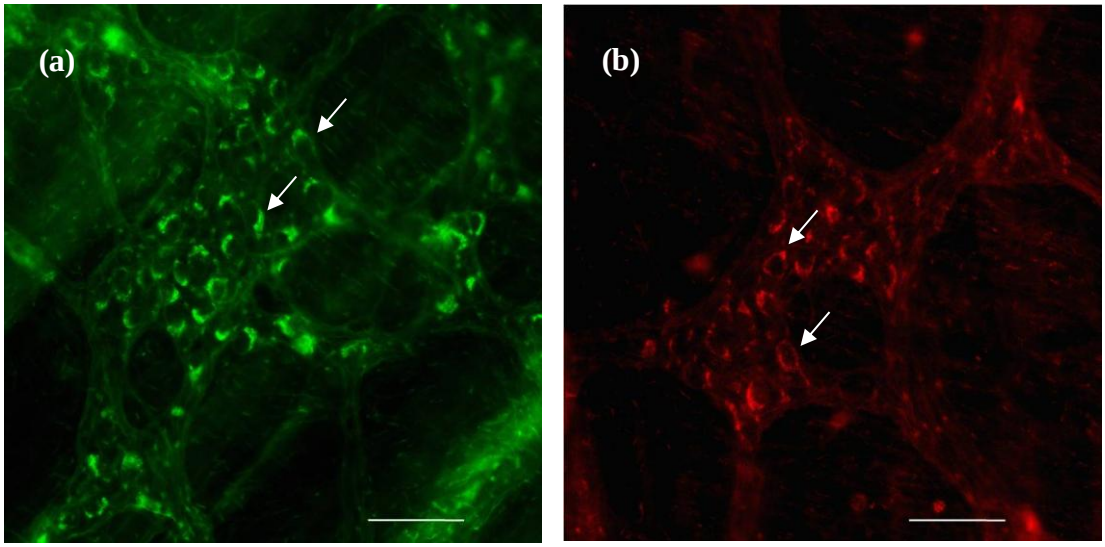



(c) Whole nodose ganglia



**Supplementary Figure S3 The weekly body weight gain, expression of NPW and GPR7 mRNA in the gastric mucosa and nodose ganglia respectively in 20 and 32-week-old female mice**

(a) The weekly body weight gain of mice in 20 and 32-week-old female mice ($N$ = 20). (b) Expression of NPW mRNA relative to β-actin in gastric mucosa of 20 ($N$ = 5) and 32 ($N$ = 5) week old mice. (c) Expression of GPR7 mRNA relative to β-tubulin in whole nodose ganglia in 20 ($N$ = 5) and 32 ($N$ = 5) week old mice.




**Supplementary Figure S4 GPR7 immunohistochemistry in whole-mount mouse stomach myenteric plexus**

(a) GPR7-labelled cell bodies using secondary antibody conjugated to Alexa Fluor 488.
(b) GPR7-labelled cell bodies using secondary antibody conjugated to Alexa Fluor 568.

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