Chronic disruption of body weight but not of stress peptides or receptors in rats exposed to repeated restraint stress

Ruth B.S. Harris,a,⁎ Julie Palmondonb, Stephen Leshina, William P. Flatta, Denis Richardb

a Department of Foods and Nutrition, Dawson Hall, University of Georgia, Athens, GA 30605, USA
b Department of Anatomy and Physiology, Université Laval, Québec, Canada G1K 7P4

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Abstract

Rats exposed to restraint stress for 3 h on each of 3 days lose weight and do not return to the weight of their non-stressed controls for extended periods of time. Studies described here demonstrate that the initial weight loss is associated with increased energy expenditure and reduced food intake on the days of restraint but that there is no difference between stressed and control rats once stress ends. The failure to compensate for this energy deficit accounts for the sustained reduction in weight which lasts for up to 80 days after the end of restraint. In an additional experiment, in situ hybridization was used to measure mRNA expression of corticotrophin releasing factor (CRF) and CRF receptors in hypothalamic nuclei, of urocortin (UCN) in the Edinger Westphal nucleus and of UCN III in the rostral perifornical area and medial amygdaloidal nucleus. Immediately after the second 3 h bout of restraint stress, there was a significant increase in expression of UCN in the Edinger Westphal nucleus and of CRF-R1 in the paraventricular nucleus of the hypothalamus and a less pronounced decrease in CRF-R2 expression in the ventromedial nucleus of the hypothalamus. There were no differences in expression of stress-related peptides or their receptors 40 days after the end of repeated restraint. These results suggest that the sustained reduction in body weight and increased responsiveness to subsequent stressors in rats that have been exposed to repeated restraint are not associated with prolonged changes in mRNA expression of CRF receptors or their ligands.

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Introduction

We have previously reported that rats exposed to 3 h of restraint stress lose weight on the day of restraint and do not return to the weight of their non-stressed controls for at least 10 days (Rybin kto et al., 1997) and that, when the restraint stress is repeated on each of 3 days (repeated restraint), the amount of weight that is lost is increased (Harris et al., 2002). We have also reported that the difference in weight between control and stressed animals is maintained for at least 40 days after the end of repeated restraint (Harris et al., 1998) and here we show that the difference is maintained for as long as 11 weeks after the end of restraint. Therefore, although the stressed rats start to gain weight at the same rate as their non-stressed controls once the restraint has ended, they do not reach the same weight as the controls. The stress-induced weight loss is small, ranging from 5 to 15% of body weight in different experiments (Harris et al., 1998, 2002), but it is statistically significant. It is also significant from a physiological perspective because weight loss that is induced by other types of interventions, such as food restriction (Harris et al., 1986), is quickly recovered once the experimental intervention is removed.

Weight loss has to result from a negative balance between energy expenditure and intake. In the repeated restraint stress model, the rats are hyperthermic during restraint stress (Harris et al., 2002), and the second experiment described here confirms that energy expenditure is elevated during the time that the rats are in the restraining tubes. On the days of restraint stress, restrained rats have a significantly lower energy intake than their non-stressed controls, and, once stress ends, the energy intake of the restrained rats returns to that of the controls, but there is no attempt to overeat to compensate for the energy deficit experienced during restraint (Harris et al., 1998). This implies that the regulatory mechanisms that normally would promote hyperphagia to allow a full recovery of body weight...
are inactive in these animals. This may either be because stress somehow modifies the pathways that would normally sense and respond to a reduction in weight or because activation of stress-related pathways acts in opposition to the mechanisms that would normally promote a recovery of weight to normal levels. Therefore, it may be assumed that, at some level of the regulatory organization, the initial weight loss and/or the sustained reduction in weight are associated with increased release of stress-related peptides such as CRF or the urocortins (UCN, UCN II, UCN III) (Latchman, 2002; Pellemounter et al., 2004; Rivest et al., 1989). It is well established that both acute and chronic administration of CRF inhibit food intake (Hotta et al., 1991). The effect appears to be mediated by inhibiting the orexigenic action of neuropeptide Y (NPY) (Heinrichs et al., 1992) and by promoting the expression of proopiomelanocortin (POMC) (Hotta et al., 1991) which is the precursor of a number of peptides including alpha melanocyte stimulating hormone (αMSH). αMSH is an agonist of central melanocortin receptors (MC4 and MC3) which inhibit food intake and increase energy expenditure (Voisey et al., 2003).

Administration of exogenous UCN, UCN II or UCN III also inhibits food intake (Inoue et al., 2003; Ohata and Shibasaki, 2004; Spina et al., 1996), although the response to UCN II and to UCN III is delayed by several hours, contrasting with the early inhibition of food intake by CRF or UCN. Projections from UCN III neurons overlap with CRF receptors in areas that include the hypothalamus (Li et al., 2002), whereas UCN neurons do not appear to project to the hypothalamus, an area of the brain that is classically associated with the regulation of food intake, but do extend to the nucleus of the solitary tract in the brain stem (Bittencourt et al., 1999), an area that has been shown capable of mediating the anorectic effect of centrally administered UCN (Daniels et al., 2004). Stress responses that are initiated by CRF or UCNs are mediated by two major subtypes of receptors: CRF-R1 and CRF-R2. Studies with knockout mice and selective receptor antagonists suggest that stress-induced hypophagia is largely dependent upon activation of CRF-R2 (Cullen et al., 2001), whereas stimulation of thermogenesis (Cullen et al., 2001), activation of the hypothalamic–pituitary adrenal axis (Pellemounter et al., 2004; Timpl et al., 1998) and anxiety-type behavior (Contarino et al., 1999) may be dependent upon CRF-R1.

The specific mechanisms responsible for the sustained reduction in weight in rats that have been exposed to repeated restraint stress, including the initiator of the response and the down-stream pathways activated by the initiator, have not been identified. Observations that rats that have been exposed to repeated restraint stress are hypersensitive to novel stressors several weeks after the restraint stress has ended (Harris et al., 2004) suggest that, in addition to the chronic down-regulation of body weight, there also is a chronic change in the reactivity of stress-related pathways. The sustained loss of weight is not specific to repeated restraint because others have reported a chronic reduction in body weight of rats exposed to social defeat (Meerlo et al., 1996), immobilization (Valles et al., 2000) and in rats and mice exposed to chronic mild stress (Harris et al., 1997; Michel et al., 2005). In addition, it is well documented that chronic stress leads to an increased endocrine response to a novel stressor (Akana and Dallman, 1997; Bhatnagar and Dallman, 1998).

The experiments described here investigated different aspects of the repeated restraint model. As noted above, we have now demonstrated that the restrained rats do not regain the weight they lose during stress even 80 days after the end of restraint. Secondly, we have measured energy expenditure of the rats during and after repeated restraint to determine whether there is a sustained elevation of expenditure that may contribute to the reduced weight of the rats. Finally, central expression of stress-related peptides and their receptors has been measured during and after repeated restraint to determine whether chronic changes in expression of these proteins contribute to the sustained reduction in weight by inhibiting or opposing activity of pathways that would normally return body weight to that of non-stressed control rats.

Methods

All experimental procedures involving animals were approved by the University of Georgia Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experiment 1: long-term recovery of body weight following repeated restraint stress

Forty male Sprague–Dawley rats (Harlan Sprague–Dawley, TX), weighing 310–330 g, were housed in individual hanging wire-mesh cages in a temperature-controlled room maintained at 23 ± 2°C, approximately 55% humidity, with the lights on for 12 h per day from 7:00 a.m. The rats had free access to water and to food (Purina Rodent Chow 5012, Purina Mills, MO) at all times except during the hours of restraint stress. After 1 week of adaptation to the housing conditions, body weights and food intakes, corrected for spillage, were recorded daily for 7 days. The rats were then divided into two weight-matched groups, one was the non-stressed Control group and the other was subjected to repeated restraint stress. For repeated restraint, the rats were removed from their home cage and placed in plastic restraining tubes (21.6 cm long, 6.4 cm diameter: Plas Labs, MI) in an experimental room. Control rats were moved to the same room and placed in shoe-box cages with bedding but without food or water for the period of restraint. The rats were restrained for 3 h, starting at 8:00 a.m., on each of 3 consecutive days. At the end of restraint, the animals were returned to their home cages with free access to food and water. Daily measures of food intake and body weight continued for 36 days after the start of restraint, and then body weight was recorded once each week. The rats were killed 80 days after the end of restraint stress. Wet weights of adrenal glands and thymus and carcass weights were recorded.

Experiment 2: energy expenditure of rats exposed to repeated restraint stress

Twelve male Sprague–Dawley rats were given free access to chow and water and were adapted to handling for 1 week. The rats were then housed in individual metabolic cages in an indirect calorimeter that has been described in detail previously (Loh et al., 1998; Wang et al., 1999). They were allowed to adapt to the calorimeter for 1 day, and then energy expenditure was measured continuously for the rest of the experiment. Body weights, food intakes and water intakes of the rats were recorded daily. After 6 days of baseline measurements, the rats were divided into two weight-matched groups, and half of the rats were placed in restraining tubes for 3 h inside the calorimeter cages. This was repeated on each of the next 2 days. Food and water were removed from the cages of control, non-stressed rats for the duration of restraint. Energy
Experiment 3: central mRNA expression of CRF, UCN and CRF receptors in rats exposed to repeated restraint stress

Thirty Sprague–Dawley rats were housed individually as described for Experiment 1. They were acclimated to the environment and to handling procedures for a week, and daily body weights and food intakes were recorded for 5 days. Sixteen of the rats (Acute) were divided into two weight-matched groups, and one was exposed to repeated restraint stress. Immediately at the end of 3 h restraint on the second day of stress, the rats were anesthetized with 90 mg/kg ketamine and 10 mg/kg xylazine and then perfused intracardially with 200 ml ice-cold saline followed by 500 ml 4% parafomaldehyde solution. These rats were killed after two rather than three periods of restraint because weight loss on the first 2 days of repeated restraint is routinely greater than on the third day of restraint (Harris et al., 2002), therefore, we anticipated that acute changes in mRNA expression would be greater on Day 2 than Day 3 of restraint. The brains were collected and stored in 4% paraformaldehyde solution at 4°C. The remaining 14 rats (Chronic) were divided into two weight-matched groups. One was subjected to repeated restraint (3 h of restraint on each of 3 days), and the other was a control group. Daily body weights and food intakes were recorded for 40 days after the end of restraint when the rats were anesthetized and perfused with parafomaldehyde for collection of the brains which were stored in 4% paraformaldehyde solution until slicing.

For in situ hybridization, the brains were transferred to a 4% parafomaldehyde, 10% sucrose solution for 12 to 16 h before sectioning. Brain sections (30 μm) were cut using a freezing Vibriamore and stored in cold sterile cryoprotectant solution (50 mM phosphate buffer, 30% ethylene glycol, 20% glycerol). For each mRNA that was evaluated, 1 of every 5 sections was mounted on a poly-l-lysine-coated slide and desiccated overnight under vacum. The sections were then fixed for 20 min in 4% parafomaldehyde, digested for 30 min at 37°C with proteinase K (10 μg/ml), washed in 1 ml Tris–HCl containing 50 mM EDTA, pH 8.0) acetylated with acetic anhydride (0.25% in 1 M Triethanolamine, pH 8.0) and dehydrated through increasing concentrations of ethanol (50, 70, 90 and 100%). After vacuum drying for at least 2 h, hybridization solution containing antisense 32P-labeled cRNA probe (107 cpm/μl) was spotted onto each slide. The slides were sealed with a coverslip and incubated overnight at 60°C in a slide warmer. The next day, the coverslips were removed, and slides were rinsed four times with 4× saline–sodium citrate (SSC), digested for 30 min at 37°C with RNase A (20 μg/ml in 10 mM Tris, 500 mM NaCl, 1 mM EDTA) and dehydrated through increasing concentrations of ethanol. After drying for 2 h in a vacuum oven, the slides were exposed to an X-ray film for 24 h. After removal from the autoradiography cassette, the slides were defatted in xylene and dipped in NTB-2 nuclear emulsion (Eastman Kodak, NY). The slides were exposed for 7 min before being developed in D19 developer (Eastman Kodak) for 3.5 min at 14–15°C and fixed in rapid fixer (Eastman Kodak) for 5 min. Tissues were rinsed in running distilled water for 1–2 h, counterstained with 0.25% thionin, dehydrated with increasing concentrations of ethanol, cleared in xylene and coverslipped with DPX (Sigma-Aldrich, MO). The hybridization signals revealed on NTB-2-dipped nuclear emulsion slides were analyzed and quantified under a light microscope equipped with a black and white video camera coupled to a Macintosh computer using Image software (Version 1.55 non-FTP, Wayne Rasband, NIH, MD). The optical density for the hybridization signal was measured under dark field illumination at a magnification of ×25. Brain sections from the different rats were matched for rostrocaudal levels as closely as possible. The OD determinations were performed bilaterally on the three to five sections on each side of the brain for each animal, and the OD values obtained were averaged to provide an individual score for each rat. The OD for each specific region was corrected for the average background signal, which was determined by sampling unlabeled areas outside of the areas of interest. The boundaries for each nucleus were determined under brightfield illumination. For each area of the brain that was analyzed, the densitometric analyses were performed on all of the animals without changing the light source of the microscope. Preliminary analysis of the NTB-2 nuclear emulsion-dipped slides was achieved by autoradiography using a standardized calibration bar (Eastman Kodak) (data not shown).

Areas quantified and hybridization probes

Based on reports of distribution of the mRNA for stress-related peptides (Sawchenko et al., 1993; Vaughan et al., 1995), their receptors (Van Pett et al., 2000) or the pattern of projection of their neurons (Bittencourt and Sawchenko, 2000; Li et al., 2002), together with information on the involvement of specific areas of the brain in the control of food intake or energy expenditure, the following measurements were made: UCN mRNA expression in the Edinger Westhal nucleus, CRF and CRF-R1 in the paraventricular nucleus of the hypothalamus (PVN), CRF-R2 mRNA expression in the ventromedial nucleus of the hypothalamus (VMH) and UCN III mRNA in rostral perifornical area and the medial amygdaloid nucleus.

The CRF cRNA probe was generated from the 1.2 kb EcoRI fragment of rat CRF cDNA (Dr. K. Mayo, Northwestern University, IL) subcloned into a pGEM-4 vector (Promega, WI) and linearized with HindIII/SpeI and EcoRI/T7 for antisense and sense probes, respectively. The CRFR1 cRNA was generated from 1.3 kb PstI fragment of the rat CRFR1 cDNA (Dr. W. Vale, The Salk Institute, CA) subcloned into pBluescript-SKII plasmid (Strategene, CA) and linearized with BamHI/T7 and EcoRI/T3 for antisense and sense probes, respectively. The CRF receptor II cRNA was generated from the 275 bp EcoRI fragment of rat CRF receptor II cDNA (Dr. T.W. Lovenberg, Neurocrine Bioscience Inc., CA), subcloned into pBlueScript vector (Strategene) and linearized with EcoRI/T3 and BamHI/T7 for antisense and sense probes, respectively. The UCN cRNA probe was generated from 600 bp EcoRI fragment of rat UCN cDNA (Dr. W. Vale) subcloned into pBlueScript vector and linearized with Smal/T7 and HindIII/T3 for antisense and sense, respectively. The DNA fragment of 495 bp corresponding to the almost complete coding sequence of the reported mouse UCN III mRNA (nucleotide 1–495, GeneBank accession no. AF361944) was amplified by PCR from a cDNA using a pair of 18-bp oligonucleotide primers complementary to nucleotides 1–18 (5′-ATGCTGTAGCCCACTAC-3′) and 475–494 (5′-TACTCTTCTTCTCCACAGTCTG-3′) and labeled with γ-32P-ATP. The cDNA were subcloned into pGEM-T vector (Promega) and linearized with EcoRI and SalI/T7 for antisense and sense probes, respectively.

The specificity of each probe was confirmed by the absence of a positive signal in sections hybridized with sense probe. Radioactive riboprobes were synthesized by incubation of 250 ng linearized plasmid in 10 mM NaCl, ATP/CTP/GTP/a-35S-UTP, 40 μg/ml RNAase (Promega, WI) and 20 U of either T7, T3 or T3 RNA polymerase for antisense probe, for 60 min at 37°C. The DNA templates were treated with 100 μl of DNAase solution (1 μl DNase, 5 μl of 5 mg/ml tRNA, 94 μl of 10 mM Tris/10 mM MgCl2). The preparation of the riboprobe was completed through a phenol-chloroform extraction and ammonium acetate precipitation.

Statistical analysis

Repeated measures of body weight, food intake, water intake or energy expenditure were compared by repeated measures analysis of variance with post-hoc comparison of specific groups by Duncan’s multiple range test. Single measures were compared by unpaired t test (Experiments 1 and 2) or by two-way analysis of variance with post-hoc Duncan’s multiple range test (Experiment 3). All analyses were performed using Statistica Software (StatSoft, OK).

Results

Experiment 1

Rats exposed to repeated restraint stress lost weight on the days of restraint, and, although they gained weight once stress ended, they did not return to the weight of control rats during the post-stress period (Fig. 1A). The amount of weight that was lost during restraint stress was relatively small, but the weights of the two groups of rats were significantly different from the first day of restraint until 25 days after the end of restraint (RR: \( P < 0.007 \), Day: \( P < 0.0001 \), Interaction: \( P < 0.006 \)). The difference in average body weights was 14 g
immediately after the end of restraint and was 18 g at the end of the study, therefore, the loss of significance was not due to the weight of the restrained rats catching up to that of the controls but due to increased variability within the groups and the difference in weight representing a smaller proportion of the weight of the rats. Food intake was inhibited on the days of restraint and 1 day after the end of restraint and then returned to control levels (Fig. 1B: RR: \( P < 0.002 \), Time: \( P < 0.001 \), Interaction: \( P < 0.04 \)). There were no differences in adrenal or thymus weights of the rats at the end of the experiment (data not shown).

Experiment 2

Rats exposed to restraint stress showed a significant increase in energy expenditure during the 3 h of restraint stress, but then expenditure returned to control levels as soon as the stress ended (Fig. 2A). There were no differences in energy expenditures of the restrained and control rats on the 3 days immediately following restraint stress (Fig. 2B). On stress days, respiratory quotient (RQ) was lower in restrained than control rats during the second half of the period of restraint (Fig. 3A), for several hours after the end of restraint and for about 90 min in the middle of the dark period. There were no differences in RQ of stressed and control rats on the days immediately following the end of restraint (Fig. 3B). The two groups of rats weighed the same on the morning before the first restraint stress (Controls: 363 ± 7 g, Restrained: 362 ± 6 g). The restrained rats lost weight on the 3 days of stress and then started to regain weight at the same rate as controls so that they weighed less than controls at the end of the calorimetry measures (Controls: 376 ± 5 g, Restrained: 362 ± 6 g). Food intake was inhibited in stressed rats on the days of restraint but returned to control levels during the post-stress period (Restrained period: Controls: 19.9 ± 0.8, Restrained: 15.8 ± 0.6 g/rat/day; Post-stress period: Controls: 19.1 ± 0.8, Restrained 18.9 ± 0.7 g/rat/day; RR: \( P < 0.02 \), Time
period: NS, Interaction: \( P < 0.02 \)). There was no significant effect of restraint stress on water intake, even though food intake was inhibited (data not shown).

**Experiment 3**

As in previous studies, the Chronic restrained rats lost weight on the 3 days of restraint stress and then gained weight at the same rate as control animals (data not shown). These rats ate significantly less than the controls on the days of restraint, but food intake returned to control levels once restraint ended (data not shown).

In situ hybridization showed significant increases in UCN mRNA expression in the Edinger Westphal nucleus (Duration: NS, RR: NS, Interaction: \( P < 0.07 \)) and CRF-R1 in the PVN (Duration: NS, RR: \( P < 0.008 \), Interaction: \( P < 0.009 \)) of Acute restraint rats compared with their controls. When CRF-R2 mRNA expression in VMH was considered across all four groups in the study, there was a non-significant inhibition of expression in the Acute stress rats. When only the two Acute groups of rats were compared by a two-tailed \( t \) test, then the difference reached statistical significance (\( P < 0.05 \)). There were no differences in mRNA expression of any of the stress-related peptides or of the CRF receptors measured in the Chronic restraint rats compared with their controls (Fig. 4). There were significant elevations of UCN and UCN III mRNA expression in Chronic compared with Acute rats (Duration: \( P < 0.003 \), RR: NS, Interaction: NS), but these differences were not specific to the stressed animals.

**Discussion**

The three experiments described here provide new information on the mechanisms that contribute to the stress-induced weight loss of rats exposed to repeated restraint stress. Previously, we had reported that rats exposed to repeated restraint did not recover the weight lost during stress for up to 40 days after stress ended (Harris et al., 1998). The first experiment described here extends those observations and demonstrates that the difference in weight between restrained rats and their non-stressed controls is maintained for 11 weeks after the end of restraint. The difference in weight of restrained and control rats was the same 80 days after the end of stress as on the day after the end of stress. There was no evidence in this experiment that the rats made any attempt to compensate for the reduced energy intake or weight loss during stress by overeating or by reducing energy expenditure after the stress ended. This is consistent with our previous observations (Harris et al., 1998) and suggests that restraint stress disrupts the normal feedback regulatory mechanisms that allow an animal to compensate for disturbances to the metabolic equilibrium that determine body weight. We have previously reported that, although the difference in body weight between control and restrained animals does not change during the post-
stress period, the composition of the difference in weight between control and restrained rats transitions from an initial loss of lean tissue to a reduction in both lean and fat tissue (Zhou et al., 1999) and is associated with shifts in adipocyte and hepatic glucose uptake and utilization (Zhou et al., 1999, 2001). These observations suggest that the animal is sensitive to the change in proportional body composition but not to the reduction in total body mass.

The second study described here showed that energy expenditure was increased in stressed rats during restraint but that it returned to control levels as soon as the stress ended. Stress also reduced the RQ of the rats, indicating an increase in fatty acid and/or protein oxidation over carbohydrate oxidation, confirming previous observations in lean and obese Zucker rats (Duclos et al., 2005). We have previously found that restraint stress causes an acute hyperthermia in rats (Harris et al., 2002). One of the primary thermogenic tissues in rats is brown adipose tissue (Cannon and Nedergaard, 2004), and the increase in fatty acid oxidation implied by the reduction in RQ during restraint stress may have been associated with activation of brown fat uncoupling pathways in addition to promotion of lipid mobilization and metabolism that would be expected in response to the adrenergic and adrenal response to stress (Duclos et al., 2005; Farias-Silva et al., 1999). It is well established that activation of the sympathetic nervous system outflow to brown adipose tissue increases thermogenesis by activating uncoupling protein one (UCP1) and allowing proton flux into the mitochondrial matrix without coupling it to the production of ATP (see Adams, 2000 for review). This movement of protons down a concentration gradient releases heat and stimulates fatty acid oxidation to restore the proton gradient across the inner mitochondrial membrane. The rapid elevation of energy expenditure in rats exposed to 3 h of restraint suggests that the response was due to increased activity of existing UCP1 in brown adipose tissue, rather than a change in thermogenic capacity of the tissue, which would be expected in conditions that caused a chronic activation of thermogenesis (Zaninovich et al., 2002). The reduced RQ was sustained for

![Fig. 3. Respiratory quotient (RQ) of male Sprague–Dawley rats in Experiment 2. Top panel shows the average RQ for the 3 days on which the rats were restrained. Bottom panel shows the average RQ of the rats for the 3 days immediately following the end of restraint. Data are means ± SEM for groups of 6 rats. Asterisks indicate time points at which RQ of restrained rats was significantly ($P < 0.05$) different from that of controls.](image)
Fig. 4. Quantification of stress-related peptides and receptors by in situ hybridization in Experiment 3. Within each panel, the bar graphs represent the average (+SEM) density of expression for groups of 6 to 8 rats, micrographs show representative sections for hybridization, and the schematic indicates the area quantified. Values for mRNA expression of a specific protein that do not share a common superscript are significantly different at $P < 0.05$ determined by two-way ANOVA and post-hoc Duncan’s multiple range test. The asterisk in panel F indicates a significant difference between acute control and restrained rats determined by $t$ test.
several hours after the end of restraint, even though energy expenditure had returned to normal, which implies that some of the changes in cellular metabolism during stress took longer to reverse than the whole animal requirement for increased heat production and energy expenditure and may have been occurring independently of activation of brown fat thermogenesis. In addition to the delay in correcting RQ at the end of restraint, there was a time period of about 90 min in the middle of the dark phase in which both the energy expenditure and the RQ of restrained rats were lower than that of controls. We did not measure the circadian pattern of feeding of the rats in this study and cannot determine whether the transient drop in RQ was associated with hypophagia during the dark period. Despite the changes in energy expenditure and RQ of the rats on the days of restraint, there was no difference in either measure during the 3 days that followed restraint stress. Therefore, we have to assume that the maintenance of a reduced weight in the restrained animals is not accounted for by a chronic significant elevation of energy expenditure in the presence of a normal food intake.

The short-lived elevations of energy expenditure during stress and the reduction in food intake, which may last for 48 h after the stress has ended, result in a temporary state of negative energy balance that causes weight loss. The rats rapidly normalize their intake and expenditure but appear to be insensitive to the reduction in their body weight at the end of restraint and maintain energy balance to defend this new weight rather than responding by overeating to compensate for the loss. This chronic reduction in body weight has been observed in other models of acute stress, such as immobilization (Valles et al., 2000) and social defeat (Meerlo et al., 1996), and is not specific to restraint stress. The final study described here investigated whether there were acute or chronic changes in the level of expression of either stress-related peptides or their receptors that may contribute to the reduced body weight and increased stress responsiveness of restrained rats during the post-stress period. The mechanisms that allow the reduced weight to be maintained once stress has ended or that are responsible for the enhanced endocrine response to mild stress and the reduction in food intake, which may last for 48 h after the stress has ended, result in a temporary state of negative energy balance that causes weight loss. The rats

the end of restraint, when body weight remained reduced in restrained rats.

There were acute effects of restraint stress on the expression of CRF-R1 mRNA in the PVN and of UCN mRNA expression in the Edinger Westphal nucleus of rats killed at the end of the second 3 h restraint. The increase in UCN, which is an agonist for both CRF-R1 and CRF-R2, would be consistent with a state in which food intake is inhibited and thermogenesis is increased. Activation of CRF-R1 has been associated with the HPA axis response to stress and increased anxiety-type behavior, whereas activation of CRF-R2 may down-regulate anxiety (Balle and Vale, 2004). Therefore, the observed changes in receptor mRNA expression would be consistent with increased anxiety and release of corticosterone in the restrained rats during the 3 h of restraint. Neither of these parameters was measured in this study, although we have previously demonstrated a robust corticosterone response to restraint stress (Harris et al., 2004). Others have reported that both CRF and CRF-R1 in the PVN are induced by stress (Luo et al., 1994; Makino et al., 1995; Richard et al., 1996; Timofeeva et al., 2003). The rate of change in CRF-R1 mRNA expression appears to be slow as Van Pett et al. (2000) found a 2 h delay in induction of expression following acute hemorrhage or foot shock and a maximal increase in expression 4 to 6 h after stress. Inaki et al. (2001) demonstrated that PVN CRF-R1 mRNA expression co-localized with CRF mRNA, indicating a positive feedback loop in which CRF induced its own receptors. More recently, it has been reported that the CRF-R1 gene expression is regulated by both CRF (Mansi et al., 1996) and UCN in a positive feedback manner (Parham et al., 2004). In this study, we did not find any significant increase in PVN CRF mRNA expression, but there was a substantial increase in UCN mRNA expression in the Edinger Westphal nucleus, suggesting that release of this neurotransmitter could have been responsible for the increased hypothalamic CRF-R1 expression because the PVN receives projections of UCN immunoreactive neurons from the Edinger Westphal nucleus (Bittencourt et al., 1999). In addition, although UCN mRNA expression was measured in the Edinger Westphal nucleus, the site at which mRNA expression is greatest, it also is expressed at low levels in other brain areas, including the PVN (Bittencourt et al., 1999), and restraint stress has been shown to increase PVN UCN mRNA expression (Shi et al., 2000). Alternatively, there may have been an early increase in CRF expression that initiated the change in receptor expression. Others have reported an early increase in heteronuclear CRF mRNA expression in rats (Kovacs and Sawchenko, 1996), whereas tissue was collected at the end of 3 h of restraint stress in this study. Infusion studies have demonstrated that both UCN II and UCN III inhibit food intake but that the response is not apparent until several hours after the infusion. We did not find any effect of restraint stress on UCN III mRNA expression in this study, and we only measured 24 h food intake, therefore, it is not possible to determine whether the inhibition of food intake in stressed rats, compared with controls, occurred at specific time points in relation to the initiation of restraint.
At the end of the 3 h of restraint on day two of restraint, CRF-R₂ mRNA was down-regulated in the VMH of restrained rats compared with their controls. There is dense innervation of the VMH by UCN III reactive fibers (Li et al., 2002), but we did not find any change in UCN III mRNA expression in response to restraint stress in the two areas that were measured in this study. CRF-R₂ has been implicated in stress-induced anorexia (Contarino et al., 1999) and in the inhibition of food intake caused by central infusions of either CRF or UCN (Smagin et al., 1998). Although the VMH is responsive to changes in energy balance (Li et al., 1994), the current view is that the VMH is not directly involved in the regulation of either food intake (Gold, 1973) or the sympathetic outflow to brown adipose tissue (Bamshad et al., 1999), which contributes to increases in energy expenditure in rodents. The changes in VMH receptor expression may, however, be considered indicative of changes in expression in other tissues. Others have reported that pituitary CRF-R₂ mRNA expression is down-regulated by restraint stress, glucocorticoids and endotoxins (Kageyama et al., 2003) and that cardiac CRF-R₂ mRNA is inhibited by UCN and by glucocorticoids (Asaba et al., 2000). Interestingly, CRF-R₂-deficient mice do not show the normal degree of hyperphagia following food deprivation (Bale et al., 2000), a situation that has some parallels to the failure of restrained rats to compensate for stress-induced weight loss. In addition, starvation (Makino et al., 1998; Timofeeva and Richard, 1997) or repeated immobilization stress (Makino et al., 1999) has been shown to reduce expression of CRF-R₂, suggesting that the reduced receptor expression provides a compensatory mechanism to counteract the metabolic actions of CRF and of the urocortins.

The changes in UCN and CRF receptor expression in rats killed immediately after restraint stress suggest that activation of the CRF or UCN systems may be directly associated with the reduction in food intake and increase in energy expenditure that is observed on the day of restraint. The failure to find any sustained changes in expression of CRF-related peptides or CRF receptors suggests that, if the stress-related peptides are responsible the sustained down-regulation of body weight, then it may be due to modification of a down-stream pathway rather than chronic up-regulation of CRF or UCN. Others have reported that both NPY and the melanocortin system mediate CRF-induced inhibition of food intake (Heinrichs et al., 1992; Hotta et al., 1991). In previous experiments, we did not find any changes in mRNA expression of NPY immediately after the end of restraint (Rybkina et al., 1997) but that mice that ectopically overexpress agouti protein, an antagonist of melanocortin receptors, demonstrate an exaggerated stress response (Harris et al., 2001). This second set of results would not support the concept of stress-induced hypophagia being mediated by activation of melanocortin receptors, but further studies are needed to determine whether compensatory systems in the transgenic mice interfere with the stress response. An association between the sustained response to stress in restrained rats and the melanocortin system is attractive because of the observation that a single application of the melanocortin receptor antagonist agouti-related protein (AgRP) can stimulate food intake for up to 4 days (Hagan et al., 2000). Thus, there is precedent for an association between acute changes in melanocortin receptor activity and long-term responses. In the study reported here, there were significant increases in the levels of expression of UCN, UCN III and CRF-R₂ in rats that were killed 40 days after restraint compared with those killed at the end of the second 3 h of restraint stress, but none of these differences was specific to the restrained rats and must have been due to the age of the rats or environmental conditions at the time of sacrifice. The measures made in this experiment do not exclude the possibility of an increased sensitivity to baseline levels of stress-related peptides in rats that had been exposed to repeated restraint. We have found that rats subjected to repeated restraint stress show an exaggerated endocrine response to a subsequent mild stress (Harris et al., 2004), and, if the rats are hypersensitive to stress-related peptides, then it is possible that there also would be an exaggerated energetic response to small stressors in terms of body weight regulation.

In summary, the results from the three studies described here demonstrate that the reduction in body weight that is induced by repeated restraint stress is partially due to an increase in energy expenditure during stress that is not compensated for by either a decrease in expenditure or an increase in food intake during the post-stress period. Therefore, the difference in weight of control and stressed rats is maintained for extended periods of time. If we could identify the mechanisms responsible for this sustained reduction in weight, then it may provide new understanding of the events that are normally involved in the reversal of weight loss in a majority of individuals who lose weight (Wing and Hill, 2001). Measurement of levels of mRNA expression of stress-related peptides and their receptors indicated acute but opposing changes in expression of CRF-R₁ and CRF-R₂ in the hypothalamus and a substantial increase in UCN mRNA expression in the Edinger Westphal nucleus. There was no evidence of a chronic effect of stress on the expression of the peptides or receptors that might contribute to the sustained reduction in weight or the increased stress responsiveness (Harris et al., 2004) of rats that have been exposed to repeated restraint stress.

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References


