

Sex differences in the effects of chronic stress and food restriction on body weight gain and brain expression of CRF and relaxin-3 in rats

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This study investigated sex-specific effects of repeated stress and food restriction on food intake, body weight, corticosterone plasma levels and expression of corticotropin-releasing factor (CRF) in the hypothalamus and relaxin-3 in the nucleus incertus (NI). The CRF and relaxin-3 expression is affected by stress, and these neuropeptides produce opposite effects on feeding (anorexigenic and orexigenic, respectively), but sex-specific regulation of CRF and relaxin-3 by chronic stress is not fully understood. Male and female rats were fed *ad libitum* chow (AC) or *ad libitum* chow and intermittent palatable liquid Ensure without food restriction (ACE), or combined with repeated food restriction (60% chow, 2 days per week; RCE). Half of the rats were submitted to 1-h restraint stress once a week. In total, seven weekly cycles were applied. The body weight of the RCE stressed male rats significantly decreased, whereas the body weight of the RCE stressed female rats significantly increased compared with the respective control groups. The stressed female RCE rats considerably overate chow during recovery from stress and food restriction. The RCE female rats showed elevated plasma corticosterone levels and low expression of CRF mRNA in the paraventricular hypothalamic nucleus but not in the medial preoptic area. The NI expression of relaxin-3 mRNA was significantly higher in the stressed RCE female rats compared with other groups. An increase in the expression of orexigenic relaxin-3 and misbalanced hypothalamic-pituitary-adrenal axis activity may contribute to the overeating and increased body weight seen in chronically stressed and repeatedly food-restricted female rats.

Keywords: c-fos, chronic stress, corticosterone, CRF, feeding, female, food restriction, HPA axis, male, relaxin-3

Received 5 June 2012, revised 5 November 2012, 10 December 2012 and 1 February 2013, accepted for publication 17 February 2013

Stress triggers dissimilar behavior responses and particular activation of the hypothalamic-pituitary-adrenal (HPA) axis in male and female rats (Maren *et al.* 1994; Padilla *et al.* 2009; Simpson & Kelly 2012; Taylor *et al.* 2000). The central component of the HPA axis includes the neurons of the paraventricular hypothalamic nucleus (PVN) that produce corticotropin-releasing factor (CRF), which, via the adrenocorticotrophic hormone (ACTH), stimulates synthesis and release of glucocorticoids (cortisol in humans and corticosterone in rodents) from the adrenal glands (Dallman *et al.* 1994; Herman *et al.* 1996; Sawchenko *et al.* 2000). Glucocorticoids downregulate CRF expression in the PVN. The CRF decreases food intake, and pretreatment with CRF antagonists powerfully reverses stress-induced anorexia (Krahn *et al.* 1986; Smagin *et al.* 1999). Conversely, glucocorticoids promote food intake that helps restore the energy resources depleted during the stress response (Dallman *et al.* 2004; Takeda *et al.* 2004). In the hypothalamus, CRF expression has also been detected in the medial preoptic area (MPOA) (Swanson *et al.* 1983). Interestingly, CRF decreased food intake when injected directly into the PVN (Krahn *et al.* 1988) but not to other hypothalamic areas, including the lateral hypothalamus (Krahn *et al.* 1988) and MPOA (Dagnault & Richard 1997).

The hormones of the HPA axis play an important role in regulating food intake, and imbalance of the HPA axis may lead to the development of eating disorders. For example, basal hyperactivity and higher stress-induced reactivity of the HPA axis have been reported for bulimia nervosa and binge eating (Bruce *et al.* 2012; Gluck *et al.* 2004; Hudson *et al.* 1983), eating disorders whose prevalence is more than three times higher in women compared with men (Hoek & Van Hoeken 2003; Hudson *et al.* 2007; Jara *et al.* 2009; Woodside *et al.* 2001).

In addition to glucocorticoids, hypothalamic CRF expression is regulated by many neurotransmitters and neuropeptides, including gamma-aminobutyric acid, norepinephrine, serotonin, oxytocin and neuropeptide Y (Boisvert *et al.* 2011; Bulbul *et al.* 2011; Cole & Sawchenko 2002; Dallman *et al.* 1995; Kageyama & Suda 2010; Suda *et al.* 1993; Yao & Denver 2007). The recently discovered neuropeptide relaxin-3 (Bathgate *et al.* 2002; Burazin *et al.* 2002) may potentially regulate CRF expression. Acute intracerebroventricular (icv) administration of relaxin-3 increased CRF and c-fos expression in the PVN (Watanabe *et al.* 2011a). In addition, icv and intra-PVN injections of relaxin-3 increased plasma ACTH and corticosterone levels within minutes (McGowan *et al.* 2007; Watanabe *et al.* 2011a). Relaxin-3 is a member of the insulin-like peptide family (Bathgate *et al.* 2006; Wilkinson *et al.* 2005). In contrast to other known relaxins, relaxin-3

is abundant in the brain, but not in the female reproductive tissues such as ovary and uterus (Bathgate *et al.* 2002; Liu *et al.* 2003a). In the rat, mouse and primate brain, relaxin-3 is strongly expressed in the neurons of the nucleus incertus (NI) (Burazin *et al.* 2002; Ma *et al.* 2009; Smith *et al.* 2010; Tanaka *et al.* 2005). From the NI, the relaxin-3 neurons largely project to forebrain areas, including the hypothalamus, where relaxin-3 activates its cognate receptor, the relaxin-family peptide 3 (RXFP3) receptor, whose strong expression was detected in the PVN in rat and mouse brain (Chen *et al.* 2005; Ma *et al.* 2007; Smith *et al.* 2010). Expression of relaxin-3 mRNA, heteronuclear RNA and immunoreactivity in the NI was upregulated by stress (Banerjee *et al.* 2010; Tanaka *et al.* 2005), and relaxin-3 strongly increased food intake (Hida *et al.* 2006; McGowan *et al.* 2005, 2006; Tanaka 2010). The levels of relaxin-3 expression in the brain assessed with reverse transcription polymerase chain reaction (RT-PCR) in male and female mice appear to be similar at basal conditions (Bathgate *et al.* 2002). However, the regulation of relaxin-3 expression by stress and diet in different sexes is not yet clear. In addition, there is no consensus regarding the expression of hypothalamic CRF in male and female rats because higher, lower or no difference in PVN CRF expression in rats of different sexes has been reported (Almeida *et al.* 1997; Duncko *et al.* 2001; Givalois *et al.* 1997; Patchev *et al.* 1995). Investigating the reactivity of the HPA axis to physical stress and metabolic challenges may help to understand sex-specific vulnerability in the development of eating disorders.

This study was designed to investigate the sex-specific effects of repeated stress and dieting on body weight, food intake, HPA axis activity and CRF and relaxin-3 expression in the brains of male and female rats. The results showed that chronic stress and food restriction produced opposite effects on body weight gain in different sexes. Chronically stressed and repeatedly food-restricted female rats with intermittent access to highly palatable liquid Ensure had hyperphagia and increased body weight and showed imbalanced activity of the HPA axis with a significant increase in plasma corticosterone levels and a decrease in PVN CRF expression as well as significant increase in relaxin-3 expression in the NI.

Methods

Animals

Male ($n=36$) and female ($n=44$) Sprague Dawley rats of the same age (8 weeks) were purchased from the Canadian Breeding Laboratories (St-Constant, QC, Canada). All rats were housed in the same room in individual plastic cages lined with wood shavings and maintained on a 12:12 h dark–light cycle (lights on between 0600 and 1800 h), with ambient temperature of $23 \pm 1^\circ\text{C}$, free access to tap water and the standard laboratory rat diet (Rat/Mouse/Hamster chow; 1000 Formula; 12.9 kJ/g; Agway Prolab, Syracuse, NY, USA), unless otherwise specified. All rats were cared for and handled according to the *Canadian Guide for the Care and Use of Laboratory Animals*, and this protocol was approved by our institutional animal care committee.

Weekly treatment cycles

During the first week, all rats were fed *ad libitum* chow and were not subjected to stress. After the first week, the rats were randomly divided into two cohorts.

The first rat cohort (12 male and 12 female rats, fed chow) was fed *ad libitum* chow (AC groups). The male and female rats in the first cohort were randomly divided into non-stressed groups (male, ACm-NS and female, ACf-NS; $n=6$) and groups submitted to 1-h restraint stress per week (male, ACm-S and female, ACf-S; $n=6$).

The rats of the second cohort (24 male and 32 female rats, fed chow and Ensure) were fed *ad libitum* chow (ACE groups), or were food-restricted for 2 days (first and second days of the weekly cycles; RCE groups; Fig. 2a) when the food-restricted rats were provided with 60% of the chow eaten by the control, non-stressed *ad libitum*-fed groups of the same sex. After food restriction, all groups in the second cohort received *ad libitum* chow until the following food restriction in the next weekly cycle as well as 2-h access to high-lipid high-carbohydrate chocolate-flavored liquid Ensure (Abbott Nutrition, Saint-Laurent, QC, Canada; energy 1.06 kcal/ml; proteins 15.0 kcal%; carbohydrates 61.0 kcal%; lipids 24.0 kcal%; cacao powder, 12 vitamins and 17 minerals) on the first and second days of refeeding after food restriction (third and fourth days of the weekly cycles). On the fifth and sixth days of the weekly cycles ('recovery' days), all rats in the second cohort received only *ad libitum* chow until the stress session. On the seventh day of the weekly cycles, the rats were non-stressed (male ACEm-NS, RCEm-NS, $n=6$; female ACEf-NS, RCEf-NS, $n=8$) or stressed (male ACEm-S, RCEm-S, $n=6$; female ACEf-S, RCEf-S, $n=8$) by 1-h restraint stress. Rodent sling suits secured in rodent sling frames (VWR International, Montreal, QC, Canada) were used for the restraint stress sessions performed in a separate sound-proofed room. During the stress sessions, the non-stressed rats remained in their home cages without food to mimic the feeding conditions of the stressed rats, which did not eat during 1-h stress. The stress and feeding paradigms used in this study were similar to those used in our previous study (Martin & Timofeeva 2010). Immediately after the stress session, all rats in the second cohort had 2-h access to chocolate Ensure and chow. After this 2-h post-stress feeding, the following cycle with the next food restriction began (Fig. 2a). In total, seven cycles of weekly treatments were carried out, and all rats in the first and second cohorts were killed immediately after the last (the seventh) stress session at the beginning of light onset. The female rats were killed at the diestrus phase of the estrous cycle.

Body weight and food intake were measured every day. Chow and Ensure intake was measured by subtracting the amount of chow and Ensure solution remaining from the fixed amount provided to the rats. Food spillage was carefully calculated and accounted for in the measurements.

Determination of the estrous cycle

Vaginal smears were collected in the morning (0700 h) and then stained with methylene blue (Sigma, Oakville, ON, Canada). The phases of the estrous cycle were identified according to the morphological changes in the vaginal smear. Diestrus was defined as the phase when the vaginal smear predominantly contained leukocytes. Proestrus, the following phase, contained leukocytes and nucleated epithelial cells in the smear. At estrus, which followed proestrus, the smear contained predominantly enucleated epithelial cells. Finally, metestrus was identified by the presence of enucleated epithelial cells and leukocytes.

Plasma corticosterone determination

The intracardial blood samples were taken in anesthetized rats immediately before the intracardial perfusion with saline. Plasma corticosterone was determined in duplicates with a Corticosterone Double antibody ^{125}I Radioimmunoassay Kit for rats and mice (MP Biomedicals, Solon, OH, USA; sensitivity, 7.7 ng/ml; interassay coefficient of variation, 6.5–7.2%).

Brain preparation

Brains were prepared as previously described (Poulin & Timofeeva 2008). Briefly, rats were rapidly anesthetized (60 mg/kg ketamine plus 7.5 mg/kg xylazine) and perfused intracardially with 200 ml of saline followed by 500 ml of paraformaldehyde (4%) solution. The brains

were removed at the end of perfusion and kept in paraformaldehyde for an additional period of 7 days. The brain were then transferred to a solution containing paraformaldehyde (4%) and sucrose (20%) before being cut 12 h later using a sliding microtome (HistoSlide 2000, Heidelberg, Germany). Thirty-micron-thick sections were collected and stored at -30°C in a cold sterile cryoprotecting solution containing sodium phosphate buffer (50 mM), ethylene glycol (30%) and glycerol (20%).

In situ hybridization for CRF, c-fos and relaxin-3

The CRF, c-fos and relaxin-3 mRNAs expression levels were measured with *in situ* hybridization as previously described (Martin & Timofeeva 2010; Mitra *et al.* 2011). Briefly, the sections were mounted on poly-L-lysine-coated slides and were fixed for 20 min in paraformaldehyde (4%), digested for 30 min at 37°C with proteinase K (10 $\mu\text{g}/\text{ml}$ in 100 mM Tris-HCl containing 50 mM EDTA, pH 8.0), acetylated with acetic anhydride (0.25% in 0.1 M triethanolamine, pH 8.0) and dehydrated through graded concentrations of alcohol. Sections were incubated overnight with antisense ^{35}S -labeled cRNA probes (10⁷ cpm/ml) of CRF [generated from the 1063-bp fragment of rat CRF cDNA (Dr K. Mayo, Northwestern University, Evanston, IL, USA)], c-fos [generated from the 2116-bp fragment of rat c-fos cDNA (Dr I. Verma, the Salk Institute, La Jolla, CA, USA)] or relaxin-3 [generated from the 436-bp fragment of rat relaxin-3 cDNA (gene bank NM_170667); the probe included a 17-452 bp sequence of the complete 470-bp rat relaxin-3 cDNA; sense primer: 5' GCTGATCATGGCAACTCGGG 3'; antisense primer: 5' CAGATCCTAGCACAAAGCTGC 3'] at 60°C . Slides were rinsed with sodium chloride-sodium citrate solution, digested with RNase-A (20 $\mu\text{g}/\text{ml}$), washed in descending concentrations of sodium chloride-sodium citrate solutions and dehydrated in ethanol gradient. Slides were defatted in toluene, dipped in NTB2 nuclear emulsion (Eastman Kodak, Rochester, NY, USA) and exposed for 1 week before being developed. Slides were examined with dark-field microscopy using an Olympus BX61 microscope (Olympus Canada, Richmond Hill, ON, Canada). Images were acquired with a DVC-2000C digital camera (DVC Company Inc., Austin, TX, USA) and analyzed with Stereo Investigator software version 9.13 (MBF Bioscience, Williston, VT, USA). The system was calibrated for each set of analyses to prevent saturation of the integrated signal. Mean optical density (OD) was obtained by taking measurements of the pixels of the positive hybridization signal on two to four sections of the parvocellular and magnocellular parts of the PVN (PVNp and PVNm, respectively; 1.6–1.9 mm caudal to the bregma), the MPOA (0.8–0.9 mm rostral to the bregma) and the pars compacta and pars dissipata of the NI (NIc and NId, respectively; 9.6–9.8 mm caudal to the bregma) and subtracting the background readings taken from the areas immediately surrounding the region analyzed.

Statistical analysis

Results are presented as mean values \pm standard errors of the mean. Repeated-measures analysis of variance (ANOVA) was used to detect the main and interactive effects of the diet, stress and day of experiment on the body weight of the male and female rats in the first and second cohorts. The chow intake for the first week was analyzed conjointly for the first and second rat cohorts with one-way ANOVA. A regression analysis tested correlations between body weight and chow intake for all rats during the first week of the experiment. Two-way ANOVA was used to detect the main and interactive effects of stress (stressed and non-stressed rats) and gender (male and female rats) on the chow intake of the first rat cohort; three-way ANOVA was used to detect the main and interactive effects of stress, gender and diet (food-restricted and fed *ad libitum*) on the chow and Ensure intake of the second rat cohort during the third to eighth weeks of the experiment. Plasma corticosterone and OD measurements were analyzed using three-way ANOVA to show the main and interactive effects of stress, gender and diet. *Post hoc* comparisons between groups were realized using Fisher's protected least significant difference (PLSD). Results were considered significant with *P*-values < 0.05 . Each experimental group in the first cohort included six rats. In the second cohort, the male

and female groups included six and eight rats, respectively. Statistical analysis was performed using StatView5 and JMP4 software (SAS Institute, Raleigh, NC, USA).

Results

Body weight

Body weight of the first cohort (AC groups)

At the beginning of the experiment (day 1), the body weight of the chow-fed groups was 209.8 ± 2.3 g and 240.8 ± 4.1 g for the female and male rats, respectively. The male rats' higher body weight compared with that of their female counterparts of the same age corresponds to the normal body weight range for Sprague Dawley rats (Council 1995).

Repeated-measures ANOVA of the body weight of the chow-fed male rats showed significant effects of day ($F_{56,285} = 362.6$; $P < 0.0001$) and stress ($F_{56,285} = 7.6$; $P = 0.0331$) as well as significant interaction between day and stress ($F_{56,285} = 8.1$; $P < 0.0001$) (Fig. 1a).

Repeated-measures ANOVA of the body weight of the chow-fed female rats showed significant effect of day ($F_{56,285} = 111.7$; $P < 0.0001$) but not stress ($F_{56,285} = 0.3$; $P = 0.5862$) or interactive effects of day and stress ($F_{56,285} = 0.2$; $P = 0.9999$).

Post hoc analyses did not show significant difference between the ACf-NS and ACf-S rats at any day of the experiment (Fig. 1b). In contrast, the body weight of the ACm-S rats was significantly lower compared with the body weight of the ACm-NS rats on days 23–35, 37–40 and 42–57 ($P < 0.05$) (Fig. 1a). Therefore, the body weight of male, but not female, chow-fed rats persistently decreased after the second stress session.

Body weight of the second cohort (ACE and RCE groups)

At the beginning of the experiment (day 1), the female rats' body weight was 211.9 ± 1.5 g, and the male rats' body weight was 238.2 ± 2.2 g.

Repeated-measures ANOVA of the body weight of the male rats showed significant effect of day ($F_{56,285} = 887.5$; $P < 0.0001$) as well as significant interactions between day and diet ($F_{56,285} = 4.8$; $P < 0.0001$), between day and stress ($F_{56,285} = 5.5$; $P < 0.0001$), but not between day, diet and stress ($F_{56,285} = 0.7$; $P = 0.92$) (Fig. 2b).

Repeated-measures ANOVA of the female rats' body weight showed significant effect of day ($F_{56,399} = 593.5$; $P < 0.0001$) as well as significant interactions between day and diet ($F_{56,399} = 3.2$; $P < 0.0001$), between day and stress ($F_{56,399} = 1.9$; $P < 0.05$) and between day, diet and stress ($F_{56,399} = 4.7$; $P < 0.0001$) (Fig. 2c).

Post hoc analyses did not show significant differences between the ACEm-NS, ACEm-S and RCEm-NS groups or the ACEf-NS, ACEf-S and RCEf-NS groups. Conversely, the body weight of food-restricted chronically stressed groups gradually decreased in male rats (RCEm-S; Fig. 2b) but consistently increased in female rats (RCEf-S; Fig. 2c) compared with their respective control groups. In fact, the body weight of the RCEm-S rats was significantly lower

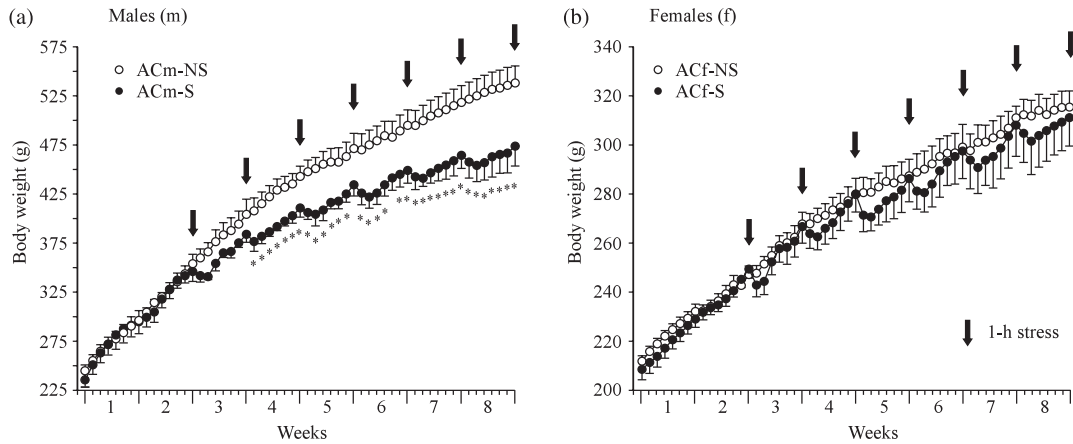


Figure 1: Body weight and weekly treatment cycles. The body weight of male (a) and female (b) chow-fed (first cohort) rats. The rats were stressed (-S) or not (-NS) with 1-h restraint stress (shown by arrows) applied once a week. All rats were killed immediately after the seventh stress session. *Significantly ($P < 0.05$, repeated-measures ANOVA followed by Fisher's PLSD) different from non-stressed rats in the same gender. Each group included six rats.

compared with the ACem-NS rats on days 31–33 and 36–57 ($P < 0.05$). In addition, the body weight of the RCEm-S rats was significantly lower compared with the ACem-S rats on days 38, 39, 46, 53, 56 and 57 ($P < 0.05$). Moreover, body weight of the RCEm-S rats was significantly lower ($P < 0.05$) compared with that of the RCEm-NS rats on days 55–57 (Fig. 1b). Conversely, the body weight of the RCEf-S rats was significantly higher compared with the RCEf-NS rats on days 41, 43, 47–50 and 54–57 ($P < 0.05$). In addition, the body weight of the RCEf-S rats was significantly higher compared with the ACEf-S rats on days 49–50 and 55–57 ($P < 0.05$). Finally, the body weight of the RCEf-S rats was significantly higher compared with the ACEf-NS rats on days 55–57 ($P < 0.05$).

Therefore, after 3 weeks of treatment (more specifically, after three weekly stress sessions and four periods of food restriction), the body weight of the chronically stressed food-restricted male rats appeared to be significantly lower compared with that of the non-stressed, *ad libitum*-fed male rats. At the end of the seven weekly cycles (seven stress and seven food restriction), the body weight of the RCEm-S rats was significantly lower compared with all other male groups (Fig. 2b; Table 3). In contrast, after 4 weeks of treatment (more specifically, after four weekly stress sessions and five periods of food restriction), the body weight of the RCEf-S rats started to be significantly higher compared with the food-restricted non-stressed female rats. At the end of the seven weekly cycles (seven stress and seven food restriction), the body weight of the female food-restricted chronically stressed rats was significantly higher compared with all other female groups (Fig. 2c; Table 3).

Food intake

Chow intake during the first week of the experiment

The first rat cohort (the AC groups) was fed *ad libitum* chow for the total duration of the experiment. The second rat cohort (the ACE and RCE groups) was fed *ad libitum* chow

for the first week. Figure 3 shows the daily chow intake during the first week of the experiment for all the rats in the first and second cohorts. Female rats at the estrus phase, but not at metestrus or proestrus, consumed a significantly lower ($P < 0.05$) daily amount of chow compared with that eaten by the rats at diestrus at the same day (Fig. 3a). During the first week, male rats consumed on average 120% of the chow eaten by female rats, but the body weight of the male rats was on average 30% higher than that of the female rats. A regression analysis showed a positive correlation between body weight and food intake ($r^2 = 0.38$; $P < 0.0001$) for rats during the first week of the experiment (Fig. 3b). Accordingly, food intake measurements taken over the weekly treatment cycles were corrected to body weight.

Food intake by the first cohort of rats

Analyses showed particular effects of stress on the chow intake of the male and female rats in the first cohort (Table 1). ANOVA showed the significant effects of stress ($F_{1,20} = 12.1$; $P = 0.0023$) and gender ($F_{1,20} = 5.6$; $P = 0.0266$) but not interactive effects of these factors ($F_{1,20} = 0.1$; $P = 0.7257$) on 24-h chow intake after the acute, first stress session (third week of the experiment). *Post hoc* analyses showed a significant decrease in 24-h chow intake after the first stress in male ($P = 0.0133$) and female ($P = 0.0387$) rats (Table 1).

Analyses of the 24-h chow intake after the fifth and sixth stress sessions (seventh and eighth weeks of the experiment) showed significant effects of stress ($F_{1,20} = 8.2$; $P = 0.0094$) and gender ($F_{1,20} = 18.0$; $P = 0.0004$), but these factors did not have interactive effects ($F_{1,2} = 1.4$; $P = 0.2516$). The *post hoc* analyses showed a significant stress-induced decrease in 24-h chow intake by male ($P = 0.0096$) but not female ($P = 0.2458$) rats after the fifth and sixth stress session (Table 1).

During the 'recovery' days (fifth and sixth days of the weekly treatment cycles), the male and female rats did not show effects of stress on chow intake in the week following

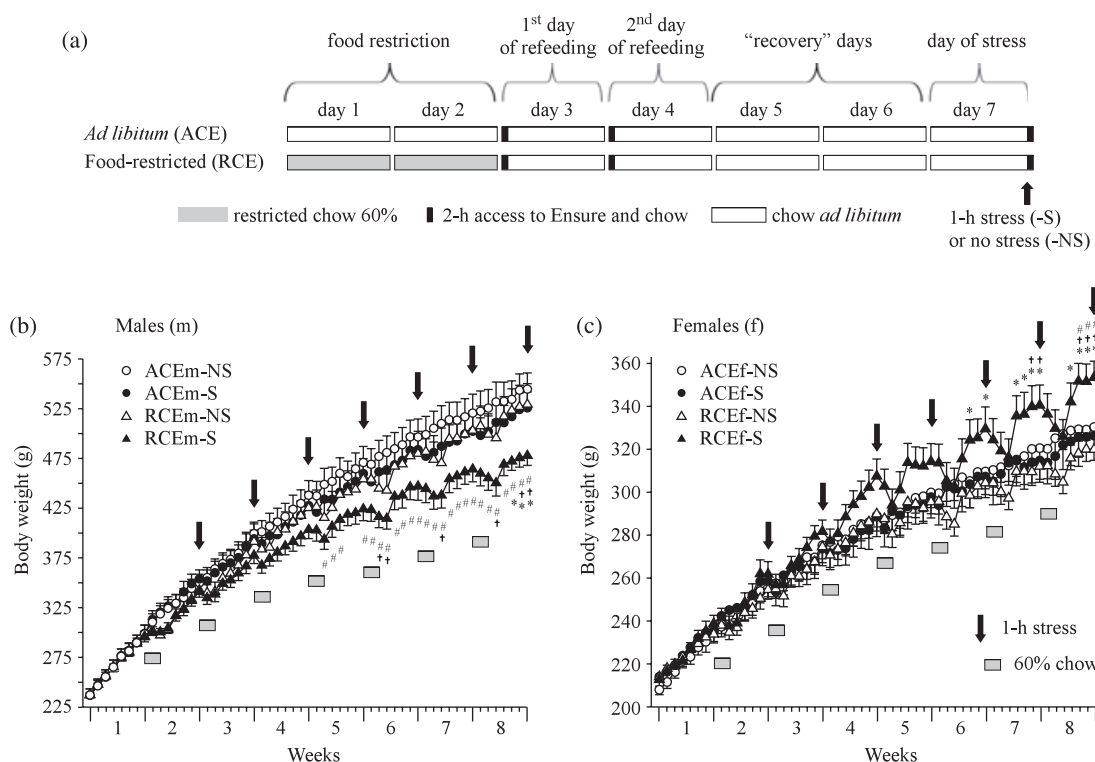


Figure 2: Body weight and weekly treatment cycles. The body weight of male (b) and female (c) chow-and-Ensure-fed (second cohort) rats. (a) Schematic representation of the weekly treatment cycles. During the first week, all rats were fed *ad libitum* chow. From the second week, all rats had in each week three 2-h access to chocolate Ensure on the third day (first day of refeeding), fourth day (second day of refeeding) and seventh day (immediately after stress) of the weekly cycle. On the first and second days of the weekly cycles, the rats were fed *ad libitum* chow (ACE groups) or were submitted to food restriction (RCE groups) consisting of 60% of chow ingested by the non-stressed non-food-restricted rats of the same sex. On the seventh day of the weekly cycles, the rats were stressed (-S) or not (-NS) with 1-h restraint stress followed by 2-h access to chow and Ensure for all rats. The rats were killed immediately after the seventh stress session. (b) The body weight of male rats over the 8 weeks of the experiment. (c) The body weight of female rats over the 8 weeks of the experiment. *Significantly ($P < 0.05$, repeated-measures ANOVA followed by Fisher's PLSD) different from the food-restricted non-stressed rats within the same gender. †Significantly different from the *ad libitum*-fed stressed rats within the same gender. #Significantly different from the *ad libitum*-fed non-stressed rats within the same gender. Each group of male rats included six rats and each group of female rats included eight rats.

the first stress session (third week of the experiment). However, female rats consumed a lower amount of chow per gram of their body weight compared with their male counterparts (ANOVA: stress effects $F_{1,20} = 0.5$; $P = 0.4805$; gender effects $F_{1,20} = 17.9$; $P = 0.0004$; gender and stress interaction $F_{1,20} = 0.1$; $P = 0.7759$) (Table 1). ANOVA and *post hoc* analyses did not show significant effects of stress ($F_{1,20} = 1.3$; $P = 0.2532$) on chow intake on the non-stressed days (fifth and sixth days of the weekly treatment cycles) during the seventh and eighth weeks of the experiment. However, the gender effect ($F_{1,20} = 5.8$; $P = 0.0259$) but not gender and stress interaction ($F_{1,2} = 0.2$; $P = 0.6475$) was significant because of the decrease in chow intake per gram of body weight in the male rats. In general, over the time, the male but not female rats showed a substantial decrease in chow intake per gram of their body weight. Because a decrease in chow intake per gram of body weight during the experiment was seen in stressed and non-stressed male

rats, this decrease was seemingly dependent on the different general dynamics of body weight gain in male and female rats.

Therefore, the first acute stress session significantly decreased 24-h chow intake in male and female rats. The chronically stressed male but not female rats showed a persistent decrease in 24-h chow intake after the stress session (Table 1).

Food intake of the second rat cohort

During the weekly treatment cycles (Fig. 2a), on the food-restricted days (first and second days of the weekly treatment cycles), the food-restricted groups of the second rat cohort received 60% of the chow eaten by the control, *ad libitum*-fed non-stressed rats. On the first and second refeeding days (third and fourth days of the weekly treatment cycles, respectively), all rats received *ad libitum* chow and 2-h daily access to Ensure. For the following 'recovery' (fifth and sixth

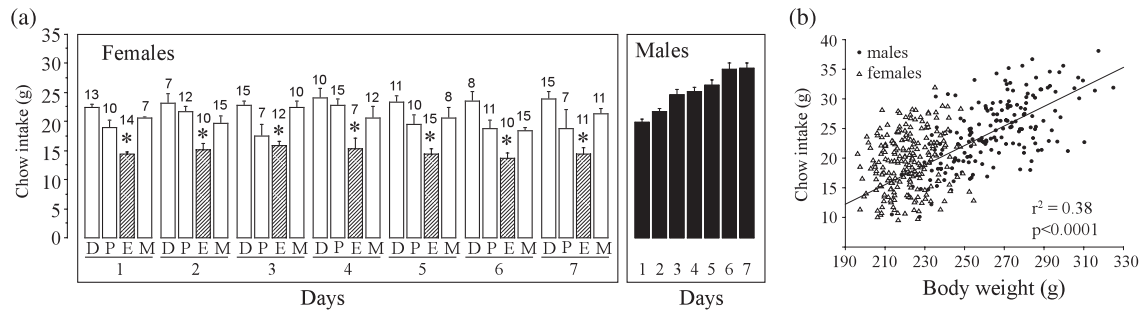


Figure 3: Chow intake during the first week of the experiment by the first and second rat cohorts. (a) Chow intake for each day of the first week by the female ($n=44$) and male ($n=36$) rats. Analyses of the female rats' chow intake were performed taking into account the phases of the estrous cycle (D, diestrus; P, proestrus; E, estrus; M, metestrus). The numbers above the bars of the female rats' chow intake correspond to the number of rats for each estrus phase of the corresponding day. (b) The correlation between daily chow intake and body weight for all rats during the first week. *Significantly ($P < 0.05$, one-way ANOVA followed by Fisher's PLSD) different from the rats in the diestrus phase on the same day.

Table 1: Chow intake (mg/g body weight) by the first cohort of rats (AC groups)

	ACm-NS	ACm-S	ACf-NS	ACf-S	ANOVA
24-h chow intake after stress					
First stress (third week)	76.9 ± 2.0	62.7 ± 2.1*	66.7 ± 5.5	55.2 ± 3.9*	S, G
Fifth and sixth stress (seventh and eighth weeks)	54.6 ± 1.5	46.2 ± 1.6*	60.8 ± 3.1	57.4 ± 1.4	S, G
24-h chow intake during 'recovery' days (fifth and sixth days of the weekly cycle)					
Third week	78.4 ± 1.7	75.3 ± 3.8	64.3 ± 3.9	63.0 ± 2.4	G
Seventh and eighth weeks	54.3 ± 0.8	52.8 ± 1.2	60.3 ± 2.5	56.9 ± 2.9	G

Two-way ANOVA followed by Fisher's PLSD showed the main and interactive effects of stress (S; stressed vs. non-stressed) and gender (G; male vs. female) on chow intake by the first cohort of rats.

*Significantly ($P < 0.05$) different from non-stressed rats in the same gender and at the same time.

days of the weekly cycles, all rats were maintained on *ad libitum* chow. On the seventh day of the weekly cycles, the rats were fed *ad libitum* chow until they were stressed (-S groups) or not (-NS groups) by 1-h restraint stress followed by 2-h access to Ensure and chow. The rats were not hungry at stress because they ate *ad libitum* chow 3 days, before the stress session. The rats' Ensure and chow intake was measured for all periods, but when Ensure was available, the rats did not eat chow, or the amount of chow they ate was minimal.

ANOVA showed significant effect of stress ($F_{1,48} = 4.1$; $P = 0.0473$) and gender ($F_{1,48} = 5.1$; $P = 0.0287$), but not diet ($F_{1,48} = 0.1$; $P = 0.7731$), and these factors had no interactive effects on the 2-h Ensure intake after the first stress session in the third week of the experiment (Table 2). *Post hoc* analyses showed that the 2-h Ensure intake after the first stress session was significantly lower ($P = 0.0287$) in the RCEm-S rats compared with the RCEm-NS rats. The rats' access to Ensure after the first stress session was the third access to Ensure (after two 2-h Ensure intake on the first and second refeeding days in the second week); therefore, the taste of Ensure was familiar to all the rats at the first stress session.

The 2-h Ensure intake after the fifth and sixth stress sessions was similar between the groups. According to ANOVA, gender ($F_{1,48} = 2.5$; $P = 0.1215$), stress ($F_{1,48} = 0.9$;

$P = 0.3475$) and diet ($F_{1,48} = 0.3$; $P = 0.6089$) did not have significant main and interactive effects. Therefore, female rats consumed the maximum amount of Ensure from the beginning and did not show a further increase in Ensure intake during the post-stress 2-h access. Male rats showed a considerable increase in their 2-h Ensure intake that was significantly lower compared with female rats at the beginning and became comparable to that of the female rats at the end of the experiment (Tables 2 and 3). In the food-restricted male rats but not other rats, anorectic effects on the 2-h Ensure intake after the first stress session were observed, but these anorectic effects were blunted in chronically stressed RCEm-S rats (Table 2).

The effects of stress and gender were not significant for the refeeding days (Table 2). In general, during the first refeeding days the food-restricted rats overate chow (ANOVA diet effect for the third week: $F_{1,48} = 57.6$; $P < 0.0001$; for the seventh and eighth weeks: $F_{1,48} = 81$; $P < 0.0001$), whereas during the second refeeding days on the third week they overate Ensure (ANOVA diet effect: $F_{1,48} = 4.2$; $P = 0.0465$), and in the seventh and eighth weeks they ingested more chow (ANOVA diet effect: $F_{1,48} = 14.6$; $P = 0.0004$). Therefore, during refeeding hunger was a predominant factor affecting eating in both sexes.

In the first post-stressed week (third week of the experiment), the food-restricted rats consumed more chow

Table 2: Chow (mg/g body weight) and Ensure (μl/g body weight) intake by the second cohort of rats (ACE and RCE groups)

	ACEm-NS	ACEm-S	RCEm-NS	RCEm-S	ACEf-NS	ACEf-S	RCEf-NS	RCEf-S	ANOVA
2-h Ensure after stress									
First stress (third week)	63.4 ± 6.6	57.3 ± 3.5	75.4 ± 11.8	56.6 ± 11.1*	87.6 ± 6.1	69.0 ± 9.1	80.1 ± 10.2	72.4 ± 6.1	S, G
Fifth and sixth stress (seventh and eighth weeks)	82.4 ± 3.3	79.7 ± 5.9	82.6 ± 4.8	75.3 ± 1.8	84.1 ± 3.2	87.6 ± 6.7	87.6 ± 4.3	81.6 ± 3.1	
2-h Ensure on the first day of refeeding									
Third week	63.6 ± 5.2	68.3 ± 6.6	71.6 ± 9.1	66.1 ± 9.0	69.9 ± 6.6	74.8 ± 3.9	81.8 ± 6.0	79.0 ± 5.9	
Seventh and eighth weeks	59.5 ± 5.6	54.6 ± 5.2	53.5 ± 8.1	49.8 ± 3.7	59.3 ± 4.1	61.3 ± 6.8	65.7 ± 4.2	63.9 ± 5.9	
2-h Ensure on the second day of refeeding									
Third week	67.6 ± 9.1	58.7 ± 3.4	81.5 ± 6.0	70.7 ± 5.1	77.1 ± 5.3	70.6 ± 8.3	81.9 ± 5.4	76.1 ± 4.3	D
Seventh and eighth weeks	59.1 ± 4.4	56.6 ± 4.1	58.5 ± 7.8	54.8 ± 4.3	66.2 ± 2.3	74.2 ± 7.2	69.4 ± 8.0	58.3 ± 5.9	
24-h chow intake on the first day of refeeding									
Third week	58.8 ± 2.0	56.2 ± 1.7	81.4 ± 4.1 [†]	73.6 ± 4.3 [†]	60.2 ± 2.3	59.3 ± 3.6	72.5 ± 2.3 [†]	73.3 ± 3.0 [†]	D
Seventh and eighth weeks	38.6 ± 2.3	39.6 ± 1.6	54.1 ± 1.9 [†]	55.8 ± 2.5 [†]	45.5 ± 1.9	46.7 ± 2.0	59.4 ± 1.3 [†]	62.7 ± 3.8 [†]	D
24-h chow intake on the second day of refeeding									
Third week	56.0 ± 3.1	55.8 ± 2.7	54.5 ± 3.3	57.4 ± 2.9	56.4 ± 2.7	52.9 ± 3.2	51.7 ± 1.3	55.1 ± 4.1	
Seventh and eighth weeks	41.8 ± 2.3	45.0 ± 1.0	47.5 ± 1.6	49.2 ± 0.9	47.6 ± 1.6	42.5 ± 2.2	50.1 ± 2.6	53.6 ± 2.8 [†]	D
24-h chow intake during 'recovery' days (fifth and sixth days of the weekly cycles)									
Third week	70.3 ± 1.2	69.2 ± 2.7	74.8 ± 2.1	70.9 ± 2.8	62.9 ± 2.3	63.9 ± 3.1	68.2 ± 2.5	73.6 ± 2.9 [†]	D, G
Seventh and eighth weeks	54.2 ± 0.7	58.1 ± 1.0	60.7 ± 1.2 [†]	56.7 ± 1.8	63.7 ± 1.3	63.6 ± 1.8	62.7 ± 1.9	72.9 ± 2.3 ^{*,†,‡}	G, D, S, G × S, G × D × S

Three-way ANOVA followed by Fisher's PLSD showed the main and interactive effects of stress (S; stressed vs. non-stressed), diet (D; *ad libitum* fed vs. food-restricted) and gender (G; male vs. female rats) on chow and Ensure intake.

*Significantly different from non-stressed rats in the same gender and the same feeding conditions.

[†]Significantly ($P < 0.05$) different from ACE rats in the same gender and the same stressful condition.

[‡]Significantly different from all other groups.

during the 'recovery' days (fifth and sixth days of the weekly treatment cycles) (ANOVA diet effect: $F_{1,48} = 7.9$; $P = 0.0071$). In addition, ANOVA showed significant effects of the gender ($F_{1,48} = 4.8$; $P = 0.0324$) because in general, male rats consumed more chow per gram of body weight compared with female rats. *Post hoc* analyses did not show significant differences between the male groups, whereas the female rats in the RCEf-S group consumed significantly more chow compared with the ACEf-S rats (Table 2). The main effect of stress ($F_{1,48} = 0.03$; $P = 0.8616$) and the interactive effects of stress, gender and diet were not significant.

Chow intake during the 'recovery' (fifth and sixth) days of the weekly treatment cycles in the seventh and eighth weeks of the experiment showed significant main effects of diet ($F_{1,48} = 7.6$; $P = 0.0083$), gender ($F_{1,48} = 45.2$; $P < 0.0001$) and stress ($F_{1,48} = 4.1$; $P = 0.0498$) as well as significant gender × stress ($F_{1,48} = 4.3$; $P = 0.0434$) and gender × diet × stress interactions ($F_{1,48} = 13.5$; $P = 0.0006$). *Post hoc* analyses showed a significant increase in the chow intake by RCEm-NS rats compared with the ACEm-NS rats ($P = 0.0168$) as well as a significantly higher intake of chow by RCEf-S rats compared with all other groups ($P < 0.0001$

vs. ACEm-NS, ACEm-S, RCEm-NS, RCEm-S and RCEf-NS; $P = 0.0002$ vs. ACEf-NS and ACEf-S).

Tables 1–3 show the average food intake for female rats at all phases of the estrous cycle. When considered separately, during the non-estrus phases (metestrus, diestrus and proestrus) the female rats had higher food intake compared with the estrus phases, but during the non-estrus and estrus phases, the rats followed the same tendencies (data not shown) detected for the average values.

HPA axis activity

The plasma levels of corticosterone and brain expression of mRNAs were estimated in all rats after seven weekly cycles of stress and refeeding. The stressed rats were killed immediately after the last (the seventh) stress session simultaneously with their respective non-stressed control groups. The blood samples were taken in anesthetized rats by intracardial puncture. This procedure may probably affect the basal levels of corticosterone. However, the procedure was similar for all rats in order to obtain comparable data, and the time between anesthesia, intracardial blood samples and beginning of intracardial perfusion was shortened to a

Table 3: Major differences between repeatedly food-restricted chronically stressed male (RCEm-S) and female (RCEf-S) rats

	RCEm-S	RCEf-S	RCEf-S vs. RCEm-S (%)
2-h Ensure after acute (first) stress	56.6 ± 11.1	72.4 ± 6.1	+(27.9 ± 10.9)%
2-h Ensure after chronic (fifth and sixth) stress	75.3 ± 1.8	81.6 ± 3.1	+(8.4 ± 3.6)%
24-h chow on the days of 'recovery' from stress and food restriction in chronically stressed (seventh and eighth weeks) rats	56.7 ± 1.8	72.9 ± 2.3	+(28.4 ± 3.9)%
Relative body weight gain	90.2% ± 2.0%	111.1% ± 2.2%	
Plasma corticosterone	800.1 ± 38.9	1105.6 ± 96.7	+(38.2 ± 12.8)%
CRF mRNA in the PVNp	54.8 ± 3.7	39.5 ± 2.9	-(28.1 ± 5.6)%
c-fos mRNA in the PVNp	30.9 ± 3.4	20.8 ± 2.5	-(32.6 ± 8.7)%
c-fos mRNA in the PVNm	28.9 ± 4.8	19.0 ± 1.9	-(34.2 ± 6.8)%
CRF mRNA in the MPOA	14.3 ± 1.7	24.9 ± 1.5	+(73.8 ± 11.3)%
Relaxin-3 mRNA in the Nlc	43.9 ± 4.6	64.2 ± 4.3	+(46.0 ± 9.8)%
Relaxin-3 mRNA in the Nld	15.7 ± 0.8	20.4 ± 2.1	+(29.2 ± 14.3)%

Ensure: µl/g body weight; chow: mg/g body weight; relative body weight gain is expressed as a percentage of the starting body weight normalized to the body weight of non-stressed group; plasma corticosterone: ng/ml; CRF, c-fos and relaxin-3 mRNAs: optical density of the positive hybridization signal. PVNp and PVNm – parvocellular and magnocellular parts of the paraventricular hypothalamic nucleus, respectively; MPOA – medial preoptic area; Nlc and Nld – pars compacta and dissipata of the nucleus incertus, respectively.

few minutes to minimize the impact of anesthesia and blood sampling on the measured variables.

Plasma corticosterone

ANOVA showed the significant effect of gender ($F_{1,68} = 13.5$; $P = 0.0005$), stress ($F_{1,68} = 28.6$; $P < 0.0001$) and diet ($F_{1,68} = 18.7$; $P < 0.0001$) but not the interactive effects of these factors on the plasma corticosterone levels (Fig. 4a). *Post hoc* analysis showed that stress significantly increased the corticosterone levels in chow-fed male ($P = 0.0041$, ACm-S vs. ACm-NS) and female ($P = 0.0072$, ACf-NS vs. ACf-S) rats. In the ACE and RCE groups, the corticosterone levels were not significantly different between the stressed and non-stressed rats. The corticosterone levels of the ACE rats were not significantly different from those of the AC rats in the basal non-stressful conditions ($P = 0.1805$, ACEm-NS vs. ACm-NS; $P = 0.0777$, ACEf-NS vs. ACf-NS). Conversely, the corticosterone levels of the non-stressed RCE rats were significantly higher compared with that of the non-stressed AC counterparts ($P = 0.0167$, RCEm-NS vs. ACm-NS; $P < 0.0001$, RCEf-NS vs. ACf-NS). The RCEf-S rats had significantly higher corticosterone levels compared with the ACf-S rats ($P = 0.0013$). In the stressful and non-stressful conditions, female RCE rats had significantly higher corticosterone levels compared with their male RCE counterparts ($P = 0.0026$, RCEm-NS vs. RCEf-NS; $P = 0.0096$, RCEm-S vs. RCEf-S).

Therefore, the stress-induced increase in plasma corticosterone levels was significant in the chow-fed groups but not in the chow-and-Ensure-fed rats. Food restriction significantly increased corticosterone plasma levels. Higher plasma corticosterone levels were observed in the female food-restricted rats.

CRF mRNA expression in the PVNp

The CRF mRNA expression in the PVNp was affected by gender (ANOVA $F_{1,68} = 5.1$; $P = 0.0264$), stress ($F_{1,68} = 11.4$;

$P = 0.0012$) and diet ($F_{2,68} = 5.8$; $P = 0.0046$). The interaction of gender and diet was also significant ($F_{2,68} = 4.7$; $P = 0.0125$) (Fig. 4b). *Post hoc* analysis showed a significant difference between the ACm-NS and ACm-S rats ($P = 0.0381$). The difference between the stressed and non-stressed ACem and RCEm rats was not significant. In the female rats, the difference in CRF mRNA expression between the stressed and non-stressed groups did not reach levels of significance in any feeding condition. In contrast, the food-restricted non-stressed female rats had significantly lower CRF expression levels compared with all others except the RCEf-S groups (RCEf-NS vs. ACm-NS, $P = 0.0263$; vs. ACm-S, $P < 0.0001$; vs. ACEm-NS, $P = 0.0418$; vs. ACEm-S, $P = 0.0003$; vs. RCEm-NS, $P = 0.0417$; vs. RCEm-S, $P < 0.0001$; vs. ACf-NS, $P = 0.0043$; vs. ACf-S, $P < 0.0001$; vs. ACEf-NS, $P = 0.0102$ and vs. ACEf-S, $P = 0.0029$). Food-restricted stressed female rats had significantly lower CRF mRNA expression levels compared with all stressed male groups (RCEf-S vs. ACm-S, $P = 0.0003$; vs. ACEm-S; $P = 0.0029$ and vs. RCEm-S, $P = 0.0010$) and compared with the stressed and non-stressed female AC and ACE groups (RCEf-S vs. ACf-NS, $P = 0.0297$; vs. ACf-S, $P = 0.0009$; vs. ACEf-NS, $P = 0.0402$ and ACEf-S, $P = 0.0246$).

These results showed that restraint stress significantly increased CRF mRNA expression in chow-fed male rats but not in other groups. The levels of CRF mRNA expression in the RCEf-S rats were significantly lower compared with other stressed groups (Fig. 5).

c-fos mRNA expression in the PVN

Lower CRF mRNA expression in the PVN of the female food-restricted rats was accompanied by lower stress-induced induction of expression of c-fos mRNA in the PVN of the stressed food-restricted female rats (Figs. 4c,d,6). The levels of c-fos mRNA expression were analyzed in the parvocellular and magnocellular parts of the PVN, which show high density of the relaxin-3-immunopositive terminals

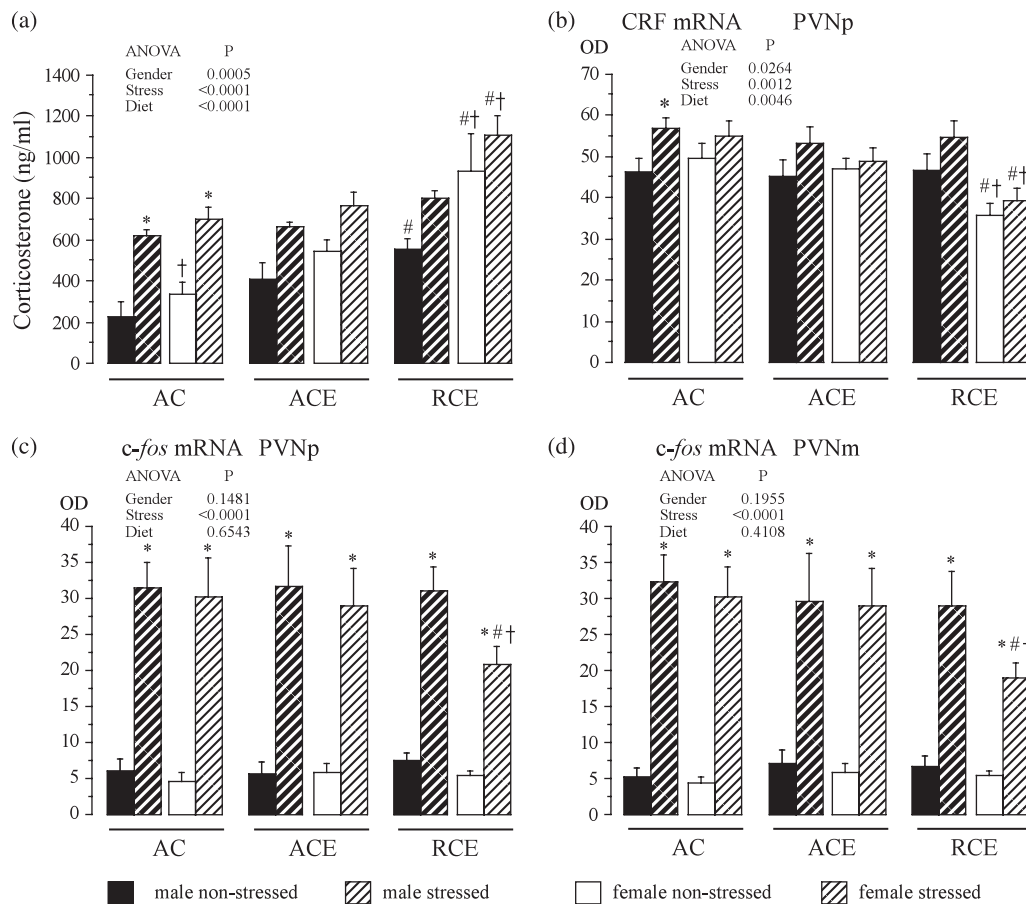


Figure 4: The activity of the hypothalamic-pituitary-adrenal (HPA) axis in male and female rats. (a) Corticosterone plasma levels. (b) The optical density (OD) of the positive hybridization signal of CRF mRNA in the parvocellular part of the paraventricular hypothalamic nucleus (PVNp). (c) The OD of the positive hybridization signal of *c-fos* mRNA in the PVNp. (d) The OD of the positive hybridization signal of *c-fos* mRNA in the magnocellular part of the paraventricular hypothalamic nucleus (PVNm). The histograms represent male and female stressed and non-stressed rats in the first cohort, chow-fed rats (AC) and the second cohort, *ad libitum* (ACE) or food-restricted (RCE) chow-and-Ensure-fed rats. *Significantly ($P < 0.05$, three-way ANOVA followed by Fisher's PLSD) different from non-stressed rats in the same gender and feeding condition. #Significantly different from the AC group in the same gender and stressful condition. †Significantly different from the male rats in the same feeding and stressful condition.

and high levels of RXFP3 expression (Ma *et al.* 2007; Tanaka 2010). In general, the *c-fos* mRNA transcript levels were barely detectable in the non-stressed rats. The final, seventh session of restraint stress triggered significant induction of *c-fos* mRNA expression in the PVNp ($P < 0.0001$, ACm-S vs. ACm-NS, ACEm-S vs. ACEm-NS, RCEm-NS vs. RCEm-S, ACf-S vs. ACf-NS and ACEf-NS vs. ACEf-S; $P = 0.0004$, RCEf-S vs. RCEf-NS) and PVNm ($P < 0.0001$, ACm-S vs. ACm-S, ACEm-S vs. ACEm-NS, ACf-S vs. ACf-NS and ACEf-S vs. ACEf-NS; $P = 0.0002$, RCEm-S vs. RCEm-NS; $P = 0.0028$, RCEf-S vs. RCEf-NS) in all rat groups (Fig. 4c,d). In the PVNp, ANOVA showed significant effects of stress ($F_{1,68} = 138.4$; $P < 0.0001$), but not diet ($F_{2,68} = 0.4$; $P = 0.6543$), gender ($F_{1,68}$; $P = 0.1481$) or interactive effects. Similarly, in the PVNm ANOVA showed significant effect of stress ($F_{1,68} = 120.7$; $P < 0.0001$), but not diet ($F_{2,68} = 0.9$; $P = 0.4108$), gender ($F_{1,68} = 1.7$; $P = 0.1955$) or interactive

effects. In the PVNp, *post hoc* analysis showed significantly lower levels of *c-fos* mRNA expression in stressed female food-restricted rats compared with the stressed male groups ($P = 0.0218$, RCEf-S vs. ACm-S; $P = 0.0183$, RCEf-S vs. ACEm-S; $P = 0.0276$, RCEf-S vs. RCEm-S) and the stressed female *ad libitum* chow-fed rats ($P = 0.0416$, RCEf-S vs. ACf-S). In the PVNm, the levels of *c-fos* mRNA expression were significantly lower in the RCEf-S group compared with all other stressed groups ($P = 0.0051$, RCEf-S vs. ACm-S; $P = 0.0289$, RCEf-S vs. ACEm-S; $P = 0.0399$, RCEf-S vs. RCEm-S; $P = 0.0215$, RCEf-S vs. ACf-S; $P = 0.0252$, ACEf-S vs. RCEf-S).

CRF mRNA expression in the MPOA

The levels of CRF mRNA expression in the MPOA were significantly higher in female rats (ANOVA gender effect:

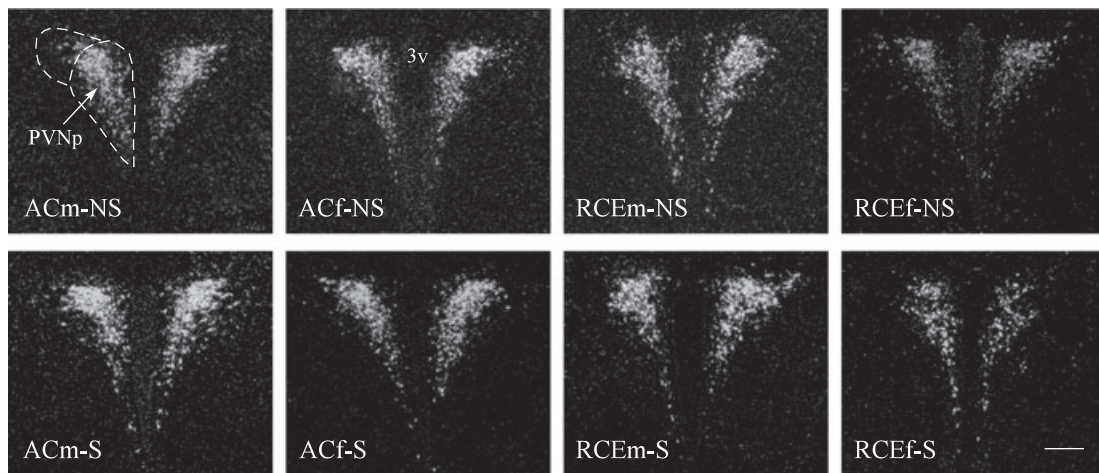


Figure 5: Food-restricted female rats showed low levels of CRF mRNA expression in the parvocellular part of the paraventricular hypothalamic nucleus (PVNp). Dark-field photomicrographs of the coronal brain sections depict the expression of CRF mRNA in the PVNp of non-stressed (-NS) or stressed (-S) male (ACm) and female (ACf) *ad libitum* chow-fed rats and male (RCEm) and female (RCEf) food-restricted chow-and-Ensure-fed rats. 3v, third ventricle. The scale bar corresponds to 300 μ m.

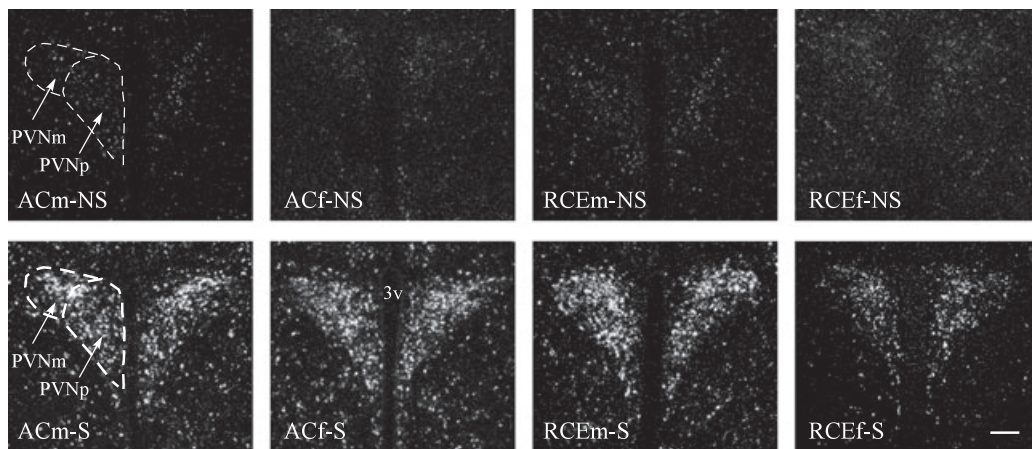


Figure 6: Food-restricted chronically stressed female rats showed low levels of *c-fos* mRNA expression in the parvocellular (PVNp) and magnocellular (PVNm) parts of the paraventricular hypothalamic nucleus. Dark-field photomicrographs of the coronal brain sections show the expression of *c-fos* mRNA in the non-stressed (-NS) or stressed (-S) male (ACm) and female (ACf) *ad libitum* chow-fed rats and male (RCEm) and female (RCEf) food-restricted chow-and-Ensure-fed rats. 3v, third ventricle. The scale bar corresponds to 200 μ m.

$F_{1,68}=45.7$; $P<0.0001$) in all experimental conditions compared with male rats (Figs. 7a,8). Chronic stress strongly increased CRF mRNA expression in the MPOA (ANOVA stress effect: $F_{1,68}=8.9$; $P=0.0038$). ANOVA did not show a significant effect of diet ($F_{2,68}=2.7$; $P=0.0707$) or interactive effects of diet, gender and stress on CRF mRNA expression in the MPOA. However, *post hoc* analysis showed that in contrast to inhibition of the expression of CRF mRNA in the PVNp (Fig. 4b), food restriction did not decrease the levels of CRF mRNA expression in the MPOA of female rats. Quite the contrary, the levels of the CRF transcript in the MPOA of the RCEf-NS rats were significantly higher compared with the ACf-NS rats ($P=0.0309$).

Therefore, the levels of CRF mRNA expression in the MPOA were significantly higher in female rats compared with male rats. Chronic stress increased CRF mRNA expression in the MPOA. In addition, repeated food restriction led to sex-specific increases in the levels of MPOA CRF expression in female rats (Fig. 7a; Table 3).

Relaxin-3 mRNA expression in the NI

ANOVA showed significant effects of diet ($F_{2,68}=6.4$; $P=0.0030$), gender ($F_{1,68}=8.7$; $P=0.0045$) and stress ($F_{1,68}=6.3$; $P=0.0145$), as well as significant interactive effects of diet and gender ($F_{2,68}=3.5$; $P=0.0365$) on the

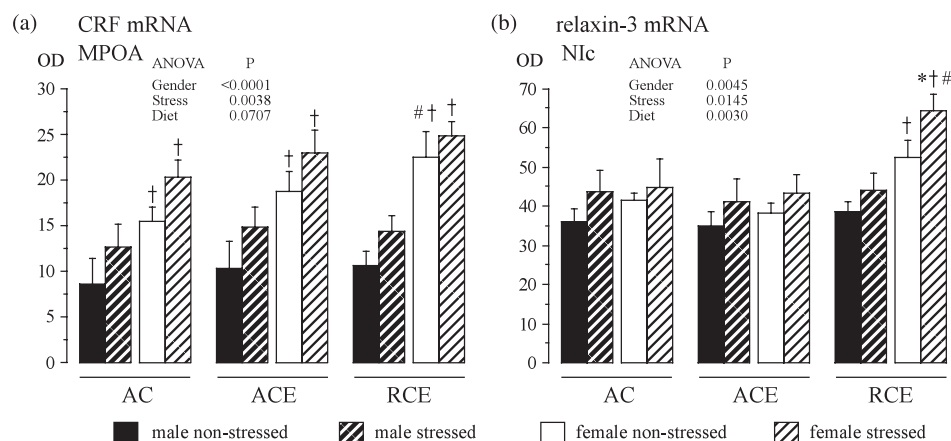


Figure 7: Expression of corticotropin-releasing factor (CRF) in the medial preoptic area (MPOA) and relaxin-3 in the nucleus incertus. (a) The optical density (OD) of the positive hybridization signal of CRF mRNA in the MPOA. (b) The OD of the positive hybridization signal of relaxin-3 mRNA in the pars compacta of the nucleus incertus (Nlc). The histograms represent male and female stressed and non-stressed rats in the first cohort, chow-fed rats (AC) and the second cohort, *ad libitum* (ACE) or food-restricted (RCE) chow-and-Ensure-fed rats. *Significantly ($P < 0.05$, three-way ANOVA followed by Fisher's PLSD) different from non-stressed rats in the same gender and feeding condition. #Significantly different from the AC group in the same gender and stressful condition. †Significantly different from the male rats in the same feeding and stressful condition.

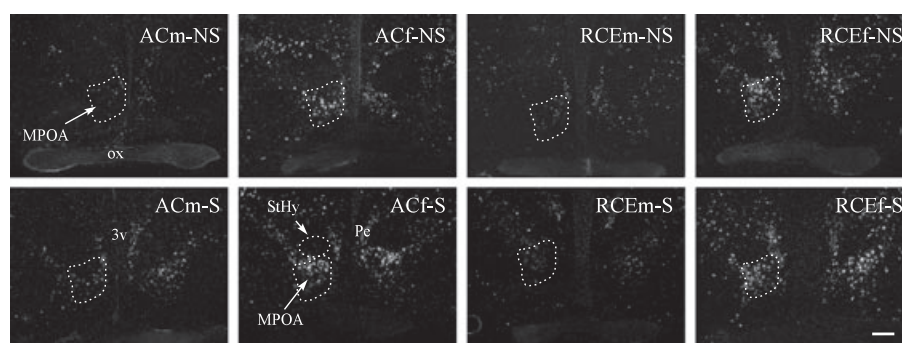


Figure 8: Expression of corticotropin-releasing factor (CRF) in the medial preoptic area (MPOA.) Dark-field photomicrographs of the coronal brain sections show the expression of CRF mRNA in the non-stressed (-NS) or stressed (-S) male (ACm) and female (ACf) *ad libitum* chow-fed rats and male (RCEm) and female (RCEf) food-restricted chow-and-Ensure-fed rats. 3v, third ventricle; ox, optic chiasm; Pe, periventricular hypothalamic nucleus; StHy, striohypothalamic nucleus. The scale bar corresponds to 300 μ m.

levels of relaxin-3 expression in the Nlc (Figs. 7b,9). In fact, stress and food restriction increased relaxin-3 expression in the Nlc, but this increase was significantly higher in female rats compared with male rats. *Post hoc* analysis showed that relaxin-3 levels were significantly higher in the RCEf-NS rats compared with the RCEm-NS ($P = 0.0300$) rats. In addition, stress significantly increased relaxin-3 expression in the RCEf-S rats to levels significantly higher than those of all other groups ($P < 0.0001$, RCEf-S vs. ACm-NS, ACem-NS, RCEm-NS and ACEf-NS; $P = 0.0015$, RCEf-S vs. ACm-S; $P = 0.0008$, RCEf-S vs. ACEm-S; $P = 0.0009$, RCEf-S vs. RCEm-S; $P = 0.0005$, RCEf-S vs. ACf-NS; $P = 0.0025$, RCEf-S vs. ACf-S; $P = 0.0002$, RCEf-S vs. ACEf-S; $P = 0.0459$, RCEf-S vs. RCEf-NS).

In the Nld (Fig. 9), ANOVA showed significant effects of stress ($F_{1,68} = 8.0$; $P = 0.0061$), gender ($F_{1,68} = 7.9$;

$P = 0.0066$) and diet ($F_{2,68} = 5.3$; $P = 0.0072$) on the levels of relaxin-3 mRNA expression. However, the interactive effects of these factors were not significant. Generally, the regulation of relaxin-3 mRNA expression in the Nld showed the same tendency detected for the Nlc. The stress- and food-restriction-induced increase in relaxin-3 expression was higher in female rats. *Post hoc* analysis showed a significant increase in the levels of relaxin-3 mRNA expression in the Nld in the RCEf-S rats compared with the RCEm-S rats ($P = 0.0353$; Table 3).

Discussion

This study was designed to investigate sex differences in the effects of chronic stress, food restriction and intermittent

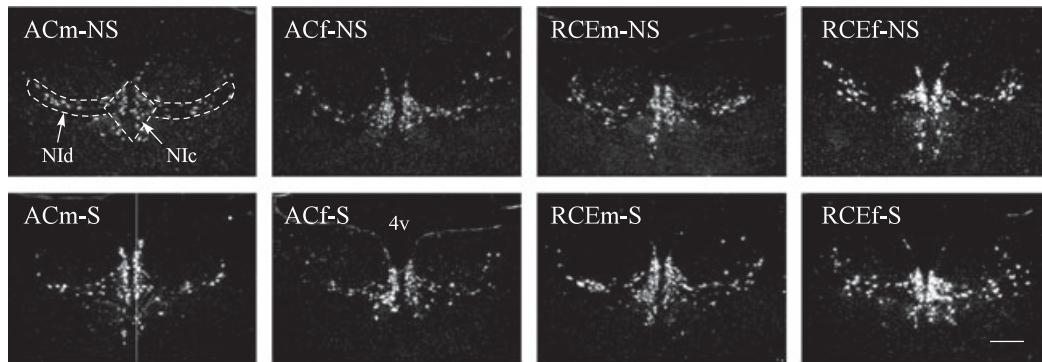


Figure 9: Food-restricted chronically stressed female rats showed high levels of relaxin-3 mRNA expression in the nucleus incertus. Dark-field photomicrographs of the coronal brain sections depict the expression of relaxin-3 mRNA in non-stressed (-NS) or stressed (-S) male (ACm) and female (ACf) *ad libitum* chow-fed rats and male (RCEm) and female (RCEf) food-restricted chow-and-Ensure-fed rats. 4v, fourth ventricle. NId – pars dissipata and Nlc – pars compacta of the nucleus incertus. The scale bar corresponds to 300 μ m.

access to palatable food on food intake regulation. The data showed that repeated restraint stress considerably decreased body weight gain in male but not female *ad libitum* chow-fed rats. Intermittent access to Ensure blunted the anorectic effects of stress in male rats. However, when the male chronically stressed chow-and-Ensure-fed (RCEm-S) rats were submitted to repeated food restriction, their body weight gain persistently decreased compared with the non-stressed respective controls. Conversely, the body weight gain of chronically stressed chow-and-Ensure-fed female rats (RCEf-S) repeatedly subjected to food restriction significantly increased compared with the non-stressed controls (Table 3). The chronically stressed food-restricted female rats had significantly increased chow intake compared with all other groups during the days of 'recovery' from stress and food restriction. The plasma corticosterone levels and relaxin-3 mRNA expression in the NI were significantly increased, whereas CRF and *c-fos* mRNAs expression in the PVN was significantly decreased in female chronically stressed food-restricted rats compared with male rats in similar conditions.

Male and female chow-fed rats (first cohort) had significantly decreased 24-h chow intake after the first, acute stress. The effect of the acute stress was brief, and within the same week, the stressed and non-stressed rats consumed comparable amounts of food on the non-stress days. Male but not female chow-fed rats showed a persistent anorectic effect in response to repeatedly applied stress. This persistent anorectic effect of chronic stress in the male rats was reflected by a significant decrease in the body weight gain of the male but not female rats. In a previous study, we showed a similar significant decrease in the body weight of chow-fed chronically stressed male rats (Martin & Timofeeva 2010). The present results are also in agreement with the earlier reports showing that chronic mild stress significantly reduced growth in male rats while having little or no impact on female growth (Duncko *et al.* 2001; Lin *et al.* 2008, 2009; Trentani *et al.* 2003; Westenbroek *et al.* 2003, 2004, 2005).

The second, chow-and-Ensure-fed rat cohort had 2-h access to Ensure immediately after stress, in a non-hungry

state, and on the first and second days of refeeding after 2 days of food restriction. Intermittent access to a palatable diet, such as sugar, sweet cookies or lard, may trigger development of binge-like eating of palatable foods, and repeated food restriction and stress may exacerbate this behavior (Avena *et al.* 2008b; Boggiano & Chandler 2006; Corwin 2004; Corwin & Buda-Levin 2004; Cottone *et al.* 2008, 2012; Hagan & Moss 1997; Hagan *et al.* 2002; Martin & Timofeeva 2010). In agreement with the previous reports (Kinzig *et al.* 2008; Martin & Timofeeva 2010), adding palatable Ensure to the diets effectively blunted the anorectic effects of stress in male rats. In addition, the male rats showed a considerable escalation in Ensure intake throughout the weekly cycles that corroborates the significant increased intake of palatable food by intermittent access to this food (Alsiö *et al.* 2012; Avena *et al.* 2008a, 2008b; Corwin 2006; Corwin *et al.* 2011; Corwin & Wojnicki 2006; Cottone *et al.* 2012; Hagan *et al.* 2002; Levine *et al.* 2003; Mitra *et al.* 2010, 2011). However, the female rats from the beginning of the experiment reached their maximal consumption of Ensure, and their consumption did not increase furthermore. The present experiment did not show a specific increase in Ensure intake induced by stress. The particular composition of Ensure, highly palatable high-fat high-carbohydrate balanced (containing vitamins and minerals) liquid food, may explain some discrepancies between our data and other reports showing that highly palatable but not balanced foods produced stress-induced bingeing (Boggiano & Chandler 2006; Boggiano *et al.* 2005; Hagan *et al.* 2002).

Food restriction significantly increased chow intake during the first day of refeeding in all food-restricted rats. The chronically stressed food-restricted female rats (RCEf-S) continued overeating chow during the 'recovery' days. This persistent overeating by the chronically stressed food-restricted female rats was reflected in a significant increase in the body weight gain of these rats.

Acute stress activates an extensive neuronal network (De Kloet *et al.* 2005; McEwen 1998; Timofeeva *et al.* 2003)

converging at the parvocellular division of the PVN (Herman *et al.* 2003; Sawchenko *et al.* 2000), the brain component of the HPA axis. Assessment of HPA axis activity has shown that non-stressed chow-fed female rats had higher basal levels of corticosterone compared with the male littermates. Conversely, a significant increase in CRF mRNA expression in response to chronic restraint stress was observed in male but not female chow-fed rats. The present results are in agreement with the numerous reports showing higher basal corticosterone plasma levels in female compared with male rats (Babb *et al.* 2013; Duncko *et al.* 2001; Iwasaki-Sekino *et al.* 2009; Patchev *et al.* 1995; Seale *et al.* 2004b; Sterrenburg *et al.* 2011). However, the diurnal and ovarian cycles must be taken into account because corticosterone measurements in daytime and at estrus did not show sex-specific differences (Atkinson & Waddell 1997; Patchev *et al.* 1995). Important to note, the stressed groups of this study were repeatedly stressed by weekly 1-h restraint stress during 7 weeks. Dependently on the type of stress, acute stress may strongly activate the HPA axis of female (Babb *et al.* 2013; Iwasaki-Sekino *et al.* 2009; Seale *et al.* 2004a; Weinstock *et al.* 1998) or male rats (Sterrenburg *et al.* 2012; Zavala *et al.* 2011). However, chronic stress may blunt sex-specific activation of the PVN neurons (Zavala *et al.* 2011) or induce significantly higher expression of CRF and *c-fos* mRNAs in the PVN in male rats (Duncko *et al.* 2001; Sterrenburg *et al.* 2011). The present data are in agreement with the previous reports showing lower production of CRF in the PVN in female chronically stressed rats compared with the male counterparts (Duncko *et al.* 2001; Sterrenburg *et al.* 2011).

Given the strong CRF anorectic effects (Bell *et al.* 1998; Bjénning & Rimvall 2000; Hotta *et al.* 1991; Jones *et al.* 1999; Pellemounter *et al.* 2000; Richard *et al.* 2002; Rothwell 1990; Semjonous *et al.* 2009; Smagin *et al.* 1998; Stengel *et al.* 2009; Tanaka *et al.* 2009; Vergoni *et al.* 1999), the hyperactivation of the CRF PVNp neurons in response to repeated restraint stress in male but not female rats may explain the sustained decrease in body weight gain and the persisting anorectic effects of stress during the weekly treatments in the male chow-fed rats. This anorectic stress effect was abolished in the male *ad libitum*-fed rats by adding palatable Ensure to their diet. Diets including sweet palatable ingredients may downregulate the induction of CRF expression in the PVNp (Dallman *et al.* 2003; Laugero *et al.* 2001; Martin & Timofeeva 2010). Indeed, the chow-and-Ensure-fed male and female rats did not show a significant increase in PVNp CRF mRNA levels in response to stress.

Repeated food restriction reversed the blunting effects of palatable Ensure on the body weight loss induced by chronic stress in male rats. Indeed, the RCEm-S rats had significantly decreased body weight gain compared with the non-stressed control group. These data are in agreement with the significantly lower body weight and cumulative food intake reported in food-restricted chronically stressed male rats compared with the control groups that were only chronically stressed or only food-restricted (Harris *et al.* 2002). Harris *et al.* (2002) have shown blunted hyperphagia occurred after the end of stress and food restriction in chronically stressed food-restricted male rats. Similarly, our

experiments showed blunted hyperphagia of the chronically stressed RCEm-S rats (a significant increase in food intake in the RCEm-NS but not in the RCEm-S rats compared with their respective *ad libitum*-fed control groups) on the days of 'recovery' from stress and food restriction. It seems that CRF and relaxin-3 were not implicated in the blunted hyperphagia of the RCEm-S rats because the expression of these neuropeptides in the RCEm-S rats was not significantly different compared with other male rats. Similarly, Harris *et al.* (2006) have shown that the persistent decrease in body weight in male rats with a history of chronic restraint stress was not accompanied by alterations in the expression of CRF or its receptors. Blunted hyperphagia and the decrease in body weight gain in the RCEm-S rats may depend on activation of the brain melanocortin system producing strong anorectic and thermogenic effects (Haynes *et al.* 1999; Richard *et al.* 2010; Skibicka & Grill 2009; Song *et al.* 2008). Restraint stress increases body temperature and energy expenditure, which is not compensated by either a decrease in expenditure or an increase in food intake during the post-stress period in male rats (Harris *et al.* 2002, 2006). The sex differences in stress-induced activation of the brain melanocortin system and its differential implication in the thermogenic and anorectic stress effects in male and female rats require further investigation.

Female food-restricted rats showed higher levels of basal and stress-induced plasma corticosterone compared with the male food-restricted groups. This result is in agreement with a higher increase in the levels of plasma corticosterone reported for the food-restricted female rats compared with male counterparts (Martin *et al.* 2007). Conversely, the female food-restricted rats had significantly lower basal and stress-induced levels of PVNp CRF mRNA expression compared with the respective male groups. Accordingly, food-restricted female rats showed significantly lower stress-induced expression of *c-fos* mRNA in the PVN compared with other groups. A transcript of immediate-early gene *c-fos* is a marker of persistent neuronal activation, and the Fos protein is implicated in transactivation of the CRF gene (Yao & Denver 2007). Lower stress-induced CRF and *c-fos* PVNp expression as well as higher plasma corticosterone levels may facilitate the overeating seen in the chronically stressed food-restricted female rats. Further experiments involving adrenalectomized rats with gradual low-to-high corticosterone replacement may examine the implication of glucocorticoids in the development of the overeating and overweight phenotype of the RCEf-S rats. However, because an increase in corticosterone and a decrease in PVN CRF mRNA levels were detected in non-stressed and stressed food-restricted female rats, an imbalance in the HPA axis activity in female food-restricted rats did not explain why stressed but not non-stressed food-restricted female rats overate chow and persistently gained weight.

The present model of chronically stressed hyperphagic female rats may presume the involvement of a brain orexigenic factor activated by stress. Restraint stress did not affect the levels of orexigenic neuropeptide Y mRNA expression in the rat hypothalamus (Rybkin *et al.* 1997), but water-restraint stress significantly increased expression of relaxin-3 mRNA in the NI (Tanaka *et al.* 2005). Relaxin-3 is a

member of the insulin-like peptide family strongly expressed in the neurons of the NI (Bathgate *et al.* 2002; Burazin *et al.* 2002; Liu *et al.* 2003a; Wilkinson *et al.* 2005). Intra-PVN administration of relaxin-3 significantly increased food intake in satiated rats within hours after injection (McGowan *et al.* 2005). Chronic intrabrain injection of relaxin-3 significantly increased the cumulative food intake, body weight and epididymal fat mass (Hida *et al.* 2006; McGowan *et al.* 2006). Relaxin-3 KO mice showed resistance to an increase in body weight, adiposity and plasma leptin and insulin levels observed in wild-type mice maintained on a moderately high-fat (6%) diet (Sutton *et al.* 2009). Other independently inbred relaxin-3 KO mice colonies displayed a normal metabolic phenotype but altered anxiety-related behavior (Watanabe *et al.* 2011b) and circadian hypoactivity (Smith *et al.* 2012). Genotyping of human relaxin-3 (*RLN3*) and *RXFP3* genes showed several associations between polymorphism in these genes and hypercholesterolemia, obesity and diabetes (Munro *et al.* 2011).

The terminals of the relaxin-3 neurons are broadly distributed in the brain, including the PVN, where relaxin-3 is released from the dense-cored vesicles and binds with high affinity to the RXFP3 receptor (Liu *et al.* 2003b; Ma *et al.* 2007; Tanaka *et al.* 2005; Wilkinson & Bathgate 2007). Activation of the RXFP3 receptor triggers the protein kinase C–signal-regulated kinase (ERK) 1/2 pathway (van der Westhuizen *et al.* 2007), which may facilitate transcription of CRF in the PVNp neurons (Blume *et al.* 2009). Potential stimulation of CRF expression by relaxin-3 was suggested by the increased expression of PVN CRF mRNA after icv administration of relaxin-3 (Watanabe *et al.* 2011a). Relaxin-3 icv and intra-PVN administration also increased the plasma ACTH and corticosterone levels within minutes, suggesting activation of the HPA axis (McGowan *et al.* 2007; Watanabe *et al.* 2011a).

This study has shown that relaxin-3 mRNA expression in the NI was significantly increased by restraint stress. This result is in agreement with a reported increase in the NI in the levels of relaxin-3 mRNA in water-restraint stress (Tanaka *et al.* 2005) and the increased levels of heteronuclear RNA, mRNA and relaxin-3 immunoreactivity in repeated forced swim (Banerjee *et al.* 2010) as well as activation of the NI by different stressful conditions [for review see (Ryan *et al.* 2011)]. ANOVA also showed a significant effect of diet on the relaxin-3 mRNA expression in the NI. Seven weekly cycles of food restriction increased relaxin-3 expression. This upregulation of relaxin-3 expression by food restriction mirrors the usual increase in the expression of orexigenic neuropeptides by a negative energy balance (Harrold & Halford 2006; Morton & Schwartz 2001). The significant interactive effects of diet and gender on the levels of relaxin-3 expression in the Nlc showed sex-specific regulation of relaxin-3 by food restriction. In fact, food restriction induced significantly stronger expression of relaxin-3 in female compared with male RCE rats. The precise mechanisms of sex-specific regulation of relaxin-3 expression by food restriction are not yet known. Sexual dimorphism at the hormonal plasma levels in response to negative energy balance shown, for example, for plasma estradiol, testosterone, corticosterone and ghrelin (Gayle

et al. 2006; Martin *et al.* 2007), as well as modulation of estrogen anorectic effects by food restriction (Sieck *et al.* 1978) may contribute to sex-specific regulation of relaxin-3 expression by food restriction. Chronic restraint stress led to additional upregulation of the expression of relaxin-3 mRNA in the Nlc of RCEf-S rats to the significantly higher levels compared with all other groups. Interestingly, the body weight gain of the RCEf-S rats considerably increased during the weekly cycles of repeated food restriction and stress, and the RCEf-S rats showed significant hyperphagia during the 'recovery' (following stress and food restriction) days. Given the orexigenic and obesitogenic effects of relaxin-3 (Hida *et al.* 2006; Sutton *et al.* 2009), a significant increase in relaxin-3 expression in the NI of the RCEf-S rats may contribute to the development of the hyperphagic and overweight phenotype of these rats. The orexigenic effect of relaxin-3 is effectively blocked by pretreatment with antagonists of the RXFP3 receptor, a cognate receptor of relaxin-3 (Ganella *et al.* 2012; Haugaard-Kedstrom *et al.* 2011; Kuei *et al.* 2007; Shabanpoor *et al.* 2012). However, acute and chronic administration of the RXFP3 antagonists alone did not produce anorectic effects in male rats (Haugaard-Kedstrom *et al.* 2011; Kuei *et al.* 2007; Sutton *et al.* 2009). This ineffectiveness of relaxin-3 antagonists was probably dependent on the low basal activity of relaxin-3 in the models used. The chronically stressed food-restricted female rats with increased levels of relaxin-3 expression may represent an interesting model to test the anorectic effects of relaxin-3 antagonists. Reversing the overeating and overweight phenotype of the RCEf-S rats with RXFP3 antagonists would further suggest the involvement of relaxin-3 in food restriction- and chronic stress-induced overeating in female rats.

The relaxin-3-positive NI neurons express the type 1 CRF receptor (Tanaka *et al.* 2005), and this receptor mediates the stress-induced increase in relaxin-3 expression in the NI (Banerjee *et al.* 2010). Because CRF expression in the PVNp was decreased in the repeatedly food-restricted female rats, this source of CRF seemingly was not implicated in the triggering of relaxin-3 overexpression in the RCEf-S rats. Examination of CRF expression in the MPOA has shown that the CRF transcript levels were increased by stress and were significantly higher in female rats compared with male rats. Repeated food restriction led to an additional increase in CRF mRNA expression in the MPOA of female rats. Our data are in agreement with the lower content of CRF protein in the MPOA in male compared with female rats (McDonald *et al.* 1994). Indeed, female rats showed numerous intensely stained CRF-immunoreactive neurons in the MPOA, whereas male rats, even treated with colchicine, displayed few CRF-immunopositive MPOA neurons (McDonald *et al.* 1994). The MPOA neurons directly project to the dorsal tegmentum including the NI (Simerly & Swanson 1988; Swanson 1976). In the MPOA, CRF does not produce anorexia (Dagnault & Richard 1997), but mediates the antireproductive effects of various stressors by inhibiting the hypothalamic-pituitary-gonadal axis (Rivest *et al.* 1993). Further investigations should be undertaken to investigate whether MPOA CRF may be implicated in activating relaxin-3 expression in female food-restricted chronically stressed rats. In addition,

the mechanisms for the opposite regulation of relaxin-3 and PVNp CRF expression in food-restricted female rats require further clarification of the long-term dynamics of the expression of these two neuropeptides. First, the long-term effect of relaxin-3 on the expression of PVNp CRF may differ from the acute effects. Second, a persistent increase in relaxin-3 expression in female food-restricted rats may lead in the long run to the chronic hyperactivity of the HPA axis and a substantial and persistent increase in the corticosterone plasma levels that would inhibit the expression of PVNp CRF.

In summary, chronic stress and repeated food restriction increased body weight gain in female but not in male rats. The chronically stressed food-restricted female rats showed hyperproduction of plasma corticosterone and hypoproduction of PVNp CRF. In addition, female chronically stressed food-restricted rats expressed higher levels of relaxin-3 in the NI and CRF in the MPOA. The central imbalance in lower production of anorexigenic neuropeptide CRF in the PVNp and higher production of orexigenic relaxin-3 in the NI may favor overeating and increased body weight gain in chronically stressed food-restricted female rats.

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Acknowledgments

We thank Jessica Martin and Sergiu Ftomov for technical assistance. This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) and Canadian Institutes of Health Research (CIHR). E.T. is a scholar of Fonds de la Recherche en Santé du Québec (FRSQ).