Ashdin Publishing
Neuroenterology
Vol. 1 (2012), Article ID 235603, 8 pages
doi:10.4303/ne/235603

Research Article

Stressful Events in Early Life Lead to Aberrant Ghrelin and Appetite Regulation to Stress in Adulthood

Cynthia K. Y. Cheung, Ying Ying Lee, Siew C. Ng, Fung Chun Lam, Wai Tak Law, Jun Yu, Joseph J. Y. Sung, Francis K. L. Chan, and Justin C. Y. Wu

Institute of Digestive Disease, The Chinese University of Hong Kong, No. 12 Chak Cheung Street, Shatin, N.T., Hong Kong
Address correspondence to Cynthia K. Y. Cheung, ckycyn@gmail.com

Received 19 June 2012; Revised 6 July 2012; Accepted 26 July 2012

Abstract *Background.* Stressful events in early life predispose to the development of eating disorders in adulthood. This study investigates how neonatal maternal separation (NMS) affects satiety and ghrelin secretion in adulthood. *Methods.* Sprague-Dawley rats underwent NMS and controls were without NMS. Experiments were conducted on day 60: (1) water avoidance stress (WAS); (2) feeding after overnight fasting; (3) feeding after overnight fasting and WAS. Blood samples, gastric and hypothalamic tissues expression were collected for ghrelin analysis. *Results.* (1) MS rats had a higher basal ghrelin. After WAS, MS rats had enhanced ghrelin. (2) A higher initial calorie intake and lower postprandial gastric ghrelin protein in MS are observed without difference in overall calorie intake. (3) MS had symptoms of binge eating and early satiation. Overall reduction of calorie intake was observed until 48 hours in MS. *Conclusion.* Stressful events in early life led to aberrant ghrelin profile and early satiation in response to stressful experience in adulthood.

Keywords Please provide the keywords

1 Background

Stressful events in early life (such as childhood physical or sexual abuse) have been implicated as a risk factor of functional GI disorders and eating disorders such as bulimia spectrum disorders (BSDs), Bulimia Nervosa (BN), and Binge Eating Disorder (BED) [7,8,11,40]. Studies showed that the dysfunction of hypothalamic-pituitary-adrenal (HPA) [9,14,18,26] and serotonergic systems [3,31,32] may be involved in the pathogenesis of eating disorders.

Neonatal maternal separation (NMS) had been established as a model of stressful events experienced in childhood. It had shown a permanent alternation of the HPA axis with adverse consequences such as anxiety-like behaviors [16,17,24,38,39,43]. NMS had been proposed as an animal model for various gastrointestinal disorders. Studies showed NMS as a model of Irritable bowel syndrome with visceral hyperalgesia, somatic analgesia, and increased colonic motility in response to acute psychological stressors [5]. Jahng et al. further proposed NMS as an animal model of eating disorders with sustained hyperphagia and anxiety-/depression-like behaviors [12].

In order to account for some of the characteristics in eating disorders, a series of experiments had been carried out using neonatal maternal separation as an animal model. Ghrelin expression has been analyzed in detail in our experiment. Ghrelin is a 28-amino acid peptide cleaved from a larger precursor, preproghrelin. It is a growth hormone-releasing acylated peptide as endogenous ligand for the growth hormone secretagogue receptor (GHS-R) [13]. It is produced by A-like cells and localized mainly in the oxyntic mucosa of the stomach. It stimulates appetite and body weight by chronic administration of ghrelin. It can also increase body weight in many species by food intake, energy expenditure, and fuel utilization [2,20,21,22,37,41,42]. Circulating total ghrelin increases after fasting and decreases after feeding or oral glucose administration [6, 37]. Ghrelin has also been reported to be involved in anxiety regulation in response to stressful stimuli. In rodents, the administration of ghrelin induces anxiogenic behavior [1,23].

Heighted gut-brain ghrelin responses are often present in many eating disorders such as Anorexia nervosa and Prader-Willi Syndrome [34,35,36]. However, the impact of early stress on ghrelin activity and appetite regulation remains unclear. This study aimed to investigate the effects of stressful events in early life on ghrelin profile and appetite regulation in adulthood using a neonatal maternal separation stress rat model. We hypothesized that stressful experiences in early life lead to aberrant ghrelin response to fasting and acute psychological stress, which affects the satiety and appetite regulation.

2 Methods

2.1 Animals

Neonatal maternal separation (NMS) of newborn rats has been developed as a model of early life stress that leads to

Table 1: 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

the permanent changes in the central nervous system and stress-induced visceral hyperalgesia [5].

Sprague-Dawley neonates were obtained 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, we used only male pups in our experiments. The litters were randomly assigned 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, inclusive, litters were exposed to a 180-min period of maternal separation daily. The manipulation commenced at 0900 with the removal and placement of the dams into separation cages, whereas the litters were removed as a group into an isolation cage in an adjacent thermoregulated room maintained at 20 °C. After 180 minutes, litters were returned to their maternity cage with the foster dam. Eight pups were fostered as a group by a dam housed in a standard cage containing 2.5 cm of wood chip bedding material. Animals were housed on a 12:12-h light-dark cycle with access to food and water ad libitum. Table 1 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 conducted according to the institutional ethics guidelines and in conformation with the requirements of the animal experimentation and ethics committee of the institution (Ref No. 08/013/MIS) [27].

2.2 Experiment 1: acute psychological stress with water avoidance stress (WAS)

A number of 10 MS and 10 NH rats had free access of food before they were anesthesized with isoflurane (Baxter international Inc., Deerfield, USA) and underwent one hour of water avoidance stress test (WAS) on day 60 [15]. Twenty minutes before WAS, a venous blood sample was collected in EDTA tube from the tail of the rats under anesthesia. Following recovery from anesthesia, the rats were placed on a 1 cm high platform ($\varnothing = 8$ cm), elevated 8 cm above the bottom of a transparent tank ($h = 45$ cm, $w = 25$ cm, $l = 45$ cm) that contained water at 25 °C. The experiments were carried out between 8 and 10 am. The rats were confined on the water surrounded platform for one hour. Immediately after 1 hour of WAS, a second blood sample was collected from the heart of the rats after renewed anesthesia by the injection of sodium pentobarbital before they were sacrificed.

An additional 10 rats from each group were used as non-stressed controls. These rats were anesthesized and allowed to regain consciousness in their respective cages and stayed in their cages for 1 h as sham controls.

2.3 Experiment 2: satiety test after fasting

On day 59, 10 NH and 10 MS rats underwent 18-h overnight fast before satiety test was performed on day 60. Each rat was anesthesized 1 h (−60 min) before the satiety test. Fasting venous blood sample was then collected from the tail at full anesthesia (−15 min). The rats were allowed to recover from the anesthesia for 30 min to regain full consciousness (−30 min to 0 min). Satiety test began (0 min) when each rat was then exposed to six repeated 5-min cycles that consisted of 3 min of feeding and 2 min of food withdrawal [30]. The amount of food ingested in each cycle was monitored (3 min, 8 min, 13 min, 18 min, 23 min, and 28 min) and the calorie intake served as a surrogate of satiety function. A postprandial blood sample was collected immediately after the 6-cycle feeding under renewed anesthesia (28 min). A third blood sample was collected (60 min) after the second blood sample from the heart after a renewed anesthesia by injection of sodium pentobarbital before they were sacrificed.

2.4 Experiment 3: prolonged satiety test after fasting and WAS

On day 59, 10 NH and 10 MS rats underwent 18-h overnight fast followed by WAS. After anethesia with isoflurane, each rat underwent an hour of WAS. Fasting venous blood sample from tail (0 h) and postprandial blood samples (1, 3, 24, and 48 h) were collected respectively. Food was given ad libitum to the rats and the calorie intake was monitored regularly at 1, 3, 24, and 48 h.

2.5 Plasma ghrelin assay

Plasma acylated ghrelin concentration was determined by a commercially available colorimetric enzyme immunoassay acylated ghrelin kits (Cayman Chemical, Ann Arbor, MI, USA). Blood samples were collected in an ice-cooled 1.3 mL micro tube (Sarstedt AG & Co., Germany) containing EDTA. P-hydromercuribenzoic acid was added proportionally to prevent degradation of the acylated ghrelin. Acidification after centrifugation was used to further stabilize the acylated ghrelin. The supernatant from the acylated ghrelin was stored at −20 °C until assay.

2.6 Analysis of mRNA gene expression by real-time quantitative PCR

Fresh glandular gastric body and hypothalamic tissues were harvested for the measurement of ghrelin gene expression. The tissues were frozen under −80 °C until use. Total RNA was extracted from stomach and brain tissues using TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA).

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 min, 95 °C for 10 min, followed by 40 cycles
Ghrelin (anti-sense)	gct gct ggt act gag ctc ct	of 95 °C for 15 s, and 60 °C for 1 min
β-actin (sense)	tgt cac caa ctg gga cga ta	
β-actin (anti-sense)	ggg gtg ttg aag gtc tca aa	

Residual DNA was removed by DNase I (Invitrogen). RNA concentrations were measured spectrophotometrically at 260 nm with 1 μg of total RNA for each sample. Samples were first DNA digested by RQ1 RNase-free DNase (Promega corporation, Madison, WI, USA) and reverse transcription was performed by MMLV (Promega corporation, Madison, WI, USA) and random primers (Invitrogen, Karlsruhe, Germany). Primers were designed by Primer3 (NIH., US) and primer express software (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using ABI 7900 Fast Real-Time PCR system for sequence detection (Applied Biosystems). A 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.

2.7 Analysis of protein expression by western blot

Gastric body and hypothalamic tissues were harvested and stored immediately at −80 °C until use. The tissues were crushed under liquid nitrogen and homogenized with a Polytron mixer in the immersion of T-PER tissue protein extraction reagent (Thermo Fisher scientific Inc., Waltham, USA) with complete protease inhibitor cocktails tablets (F. Hoffamnn-LA Roche Ltd., Basel, Switzerland) for protein extraction. The protein concentration was detected by Bio-Rad protein Assay. For the detection of ghrelin, 40–50 μg/lane was used in electrophoresis on 16% tricine gel. Gels were electroblotted onto 0.2 μm PVDF membranes (Millipore, Billerica, USA). The membranes were blocked overnight on 5% slim milk in 5 mL of PBS-T at 4 °C. Primary antibody of rabbit anti-rat ghrelin (Santa Cruz biotechnology Inc., CA, USA) was probed overnight at dilution of 1:400–500 compared with primary antibody of rabbit anti-rat GAPDH (Abcam) probing overnight together at a dilution of 1:5000. After washing thrice with PBS-T, a secondary antibody of anti-rabbit IgG (Abcam Inc., USA) at a dilution of 1:5000 stored for one hour at room temperature (25 °C) was used. After repeated washing of PBS-T, immuocomplexes were visualized by Immobilion western chemiluminescent HRP substrate (Millipore) with X-ray film. It was quantified with the relative intensity of the bands in preproghrelin in ratio with bands of GAPDH by Quantity One (Bio-Rad).

2.8 Statistical analyses

Data were presented as mean ±SEM with indicated number (n) of experiments. Statistical analyses were performed by SPSS 15.0 (SPSS, Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad software, Inc., San Diego, CA, USA). Comparisons between two groups were performed by unpaired Student's t-test or Mann-Whitney U-test as deemed appropriate. 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 $P < .05$ was considered as statistically significant.

3 Results

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 on day 22 (MS: 38.95 ± 0.51 g, NH: 42.54 ± 0.52 g, $P < .001$) and adulthood on day 60 (MS: 270.49 ± 2.89 g, NH: 288.79 ± 2.49 g, $P < .001$) compared with NH rats.

3.1 Experiment 1

3.1.1 Effects of WAS on ghrelin mRNA and protein expression

In MS rats, there was a significant increase in baseline production of mRNA ghrelin from the hypothalamus as quantified on RT-PCR when compared with NH rats (MS: 1.012 ± 0.098, NH: 0.618 ± 0.071, $P = .009$) under sham-stressed conditions. Following WAS, there was a non-significant trend of increase in hypothalamic mRNA ghrelin in both groups. However, the increment was significantly higher in MS rats compared to NH rats (repeated measures of ANOVA, $P = .015$ for rat groups and $P = .048$ for WAS exposures). Western blot also demonstrated a consistent increase in gastric preproghrelin in both MS and NH rats after WAS compared to sham stressed controls. Furthermore, MS rats had significantly a higher gastric preproghrelin expression after WAS compared to NH rats (MS: 4.847 ± 1.008, NH: 1.860±0.302, $P = .022$) (Figures 1(a) and 1(b)).

3.1.2 Effect of WAS on plasma AG expression

MS rats had significantly a higher baseline plasma acylated ghrelin level (MS: 141.6 ± 28.92 pg/mL, NH: 97.69 ± 38.21 pg/mL, $P = .014$) than NH rats before WAS. After WAS, MS rats, but not NH rats, showed a further significant

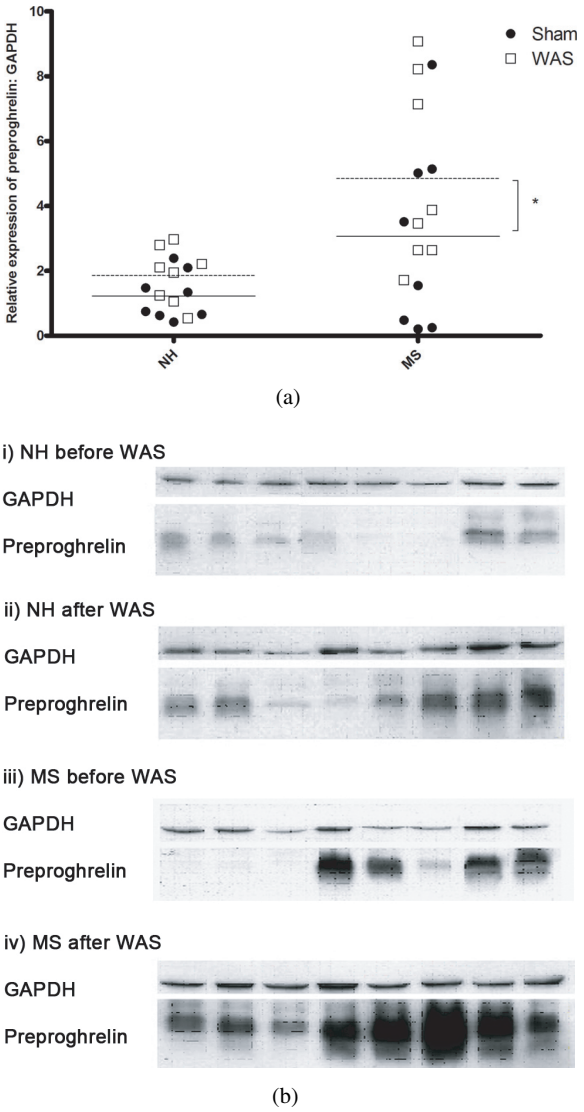

Figure 1: (a) Gastric preproghrelin protein in NH and MS rats assessed in experiment 1. Mean ±SEM, $n = 8$, $^*P < .05$. Enhanced expression of gastric preproghrelin after stress in MS rats was observed (repeated measures of ANOVA, $P = .003$ for rat groups). (b) Gastric preproghrelin protein in NH and MS rats assessed in experiment 1 by Western Blot.

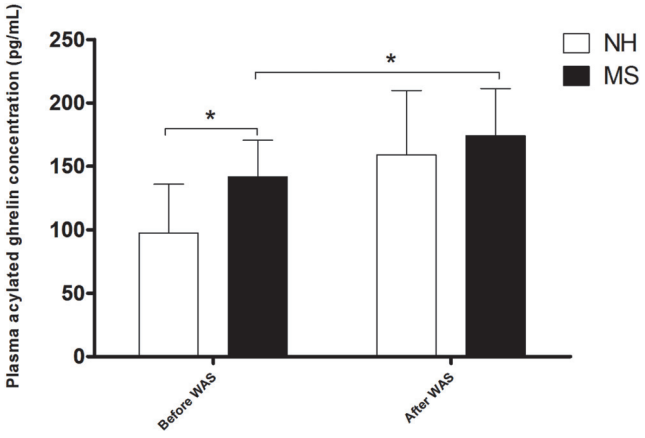

Figure 2: Plasma acylated ghrelin concentration measured in experiment 1. Mean ±SEM, $n = 9 - 10$, $^*P < .05$. Significant higher baseline and enhanced up-regulation in plasma ghrelin in MS rats were observed (repeated measures of ANOVA, $P = .032$ for rat groups, $P = .002$ for WAS).

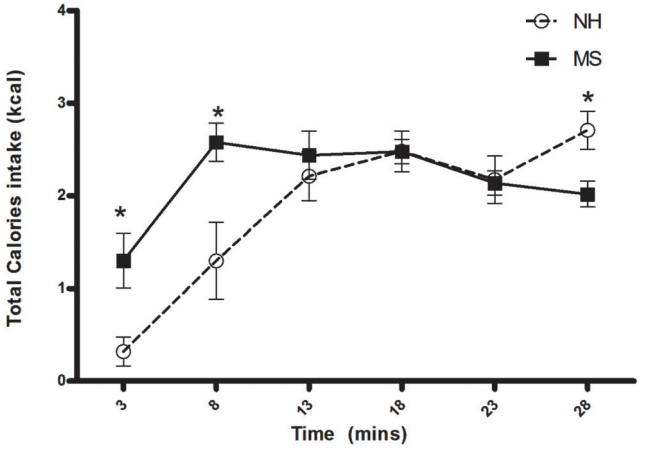

Figure 3: Total calorie intake taken in repeated feeding cycles in experiment 2. Data are expressed as mean ±SEM, $n = 9 - 10$, $^*P < .05$. MS rats ate more rapidly and later developed early satiety (repeated measures of ANOVA, $P < .001$ for interaction between rat groups and time, $P < .0001$ for time).

increase in plasma acylated ghrelin compared to baseline (pre: 141.6 ± 28.92 pg/mL, post: 173.9 ± 37.31 pg/mL, $P = .046$) (Figure 2).

3.2 Experiment 2

3.2.1 Effect of fasting on satiety

The satiety patterns between the two groups were shown in Figure 3. 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, $P = .011$) and second cycle at 8 minutes (MS: 2.578 ± 0.207 kcal, NH: 1.299 ± 0.416 kcal, $P = .019$) when compared with NH rats. However, the calorie intake of MS rats in the subsequent feeding cycles dropped abruptly and was significantly lower than that of NH rats after 6 feeding cycles at 28 min. (Repeated measures ANOVA: $P < .001$ for interaction between rat groups across time, $P < .001$ for time.) At 28 min, MS rats had significantly lower calories consumed in the last cycle (MS: 2.017 ± 0.140 kcal, NH: 2.709 ± 0.206 kcal, $P = .015$). However, the total calorie intake by both NH and MS groups over the 6-cycle feeding

was not significantly different (MS: 12.95 ± 0.597 kcal, NH: 11.19 ± 0.679 kcal, $P = .693$).

3.2.2 Effect of fasting on ghrelin mRNA and protein expression

After feeding, MS rats showed a significant postprandial suppression in the amount of mRNA encoding ghrelin in the stomach when compared with NH rats (MS: fasting only 1.427 ± 0.307, after feeding 0.895 ± 0.141; NH: fasting only 2.113 ± 0.280, after feeding 1.621 ± 0.198, $P = .014$). Within groups, both NH and MS showed down-regulation of ghrelin production in the stomach postprandially.

On Western blot, MS rats had a lower baseline preproghrelin protein than NH rats. After feeding, both groups showed an increase in the production of preproghrelin protein. Although the postprandial increment was greater in MS (fasting only: 0.221 ± 0.067; after feeding: 0.734 ± 0.113, two-fold increase $P = .005$) than NH rats (fasting only feeding: 0.529 ± 0.155; after feeding: 1.230 ± 0.120; $P = .006$), MS had less overall postprandial ghrelin protein production (MS: 0.734 ± 0.113, NH: 1.230 ± 0.120, $P = .015$) compared with NH rats.

3.2.3 Effect of fasting on plasma AG expression

In MS group, there was a significant reduction in plasma ghrelin after 6 feeding cycles at 28 min compared with baseline (fasting only: 257.05 ± 15.86 pg/mL, 28 min: 131.05 ± 15.85 pg/mL, $P < .0001$). There was a further trend of reduction in plasma ghrelin between 28 min and 1 h in MS rats (28 min: 131.05 ± 15.85 pg/mL, 1 h: 95.92 ± 12.71 pg/mL, $P = .108$). In contrast, there was no significant difference in plasma ghrelin in NH rats between duration of approximately 30 min. The overall decrease of plasma ghrelin after 1 h was more significant in MS than NH rats ($P < .0001$) (Figure 4).

Between groups at baseline, no difference in plasma ghrelin was found after prolonged fasting ($P = .519$). At 1 h, MS had a significant decrease compared to NH (MS: 95.92 ± 12.71 pg/mL, NH: 154.0 ± 14.53 pg/mL, $P = .010$).

3.3 Experiment 3

3.3.1 Effect of WAS on satiety response after fasting

The 48-hour calorie intake profile of the two groups of rats after WAS was presented in Figure 5. Although the MS rats had a significantly higher calorie intake compared with NH rats at the first postprandial hour (MS: 17.24 ± 1.10 kcal, NH: 11.95 ± 1.20 kcal, $P = .006$), they had significantly reduced cumulative calorie intake at 3 h (MS: 19.44 ± 1.50 kcal, NH: 26.49 ± 2.25 kcal, $P = .023$), 24 h (MS: 87.19 ± 3.40 kcal, NH: 109.8 ± 0.26 kcal, $P = .002$) and 48 h compared to NH rats (MS: 168.1 ± 4.76 kcal, NH: 220.8 ± 8.27 kcal, $P < .001$).

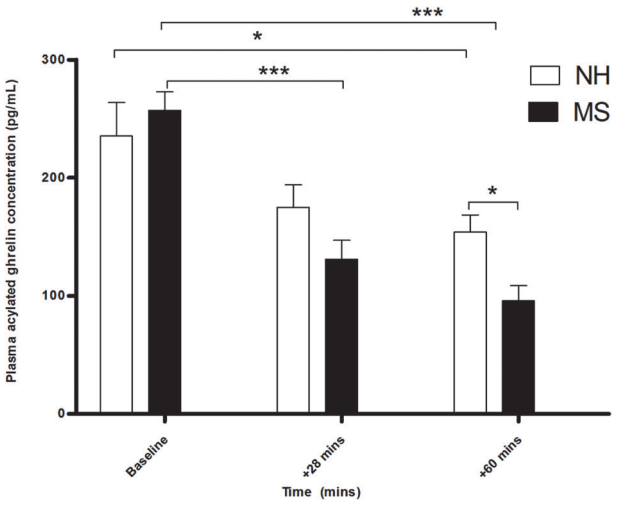

Figure 4: Plasma ghrelin concentrations measured in NH and MS rats in experiment 2. Mean ±SEM, $n = 8 - 9$, $^{***}P < .001$, $^{**}P < .01$, $^{*}P < .05$. MS showed significantly greater postprandial suppression of plasma ghrelin at 1 hour (repeated measures of ANOVA, $P = .009$ for interaction between rat groups and time, $P < .001$ for time).

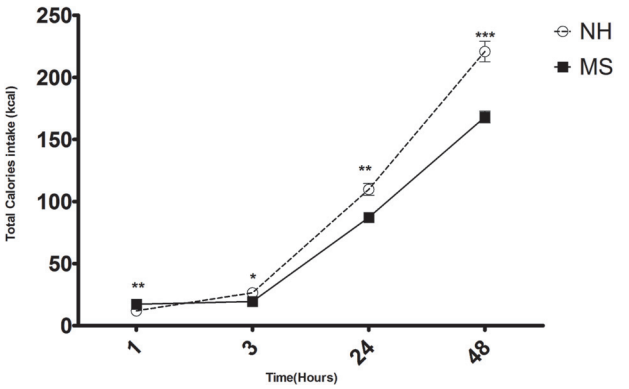

Figure 5: Total calorie intake measured in NH and MS rats in experiment 3. Data are expressed as mean ±SEM, $n = 8 - 9$, $^{**}P < .001$, $^{**}P < .01$, $^{**}P < .05$. MS rats ate more rapidly at first 1 h and later developed early satiety and significantly reduced overall food intake from 3 h to 48 h (repeated measures of ANOVA, $P < .001$ for the interaction factor between rats group and time, $P < .001$ for rat groups, $P < .001$ for time).

3.3.2 Effect of WAS on plasma AG profile after fasting

The fasting and postprandial plasma ghrelin profile of the rats was presented in Figure 6. At baseline, the plasma ghrelin level was similar between the two groups. There was a trend of reduced postprandial plasma ghrelin in NH rats but blunted responses in MS rats. At 3 h, NH rats had a significantly lower plasma ghrelin level compared to MS rats (MS: 180.8 ± 25.91 pg/mL, NH: 108.0 ± 12.60 pg/mL,

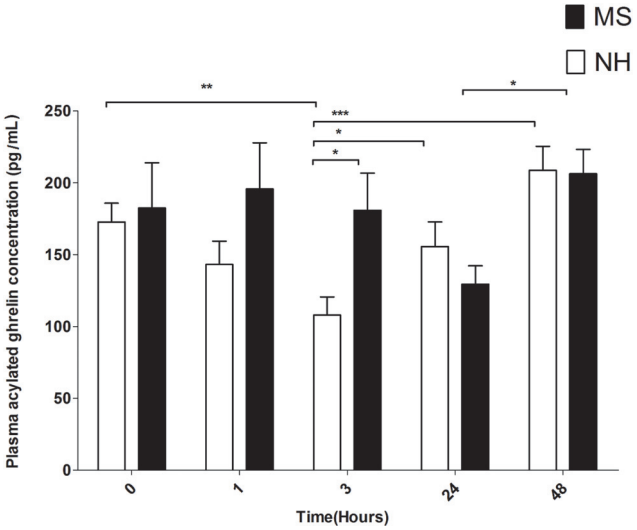

Figure 6: Plasma acylated ghrelin concentrations measured in NH and MS rats in experiment 3. Means $\pm$SEM, $n = 8-9$, $^{***}P < .001$, $^{**}P < .01$, $^{*}P < .05$. MS showed exaggerated ghrelin response up to 48 h (repeated measures of ANOVA, $P = .026$ for interaction between rat groups and time, $P = .001$ for time).

$P = .030$). However, the plasma ghrelin level restored to baseline in NH rats after 24 h, when the plasma ghrelin level in MS rats started to drop. At 48 h, there was no significant difference in plasma AG level between the two groups.

4 Discussion

This study showed that early life psychological stress in rats resulted in altered ghrelin responses and satiety function, in particularly following exposure to stressful events in adulthood. To the best of our knowledge, we have demonstrated for the potential validity of neonatal maternal separation (NMS) model for evaluation of initial binge eating and early satiation, which are common symptoms of many eating disorders such as anorexia nervosa and functional dyspepsia [19, 28].

NMS model has been shown to cause heightened anxiety in the adulthood with anxiety features such as hyper-responsiveness of HPA axis activities, hypervigilance, autonomic dysfunction, visceral hypersensitivity, and binge eating disorders [1, 12, 27, 28, 44]. Besides, hyper-responsiveness of ghrelin in NMS is compatible with the current belief of ghrelin in the role of anxiety behavior [2, 4] and it supports the notion that ghrelin dysfunction can be one of the mechanisms involved in anxiety disorder predisposed by early life adversity.

Our study has shown that early life adversity leads to aberrant feeding behavior after fasting with the characteristics of initial binge eating and early satiation. Consistent with previous studies, NMS may lead to an exaggerated feeding response to repeated fasting or refeeding challenges that may be due to increased responsiveness of the HPA axis [12, 28]. Previous studies also showed that NMS altered the preference of macronutrient and pattern of the food intake. NMS caused not only anxiety or depression-like behaviors, but also binge eating disorders after social or physiologic stressors later in life with sustained hyperphagia [12]. Additionally, sweet food intake was preferred due to NMS [30]. We expected that these changes may be associated with rapid postprandial decline in ghrelin level that led to early satiation. Our new findings shed lights on the understanding of the putative mechanisms of early life adversity involved in the development of eating disorders especially with binge eating and early satiation in adulthood. Our findings support that altered ghrelin profile may play an important role in the pathophysiology of symptoms in eating disorders. However, it is still unclear why this alteration of postprandial ghrelin developed and further studies are required to elucidate the mechanisms.

The abnormal postprandial feeding behaviors and postprandial ghrelin profile are exaggerated after additional acute psychological stress (i.e., WAS). Elucidated from our study, additional acute psychological stress not only led to exaggerated and prolonged initial binge eating related symptoms, but also development of early satiation that resulted in overall reduction in energy intake later in life in response to additional acute psychological stressors. Early satiation is a common feature in patients with many eating disorders. Besides, stress is also a common trigger of the onset of dyspeptic symptoms. These findings suggest that stress-induced dyspeptic symptoms may be mediated by abnormal ghrelin response in patients with eating disorders. Furthermore, this study lends further support to the validity and application of the NMS model as an animal model for eating disorder with postprandial distress syndrome.

Our NMS model resulted in reduction of body weight which was consistent with the study by McIntosh et al. [19] but the results are conflicting in other studies [25, 30]. The discrepancies may be attributed to differences in experimental protocol such as the species of the rats, housing conditions, procedures and the duration in neonatal handling, and nutrition components of food given. It appears that NMS causes a higher level of anxiety that may lead to response adversity towards physical and psychological stressors, resulting in abnormal secretion of ghrelin responses and aberrant appetite and satiety regulation. These changes contribute to suboptimal weight gain and lower body weight in adulthood. However, we did not investigate the metabolic rate of NMS rats during food deprivation and consumption. Repeated fasting or refeeding cycles may alter metabolic rate and reduction in energy intake [10, 12, 29, 33]. Further investigations are required to verify the inconsistencies in body weight.

5 Conclusions

In conclusion, stressful events in early life by NMS lead to hyper-responsiveness of plasma and tissue ghrelin during exposure to stressful events in the adulthood. Altered eating pattern and overall reduction in food intake were observed. These abnormalities are analogous to patients with eating disorders, particularly with history of childhood adversity. Our study may serve as the animal model with abnormal ghrelin response and also a model for investigation in potential treatment of eating disorders.

Nomenclature

AG,	acylated ghrelin
GI,	gastrointestinal
NH,	non-handling
MS,	maternal separated
NMSS,	neonatal maternal separation stress
WAS,	water avoidance stress test

Acknowledgments The authors are grateful to Prof. Leung Po Sing, School of Biomedical Science of the Chinese University of Hong Kong, for the teaching in western blotting for ghrelin detection. C. K. Y. Cheung is supported by the National Center for Complementary and Alternative Medicine (NCCAM) (grant number 1-U19-AT003266-01), research funds of Department of Medicine & Therapeutics and Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong. Its contents are solely the responsibility of the author and do not necessarily represent the official views of NCCAM.

References

[1] A. Asakawa, A. Inui, T. Kaga, H. Yuzuriha, T. Nagata, M. Fujimiya, et al., *A role of ghrelin in neuroendocrine and behavioral responses to stress in mice*, Neuroendocrinology, 74 (2001), 143–147.

[2] A. Asakawa, A. Inui, T. Kaga, H. Yuzuriha, T. Nagata, N. Ueno, et al., *Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin*, Gastroenterology, 120 (2001), 337–345.

[3] J. E. Blundell, *Serotonin manipulations and the structure of feeding behaviour*, Appetite, 7 (1986), 39–56.

[4] V. P. Carlini, M. E. Monzón, M. M. Varas, A. B. Cragnolini, H. B. Schiöth, T. N. Scimonelli, et al., *Ghrelin increases anxiety-like behavior and memory retention in rats*, Biochem Biophys Res Commun, 299 (2002), 739–743.

[5] S. V. Coutinho, P. M. Plotsky, M. Sablad, J. C. Miller, H. Zhou, A. I. Bayati, et al., *Neonatal maternal separation alters stress-induced responses to viscerosomatic nociceptive stimuli in rat*, Am J Physiol Gastrointest Liver Physiol, 282 (2002), G307–G316.

[6] D. E. Cummings, J. Q. Purnell, R. S. Frayo, K. Schmidova, B. E. Wisse, and D. S. Weigle, *A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans*, Diabetes, 50 (2001), 1714–1719.

[7] V. Folsom, D. Krahn, K. Nairn, L. Gold, M. A. Demitrack, and K. R. Silk, *The impact of sexual and physical abuse on eating disordered and psychiatric symptoms: a comparison of eating disordered and psychiatric inpatients*, Int J Eat Disord, 13 (1993), 249–257.

[8] D. T. Fullerton, S. A. Wonderlich, and B. A. Gosnell, *Clinical characteristics of eating disorder patients who report sexual or physical abuse*, Int J Eat Disord, 17 (1995), 243–249.

[9] M. E. Gluck, A. Geliebter, and M. Lorence, *Cortisol stress response is positively correlated with central obesity in obese women with binge eating disorder (BED) before and after cognitive-behavioral treatment*, Ann N Y Acad Sci, 1032 (2004), 202–207.

[10] B. Graham, S. Chang, D. Lin, F. Yakubu, and J. O. Hill, *Effect of weight cycling on susceptibility to dietary obesity*, Am J Physiol, 259 (1990), R1096–R10102.

[11] P. Groleau, H. Steiger, K. Bruce, M. Israel, L. Sycz, A. S. Ouellette, et al., *Childhood emotional abuse and eating symptoms in bulimic disorders: an examination of possible mediating variables*, Int J Eat Disord, 45 (2012), 326–332.

[12] J. W. Jahng, *An animal model of eating disorders associated with stressful experience in early life*, Horm Behav, 59 (2011), 213–220.

[13] M. Kojima, H. Hosoda, Y. Date, M. Nakazato, H. Matsuo, and K. Kangawa, *Ghrelin is a growth-hormone-releasing acylated peptide from stomach*, Nature, 402 (1999), 656–660.

[14] J. H. Koo-Loeb, N. Costello, K. C. Light, and S. S. Girdler, *Women with eating disorder tendencies display altered cardiovascular, neuroendocrine, and psychosocial profiles*, Psychosom Med, 62 (2000), 539–548.

[15] E. Kristenssson, M. Sundqvist, M. Astin, M. Kjerling, H. Mattsson, C. Dornonville de la Cour, et al., *Acute psychological stress raises plasma ghrelin in the rat*, Regul Pept, 134 (2006), 114–117.

[16] C. O. Ladd, M. J. Owens, and C. B. Nemeroff, *Persistent changes in corticotropin-releasing factor neuronal systems induced by maternal deprivation*, Endocrinology, 137 (1996), 1212–1218.

[17] D. Liu, C. Caldji, S. Sharma, P. M. Plotsky, and M. J. Meaney, *Influence of neonatal rearing conditions on stress-induced adrenocorticotropin responses and norepinepherine release in the hypothalamic paraventricular nucleus*, J Neuroendocrinol, 12 (2000), 5–12.

[18] C. Lo Sauro, C. Ravaldi, P. L. Cabras, C. Faravelli, and V. Ricca, *Stress, hypothalamic-pituitary-adrenal axis and eating disorders*, Neuropsychobiology, 57 (2008), 95–115.

[19] J. McIntosh, H. Anisman, and Z. Merali, *Short- and long-periods of neonatal maternal separation differentially affect anxiety and feeding in adult rats: gender-dependent effects*, Brain Res Dev Brain Res, 113 (1999), 97–106.

[20] N. Nagaya, T. Itoh, S. Murakami, H. Oya, M. Uematsu, K. Miyatake, et al., *Treatment of cachexia with ghrelin in patients with COPD*, Chest, 128 (2005), 1187–1193.

[21] N. Nagaya, J. Moriya, Y. Yasumura, M. Uematsu, F. Ono, W. Shimizu, et al., *Effects of ghrelin administration on left ventricular function, exercise capacity, and muscle wasting in patients with chronic heart failure*, Circulation, 110 (2004), 3674–3679.

[22] M. Nakazato, N. Murakami, Y. Date, M. Kojima, H. Matsuo, K. Kangawa, et al., *A role for ghrelin in the central regulation of feeding*, Nature, 409 (2001), 194–198.

[23] M. J. Owens and C. B. Nemeroff, *The role of corticotropin-releasing factor in the pathophysiology of affective and anxiety disorders: laboratory and clinical studies*, Ciba Found Symp, 172 (1993), 296–308.

[24] M. L. Pinheiro, V. Ferraz-de Paula, A. Ribeiro, M. Sakai, M. M. Bernardi, and J. Palermo-Neto, *Long-term maternal separation differentially alters serum corticosterone levels and blood neutrophil activity in A/J and C57BL/6 mouse offspring*, Neuroimmunomodulation, 18 (2011), 184–190.

[25] K. Ploj, E. Roman, and I. Nylander, *Long-term effects of short and long periods of maternal separation on brain opioid peptide levels in male Wistar rats*, Neuropeptides, 37 (2003), 149–156.

[26] P. Putignano, A. Dubini, P. Toja, C. Invitti, S. Bonfanti, G. Redaelli, et al., *Salivary cortisol measurement in*

normal-weight, obese and anorexic women: comparison with plasma cortisol, Eur J Endocrinol, 145 (2001), 165–171.

[27] T. H. Ren, J. Wu, D. Yew, E. Ziea, L. Lao, W. K. Leung, et al., *Effects of neonatal maternal separation on neurochemical and sensory response to colonic distension in a rat model of irritable bowel syndrome*, Am J Physiol Gastrointest Liver Physiol, 292 (2007), G849–G856.

[28] V. Ryu, J. Lee, S. Yoo, X. Gu, Y. Moon, and J. Jahng, *Sustained hyperphagia in adolescent rats that experienced neonatal maternal separation*, Int J Obes (Lond), 32 (2008), 1355–1362.

[29] M. M. Sea, W. P. Fong, Y. Huang, and Z. Y. Chen, *Weight cycling-induced alteration in fatty acid metabolism*, Am J Physiol Regul Integr Comp Physiol, 279 (2000), R1145–R1155.

[30] P. P. Silveira, C. da Silva Benetti, C. Ayres, F. Q. Pederiva, A. K. Portella, A. B. Lucion, et al., *Satiety assessment in neonatally handled rats*, Behav Brain Res, 173 (2006), 205–210.

[31] H. Steiger, *Eating disorders and the serotonin connection: state, trait and developmental effects*, J Psychiatry Neurosci, 29 (2004), 20–29.

[32] H. Steiger, L. Gauvin, M. Israël, N. Koerner, N. M. Ng Ying Kin, J. Paris, et al., *Association of serotonin and cortisol indices with childhood abuse in bulimia nervosa*, Arch Gen Psychiatry, 58 (2001), 837–843.

[33] M. B. Stein, J. R. Walker, G. Anderson, A. L. Hazen, C. A. Ross, G. Eldridge, et al., *Childhood physical and sexual abuse in patients with anxiety disorders and in a community sample*, Am J Psychiatry, 153 (1996), 275–277.

[34] M. Tanaka, T. Naruo, D. Yasuhara, Y. Tatebe, N. Nagai, T. Shiiya, et al., *Fasting plasma ghrelin levels in subtypes of anorexia nervosa*, Psychoneuroendocrinology, 28 (2003), 829–835.

[35] V. Tolle, M. Kadem, M. T. Bluet-Pajot, D. Frere, C. Foulon, C. Bossu, et al., *Balance in ghrelin and leptin plasma levels in anorexia nervosa patients and constitutionally thin women*, J Clin Endocrinol Metab, 88 (2003), 109–116.

[36] J. Tong and D. D'Alessio, *Eating disorders and gastrointestinal peptides*, Curr Opin Endocrinol Diabetes Obes, 18 (2011), 42–49.

[37] M. Tschöp, D. L. Smiley, and M. L. Heiman, *Ghrelin induces adiposity in rodents*, Nature, 407 (2000), 908–913.

[38] E. van Riel, N. G. van Gemert, O. C. Meijer, and M. Joëls, *Effect of early life stress on serotonin responses in the hippocampus of young adult rats*, Synapse, 53 (2004), 11–19.

[39] D. M. Vázquez, J. F. López, H. Van Hoers, S. J. Watson, and S. Levine, *Maternal deprivation regulates serotonin 1A and 2A receptors in the infant rat*, Brain Res, 855 (2000), 76–82.

[40] S. A. Wonderlich, T. D. Brewerton, Z. Jocic, B. S. Dansky, and D. W. Abbott, *Relationship of childhood sexual abuse and eating disorders*, J Am Acad Child Adolesc Psychiatry, 36 (1997), 1107–1115.

[41] A. M. Wren, C. J. Small, C. R. Abbott, W. S. Dhillo, L. J. Seal, M. A. Cohen, et al., *Ghrelin causes hyperphagia and obesity in rats*, Diabetes, 50 (2001), 2540–2547.

[42] Q. F. Xie, C. X. Wu, Q. Y. Meng, and N. Li, *Ghrelin and truncated ghrelin variant plasmid vectors administration into skeletal muscle augments long-term growth in rats*, Domest Anim Endocrinol, 27 (2004), 155–164.

[43] L. Zhang, V. S. Hernández, B. Liu, M. P. Medina, A. T. Nava-Kopp, C. Irles, et al., *Hypothalamic vasopressin system regulation by maternal separation: its impact on anxiety in rats*, Neuroscience, 215 (2012), 135–148.

[44] X. J. Zhang, Z. Li, W. M. Leung, L. Liu, H. X. Xu, and Z. X. Bian, *The analgesic effect of paeoniflorin on neonatal maternal separation-induced visceral hyperalgesia in rats*, J Pain, 9 (2008), 497–505.