Early Life Psychological Stress Leads to Aberrant Ghrelin and Satiety Response to Stress in Adulthood

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ABSTRACT

BACKGROUND & AIMS: Psychological stress in early childhood has been implicated in the pathophysiology of functional dyspepsia but the mechanism is unclear. This study investigates the effect of early psychological stress on the regulation of satiety function in adulthood using an animal model of neonatal maternal separation stress (NMSS).

METHODS: Sprague-Dawley (SD) rats underwent 3-hour daily maternal separation (MS) from postnatal day 2 to 22 and were weaned. The rats with no MS served as non-handling controls. Three experiments were conducted on these rats on day 60: (1) Water avoidance stress (WAS); (2): Feeding after overnight fasting and (3) Feeding after overnight fasting and WAS. Serial blood samples were collected for acylated ghrelin (AG) assay. In experiments (1) and (2), tissues from the stomach and hypothalamus were harvested additionally for evaluation of ghrelin expression. In experiments (2) and (3), calorie intake was also monitored at regular time intervals.

RESULTS: Experiment (1): MS rats had significantly higher mRNA ghrelin in hypothalamus (1.012 ± 0.098 vs 0.618 ± 0.071, $P = 0.009$) and plasma AG level

(141.6 ± 28.92 pg/mL vs 97.69 ± 38.21 pg/mL, P = 0.014) in baseline non-stressed conditions. After WAS, MS rats had further increase in plasma AG level and gastric ghrelin expression. Experiment (2): After overnight fasting, the initial calorie intake was significantly higher in MS rats (at 3 mins: 1.303 ± 0.293 kcal vs 0.319 ± 0.159 kcal, P= .011; at 8 mins: 2.578 ± 0.207 kcal vs 1.299 ± 0.416 kcal, p = 0.019) but it dropped abruptly afterward and no difference in overall calorie intake over 28 minutes was found. The postprandial plasma AG level and gastric mRNA ghrelin were significantly lower in MS rats (95.92 ± 12.71 pg/mL vs 154.01 ± 14.53 pg/mL, p = 0.010). Experiment (3): After both fasting and WAS, the MS rats had significantly higher calorie intake in the first hour (17.24 ± 1.10 kcal vs 11.95 ± 1.20 kcal, P= 0.006) but it dropped substantially afterward with significantly lower cumulative calorie intake at 3 hours (at 3 hr: 19.44 ± 1.50 kcal vs 26.49 ± 2.25 kcal, P = 0.023). The calorie intake in MS rats remained significantly lower than that of controls up to 48 hours (168.1 ± 4.76 kcal vs 220.8 ± 8.27 kcal, P< 0.001).

CONCLUSIONS: Psychological stress in early life leads to aberrant ghrelin profile and dysregulation of feeding behavior in response to acute psychological or physiological stress in adulthood.

撮要

背景及目的:

對於在生命早期所承受的心理壓力，導致成年後傾向出現功能性消化不良症候群的機理目前尚未清楚。本研究探討以動物模型測試新生期母嬰分離應激（NMSS）對成年後的飽腹感的影響。

方法:

將 SD 大鼠分成兩組：正常對照組(non-handling, NH)和母嬰分離組(maternal separation, MS)。母嬰分離組從出生的第 1-22 天每天進行三小時的母嬰分離，並於第 22 日斷乳，分籠常規飼養。在出生第 60 日，對兩組 SD 大鼠分別進行三組實驗：(1)避水應激(Water avoidance stress, WAS)；(2)隔夜禁食後餵食；(3)隔夜禁食並避水應激實驗後餵食。採集不同觀測時間點血樣做酰基化 Ghrelin (acylated ghrelin, AG)檢測。在實驗(1)和(2)，對胃和下丘腦組織進行 ghrelin 表達方面的研究；在實驗(2)和(3)，對其熱量攝取量作定期監測。

結果:

實驗(1)：MS 大鼠下丘腦組織的基礎 mRNA ghrelin 含量(1.012 ± 0.098 vs 0.618 ± 0.071, P = 0.009)和血漿 AG 含量(141.6 ± 28.92 pg/mL vs 97.69 ±

38.21 pg/mL, p = 0.014)顯著高於正常對照組。在避水應激實驗後，MS 大鼠的血漿 AG 含量和胃部 ghrelin 表達相對於正常對照組有進一步的上升。實驗(2)：隔夜禁食後，MS 大鼠於最初的熱量攝取量顯著高於正常對照組(第 3 分鐘: 1.303 ± 0.293 kcal vs 0.319 ± 0.159 kcal, P= 0.011; 第 8 分鐘: 2.578 ± 0.207 kcal vs 1.299 ± 0.416 kcal, p= 0.019)；但在 28 分鐘進食測試內其突然下降並與正常對照組的總熱量攝取量無明顯差別。MS 大鼠的餐後血漿 AG 含量和胃部 mRNA ghrelin 含量均有顯著下降(95.92 ± 12.71 pg/mL vs 154.01 ± 14.53 pg/mL, p= 0.010)。實驗(3)：在隔夜禁食並避水應激實驗後，MS 大鼠在首一小時餵食出現貪食癥，熱量攝取量顯著高於正常對照組(17.24 ± 1.10 kcal vs 11.95 ± 1.20 kcal, P = 0.006)並引起早飽現象(第 3 小時:19.44 ± 1.50 kcal vs 26.49 ± 2.25 kcal, P = 0.023)，其總熱量攝取量持續減少 (168.1±4.76 kcal vs 220.8 ± 8.27 kcal, P <0.001) 長達 48 小時。

結論:

生命早期所承受的心理壓力可導致其成年後於突發性心理或生理壓力下 ghrelin 波動及異常進食現象的產生。

LIST OF ABBREVIATIONS

ACTH	Adrenocorticophic Hormones
AG	Acylated Ghrelin
AN	Anorexia Nervosa
BN	Bulimia Nervosa
CORT	Corticosterone
CRF	Corticotropin Releasing Factor
DG	Des-acylated Ghrelin
ELISA	Enzyme Linked Immunosorbent Assay
FD	Functional Dyspepsia
GHS	Growth Hormone Secretagogue
GHS-R	Growth Hormone secretagogue Receptor
GI	Gastrointestinal
HPA	Hypothalamic Pituitary Adrenal
IBS	Irritable Bowel Syndrome
MS	Maternal Separated
NH	Non Handled
NMS	Neonatal Maternal Separation
TRP	Tryptophan

SD	Sprague-Dawley
SHRP	Stress-Hypo Responsive Period
WAS	Water Avoidance Stress
WKY	Wistar Kyoto
5HIAA	5-hydroxyindoleacetic Acid
5-HT	Serotonin

PUBLICATIONS BASED ON THE WORK IN THIS THESIS

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1 LITERATURE REVIEW

1.1 Functional Dyspepsia

1.1.1 Epidemiology and clinical features

Functional dyspepsia (FD) is a common digestive disorder which is characterized by chronic or recurrent upper abdominal symptoms without identifiable cause by conventional diagnostic means. Owing to the lack of organic abnormality and reliable biological markers, FD is a symptom-based clinical diagnosis based on the combination of various upper gastrointestinal symptoms. In Chinese population, the prevalence of functional dyspepsia has been reported to be over 10% (Lu et al. 2005). The most updated Rome III symptom criteria defines FD as persistent or recurrent pain or discomfort centered in the upper abdomen which is not relieved by defecation, not associated with a change in stool pattern and not explained by an organic disease. The pain has recurrence of once a week for at least a period of 2 months after diagnosis (Rasquin et al. 2006). The pain or discomfort can be associated with vomiting, nausea, abdominal fullness, bloating or early satiety (Thomson et al. 2003).

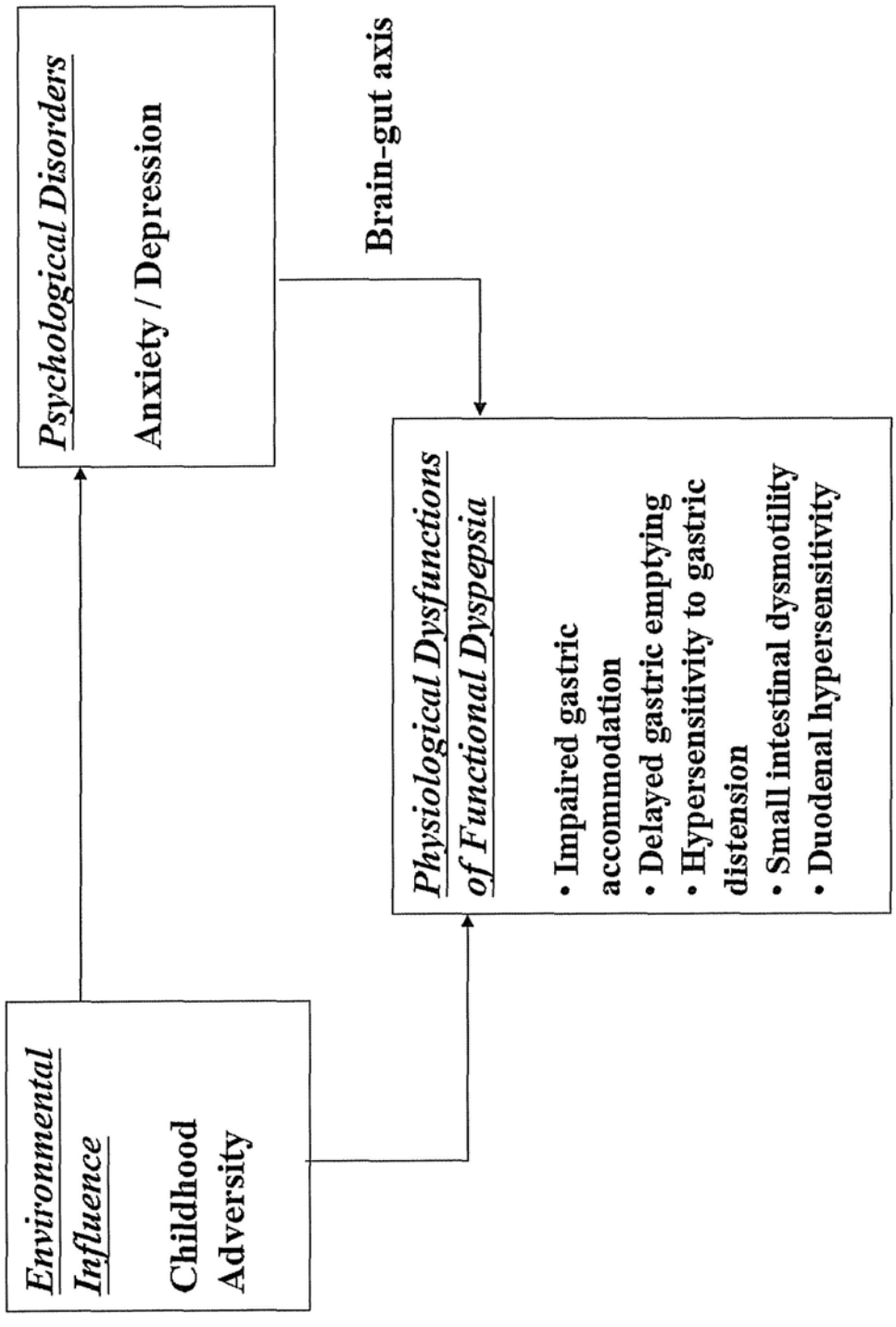
1.1.2 Pathophysiology

The pathophysiology of functional dyspepsia is poorly understood and there are a lot of speculative mechanisms. Abnormalities in gastric motor and sensory functions are common in FD patients. These abnormalities include delayed gastric emptying, impaired gastric accommodation and visceral hypersensitivity (Hoffman, Vos, & Tack 2007). FD patients have excessive sensitivity in physiological or minor noxious stimuli in both fasting and postprandial states (Thumshirn et al. 1999b) and hypersensitive to balloon distension of proximal stomach.

A biopsychosocial model proposed by Levy et al. postulates that psychological distress, childhood adversity, environmental stress and genetics can contribute to the development of functional gastrointestinal disorders (Levy et al. 2006). In recent years, it has been increasingly recognized that FD is associated with psychological disorders. FD patients were also found to be associated with anxiety and depression (Kleibeuker & Thijs 2004). However, a causative role is yet to be established even various psychological disorders are commonly associated with FD (Ammoury, Pfefferkorn, & Croffie 2009). It has

been reported that somatization and depression are the two most important determinants of severity of dyspeptic symptoms (Van Oudenhove et al. Gut 2008) Moreover, childhood adversity was also found to be associated with FD. Psychological distress was claimed to be one of the important predictor of outcome in all patients (Biggs et al. 2004;Koloski, Talley, & Boyce 2005;Olden 1998). Figure 1 illustrated the putative mechanisms proposed that contributed to the common characteristics of FD (Levy et al. 2006; Baker, Fraser, & Young 2006;Mimidis & Tack 2008).

Figure 1. Pathophysiology model of Functional Dyspepsia



1.1.3 Putative neurochemical mechanisms

1.1.3.1 Hypothalamic-pituitary adrenal axis

Hypothalamic-pituitary adrenal (HPA) axis is the major neuroendocrine system that is activated in response to actual or perceived environmental challenges. It involves a complicated network of feedback mechanisms involving the hypothalamus, pituitary gland and the adrenal glands. The HPA system regulates stress, emotions, sexuality, energy usage and immune system. Dysregulation of HPA axis was found in patients with irritable bowl syndrome (IBS) in response to acute physical stress (lumbar puncture) displaying blunted responses of plasma ACTH and cortisol expression (FitzGerald, Kehoe, & Sinha 2009). Corticotrophin releasing hormone (CRH), a mediator of HPA axis activation, has also been shown to decrease gastric motility and increase colonic motility in rats (Tache et al. 2001).

1.1.3.2 Serotonergic and adrenergic system

Serotonin is a crucial signaling molecule in the gastrointestinal tract. It is a paracrine messenger utilized by the enterochromaffin cells, which functions as sensory transducers (Gershon & Tack 2007). Serotonin is a common neurotransmitter in both central and enteric nervous systems. The serotonergic neurons are situated in the nuclei of the median raphe in brain mainly for modulation of pain from the viscera. In addition, the system also modulates other physiological activities such as mood, sleep, sex, appetite (Barnes & Sharp 1999;Gershon & Tack 2007;Gingrich & Hen 2001).

About 95% of the body's serotonin storage is located in the digestive tract. It is synthesized by the enterochromaffin (EC) of the enteroendocrine cell and serotonergic neurons of the myenteric plexus (Gershon 2000;Gershon 2004;Gershon, Drakontides, & Ross 1965;Wade et al. 1994). Serotonin is a base that is positively charged which is difficult to penetrate into the lipid bilayers of plasma membranes and therefore the inactivation of serotonin is mediated by the transporter-mediated uptake by serotonin reuptake transporter (SERT; 5-HTT) (Gershon & Tack 2007).

Dysfunction of serotonergic system has been reported in many gastrointestinal disorders. In animal studies, mice that lack SERT showed increased colonic motility and increase water content in stools (Chen et al. 2001). Reduction of SERT transcription was induced by experimental inflammation of bowel (Gershon & Tack 2007;Linden et al. 2003). SERT knockout mice were therefore considered to be a potential animal model of IBS. In human, reduction of SERT was also found in patients with IBS (Coates et al. 2004;Gershon & Tack 2007). Decrease in plasma serotonin was reported in patients with post-infectious IBS and concomitant FD (Dizdar et al. 2010). Decreased plasma and postprandial plasma serotonin was also found in FD patients (Cheung. 2011).

Noradrenergic system is located in CNS and the gastrointestinal tract. The two main types of adrenoreceptors (α and ß) are located in both pre-synaptic and post-synaptic membrane (O'Mahony et al. 2006). Noradrenergic neurons are found in pons and medulla of the brainstem. Descending fibres extend to other parts of the brainstem, spinal cord and cerebellum (O'Mahony, Dinan, Keeling, & Chua 2006).

Pharmacological modulators of adrenergic and serotonergic systems can affect gut motor function. α_2 receptor-modulating drugs profoundly affect gastric accommodation (Thumshirn et al. 1999a) and colonic tone and phasic contractility (Bharucha et al. 1997). Intracisternal administration of noradrenaline significantly reduce gastric motility in rats (Nagata & Osumi 1993). This suggests the central noradrenergic receptors are involved in the modulation of gastrointestinal motility and the dysfunction of the system may lead to gastrointestinal motility disorders. Table 1 summarizes various motor, sensory, neurochemical and psychological features of functional dyspepsia.

Table 1. Table of various motor, sensory, neurochemical and psychological features of Functional Dyspepsia

Motor	• Impaired proximal gastric accommodation • Delayed gastric emptying • Small intestinal dysmotility
Sensory	• Visceral hypersensitivity to gastric distension • Increase duodenal sensitivity to lipids or acid
Neurochemical	• HPA dysfunction with increased CRH production • Serotonergic and adrenergic system dysfunction altering gastric accommodation, colonic tone, phasic contractility, gastric motility • Urinary excretion of catecholamines in children with FD
Psychological	• Psychological disorders such as anxiety and depression • Higher prevalence in history of childhood adversity • Abnormalities with autonomic nervous system-central nervous system dysregulation

(Baker, Fraser, & Young 2006;Mimidis & Tack 2008)

1.1.4 Relationship between early life stress and functional gastrointestinal disorders

Early psychological stress events and childhood adversity such as sexual abuse have been proposed risk factors for many functional gastrointestinal disorders. These early adversities may cause psychological and physical abnormalities of the bodily functions in adulthood (Rayworth, Wise, & Harlow 2004). High prevalence of sexual, physical and emotional abuse (30%-56%) have been reported in patients with functional gastrointestinal disorders (Hill 1970;Lowman et al. 1987)

Early adverse events may precipitate heightened responses to stress in adulthood with eating disorders such as binge eating (Langenbach & Huber 2003), bulimia nervosa (Langenbach & Huber 2003;Wonderlich et al. 1997) and anorexia nervosa (Dellava et al. 2010;Favaro, Tenconi, & Santonastaso 2010;Kaye 2008;Langenbach & Huber 2003). These eating disorders are strongly associated with hyper-responsiveness of serotoninergic (Kaye 2008) and the hypothalamic-pituitary-adrenal (HPA) systems. Early life stress also causes dysregulation of HPA axis (Mello et al. 2003). It may induce a long

lasting effect on HPA axis dysfunction resulting in chronic CRH surge to stress responses. Two studies conducted by Heim et al. showed that women with abuse histories showed abnormal ACTH secretion to CRF administration, which implied altered neuroendocrine stress responses. Additionally, it has been reported that women with childhood abuse had a six-fold increase of ACTH response to a stress (Heim et al. 2001;Heim & Nemeroff 2001).

In animal studies, Coutinho et al. developed the animal model of neonatal maternal separation stress, which led to visceral hypersensitivity and anxiety-like behaviours in adulthood and 5-HT hyperactivity in central and enteric nervous system (Coutinho et al. 2006). It has also been validated as an animal model of IBS (Ren et al. 2007;Wu et al. 2010).

1.2 Animal model of functional gastrointestinal disorder

1.2.1 Existing central nervous system directed animal models VS gut directed animal models in functional gastrointestinal disorders

The development of a valid animal model for FD is difficult owing to the poor understanding of the pathophysiology. Moreover, FD is a heterogeneous condition which is characterized by several different physiological and neurochemical disturbances. As a result, there is no single universally accepted and validated animal model for FD. Furthermore, it is technically difficult to conduct quantitative measurement of visceral pain and other sensations in animals. Hence, other physiological features such as visceromotor reflex are measured as surrogate markers of visceral pain or sensory dysfunction.

In general, animal models for functional gastrointestinal disorders can be divided into CNS-directed (alternation in functions of central nervous system) and gut-directed (alternation in functions of digestive system), respectively (Mayer, Naliboff, & Chang 2001).

CNS-directed animal models are characterized by features that have high

construct and content validity for functional gastrointestinal disorders. Visceral hypersensitivity is primarily caused by functional or structural alterations in central nervous system, which include 1) sensitization of primary sensory afferents innervating the viscera, 2) hyperexcitability of spinal ascending neurons (central sensitization) receiving synaptic input from the viscera, and 3) dysregulation of descending pathways that modulate the spinal nociceptive transmission" (Sengupta 2009). Amongst various CNS-directed models, neonatal maternal separation (NMS) is one of the most extensively investigated models. Coutinho developed a rodent NMS model with visceral hyperalgesia to colonic distension (Coutinho et al. 2002). Other studies have also shown that NMS predisposes to the development of chronic pain in later life due to hyper-responsiveness of the HPA axis. (Sengupta 2009).

Another animal model that has been commonly used to evaluate the effect of acute psychological stress on gastrointestinal function is water avoidance stress test (WAS). It has been shown that WAS leads to delayed gastric emptying and small bowel transit. Furthermore, chronic stress induced by WAS increased visceral hyperalgesia, dysmotility and alteration in fecal pellet output, exaggeration of anxiety behavior, and even alteration in colonic immune activity

(Rojas-Macias et al. 2010). It has also been shown that WAS leads to up-regulation of transient receptor potential vanilloid-1(TRPV1) and ankyrin-1(TRPA1) expression in colonic afferent dorsal root ganglia (DRG). TRPV1 is involved in developing visceral hyperalgesia while TRPA1 is responsible for somatic hyperalgesia stimulated by inflammation and tissue damage (Kerstein et al. 2009;Obata et al. 2005;Yu et al. 2010).

There are limitations for CNS-directed models. First, the mechanisms of central processing of nociceptive signals from the gut are different between human and rodents (Mayer, Naliboff, & Craig 2006). Many drugs that were shown effective in suppressing visceral hypersensitivity in animal models had not been translated successfully in human studies. Second, some patients with functional gastrointestinal disorders do not have concomitant psychological comorbidities and many of them actually have predominant dysfunctions at the peripheral organs such as activated gut innate immunity in patients with post-infectious irritable bowel syndrome.

Gut-directed models involve the use of interventions at the gastrointestinal tract such as local treatment with pro-inflammatory agents or parasitic

infestations. It has been shown that administration of chemical irritants trigger chronic visceral hypersensitivity which mimics chronic functional gastrointestinal disorders such as IBS and FD. However, this model cannot fulfill the validity for the strong association between FGIDs and psychological disorders.

1.2.2 Animal model of early life stress

Maternal separation has been claimed to be the "potent naturalistic stressor" (Stanton, Gutierrez, & Levine 1988). The neonatal period from postnatal day 2 until day 14 is particularly vital for neurological development with large amount of documentation supporting that neonatal MS leads to dysregulation of the HPA axis resulting in long-term functional changes in neurohormonal system. It has been shown that NMS of newborn rats leads to permanent changes in central nervous system and the development of visceral hyperalgesia in adulthood (Carlini et al. 2007)(Coutinho et al. 2002). Recent studies also supported the validity of NMS as an animal model for eating disorders in adulthood. Furthermore, various studies also demonstrated the consequences of depression (El et al. 2006;Jahng 2010;Ladd et al. 2000) and anxiety-like symptoms after NMS (Daniels et al. 2004;Jahng 2010;Kalinichev et al. 2002).

The duration and housing conditions varied in most of the NMS studies. Studies show the postnatal development in rats' first 2 weeks of life is known as stress-hyporesponsive period (SHRP)(Cirulli et al. 1994;Kalinichev, Easterling,

Plotsky, & Holtzman 2002;Levine 2002;Schapiro, Geller, & Eiduson 1962;Schmidt et al. 2002;Schmidt et al. 2006;Vazquez 1998;vishai-Eliner et al. 1995;Walker et al. 1991). During this period, rats are unable to react to mild stressors (Lu et al. 2002;Sakly & Koch 1983;Sapolsky & Meaney 1986;Schapiro 1965;Schmidt et al. 2005;Walker et al. 1986)(Schmidt et al. 2006). There will be substantial activation of HPA axis during maternal separation when rat pups experience mild stressors of food starvation after separation from mother. Early life stress by NMS results in unrestrained production of corticotrophin-releasing factor and increased expression of its receptors (Owens & Nemeroff 1993).

Early life stress also leads to the dysregulation of serotonergic systems (Gardner et al. 2009). It has been shown that NMS leads to altered serotonergic function (Meaney & Szyf 2005;Smythe, Rowe, & Meaney 1994). Smythe et al. reported that neonatal separation permanently alters HPA responses to stress with increased 5-HT activities through the 5-HT_2 receptor pathway. NMS is also associated with increased 5-HT turnover in brain. Other changes in 5HT activities include decreased 5-HT concentration in hippocampal or frontal cortex 5-HT turnover (Smythe, Rowe, & Meaney 1994), increased 5-HT transporter expression in dorsal raphe nucleus of the brain in adulthood.

These alterations in 5HT activities may lead to dysfunctions in mood regulations. The alteration in brain serotonergic transporter expression after NMS was found to be similar to patients with depression. Altered serotonergic 1A levels have also been associated with abnormal behavioral responses to stress (Vicentic et al. 2006).

In recent years, it has been increasingly recognized that NMS may contribute to aberrant feeding behavior and satiety regulation. In addition to exaggerated anxiety and depression, it has been reported that MS rats develop hyperphagia and weight gain following social or physiologic stress challenges later in life (Ryu et al. 2008). Furthermore, MS rats lost weight more rapidly during food deprivation but regained weight more profoundly during refeeding. These observations suggested that early life stress may lead to dysregulation of behaviour and may have pathophysiological significance in the development of eating disorder (Ryu et al. 2008).

1.3 Ghrelin and appetite regulation

1.3.1 Discovery and properties

Ghrelin was first discovered in 1999 as a 28 amino-acid acylated peptide endogenous ligand of growth hormone secretagogue receptor (Kojima et al. 1999). It is produced by A-like cells and localized mainly in the oxyntic mucosa of the stomach. Active form of ghrelin mainly involves in the control of growth hormone (GH) secretion, regulation of body weight, adiposity, gastric acid secretion, gut motility and mucosal defense.

Ghrelin is produced mainly from the X/A-like cells within the oxyntic glands of the gastric fundus mucosa of the stomach. Approximately 65% of plasma ghrelin levels originated from the stomach. Besides, small intestine, brain, hypothalamus, pituitary, pancreas, kidney, lymphocytes, lung, placenta, testis and ovary also expressed ghrelin (Gualillo et al. 2003;Nogueiras, Williams, & Dieguez 2010;van der Lely et al. 2004). The widespread distribution in various tissues suggested its importance in multiple pathways involving reproduction, tumour development, glucose metabolism and immune response (Nogueiras, Williams, & Dieguez 2010).

Various derivatives of ghrelin also take crucial roles in the body. The ghrelin precursor, preproghrelin has 117 amino acids and cleaved to give rise to a 28 amino acid ghrelin peptide. With alternative splicing, it gives rise to two forms of ghrelin in des-acylated from (DG) and acylated form. Desacyl ghrelin is most abundant in body that consists 80-90% of the total circulating ghrelin. Desacyl ghrelin is proposed to be involved in cardioprotective, antiproliferative and adiopogenic activities and antagonize octanoyl-ghrelin-induced effects on insulin secretion and blood glucose levels in human (Hosoda et al. 2000;Nogueiras, Williams, & Dieguez 2010). Another 23 amino acid ghrelin-associated peptide was known as obestatin. It had been found that with peripheral and central administration, obestatin could act as antagonist against the effect of ghrelin on food intake, body weight and also gastric emptying, but not on growth hormone levels (Chartrel et al. 2007;Nogueiras, Williams, & Dieguez 2010). Further treatment with obestatin had been reported to obtain a sustained suppression of gastric emptying activity.

1.3.2 Factors that affect ghrelin secretion / metabolism

Expression of ghrelin is regulated by multiple pathways. Ghrelin expression and circulating ghrelin levels decrease with age and highly related to age-related declines in appetite, immunity and cognitive function (Jeffery et al. 2011). Lower mean 24-h acylated ghrelin levels were found in adults age of 60 years and older compared with a group of young adults at the mean age of 30 years with similar BMI (Nass, Gaylinn, & Thorner 2011). Ghrelin secretion is highly affected by food intake (Bagnasco, Kalra, & Kalra 2002;Tolle et al. 2002). Ghrelin levels also vary with hormonal cycles in female, gender, body mass, and obesity. It has also been found to be abnormal in eating disorders such as anorexia nervosa and bulimia nervosa (BN) (Ghigo et al. 2005).

1.3.3 Ghrelin receptors

The GHS-R consists of seven transmembrane domains. There are two ghrelin receptor cDNAs isolated and they are known as GHS-R type 1a and GHS-R type 1b, respectively (Howard et al. 1996;Kojima & Kangawa 2008;McKee et al. 1997;Smith et al. 1999). The alternative form of GHS-R 1b is produced by alternative splicing mechanism (Howard et al 1996).There is a COOH-terminal truncated form of 1a receptor and it is pharmacologically

inactive (Kojima & Kangawa 2008).

GHS-R mRNA is found in many parts of the brain. It is predominantly expressed in the arcuate (ARC) and ventromedial nuclei (VMN) and in the hippocampus (Guan et al. 1997;Nakazato et al. 2001)(Howard et al 1996). It can also be found in hypothalamus and pituitary, dorsal and median raphe nuclei.

Besides the brain, GHS-R is also present in many peripheral organs such as heart, lung, liver, kidney, pancreas, stomach, small and large intestines, adipose tissue and immune cells suggesting its importance and diversity of biological functions in the body" (Gnanapavan et al. 2002;Hattori et al. 2001;Kojima, Hosoda, & Kangawa 2001;Kojima & Kangawa 2008)(Guan et al. 1997, Lu et al. 2002).

1.3.4 Physiological effects of ghrelin in gastric functions

Ghrelin plays an important role in the gastrointestinal (GI) tract as a regulator of orexic behaviours such as appetite stimulation and regulation of body weight. There is marked diurnal variation in the concentration of circulating ghrelin levels, with surge of plasma levels during fasting and trough after food intake. Ghrelin expression in the stomach rises with fasting and decreases after meal within 1 hour (Cummings et al. 2001;Tschop et al. 2001a). Its synthesis can also be pharmacologically suppressed by the administration of leptin and interleukin (IL)-1ß (Madison et al. 2008;Teff et al. 2004). Ghrelin is responsible for the mediation of hunger sensation and its levels are correlated with the degree of hunger (Kojima & Kangawa 2008). Postprandially, the decrease in plasma ghrelin level is also proportional to the ingested calorie intake (Callahan et al. 2004). Studies showed that ghrelin secretion is up-regulated in patients with anorexia and cachexia (Nedvidkova et al. 2003;Otto et al. 2001;Shiiya et al. 2002) while down-regulated in patients with hyperphagia and obesity (Tschop et al. 2001b).

The role of ghrelin in food intake regulation was further supported by the

abundance of ghrelin containing neurons in the arcuate (ARC) nucleus and different regions of the hypothalamus (Kojima et al. 1999; Lu et al.2002). These neurons send excitatory signals to neuropeptide Y and agouti-related protein (AgRP) expressing neurons, which trigger the release of orexigenic peptides. At the same time, it also triggers the POMC neurons to cease the release of anorexigenic peptide (Kojima & Kangawa 2008). From the PVN, ghrelin neurons suppress the release of γ-aminobutyric acid (GABA), therefore leads to the stimulation of corticotropin-releasing hormone (CRH)-expression neurons to release ACTH and cortisol (Kojima & Kangawa 2008). The presence of GHS-R in nodose ganglion also suggests that the signals are transmitted from the stomach to the brain via vagus nerve (Date et al. 2002;Sakata et al. 2003;Zhang et al. 2004).

Systemic administration of ghrelin has been shown to stimulate food intake in both animal models and humans (Tschop, Smiley, & Heiman 2000;Wren et al. 2001)(Nakazato et al. 2001). Chronic intracerebroventricular injection of ghrelin increases overall food intake and also decreases metabolic rate, and thereby leads to increase in body weight in rats. The stimulant effect of ghrelin on appetite is probably mediated both centrally and peripherally because peripheral

administration of ghrelin also stimulates food intake although it cannot pass through the blood-brain barrier. It has also been reported in a small pilot human study that intravenous administration of ghrelin before meal can increase the daily food intake by approximately 30% (Akamizu et al. 2010).

Ghrelin is also a potent prokinetic agent, which accelerates the small bowel transit and gastric emptying (Depoortere et al. 2005;Edholm et al. 2004;Kitazawa et al. 2005;Trudel et al. 2002). Ghrelin restores fasting pattern of small bowel motor activity in fed rats (Fujino et al. 2003). The prokinetic effect of ghrelin is probably mediated by cholinergic activation because ghrelin receptors are abundant on the cholinergic neurons in the enteric nervous system (Edholm et al. 2004). Besides prokinetic effect, ghrelin is also a stimulant of gastric acid secretion (Peeters 2003;Yakabi, Kawashima, & Kato 2008) through activation of vagal pathways (Date et al. 2002)(Masuda et al. 2000).

1.3.5 Relationship between ghrelin and psychological stress

Stress can be defined as an acute threat to the homeostasis of an organism by real (physical, "interceptive"; e.g. hemorrhage, gut infection) or perceived (psychological, "interceptive") events to trigger physiological and behavioral responses that can defend the stability of the internal environment (Chrousos & Gold 1992;Selye 1998). Psychological stress is commonly believed as a predisposing factor of functional gastrointestinal (GI) disorders and its effect can be both short and long lasting (Monnikes et al. 2001). There are many different types of acute and chronic stressors used in animal models. These include immobilization, tail shock, cold stress, forced swimming, water avoidance etc.

In addition to its orexigenic effect, ghrelin has also been shown to mediate the anxiety (anxiogenic) behavior (Asakawa et al. 2001). Ghrelin mRNA expression significantly increased with tail pinch and starvation stress. The anxiogenic action of ghrelin is probably mediated by activation of HPA axis. It has been shown that administration of ghrelin stimulates CRH mRNA expression as well as ACTH and cortisol release (Nagaya et al. 2001;Wren et al. 2000). Kristenssson et al. reported that WAS can induce 5-fold increase in

plasma ACTH concentration in rat models (Kristenssson et al. 2006).

Ghrelin has also been shown to induce other stress-related metabolic changes. Lambert et al. reported that intravenous ghrelin induced a rise in plasma glucose concentration in lean individuals and cortisol levels in both lean and overweight subjects (Lambert et al. 2011).

1.3.6 Potential role of ghrelin in functional dyspepsia

The role of ghrelin in functional dyspepsia is unclear and the results are conflicting. It has been reported that circulating ghrelin levels were higher in FD patients (Lanzini et al. 2006). On the other hand, preprandial ghrelin levels have been observed to be lower in FD patients with delayed gastric emptying (Lee et al. 2009;Takamori et al. 2007). It has been reported that acylated ghrelin levels are significantly correlated with gastric emptying in FD patients with postprandial distress syndrome (Shindo et al. 2009).

A significant portion of FD patients was found with delayed gastric emptying with a sensation of postprandial fullness, nausea and vomiting (Perri et al. 1998;Sarnelli et al. 2003;Stanghellini et al. 1996). Plasma ghrelin levels have been reported to be the indicators of delayed gastric emptying in FD patients and plasma ghrelin levels have been reported to be correlated with symptom severity in FD patients (Jonsson & Theorell 1999;Shinomiya et al. 2005).

The ghrelin level has also been implicated to be associated with specific symptoms of FD. First, active ghrelin levels were found to be lower in patients with postprandial fullness and/or early satiation. Lower acylated ghrelin levels were also observed in patients with non-erosive reflux disease (NERD).

1.3.7 Laboratory methods of ghrelin assay

1.3.7.1 Blood extraction for Enzyme linked Immunosorbent Assay (ELISA)

Different blood sampling techniques have been described for plasma ghrelin extraction. Different techniques are associated with different levels of animal discomfort due to variation in handling, restraining, anaesthesia, invasiveness and the blood volume sampled (Van et al. 2001). Frequency and the volume of blood extraction must be taken into account for the weight of the animals. Circulating blood volume in rats is in the range of 50-70 ml/kg. Large volume (15-20% of circulating volume) of blood removal would lead to reduced cardiac output and blood pressure. On the contrary, frequently blood sampling in smaller volumes can lead to anaemia. Therefore, both the blood sampling frequency and volume should be minimized in order to avoid these adverse effects.

Orbital sinus blood sampling is common for volume extraction of greater than 0.5 ml in rats. However, this technique is subjected to criticism because of the animal ethical concern on the pain and tissue damage as well as the influence on the stress and locomotion of the animal (Van et al. 1992). It has

been reported this technique is acceptable only as a terminal procedure (Morton D.B et al. 1993) because of the short sampling time and less restriction on locomotion required especially during the dark period (van et al. 2000).

For extraction of non-terminal blood sampling in rats with volume of greater than 0.5 ml each, puncture at the tail vein under short-acting anaesthesia has been recommended (Morton D.B. et al. 1993). Although tail vein puncture is more time-consuming, this technique induces less pain and distress in the rats (Van et al. 2000).

1.3.7.1 Localization of ghrelin expression in stomach of rodents for Western blotting and Real time PCR

Previous studies on rats have shown that the ghrelin-producing cells are most abundant in the fundus of stomach (Sakata et al. 2002) but subsequent study revealed that the fundus of rat's stomach is located at the fore stomach, which consists primarily of squamous epithelium rather than gastric secretory mucosa. Topographic localization of the ghrelin expression in different regions of the stomach in rats were evaluated by Li et al., who reported highest concentration of ghrelin protein at the greater curvature and lesser curvature whereas no ghrelin protein was detected in fundus, pylorus and cardia (Li et al. 2011).

1.4 Issues remain unresolved

- Early life stress has been shown to be associated with functional dyspepsia in epidemiological studies. However, the pathophysiologic link between early life stress and functional dyspepsia is yet to be established.

- Early life stress has been shown to adversely affect the functions of various neuroendocrine systems such as HPA axis, adrenergic and serotonergic systems in adulthood. However, its effect on the ghrelin function remains unclear.

- Although some animal models have been developed for visceral hypersensitivity which is commonly seen in functional gastrointestinal disorders. There is still a lack of valid animal model for functional dyspepsia and other disorders of appetite regulation.

- Being a potent appetite and motility stimulant, ghrelin profile has been reported to be deranged in patients with gastric motility disorder. Yet, its role in the pathophysiology of early satiation, a common symptom of functional dyspepsia, is still unclear.

1.5 Hypotheses

1.5.1 Hypothesis for Experiment 1: Effect of neonatal maternal separation on ghrelin profile after acute psychological stress (Water avoidance stress)

Ghrelin was found to have association with stress and anxiety. Kristenssson et al. showed WAS could induce the increased in plasma ghrelin in low anxiety rat strain of SD by 85% and 40% in high anxiety rat strain of (Kristenssson et al 2006). The ghrelin expression differed between high and low anxiety strains of rodents. Human studies also revealed that ghrelin was involved in mediating plasma glucose concentration and cortisol (Lambert et al. 2011). However, the effect of NMS on the alternation of ghrelin profile after stress is still unknown. We are interested in investigating the effect of NMS that may lead to permanent change in multiple neurochemical pathways. This may result in alternation of ghrelin responses in adulthood during acute psychological stress. We hypothesize that NMS leads to the alternation in ghrelin profile in adulthood after acute psychological stress.

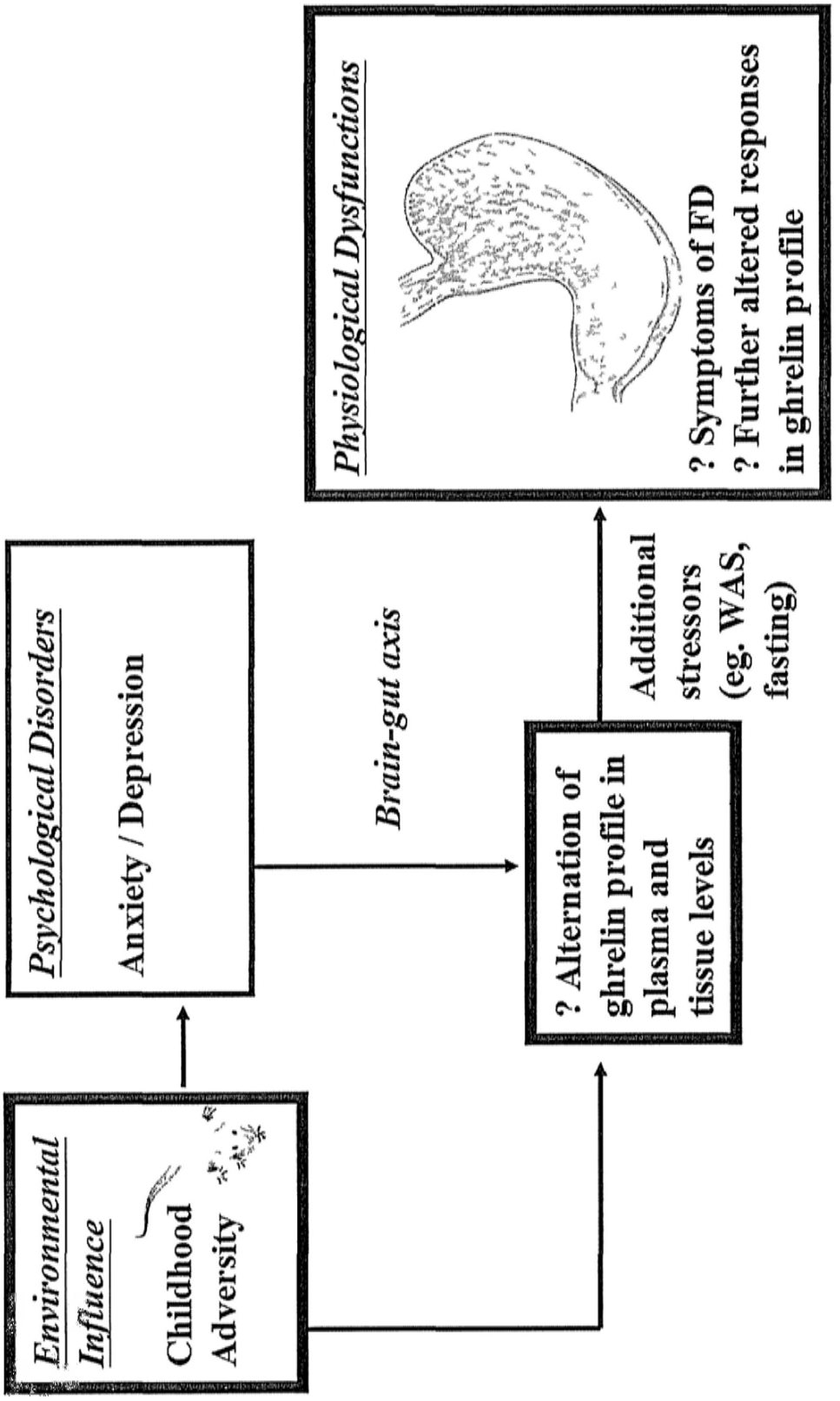
1.5.2 Hypothesis for experiment 2: Effect of neonatal maternal separation on ghrelin and satiety profile after fasting

Ghrelin has been known as an appetite stimulating hormone. On the contrary, a significant portion of functional dyspepsia patients was found with history of childhood adversity and with early satiation and postprandial fullness as predominant symptoms. We are interested to know if NMS as the induction of childhood adversity in rats leads to alternation of ghrelin profile after fasting that may lead to early satiation and postprandial fullness in adulthood which can be the common symptoms in FD patients. Therefore, we hypothesize that NMS leads to abnormal ghrelin and satiety profile after fasting stress in adulthood.

1.5.3 Hypothesis for experiment 3: Effect of neonatal maternal separation on ghrelin and satiety profile after a combination of water avoidance and fasting stress

Appetite can be altered by a combination of stressors in humans. We would also like to investigate on the effect on NMS on ghrelin and satiety profile after a combination of stressors such as water avoidance and fasting stress. Therefore, we hypothesize that NMS leads to alternation of ghrelin and satiety profile after a combination of water avoidance and fasting stress. Figure 2 demonstrated the summary of the overall proposed hypotheses in our rat model.

Figure 2. Schematic diagram of our hypotheses in the rat model



1.6 Study objectives

1.6.1 Objectives for Experiment 1: Effect of neonatal maternal separation on ghrelin profile after acute psychological stress (Water avoidance stress)

We aim to compare the plasma and ghrelin expression in stomach and brain tissues of the neonatal maternal separated rats after acute physiological stress in adulthood (water avoidance stress).

1.6.2 Objectives for experiment 2: Effect of neonatal maternal separation on ghrelin and satiety profile after fasting

We aim to compare the plasma and ghrelin expression in stomach and brain tissues of the neonatal maternal separated rats after prolonged fasting stress in adulthood.

1.6.3 Hypothesis for experiment 3: Effect of neonatal maternal separation on ghrelin and satiety profile after a combination of water avoidance and fasting stress

We aim to compare the plasma and ghrelin expression in stomach and brain tissues of the neonatal maternal separated rats after a combination of water avoidance and fasting stress in adulthood.

2. METHODOLOGIES

2.1 Materials & Chemicals

- 1.3ml micro tube (Sarstedt AG & Co., Nümbrecht, Germany)
- 0.2um PVDF membranes (Millipore, Billerica, USA)
- 2-mercaptoethanol (Merck, NJ, USA)
- Anti-rabbit IgG (Abcam Inc., MA, USA)
- Acetic Acid Glacial 100% (Scharlau, Barcelona, Spain)
- 40% Acrylamide/ Bis Solutions, 29:1 (Biorad laboratories Inc., CA, USA)
- Ammonium persulfate (APS, 0.1g/ml in double distilled water, stored at -20^0C; Merck, NJ, USA)
- Bovine Serum Albumin (SIGMA-ALDRICH, MO, USA)
- Bromophenol Blue (Amresco Inc., OH, USA)
- Chloroform (LabScan Asia Co. Ltd)
- Comassie blue R-250 Destaining solution (Biorad laboratories Inc., CA, USA)
- DNase I (Invitrogen, CA, USA)
- dNTP (10mM, Fermentas, Burlington, Canada)
- Ethanol absolute (Scharlau, Barcelona, Spain)
- Glycerol (Amresco Inc., OH, USA)

- Glycine (Amresco Inc., OH, USA)
- Hydrochloric Acid (5.0M, Merck, NJ, USA)
- Isopropanol (Merck, NJ, USA)
- Methanol (Scharlau, Barcelona, Spain)
- MMLV Reverse transcriptase (Promega corporation, WI, USA)
- MMLV 5X Buffer (Promega corporation, WI, USA)
- P-hydromercuribenzoic acid (SIGMA-ALDRICH, MO, USA)
- POWER SYBR© Green Master Mix (Applied Biosystems Inc., CA USA)
- protease inhibitor cocktails tablets (F. Hoffamnn-LA Roche Ltd., Basel, Switzerland)
- Ponceau S (Amresco Inc., OH, USA)
- Potassium Hydroxide (AnalaR BDH, PA, USA)
- Rabbit anti-rat ghrelin (Santa Cruz biotechnology Inc., CA, USA)
- Sodium Dodecyl sulfate (20%, USB Corporation, OH, USA)
- See Blue® Plus2 Pre-stained standard (Invitrogen, CA, USA)
- Slim milk (Nestle Carnation, Vevey, Switzerland)
- Sodium Chloride (Amresco Inc., OH, USA)
- Sodium Hydroxide (Merck, NJ, USA)
- T-PER tissue protein extraction reagent (Thermo Fisher scientific Inc.,

Massachusetts, USA)

- Tricine (USB Corporation, OH, USA)
- Tris-(hydroxymethyl)-aminomethane buffer (International Laboratory USA, CA, USA)
- TRIZOL® reagent (Invitrogen, CA, USA)
- Tween 20 (Amresco Inc., OH, USA)
- TEMED (SIGMA-ALDRICH, MO, USA)
- Western Protein set reagent (Biorad laboratories Inc., CA, USA)
- Ghrelin (rat acylated) EIA kit (Cayman Chemcial, MI, USA)
- Random primers (Invitrogen, CA, USA)
- RQ1 Dnase (Promega corporation, WI, USA)
- RQ1 Dnase 10X Buffer (Promega corporation, WI, USA)
- RQ1 Dnase Stop solution (Promega corporation, WI, USA)
- Rnase Inhibtior (Promega corporation, WI, USA)
- RQ1 RNase-free DNase (Promega corporation, WI, USA)
- Immobilion® western chemiluminescent HRP substrate (Millipore, MA, USA)
- Medical X-ray film – Super RX (Fujifilm, NJ, USA)

2.2 Laboratory Techniques

2.2.1 Real Time PCR for Ghrelin in tissues

2.2.1.1 Samples preparations and procedures

Preparation of samples for RNA extraction

Fresh glandular gastric body and hypothalamic tissues were harvested for the measurement of ghrelin gene expression. The tissues were frozen under -80 ^{o}C until use. Total RNA was extracted from stomach and brain tissues using TRIzol reagent. About 0.1 g of tissue was immersed in 1 ml of TRIzol using autoclaved clean glass vial. The homogenated samples were incubated for 5 minutes at room temperature. Add 0.2 ml of chloroform per 1 ml of TRIzol and shook vigorously for 15 seconds. The samples were centrifuged for 15 minutes at 4 ^{o}C for 12000g. Then the aqueous upper phrase was collected. Moreover, 0.5 ml of isopropanol was added into per 1 ml of Trizol at 4 ^{o}C for 1 hour for RNA precipitation in the aqueous phrase. Samples were further incubated 10-15 minutes at room temperature. The samples were centrifuged again for 10 minutes at 4 ^{0}C with 12000 g. Supernatant was removed and pellet was retained. 1 ml of 75 % of ethanol in DNAase RNAse free H_2O was used per 1 ml of TRIzol to wash once with the samples. After vortexing, samples were

centrifuged at 7500 g for 20 minutes at 4 ^{0}C. Samples were air dried for 5-10 minutes at room temperature, the tubes were inverted and solution was shaken off to ensure the RNA pellet was completely dry to increase solubility later. Finally, the pellet was re-dissolved into DNAse RNAse free H_2O for storage at -80 ^{0}C for long term storage.

Measurement of RNA concentration

RNA concentrations were measured spectrophotometrically at 260 nm with 1 μg of total RNA for each sample by Nanodrop ® ND1000 spectrophotometer machine using ND-1000 V.3.2.1 software. The concentration was measured by OD 260 DNA contamination and OD 280 for protein contamination. All OD 260/280 ratios of the samples are above 1.8. Concentration of RNA in the sample was formulated by the equation of OD_{260} x dilution factor ÷ 200.

Reverse transcription of mRNA expression

Residual DNA was first removed by DNase I (Invitrogen). Samples were first DNA digested by RQ1 RNase-free DNase (Promega Corporation, Madison, WI) with Dnase 10X buffer with 2 μg of mRNA in each sample. After incubation of 37 ^{0}C for 30 minutes, 1μl of Dnase stop solution was added to

constitute a total volume of 11 µl of samples. Further heating of samples at 65 ^{0}C for 10 minutes, random primers and ddH_2O were added. After heating for 5 minutes at 70 ^{0}C, the samples were cooled immediately in ice at 4 ^{0}C. Reverse transcription was performed by 1 µl of MMLV, 1.25 µl of dNTP, 0.63 µl of RNase inhibitor, 2.12 µl of ddH_2O and 5 µl of M-MLV 5X buffer per 2 µg of samples. PCR was performed by 37 °C for 2 hours, followed by 95 °C for 5 minutes and cooled at 4 °C.

Real time PCR for cDNA gene expression

Primers were designed by Primer3 (NIH., US) and primer express software (Applied Biosystems, Foster City, CA). Real-time PCR was performed using ABI 7900 Fast Real-Time PCR system for sequence detection (Applied Biosystems). 10 µl - reaction mixture was prepared with POWER SYBR© Green Master Mix (Applied Biosystems) as detection format containing total volume of 10 µl and 0.4 µl cDNA. Gene specific primers with melting curve analyzed for specificity of the amplified products are shown in table 2.

Table 2. Primers and Annealing temperatures used for Real Time PCR experiments

Primers	Primer Sequence 5' – 3'	Temperature Cycle Profile
Ghrelin (sense)	cca gca gag aaa gga aat cca	50 °C for 2 minutes, 95 °C for
Ghrelin	gct gct ggt act gag ctc ct	10 minutes, followed by 40
(anti-sense)		cycles of
ß-actin (sense)	tgt cac caa ctg gga cga ta	95 °C for 15 seconds, and 60
ß-actin (anti-sense)	ggg gtg ttg aag gtc tca aa	°C for 1 minute

2.2.2 Plasma Acylated Ghrelin ELISA detection

2.2.2.1 Buffers and solutions

P-hydroxymercuribenzoic acid in potassium phosphate (PHMB) buffer for sample preparation

10 µl of the PHMB solution was required for each 1 ml of blood collected. For preparation of PHMB, 100 mM of potassium phosphate buffer was prepared with 1.2% of 10N of NaOH added into the solution.

EIA buffer

EIA powder was used to reconstitute into 50 ml of ddH_2O and allowed to stand for 5 minutes until it was completely dissolved. Buffer was mixed thoroughly by gentle inversion.

Rat acylated ghrelin standard

The standard was prepared by reconstituting the vial of standard powder with ddH_2O. The sample was allowed to stand for 5 minutes for complete dissolution. The serial dilution started from (S1) 250 pg/ml then to (S2) 125 pg/ml, (S3), 62.5 pg/ml, (S4) 31.3 pg/ml, (S5) 15.6 pg/ml, (S6) 7.81 pg/ml, (S7) 3.91 pg/ml and (S8) 1.96 pg/ml. The standards were stored at -20 ^{o}C with a

stability of one week.

Anti-acylated Ghrelin-AchE tracer

The tracer was reconstituted with 10 ml of EIA buffer and allowed to stand for 5 minutes for complete dissolution. The tracer was stored at 4 °C for one week.

Wash Buffer

1 ml of concentrated wash buffer was diluted into 400 ml of ddH_2O. 200 µl of Tween 20 was mixed thoroughly into the solution by gentle inversion and kept at 4 °C for a stability of one week.

Ellman's Reagent

1 ml of concentrated wash buffer was diluted by 49 ml of ddH_2O. Then the Ellman's reagent powder was dissolved into the solution and kept in the dark at 4 °C and discarded on the same day.

2.2.2.2 Samples preparations and procedures

Samples preparation

Blood samples were collected by tubes of EDTA and 10 µl of PHMB was added for every 1 ml of blood collected to prevent degradation of acylated ghrelin by protease. Samples were centrifuged at 3500 rpm for 10 minutes at 4 °C. Supernatants were collected into clean tubes. 100 µl of 1N HCL per ml of collected plasma and further centrifugation was required at 3500 rpm for 5 minutes at 4 °C. Then supernatants were transferred to separate clean tubes and stored immediately at -20 °C for further assay later on.

Procedure for ELISA assay

Samples were first thawed and diluted into at least 1:5 with EIA buffer. 110 µl of the samples and a set of standards were dispensed into the respective wells. 100 µl of anti-acylated ghrelin AchE tracer was added into each well except the blank. The plate was incubated under 4 °C for 20 hours overnight. On the next day, the solution in the wells was discarded and washed with wash buffer thoroughly for 5 times. The plate was inverted gently to remove the liquid and dried by absorbent paper. After 5 times, the plate was shaken by orbital shaker for 5 minutes. Then the plate was further re-washed for 5 times with wash buffer.

After drying, 200 µl of Ellman's reagent was added into each 96 wells. The plate was incubated in darkness at room temperature with orbital shaker for 30 minutes. The plate was read at 414 nm by Biochrom Asys UVM340 microplate reader.

2.2.3 Western Blotting for Preproghrelin in tissues

2.2.3.1 Buffers and solutions

Procedures of the tricine-SDS-PAGE were modified according to Schagger H et al. 1987.

5 % Slim Milk

2.5 g of silm milk powder was dissolved into PBS-T to a volume of 50 ml. The emulsion was mildly vortexed and stored at 4 °C.

Gel Buffer

18.15 g of Tris (3.0 M) was added up to 50 ml of double distilled H_2O with final 0.3 % of SDS into the buffer. pH value was adjusted to 8.45 and kept under room temperature.

4% Stacking Gel Solution (Portion for 1 gel)

300 µl of 40 % acrylamide solution (29:1) was added with 900 µl of gel buffer in 1.8 ml of ddH_2O. After thorough mixing, 22.5 µl of APS and 4.5 µl of TEMED were added into the solution and ready for casting.

16% Resolving Gel Solution (Portion for 1 gel)

2.2 ml of 40 % acrylamide (29:1), 2.2 ml of gel buffer, 0.55 ml of 70% glycerol was added into 0.55 ml of ddH_2O. 27.5 µl of APS and 2.75 µl of TEMED were added into the solution with thorough mixing and ready for casting.

Sample Buffer

Sample buffers contained reducing properties with ingredients containing 1 g of SDS, 5 ml of glycerol, 2.56 ml of 2-mercaptoethanol, 2.13 ml of 0.5M Tris HCL (pH 6.8) with trace amount of Bromophenol Blue. The buffer was aliquoted into 0.5 ml and stored at -20 °C according to Shagger H. et al., 1987.

Stripping Buffer

Harsh stripping was added with 10 ml of 20% SDS, 12.5 ml of 0.5M Tris HCl (pH 6.8) and 77.5 ml of ddH_2O. 0.8 ml of ß-mercaptoethanol was added under the fumehood. The buffer then was kept at 4 °C.

Ponceau S Staining Solution

Ponceau S staining solution was first prepared with 10 ml of MiliQ water mixed with 0.3 ml of acetic acid and 0.033 g of Ponceau S. MiliQ water was added up to a final volume of 30ml. It was kept at room temperature.

Transfer buffer for semi-dry blotting

300 mM Tris was prepared with 100 mM of acetic acid into double distilled H_2O. pH value was adjusted to 8.6 and kept under 4 ^{o}C for storage.

Cathode buffer (10X)

Cathode buffer (10X) was prepared by 24.2 g of Tris (1.0 M) with 35.84 g of tricine (1.0 M) and add double distilled H_2O to a final volume of 200 ml with final 1 % of SDS. pH value was adjusted to 8.25 and kept in room temperature.

Anode buffer (10X)

48.4 g of Tris (2.1M) was dissolved into double distilled H_2O to make a final volume of 200 ml. pH value was adjusted to 8.9 and kept under room temperature.

2.2.3.2 Sample preparations and procedures

Sample preparation for measurement of protein concentration

Gastric body and hypothalamic tissues were extracted and stored immediately at -80 °C until use. Then the tissues were crushed during immersion in liquid nitrogen and later homogenized by a Polytron mixer by T-PER tissue protein extraction with protease inhibitor tablets. The homogenates were stored immediately by -80 °C for long term use.

Protein concentration determination

Dissolve 0.02 g of BSA into 10 ml T-PER into a concentration of 2 mg/ml. A standard curve was prepared (S1) 1.5 mg/ml, (S2) 1.25 mg/ml, (S3) 1.0 mg/ml, (S4) 0.75 mg/ml, (S5) 0.5 mg/ml, (S6) 0.25 mg/ml. A solution was made by reagent S and A by a ratio of 20 µl: 1000 µl. 5µl of standards or samples was added into each 96 wells plate. 25 µl of the solution (reagent A &S) was added into each well. Then 200 µl of reagent B was added into each well. The plate was mixed gently by 5 seconds. And the plate was read at absorbance of 750 nm. The total amount of sample homogenates loaded into each well was calculated by the concentration determined x volume loaded to get a total amount protein in each sample to be between 40-50 µg.

Casting Procedure

The casting was based on 16% acrylamide gel. 4 gels were set together under the same condition with dimensions of 7.5 x 10 x 0.1 cm. After the loading of separating gel by the use of pipette, 100 % absolute ethanol was used to avoid the contact with air and allowed to polymerize for 1.5 hour. A sharp liquid interface would be seen after complete polymerization. Ethanol was discarded with the use of absorbent paper to ensure ethanol was fully evaporated from the surface of separating gel. 4% stacking gel was freshly prepared and poured into the cast and allowed to polymerize for 1.5 hour. Then the gels were wrapped and allowed to store at 4 °C overnight for complete polymerization.

Sample loading and gel electrophoresis

Each sample buffer: sample was loaded with a ratio of 1:4. The mixture was heated immediately for 5 minutes at 100 °C as proteases and SDS in the sample buffer could cause serious degradation of the protein. Once heated, samples were kept under ice at 4 °C for immediately loading into gels or under -80 °C for long term storage. For detection of ghrelin and beta-actin, (40-50 μg/

lane) was loaded into 1.0 mm gels of 15 wells.

Cathode buffer (1X) was filled into the upper case. Gels were immersed well into the cathode buffer. The anode buffer (1X) was filled with the outer tank. For better stacking effect, at least 30 minutes of constant 90 V was applied. At least 1.5 hour was applied with constant 120 V for better separation effect. The Gel tank system was immersed into ice for prevention of overheating of the buffer solutions and acrylamide gels as well as better resolution effect.

0.2 μm PVDF membranes were immersed into 100 % methanol to activation of the membranes for incubation of 5-10 minutes. Double distilled H2O was used to rinse thoroughly after immersion in absolute methanol. The membranes were further immersed into transfer buffer and equilibrate for at least 30 minutes. 6mm stack of Whatman chromatography papers were also immersed into transfer solution for at least 1 hour.

After extraction of the gels, a piece of Whatman chromography paper was placed first followed by a piece of PVDF membrane. The gel was further stacked on top of the PVDF membrane. A glass rod was used to roll away the

bubbles trapped between the gels and PVDF membranes for better transfer. A final piece of whatman chromography paper was used to sandwich the stack for better supply of electrolytes and therefore better electroblotting effect.

The anode was placed on top of the 'sandwich' and a load was placed on top of the anode to ensure a better contact of the gels with PVDF membranes and avoid the expansion of the gel during the protein transfer.

Antibody probing and film development

After transferring the proteins from the gels to PVDF membranes, the membranes were first washed by PBS-T for 5 minutes with mild shaking. Then the membranes were allowed to have blocking of 5 % slim milk for 1 hour at room temperature. Slim milk was discarded after 1 hour with mild rinsing of PBS-T for 5 minutes. 1:400-500 of rabbit anti-rat ghrelin primary antibody was used with overnight probing at 4 ^{o}C. The membranes were washed by PBS-T thrice on the next day for duration of 10 minutes washing each. A secondary antibody of anti-rabbit IgG was probing at a dilution of 1:5000 for 1 hour with mild vortexing. The membranes were washed thrice again as previously stated.

After pre-incubating of the immobilon western chemiluminescent HRP substrate at room temperature for 10 minutes, it was added to the surface of the membranes evenly with 5 minutes of incubation at room temperature. The surplus staining solution on the surface was then discarded and the membranes were ready for X-ray film development.

Quantification of the bands were analyzed by Quantity One® (Bio-rad) using the X-ray film developed. It was measured by the intensity of the bands as relative expression of target gene : GAPDH of each individual sample.

2.3 Statistical analysis

Data were presented as mean ± SEM with indicated number (n) of experiments. Statistical analyses were performed by SPSS 15.0 (SPSS, Chicago, IL) and GraphPad Prism 5.0 (GraphPad software, Inc., San Diego, CA). Comparisons between two groups were performed by unpaired student t-test for moderate sample sizes or Mann-Whitney U-test for non-parametric tests. Comparisons in trends and interaction across groups were performed by repeated measures of ANOVA with Bonferroni post-test as the test for post-hoc comparisons. A difference with $P < 0.05$ was considered as statistically significant.

2.4 Experimental Procedures

2.4.1 Neonatal maternal separation procedure

Neonatal maternal separation (NMS) of newborn rats has been developed as a model of early life stress that leads to the permanent changes in the central nervous system and stress-induced visceral hyperalgesia.

Sprague-Dawley neonates were grown from the Laboratory Animal Services Center of The Chinese University of Hong Kong on postnatal day 2. To avoid the influence of estrogen and hormonal cycles on neurochemical stimulation on the influence of ghrelin especially, we used only male pups in our experiments. The litters were randomized to one of two rearing conditions: 1) maternal separated (MS) group or 2) control, non-handling (NH) group.

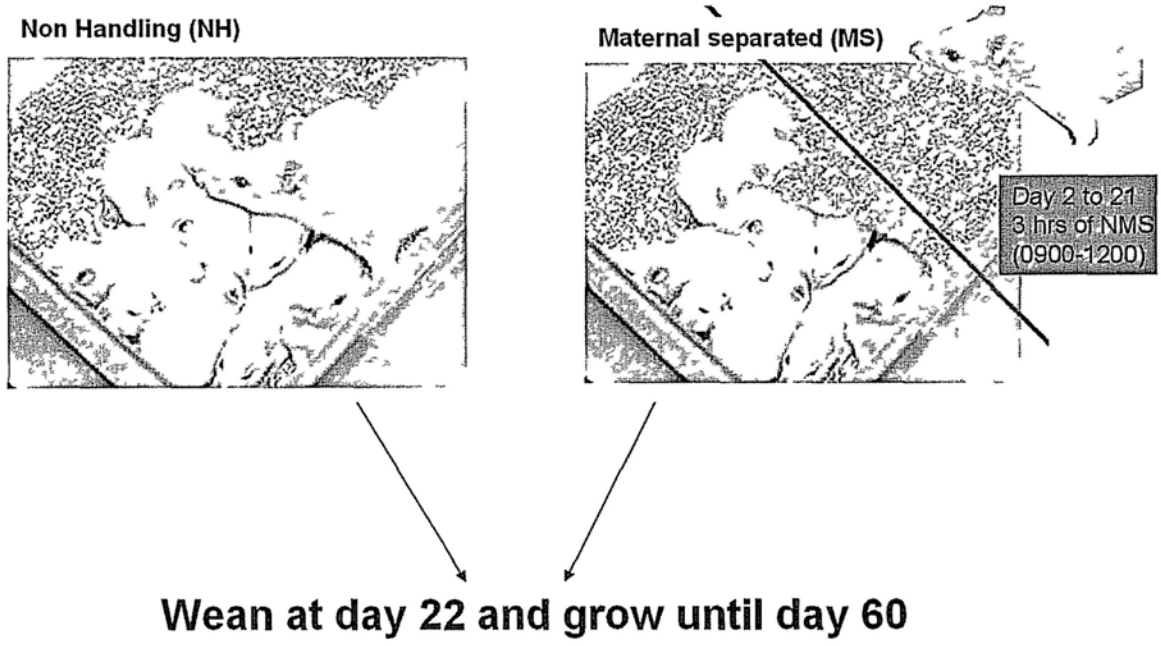
During postnatal day 2 to day 21, litters were exposed to a 180-min period of maternal separation daily (Coutinho et al. 2002). The manipulation commenced at 0900 with the removal and placement of the dams into separation cages, whereas the litters were separated in a group into an isolation cage with an adjacent thermoregulated room maintained at 20 °C. After 180 minutes, pups were returned to their maternity cage with the foster dam. Eight pups were

fostered as a group by a dam housed in a cage consisting 2.5 cm of wood chip bedding material. Rats were housed on a 12:12-h light-dark cycle with access to food and water ad libitum. Table 3. showed the nutritional components of the food given to both NH and MS rats. Litters were weaned on day 22. Animal care and experimental procedures were followed according to the institutional ethics guidelines and requirements of the animal experimentation of the institution. A simple illustration showed in figure 3.

Table 3. Nutritional composition (%) of food used in the study (Prolab RMH 2500, 5P14)

Total protein (%)	28.768
Total carbohydrate (%)	59.095
Total fat (%)	12.137
Crude Fiber (%)	5.3
Energy (kcal/g)	3.34

Figure 3 Simple Illustration on procedures in neonatal maternal separation



2.4.2 Experiment 1: Acute psychological stress with water avoidance stress (WAS)

2.4.2.1 Sample Size Calculation:

According to a similar study from Kristenssson et al. (Kristenssson et al. 2006) on acute stress response on normal SD rats and highly-anxious Wistar Kyoto (WKY) rats, the sample size required for each MS and NH group was 10 respectively. Since our study was novel on the acute stress response on MS rats, we predicted MS rats may develop highly sensitive stress response in adulthood that may resemble highly-anxious strain of rats such as Wistar Kyoto. We set the sample size to 10 in each sub-group if our hypothesis could fit.

2.4.2.2 Experimental Procedure

WAS was carried out to compare ghrelin expression in response to induced anxiety among 10 NH and 10 MS rats. Another group of 10 NH and 10 MS rats served as the controls for sham stress. It was to represent the normal NH and MS rats that were not agitated by WAS.

Blood samples were collected in EDTA tubes from the tail vein. Each rat underwent isoflurane anaesthesia with vein tail extraction. About 500 µl of

blood was collected for each sampling. Anaesthesia was used to reduce the associated stress during extraction.

After blood extraction, the controls returned to their respective cages and allowed to recover without stressful stimulation for 1 hour as a sham stress control. Each control rat was killed 1 hour after intraperitoneal injection of sodium pentobarbital. Blood samples were extracted again immediately after anaesthesia with body weight recorded.

Another group of 10 NH and 10 MS rats were put under water avoidance test. The test apparatus consisted of a Plexiglas tank (45 cm length x 25 cm width x 25 cm height) with a glass block (10 cm length x 8 cm width x 8 cm height) affixed to the center of the floor. The tank was filled with fresh room temperature water (25°C) to within 1 cm of the top of the block. Before stress was induced, blood samples were extracted by tail vein. Each rat was placed on the water-surrounded platform after isoflurane anaesthesia and allowed to recover from anaesthesia. After the rat regained its consciousness, it was exposed to water avoidance stress for 1 hour. This well-characterized test symbolized a potent psychological stressor with large elevations of ACTH and

CORT within 30 minutes as shown in various research studies. Each rat was renewed anaesthesia with intraperitoneal injection of sodium pentobarbital and killed immediately after 1 hour of WAS. The blood sampling was collected immediately after water avoidance test by tail vein. 10 NH rats and 10 MS rats were used for extraction of fresh stomach tissues from greater and lesser curvature and brain tissues. The procedures for extraction were identical as the sham group.

Ghrelin from blood plasma, hypothalamus and gastric mucosal tissues was examined to compare the variations in expression among NH and MS rats. Expression of ghrelin after WAS and in sham stress conditions was analyzed within the same group and across NH and MS groups to investigate the association with ghrelin expression after induced stress for further investigations. A diagrammatic representation of protocol 1 will be illustrated in Figure 4a and 5b.

Figure 4a. Simple illustration of procedure of experimental group in WAS

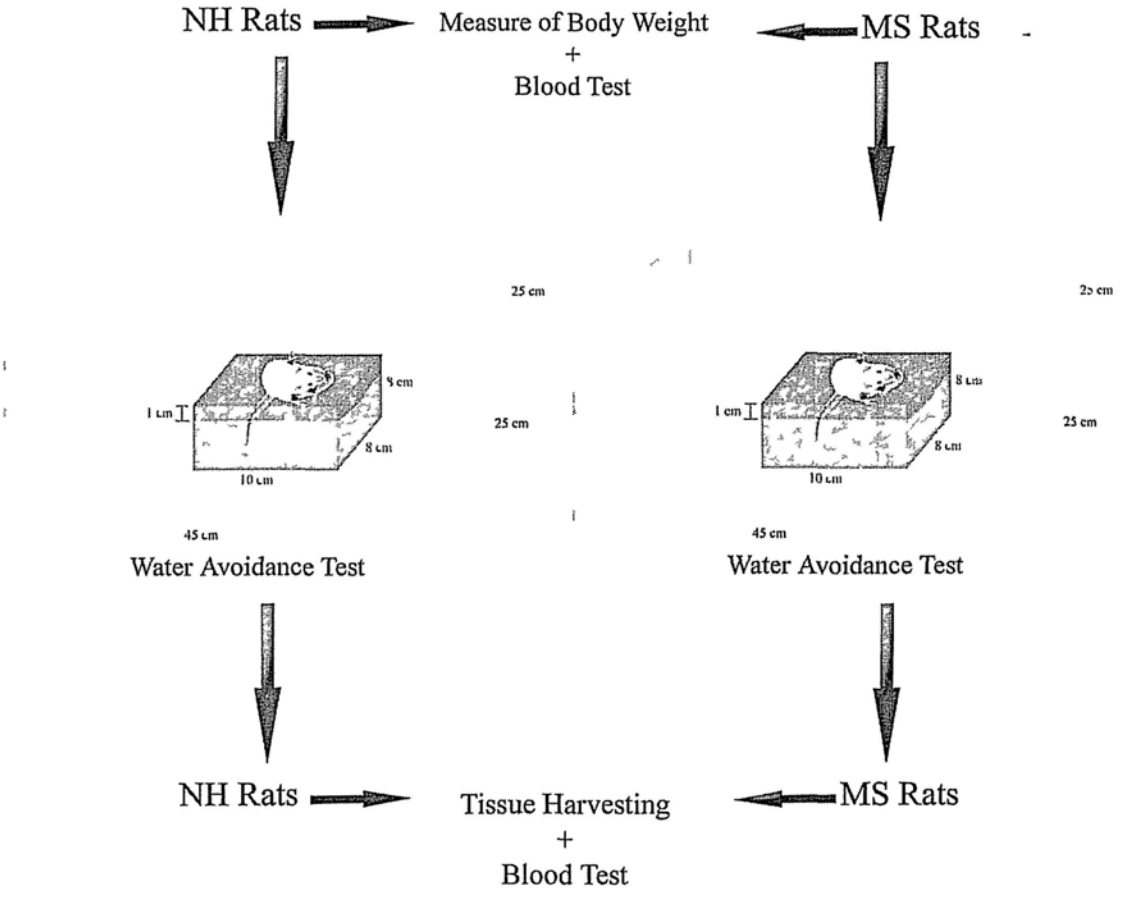
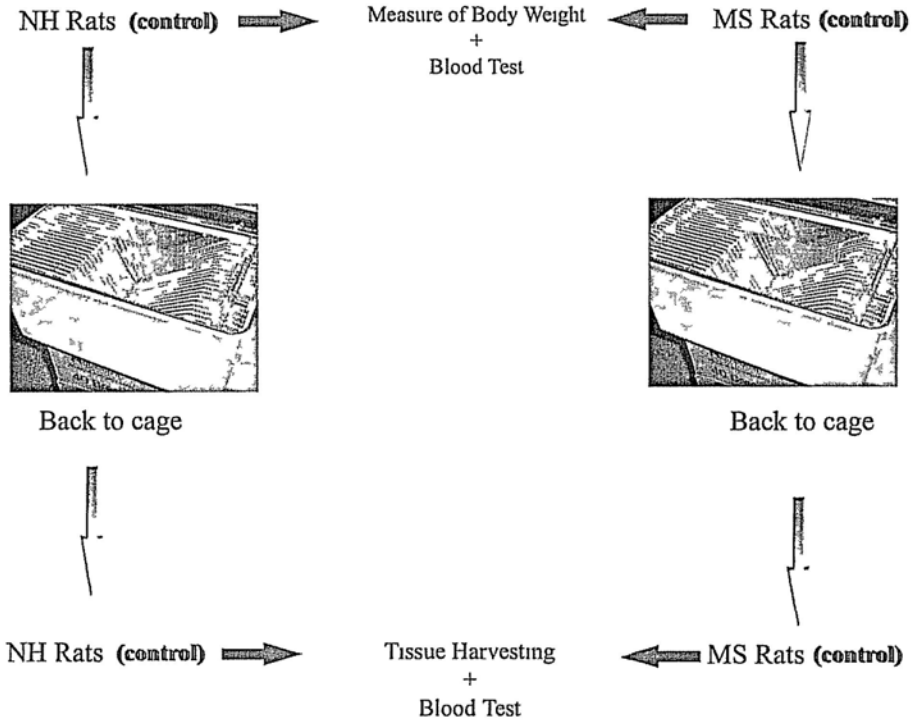


Figure 4b Simple illustration of procedure of sham group in WAS

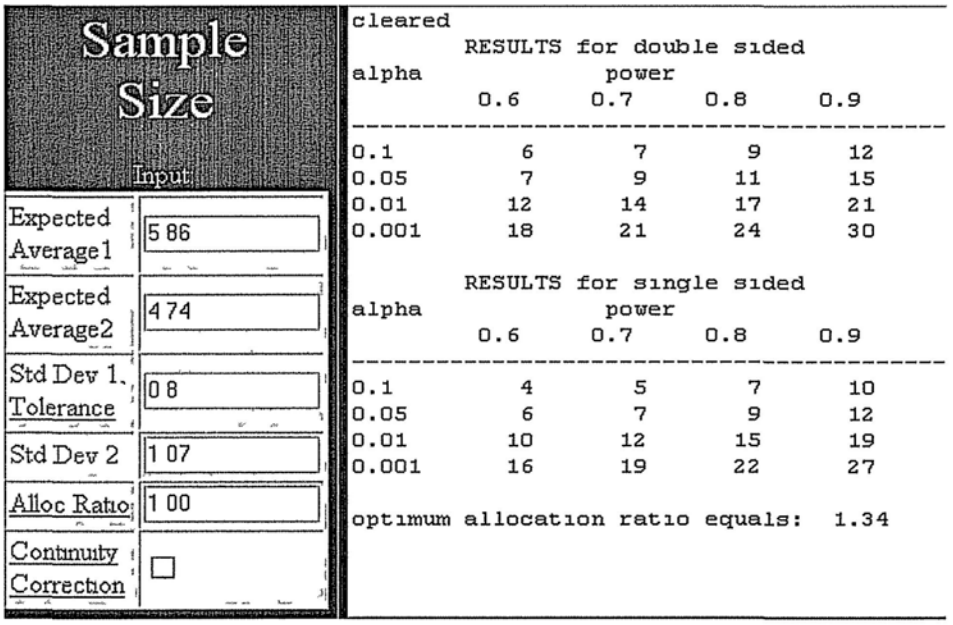


2.4.3 Experiment 2: Procedures of satiety test after fasting

2.4.3.1 Sample size calculation:

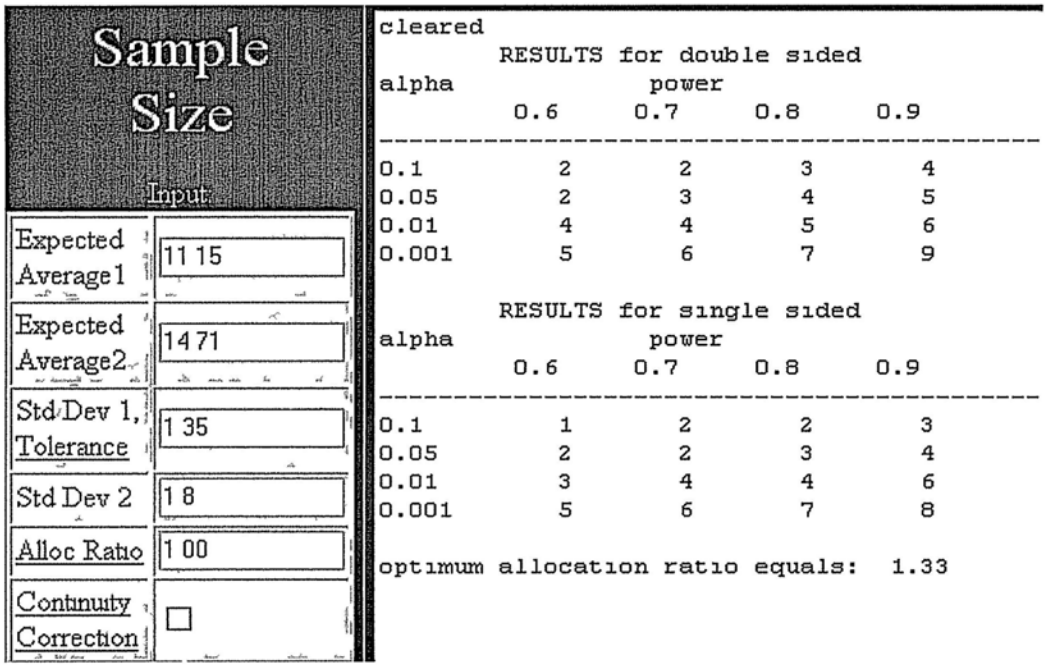
The majority of ideas on these protocols were novel. Silveira et al. (Silveira et al. 2006) had a satiety assessment in neonatally handled rats with the average food calories intake in non-handling group and maternal separation group. Using the data from Sliveira et al., the sample size for each group was calculated using the *Simple Interactive Statistical Analysis (SISA)* programme developed by Daan Uitenbroek. According to Sliveira et al., two types of food are given to the rats (Froot loops, Kellogg's and Fiber One, Nestle). For type I error $\alpha = 0.05$, type II error $\beta = 0.8$, sample size required for each MS and NH group is 11 for feeding Fiber One, Nestle. Figure 5 showed the sample size calculation generated from SISA.

Figure 5. Sample size calculation generated using the mean of food calories from Fiber One, Nestle



As for feeding by Froot Loops, Kellogg's with reference to type I error α = 0.05, type II error β = 0.8, sample size required for each MS and NH group was 4. Sample size calculation was shown in figure 6.

Figure 6. Sample size calculation generated using the mean of food calories from Froot Loops, Kellogg's



Since different nutritional products may affect the food intake of rats, we would like to recruit 10 rats each in different sub-groups and the power should be close to 80%.

2.4.3.2 Experimental procedure

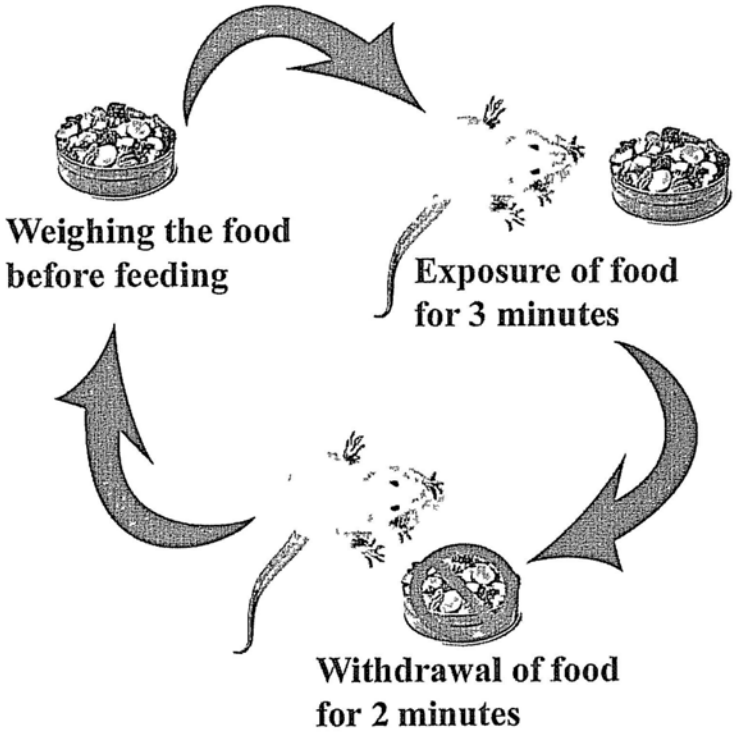
Starvation test was conducted to compare the expression of ghrelin in circulating blood, hypothalamus and gastric mucosal tissue among the NH and MS rats. All 20 NH and 20 MS rats underwent overnight fasting of 18 hours with free access of water.

Control group of 10 NH and 10 MS rats were anaesthetized with intraperitoneal injection of sodium pentobarbital. Body weight of each rat was first measured. Stomach tissues from greater and lesser curvature and brainstem tissues were harvested among 10 NH and 10 MS rats with preprandial ghrelin expression. The procedures for anaesthesia and sacrifice were the same as protocol 1. Ghrelin expression in blood plasma, hypothalamus and gastric mucosal tissue was measured to serve as the control for both NH and MS rats preprandially.

Blood samples of experimental group among 10 NH and 10 MS rats were collected before feeding by tail vein with anaesthesia. Fixed amount of food was given to each rat with an isolated container after fasting. Each rat was allowed to regain consciousness freely at their respective cage and have restrained feeding

conditions for 28 minutes. 6 exposures of food were monitored to evaluate the feeding behaviour between NH and MS rats. The cycle began with 3 minutes of food exposure. Then the next 3 minutes of food exposure began after 2 minutes of withdrawal of food. The remaining food in the container after each 3 minutes was recorded and converted into the respective nutrition content after each feeding period (Silveira et al, 2006). Therefore, the amount of nutrients each rat ingested across each interval could be measured accordingly. The simple illustration was shown in Figure 7.

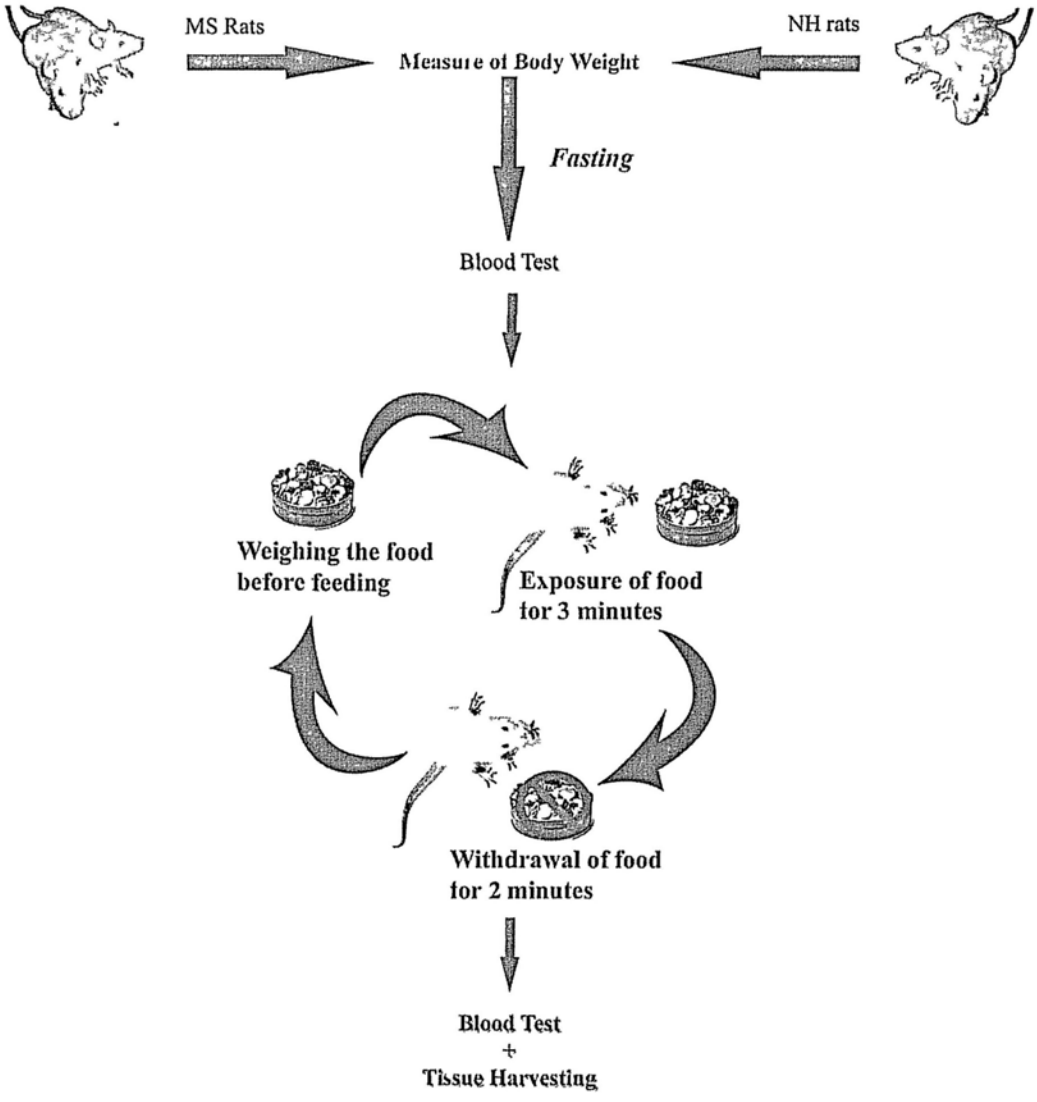
Figure 7. Diagrammatic illustration of procedure in repeated food intake to address satiety



Blood samples were collected immediately for once after 28 minutes of restrained feeding and also 1 hour after the start of the food intake just before sacrificing the rats. Plasma ghrelin profiles conducted by Overduin et al. (Overduin et al. 2005) suggested that greatest drop of plasma ghrelin was found one hour after the infusion of glucose into stomach in rats. These rats were anaesthetized with intraperitoneal injection of sodium pentobarbital for perfusion and killed immediately after the extraction of blood at 1 hour.

Circulating, gastric mucosal and hypothalamic expression of ghrelin were examined to compare the differential expression among NH and MS rats. Difference in expression of ghrelin preprandially and postprandially within the same group and across groups was studied to examine the association with ghrelin after induced starvation stress. A diagrammatic representation of protocol 2 will be illustrated in Figure 8.

Figure 8. Simple illustration of the procedure in experiment 2



2.4.4 Experiment 3: Follow up experiment: Procedures of satiety test after fasting and WAS

Another set of 10 NH and 10 MS rats were to investigate the association of satiety regulation after a combination of multiple stressors that involved WAS and starvation stress test. Each rat underwent 18 hour overnight fast before isoflurane anesthesia for WAS. Each rat regained consciousness on the block inside the water tank and allowed to stay on the block for 1 hour. Renewed anesthesia was done after 1 hour of WAS with fasting venous blood samples from tail veil collected before feeding. Food was given ad libitum to the rats with postprandial blood samples collected at 0, 1, 3, 24 and 48 hours respectively. The calorie intake was monitored regularly at the same time between intervals. A simple illustration of experiment 3 was shown in Figure 9 and table 4 showed the summary of all the experiments.

Figure 9. Illustration of the procedures in experiment 3

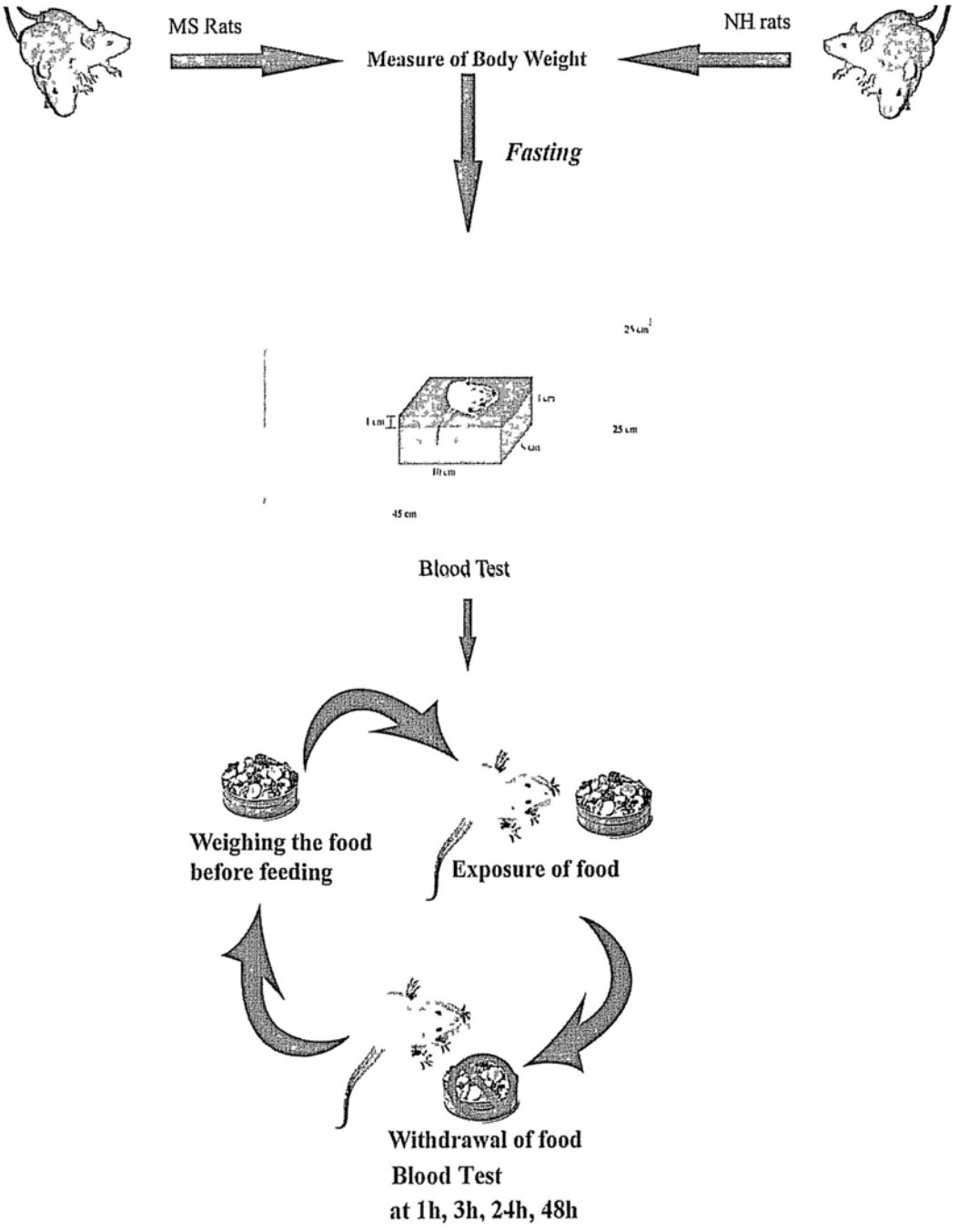


Table 4. Summary of the experiments

	Aim	Hypothesis	Design Outline	Outcome Measures	Significance of Experiment
Experiment 1	•To investigating the effect of NMS on the alternation of ghrelin responses in adulthood during acute psychological stress (WAS)	•NMS leads to the alternation in ghrelin profile in adulthood after acute psychological stress	•NMS conducted •Blood sampling taken from pre-WAS in adulthood (Day 60). •1h WAS •Immediate blood sampling and tissue extraction	•Plasma, hypothalamic and gastric ghrelin from greater and lesser curvature was assessed by various laboratory techniques	•To examine if NMS leads to alternation of anxiety-related ghrelin responses during acute physiological stress
Experiment 2	•To investigate the alternation of ghrelin profile by NMS after fasting which may result early satiation and postprandial fullness in adulthood	•NMS leads to abnormal ghrelin and satiety profile after fasting stress in adulthood	•NMS conducted •18h overnight fasting in adulthood (Day 59) •Baseline blood sampling followed by 28m of satiety test •Immediate blood sampling at 28m •Sacrifice for tissue and blood extraction at 1h	•Plasma, hypothalamic and gastric ghrelin from greater and lesser curvature was assessed by various laboratory techniques	•To examine if NMS leads to alternation of satiety responses and eating pattern through mediation of ghrelin responses after prolonged fasting
Experiment 3	•To investigate on the effect on NMS on ghrelin and satiety profile after a combination of stressors such as water avoidance and fasting stress.	•NMS leads to alternation of ghrelin and satiety profile after a combination of water avoidance and fasting stress.	•NMS conducted •18h overnight fasting in adulthood (Day 59) •1h WAS at the 17th hour of fasting (Day 60) •Food ad libitum for 48 hours •Blood sampling recorded at 0h, 1h, 3h, 24h and 48h	•Plasma, hypothalamic and gastric ghrelin from greater and lesser curvature was assessed by various laboratory techniques	•To examine if NMS leads to alternation of satiety profile and eating pattern after a combination of physical and psychological stress through mediation of ghrelin responses

RESULTS

3.1 Body weight analysis

3.1.1 Pilot studies

16 NH and 16 MS rats were used to the first set of pilot studies on experiment 1, 2 and 3. Although reduction of body weight in MS during childhood (Day 22) was not observed by the independent sample student t test (MS: 44.7 ± 1.3 g VS NH: 46.4 ± 0.6 g, $p = 0.2357$). There was a trend in reduction of body weight in adulthood (MS: 294.7 ± 7.1 g VS NH: 311.2 ± 4.3 g, $p = 0.075$). Reduction of body weight was predicted by sample size calculation in the actual studies. Figure 10a and 10b showed the boxplot analysis for pilot studies in body weight for day 22 and day 59 respectively.

Figure 10a. The boxplot analysis of body weight between NH and MS groups on day 22 (n=16).

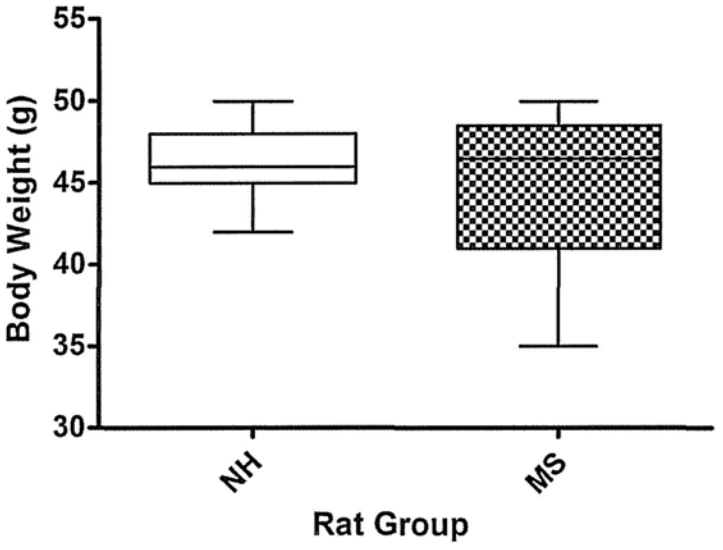
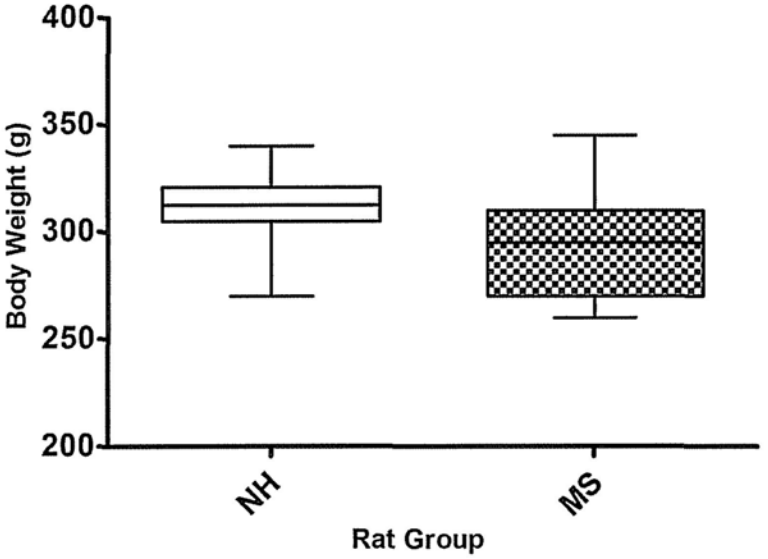


Figure 10b. The boxplot analysis on body weight between NH and MS groups on day 60 (n=16).



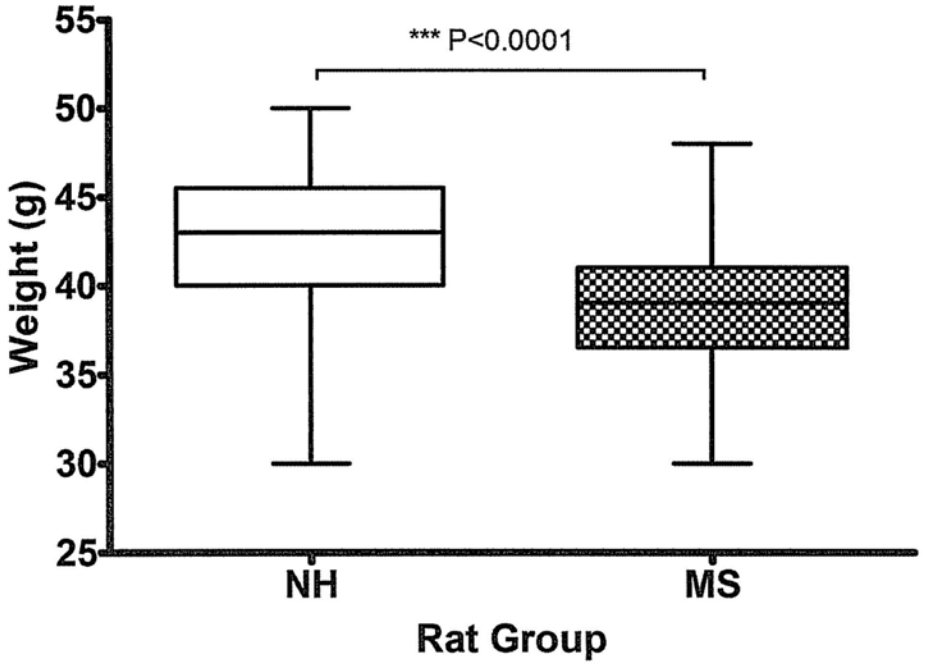
3.1.2 Actual Study

A total of 128 rats (67 NH and 61 MS rats) were used in this study. MS rats had significantly lower mean body weight at both childhood and adulthood. On day 22, independent sample t test was used to compare the mean body weight between 2 groups (MS: 39.0 ± 0.5 g, NH: 42.5 ± 0.5 g, P< 0.0001) shown in table 5 and two boxplot analyses was shown in figure 11a and 11b on day 22 and day 60 respectively.

Table 5. Body weight analysis in both NH and MS rats at childhood and adulthood. Values were expressed in mean ± SEM. MS had significant lower body weight in both childhood and adulthood.

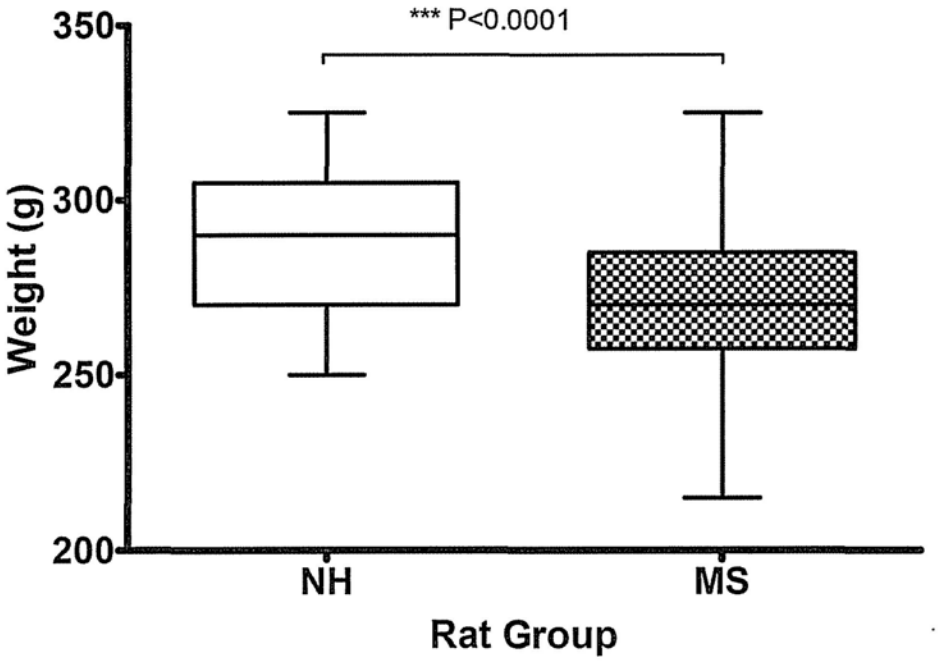
Group	Weight at day 22 (g)	Weight at day 60 (g)
MS Group (n=61)	39.0 ± 0.5	270.5 ± 2.9
NH Group (n=67)	42.5 ± 0.5	288.8 ± 2.5
P value	<0.0001	<0.0001

Figure 11a. Boxplot analysis of body weight between MS & NH groups (Day 22)



Approaching adulthood on day 60, another one sample t test was used to compare the mean body weight between 2 groups and showed that the body weight of MS was significantly lower (MS: 270.5 ± 2.9 g, NH: 288.8 ± 2.5 g, P< .0001) compared with NH rats in figure 11b.

Figure 11b. Boxplot analysis of body weight between MS & NH groups (Day 22)



3.2 Results from experiment 1

3.2.1 Effects of water avoidance stress on ghrelin mRNA and protein expression

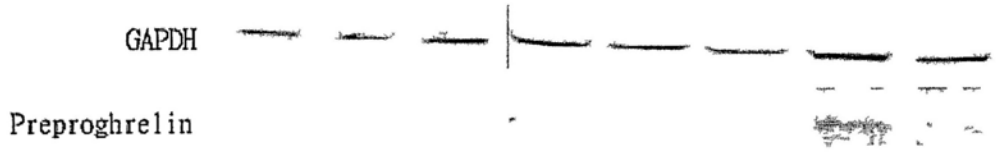
In MS rats, there was a significant increase of mRNA ghrelin from the hypothalamus in sham stress conditions as quantified on RT-PCR when compared with NH rats shown in table 6 (MS: 1.012±0.098, NH: 0.618±0.071, P= 0.009). After WAS, there was a non-significant trend in increasing hypothalamic mRNA ghrelin in both groups. However, the increment was significantly greater in MS rats than NH rats (Repeated measures of ANOVA, p= 0.015 for rat groups and p= 0.048 for WAS exposures). Western blot also showed consistent increase in gastric preproghrelin in both groups after WAS. Furthermore, MS rats had significantly higher gastric preproghrelin expression after WAS than NH rats shown in figure 12. Figure 13 and table 7 showed the mean of quantified gastric preproghrelin protein before and after WAS in both groups after normalization with housekeeping gene of GAPDH by independent samples t test (MS: 4.847±1.008, NH: 1.860±0.302, p = 0.022).

Table 6. Amount of mRNA encoding ghrelin in gastric body and hypothalamus as quantified by RT-PCR. Values were expressed in mean ± SEM. There was significant increase of ghrelin in hypothalamus at non-stressed conditions while there was no significant difference after stress in hypothalamus. No change was found in non stressed conditions and after stress in stomach between groups.

	Hypothalamus		Stomach	
Group	Sham Stress	WAS	Sham Stress	WAS
MS Group (n=7)	1.012 ± 0.098	1.468 ± 0.3138	0.566 ± 0.0744	1.141 ± 0.547
NH Group (n=7)	0.618 ± 0.071	0.9164 ± 0.1324	0.851 ± 0.115	0.566 ± 0.0744
P value	0.0088	0.1438	0.0647	0.3379

Figure 12 showed the western blot film showing the gastric preproghrelin and GAPDH in NH and MS rats before and after WAS, n=8.

NH Sham

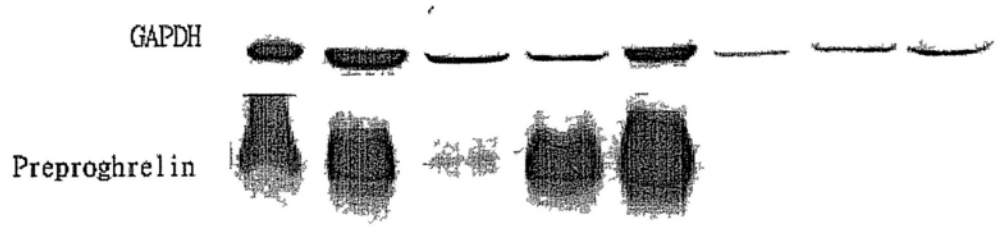


Ratio	1.474	1.343	0.749	0.659	0.624	0.425	2.388	2.099

NH WAS

Ratio	1.240	2 213	1 055	0.540	2.110	2.975	1.949	2.797

MS Sham



Ratio	1.948	2 153	1.432	7.347	3.918	0.945	0.594	0.696

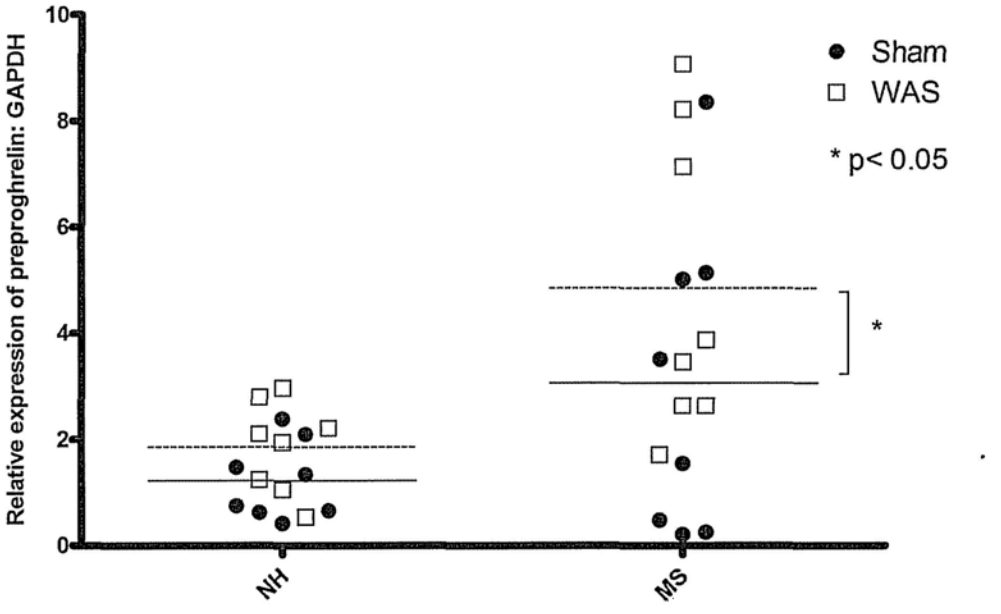
MS WAS

Ratio	2.639	2.640	1.717	3.880	8.220	9.075	7.142	3.467

Table 7 . Amount of ghrelin protein in gastric body as quantified by Western Blot. Values were in mean ± SEM. There was significant increase of ghrelin in stomach after WAS while there was no significant difference at baseline.

	Stomach	
Group	Sham	WAS
MS Group (n=7)	1.670 ± 0.438	4.847 ± 1.008
NH Group (n=8)	1.220 ± 0.258	1.860± 0.302
P value	0.3999	0.0218

*Figure 13. Gastric preproghrelin protein in NH and MS rats analyzed in experiment 1 after normalization with GAPDH by western blotting. Mean ± SEM, n=8, *p< 0.05. Increased expression of gastric preproghrelin after stress in MS rats was seen (Repeated measures of ANOVA, p = 0.003 for rat groups).*



3.2.1.2 Effect of water avoidance stress on plasma acylated ghrelin expression

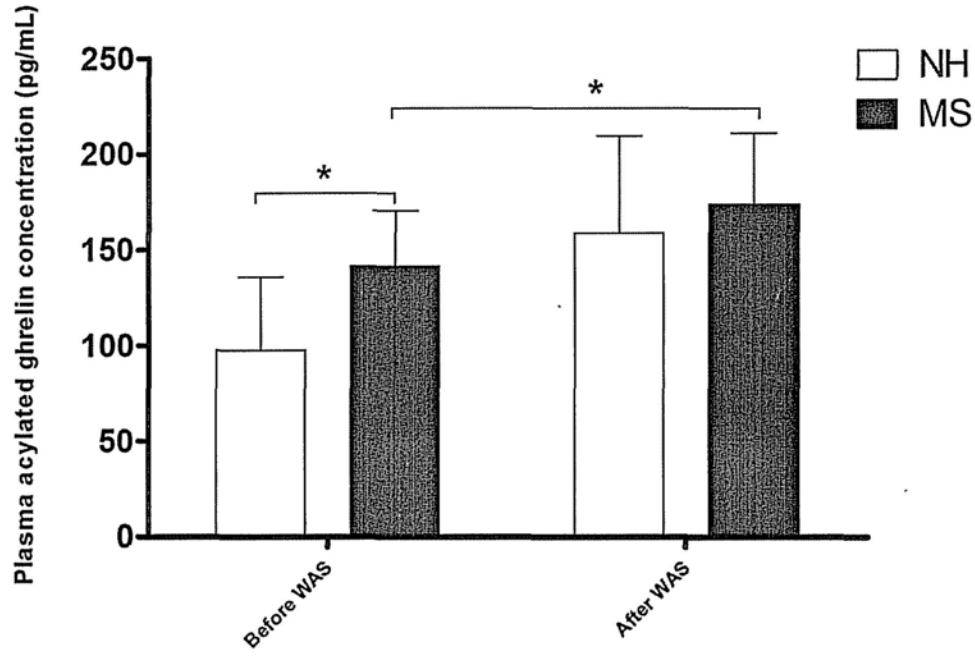
MS rats had significantly higher baseline plasma acylated ghrelin level (MS: 141.6±28.92 pg/mL, NH: 97.69±38.21pg/mL, p= 0.014) than NH rats before WAS in table 8.

Table 8. Amount of plasma acylated ghrelin as quantified by ELISA analysis. Values were in mean ± SEM. MS had significantly higher baseline and up-regulation after WAS in plasma acylated ghrelin.

Group	Pre WAS (pg/ml)	Post WAS (pg/ml)	P value
MS Group (n=10)	141.59 ± 28.92	173.92 ± 37.31	0.0457
NH Group (n=11)	97.69 ± 38.21	158.83 ± 50.85	0.1031
P value	0.0142	0.477	

After WAS, MS rats, but not NH rats, showed a further significant increase in plasma acylated ghrelin compared to baseline (pre: 141.6±28.92 pg/mL, post: 173.9±37.31pg/mL, p= 0.046) in figure 14.

*Figure 14. Plasma Acylated Ghrelin concentration assessed in experiment 1. Mean ± SEM, n=9-10, *p< 0.05. Significant increase in baseline and up-regulation in plasma ghrelin in MS rats were shown (Repeated measures of ANOVA, p= 0.032 for rat groups, p= 0.002 for WAS).*

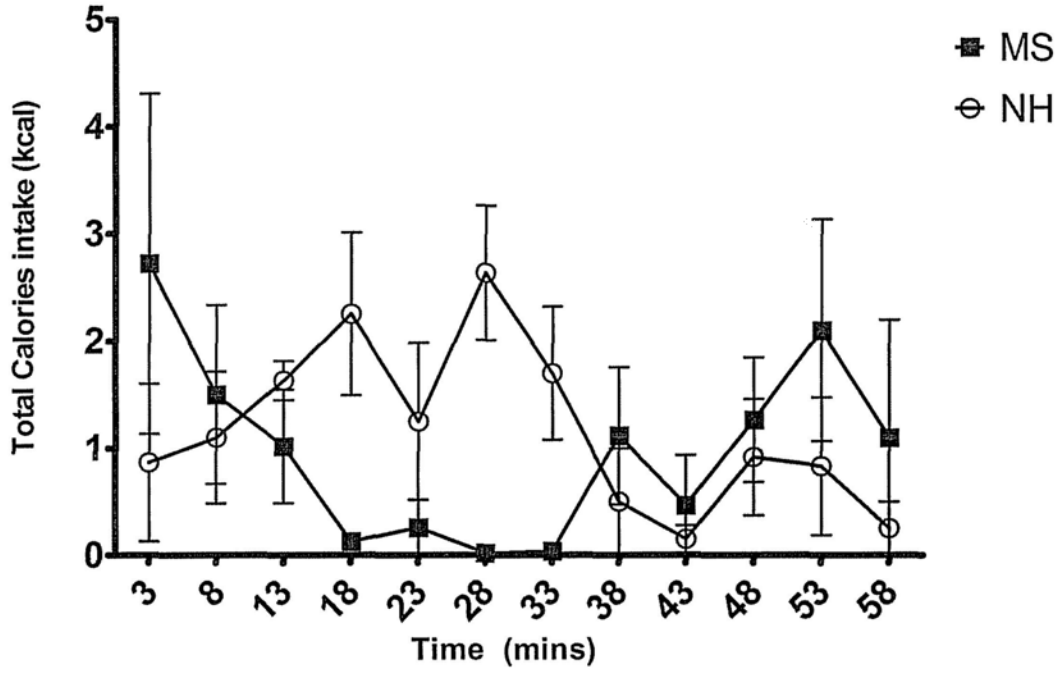


3.3 Results from experiment 2

3.3.1 Pilot study and preliminary analysis

In the pilot study, satiety assessment was done for 12 cycles that lasted for 58 minutes on 4 NH and 4 MS rats. Sustained eating was found in NH rats while fast postprandial fullness was found in MS. No significant difference was found from repeated measures of ANOVA for rat group and time factor, however there was an interaction between rat group and time (p=0.0255). Figure 15 showed the total calories intake between each interval across 58 minutes.

Figure 15. Total calories intake between NH and MS across 58 minutes



From figure 15, binge eating followed by a huge reduction of postprandial food intake up to 30 minutes was observed in MS rats. In comparison, NH started off with a relatively small intake of food then increased and reached maximum food intake at near 30 minutes. After 30 minutes, the difference in food intake was not significantly different from observations. Given the observation by the small sample size pilot study, we predicted the differential satiety abnormalities should be within the first half hour. Therefore for the actual study in experiment 2, 6 repeated feeding cycles were used.

3.3.2 Actual study

3.3.2.1 Effect of fasting on satiety regulation

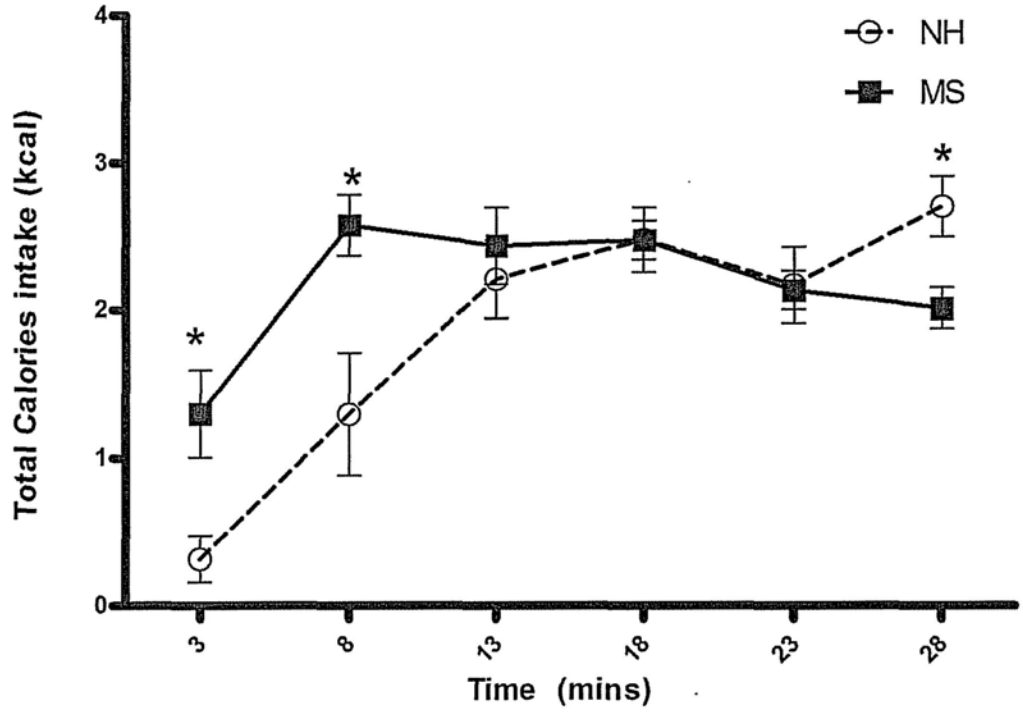
A diagram of detailed satiety assessment between the two groups was shown in figure 16. MS rats had a significantly higher calorie intake after the first feeding cycle at 3 minutes (MS: 1.303±0.293 kcal, NH: 0.319±0.159 kcal, p= 0.011) and second feeding cycle at 8 minutes (MS: 2.578±0.207 kcal, NH: 1.299 ±0.416 kcal, p = 0.019) when compared with NH rats with binge eating symptoms. However, the calorie intake of MS rats dropped abruptly in the subsequent feeding cycles and was significantly lower compared to NH rats after a total of 6 feeding cycles at 28 minutes with a sign of early satiety. (Repeated measures ANOVA: $p < 0.001$ for interaction between rat groups across time, $p < 0.001$ for time).

Particularly at 28 minutes, MS rats had significantly less calories consumed in the last cycle (MS: 2.017±0.140 kcal, NH: 2.709±0.206 kcal, p = 0.015). However, the total calorie intake by both groups over the 6-cycle feeding was non-significantly different (MS: 12.95±0.597 kcal, NH: 11.19±0.679 kcal, p = 0.693). Table 9 showed the average accumulative calorie intake after 28 minutes between NH and MS rats.

Table 9. The average total calorie intake after 28 min in MS & NH groups. Values were in mean ± SEM. Both groups had no significant difference in total calorie consumed during 28 min.

Group	Total calorie consumed (kcal)
MS group (n=10)	12.95 ± 0.597
NH group (n=9)	11.19 ± 0.679
P value	0.693

*Figure 16. Total calorie intake taken in 6 repeated feeding cycles in experiment 2. Data were expressed as mean ± SEM, n=9-10, *p< 0.05. MS rats showed binge eating symptoms and later developed early satiety (Repeated measures of ANOVA, p< 0.001 for interaction between rat groups and time, p< 0.0001 for time).*



3.3.2.2 Effect of fasting on ghrelin mRNA and protein expression

After feeding, MS rats showed a significant postprandial reduction in the amount of mRNA encoding ghrelin in the stomach than NH rats shown in table 10 (MS: Fasting 1.427 ± 0.307, after feeding 0.895 ± 0.141; NH: fasting 2.113 ±0.280, after feeding 1.621 ± 0.198, p= 0.014). Within groups, down-regulation of ghrelin production in the stomach postprandially was observed.

Table 10. Amount of mRNA encoding ghrelin in gastric body and hypothalamus as quantified by RT-PCR. Values were expressed in mean ± SEM. MS had a significant postprandial suppression of gastric ghrelin in stomach compared to NH. No change was found at baseline and after stress in hypothalamus.

	Hypothalamus		Stomach	
Group	Fasting Only	After feeding	Fasting Only	After feeding
MS group (n=7)	0.127 ± 0.008	0.127 ± 0.008	1.427 ± 0.307	0.895 ± 0.141
NH group (n=7)	0.109 ± 0.011	0.211 ± 0.071	2.113 ± 0.280	1.621 ± 0.198
P value	0.2164	0.2960	0.1296	0.0136

On Western blot, MS rats had lower baseline gastric preproghrelin protein than NH rats showed in table 11. After feeding, both groups showed an increase in the production of preproghrelin protein. Although the postprandial increment was higher in MS (Before feeding: 0.221±0.067; After feeding: 0.734±0.113, two-fold increase p= 0.005) than NH rats (before feeding: 0.529±0.155, After feeding: 1.230±0.120; p= 0.006), MS had reduced overall postprandial ghrelin protein production (MS: 0.734±0.113, NH: 1.230±0.120, p = 0.015) than NH rats.

Table 11. Amount of ghrelin protein in gastric body as quantified by Western Blot. Values were expressed in mean ± SEM. MS had significantly reduced up-regulation of preproghrelin protein after feeding.

	Stomach	
Group	Fasting Only	After feeding
MS group (n=6)	0.221 ± 0.067	0.734 ± 0.113
NH group (n=6)	0.529 ± 0.155	1.230 ± 0.120
P value	0.1181	0.0147

3.3.2.3 Effect of fasting on plasma acylated ghrelin expression

In MS group, a significant reduction in plasma ghrelin after 6 feeding cycles at 28 minutes in comparison with the baseline was observed and showed in table 12 (baseline: 257.05±15.86 pg/mL, 28 min: 131.05±15.85 pg/mL, $p < 0.0001$).

Table 12. Expression level of plasma acylated ghrelin at three intervals by ELISA . Values were in mean ± SEM. There was a trend in rapid postprandial down-regulation of plasma acylated ghrelin at 28 min and a significant suppression of plasma acylated ghrelin at 1h in MS rats.

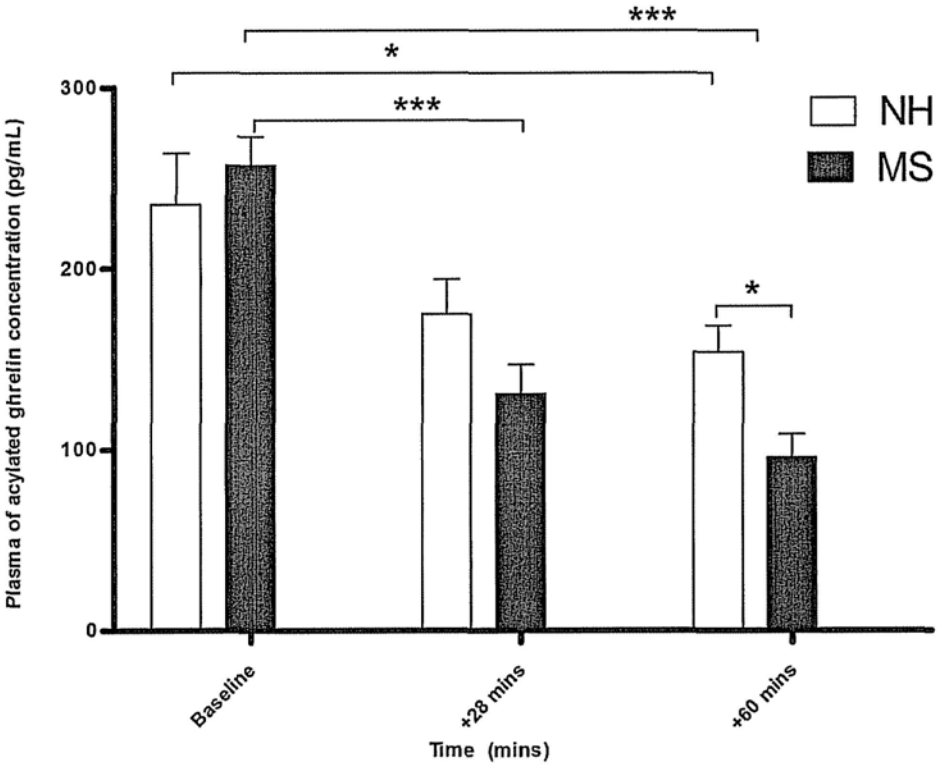
Group	Baseline (pg/ml)	28 min (pg/ml)	1 h (pg/ml)
MS group (n=8)	257.05 ± 15.86	131.05 ± 15.85	95.92 ± 12.71
NH group (n=8)	235.29 ± 28.45	174.95 ± 18.97	154.013 ± 14.53
P value	0.518	0.098	0.0101

There was a further trend in suppression of plasma ghrelin between 28 minutes and further reduction by one hour in MS rats (28 min: 131.05±15.85 pg/mL, 1 hour: 95.92±12.71 pg/mL, $p = 0.108$). In contrast, no significant difference in plasma ghrelin in NH rats between different time points was

observed. The overall reduction of plasma ghrelin after an hour was more significant in MS compared to NH rats ($p< 0.0001$) (Figure 17).

Between groups at baseline, there was no significant difference in plasma ghrelin after prolonged fasting ($p= 0.519$). However at 1h, MS had a significant decrease than NH (MS: 95.92±12.71 pg/mL, NH: 154.0±14.53 pg/mL, $p= 0.010$).

*Figure 17. Plasma AG concentrations analyzed in NH and MS rats at experiment 2. Mean ± SEM, n=8-9, ***p< 0.001, **p< 0.01, *p< 0.05. MS showed significantly higher postprandial suppression of plasma AG ghrelin at 1 hour (Repeated measures of ANOVA, p= 0.009 for interaction between rat groups and time, p< 0.001 for time).*

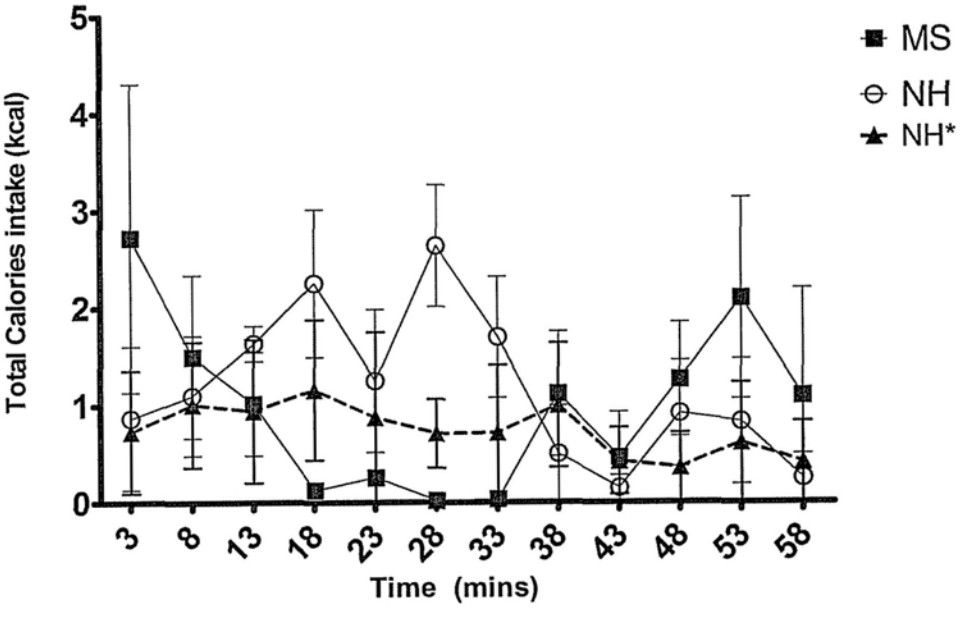


3.4 Results from experiment 3

3.4.1 Pilot study and preliminary analysis

In order to minimize the number of rats used for the study, 4 NH rats were used as the pilot studies the normal satiety assessment after WAS, a huge standard deviation was observed with either huge intake of food or non significant intake of food with the first hour. Therefore a longer period of satiety assessment was preferred to see the strong psychological effects on food intake that lasted up to 48 hours with a paper published by Fleshner et al. (Fleshner et al. 1995). Acute stressors such as tail shocks could lead to a long lasting effect on the stress modulators in the rats such as CORT and corticotropin binding globulin (CBG) that could persist until 96 hours. Therefore we predicted the effect on the change in satiety regulation on NH and MS rats could last for up to a longer period in our actual experiment. The calorie intake of the first hour was recorded and showed on figure 18.

Figure 18. Satiety assessment on the food taken between groups in pilot study of experiment 3. (NH symbolized the group of NH rats that had WAS after overnight fasting prior to satiety assessment)*

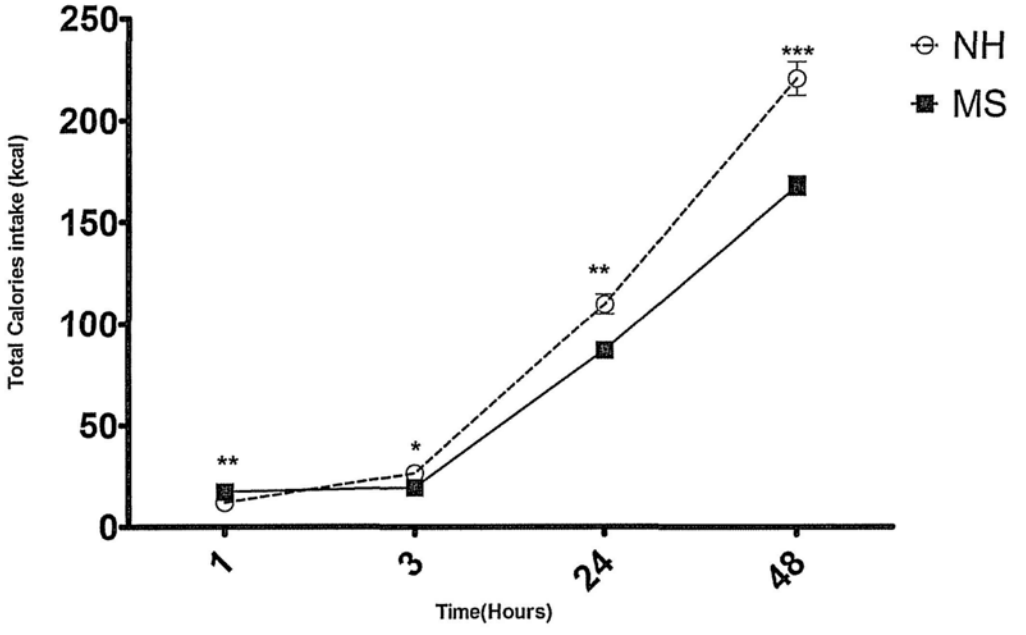


3.4.2 Actual study

3.4.2.1 Effect of water avoidance stress on satiety response after fasting

The 48-hour calorie intake satiety assessment profile of the NH and MS rats after WAS was presented in figure 19. MS rats had significantly higher calorie intake than NH rats at the first postprandial hour (MS: 17.24 ± 1.10 kcal, NH: 11.95 ± 1.20kcal, p= 0.006), but MS rats showed highly significant reduction of cumulative calorie intake at 3 hours (MS: 19.44 ± 1.50 kcal, NH: 26.49 ± 2.25 kcal, p= 0.023), 24 hours (MS: 87.19 ± 3.40 kcal, NH:109.8 ± 0.26 kcal, p= 0.002) and 48 hours compared to NH rats (MS: 168.1 ± 4.76 kcal, NH: 220.8 ± 8.27 kcal, P< 0.001).

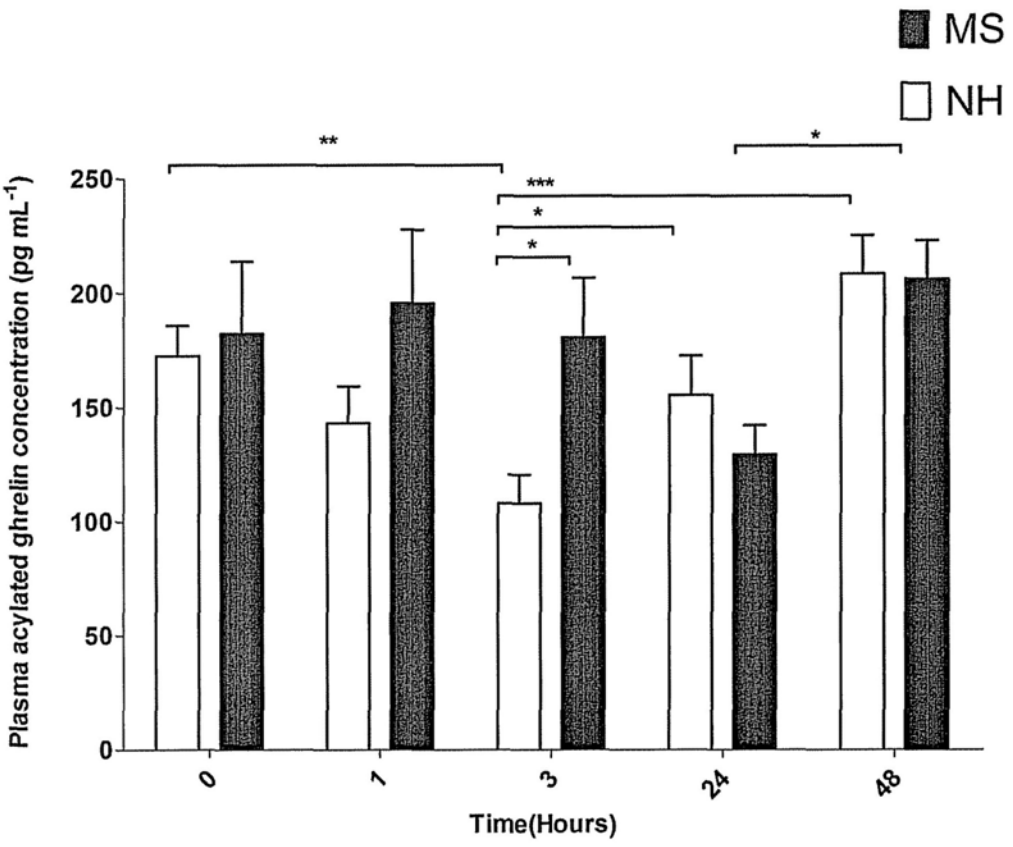
*Figure 19. Total calorie intake recorded in NH and MS rats in experiment 3. Data were expressed as mean ± SEM. n=8-9. ***p< 0.001, **p< 0.01, *p< 0.05. MS rats ate more rapidly at first hour, then developed early satiation quickly and significant reduction in overall food intake from 3 hours to 48 hours (Repeated measures of ANOVA, p< 0.001 for the interaction factor between rats group and time, p< 0.001 for rat groups, p< 0.001 for time).*



3.4.2.2 Effect of water avoidance stress on plasma acylated ghrelin profile after fasting

The fasting baseline and postprandial plasma acylated ghrelin profile of the rats was presented in figure 20. At baseline, the no difference in plasma acylated ghrelin levels between the two groups was found. However, a trend of reduced postprandial plasma acylated ghrelin in NH rats but not in MS rats was observed later on. At 3 hours, NH rats had significantly lower plasma acylated ghrelin level than MS rats (MS: 180.8 ± 25.91 pg/mL, NH: 108.0 ± 12.60 pg/mL, $p = 0.030$). However, the plasma acylated ghrelin level restored to normal level (baseline) in NH rats after 24 hours while the plasma acylated ghrelin level in MS rats started to drop but unable to return to baseline at that time. A time delay in plasma acylated ghrelin restoration to baseline level was observed in MS rats. After 48 hours, no significant difference in plasma acylated ghrelin level between the two groups was observed.

*Figure 20. Plasma acylated ghrelin concentrations measured in NH and MS rats in experiment 3. Means ± SEM, n=8-9, ***P< 0.001, **P< 0.01, *P< 0.05. MS showed exaggerated ghrelin response up to 48 hours with a time delay in restoration of acylated ghrelin back to baseline level. (Repeated measures of ANOVA, P=0.026 for interaction between rat groups and time, P= 0.001 for time)*



4. DISCUSSION

4.1 General conclusion

This study showed that early psychological stress in rats resulted in altered ghrelin responses that were associated with satiety dysfunction, in particularly following exposure to acute physical or psychological stress in adulthood. Table 13 summarizes the key findings of our experiments and figure 21 highlights our findings that provide new insights to the pathophysiological model of FD.

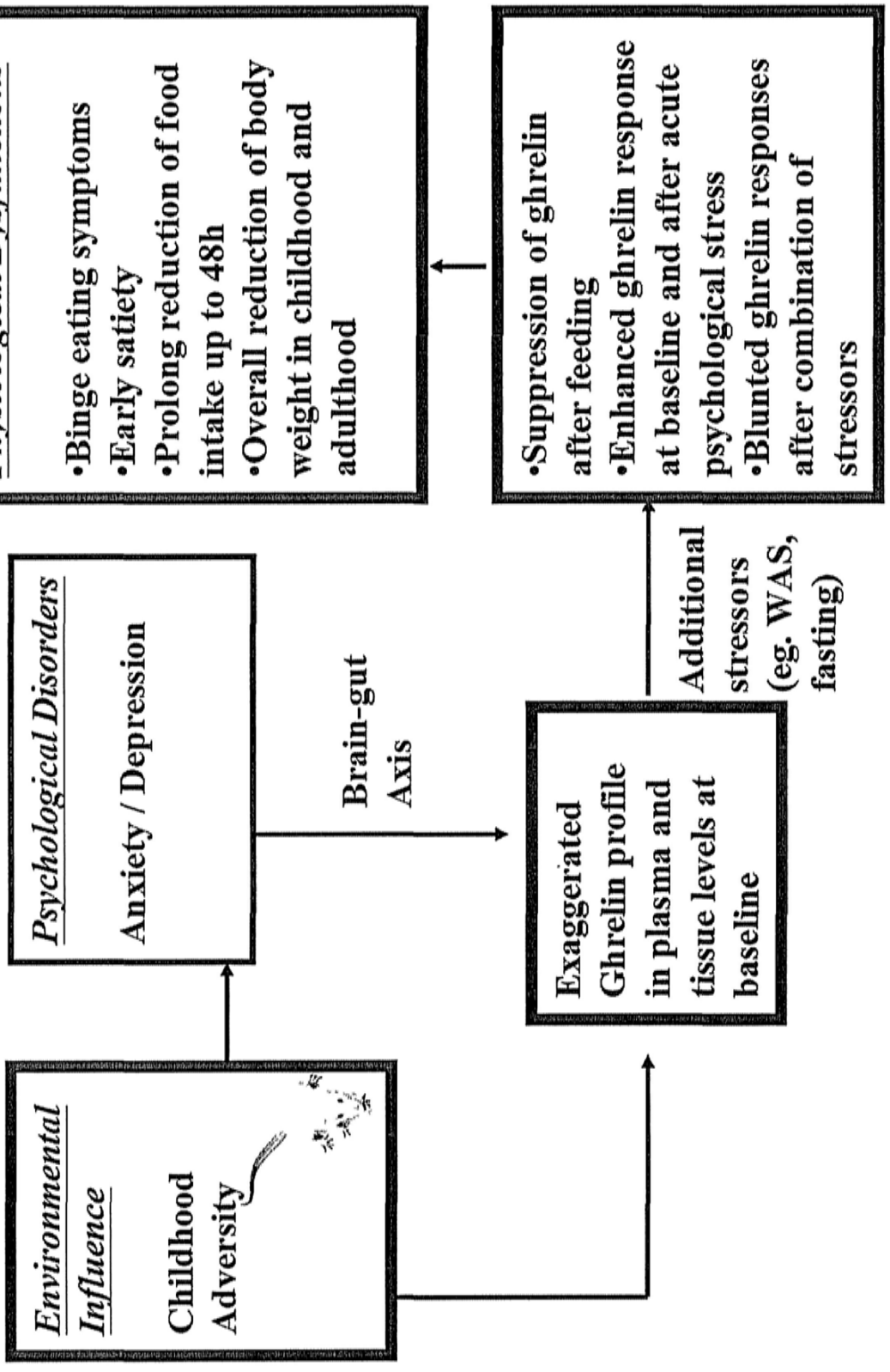
1) Neonatal maternal separated rats had higher hypothalamic mRNA ghrelin level and plasma ghrelin at baseline which was associated with further up-regulation of gastric ghrelin protein and plasma ghrelin secretion in response to an acute stressful event such as water avoidance stress test.
2) After fasting, MS rats had attenuated up-regulation of gastric ghrelin protein and greater postprandial down-regulation of mRNA ghrelin and plasma ghrelin, which was associated with initial abrupt eating and early satiation.
3) The combination of fasting and acute physiological stress resulted in higher food intake similar to binge eating symptoms followed by early satiety and subsequent prolonged appetite suppression up to 48 hours. There was also blunted response of enhanced plasma acylated ghrelin level up to 24 hours.

4) MS rats had lower body weight in both childhood and adulthood compared with NH rats. This may be attributed to the abnormal feeding behavioral response to environmental stress.

Table 13. Major findings in our experiments

	Results	Conclusion
General	•Reduction of body weight in MS rats in childhood •Reduction of body weight in MS rats in adulthood	•NMS led to reduction of body weight in both childhood and adulthood
Experiment 1	MS sham group •Increased hypothalamic mRNA in MS rats •Increased plasma acylated ghrelin in MS rats during before WAS MS WAS group •Increased gastric preproghrelin expression in MS rats after WAS	•Higher ghrelin expression showed in MS rats during non-stressed conditions •Enhanced ghrelin responses in gastric tissue and plasma after WAS in MS rats •Higher ghrelin expression maybe associated with increased anxiety in MS rats by NMS
Experiment 2	After feeding, •Increase initial calorie intake at 3m and 8m in MS rats during repeated feeding cycle •Reduction of gastric mRNA in MS rats •Decreased gastric preproghrelin in MS rats •Decreased plasma ghrelin in MS rats at 1h •No significant difference in total calorie intake	•Induction of early satiety in MS rats •Abnormal eating pattern in MS rats maybe due to reduction of ghrelin in plasma and tissue levels resulted from NMS
Experiment 3	•Increased calorie intake in MS rats after a combination of stressors at 1h •Suppression of calorie intake in MS rats at 3h, 24h and 48h •Blunted responses in plasma ghrelin expression in MS rats that lasted up to more than 24h •Returned to normal plasma ghrelin expression at 48h	•NMS led to blunted responses in ghrelin profile and irregular eating pattern during exposure to a combination of stressors •These abnormalities maybe resulted by the consequences of NMS

Figure 21. ***New findings in the pathophysiological model of FD***



4.2 Discussion

Our findings on the higher baseline hypothalamic mRNA ghrelin level and plasma ghrelin as well as heightened stress induced up-regulation of gastric ghrelin protein and plasma ghrelin secretion are compatible with previous studies (Kristenssson et al. 2006). Anxiety is associated with increased expression of plasma acylated ghrelin. At baseline, expression of circulating plasma and hypothalamic mRNA ghrelin were up-regulated. These findings suggested high anxiety levels in MS rats even in unstressed conditions. After acute psychological stress, further heightened up-regulation in plasma and gastric ghrelin protein were observed. These lend further support to the hypothesis of hyper-responsiveness of ghrelin to acute psychological stress. However, no direct mechanism was proposed in our experiment to prove the mediation of ghrelin in functional dyspepsia, further studies should be investigated.

We have also observed that MS rats showed attenuated up-regulation of gastric ghrelin protein after fasting and profound postprandial down-regulation of mRNA ghrelin and plasma ghrelin, which was associated with decreased food intake in response to physical or psychological stress. The attenuated fasting

levels and substantial postprandial down-regulation may lead to weakened stimulatory effect on appetite and thereby overall calorie intake. This is probably attributed to 5HT dysfunction in brain by the NMS. Dysregulation of hypothalamic-pituitary-adrenal (HPA) stress axis is highly associated with involvement of 5-HT neurocircuitry and psychiatric disorders (Goel & Bale 2010). 5-HT dysfunction in brain may subsequently alters the expression of plasma active ghrelin levels as supported by Nogogaki et al. (Nonogaki, Ohashi-Nozue, & Oka 2006). A negative feedback system was proposed that brain serotonin systems via 5-HT2C receptors and/or 5-HT1B receptors played an inhibitory role in regulating plasma active ghrelin levels in mice. Further investigations can be carried out to link the connections between serotoninergic system and adversity in ghrelin responses.

The rise of ghrelin up to 48h could be accounted firstly with the association in heightened anxiety after multiple stressors over a long period of time (Kristenssson et al. 2006; Asakawa et al. 2001). This surge may also be accounted for greater stimulation in gastric emptying to compensate the early satiety and delayed gastric emptying in MS rats as a pathophysiological response to exacerbation of FD as suggested by T. Nichizawa.

One unexpected finding was that the exaggerated plasma acylated ghrelin after exposure of multiple stressors contradicted to the reduction of food intake in the MS rats. This suggested further investigation required for understanding the appetite regulation when multiple stressors were involved. Since inconsistency in food intake pattern and ghrelin profiling were shown in different experiments, the complex pathways remained unclear for short and long-term energy homeostasis in rats. Larger sample size for these experiments should be required for the reproducibility of these observations. Plasma desacyl ghrelin should be investigated for its involvement in cell proliferation and adipogenesis (Bedendi et al. 2003;Broglio et al. 2004;Cassoni et al. 2001;Thompson et al. 2004)(Shinomiya et al 2005). Desacyl ghrelin was also proposed to be an indicator of both ghrelin production and secretion (Hosoda et al. 2004)(Silveira et al. 2006). Further studies on other appetite mediating proteins such as NPY and leptin are required to give more profound insights to the actual signaling pathways.

Body weight in neonatally handled rats was significantly reduced compared to controls. Studies had reported an increase (Panagiotaropoulos et al.

2004)(Shinomiya et al, 2005) or no difference (Ploj et al. 2003; Silveira et al. 2006) in body weight in neonatally handled rats. The discrepancies could be subjected to multiple conditions such as rat strains, housing conditions, handling procedures, the duration in neonatally separation and contents of the nutrients in the food given. We therefore postulated that neonatally handled rats had a higher anxiety that might lead to response adversity to stressors, hence resulted in abnormal secretion of ghrelin responses to the appetite and satiety regulation. Therefore, reduction of lower body weight resulted in adulthood.

Limitations included anesthertisa of rats before the extraction of blood in most of the procedure to reduce the stress on rats. However, the influence of ghrelin measurements during conscious and unconscious stages remains unclear, further investigations should be conducted.

4.3 Overall Conclusion

In conclusion, early childhood stress by NMS induced to heightened response in ghrelin in plasma and tissue levels during exposure to both physical and acute psychological stressors in adulthood. The abnormalities in ghrelin response are abrupt eating, early satiation and also reduced overall food intake in MS rats. These abnormalities coincided with many postprandial distress symptoms in functional dyspepsia patients with early childhood adversities. Our findings suggested that childhood adversity may predispose to the development of functional dyspepsia. And NMS in rats may provide a valid model for further studies on the pathogenesis of ghrelin system in particular to symptoms of binge eating and early satiation. This model may serve as a plausible animal model for functional dyspepsia with treatment of binge eating symptom.

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