Effect of restraint stress on food intake and body weight is determined by time of day

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Rykin, Igor I., You Zhou, J ulia Volaufova, Gennady N. Smagin, Donna H. Ryan, and Ruth B. S. Harris. Effect of restraint stress on food intake and body weight is determined by time of day. Am. J. Physiol. 273 (Regulatory Integrative Comp. Physiol. 42): R1612–R1622, 1997.—Three experiments were conducted to investigate the effect of restraint stress applied at different times of the light-dark cycle on feeding behavior and body weight of rats. Sprague-Dawley rats were restrained for 3 h in restraining tubes either at the start or the end of the light cycle. There was a significant reduction in food intake on the day of restraint and no change in food intake during a 10-day recovery period in either experiment. Reductions of food intake on the day of restraint were about the same for both restrained groups compared with their controls. When stress was applied in the evening, eating was inhibited during the first 2 h after restraint, whereas in rats restrained in the morning, feeding was suppressed twice: during the 4 h after restraint and during the first 2 h of the dark cycle. Restraint induced a significant weight loss that was greater in the rats stressed in the morning. Neuropeptide Y (NPY) levels determined at the time of food suppression for both experiments (beginning of the dark cycle) revealed an elevation of NPY in the paraventricular nucleus of rats stressed in the morning compared with other groups, but no difference in hypothalamic NPY mRNA expression. Expression of uncoupling protein mRNA in brown adipose tissue and leptin mRNA in epididymal fat, measured at the start of the dark period, was not altered by stress. There was an elevation of dopamine turnover in the hypothalamus of rats restrained at the end of light cycle, but not those restrained in the morning. These results show that restraint stress has a greater effect on metabolism and energy balance when it is applied in the morning. Additional studies are needed to elucidate mechanisms involved in the suppression of food intake 9 h after restraint.

Central mechanisms involved in the stress-induced inhibition of food intake have not been fully elucidated, but certain peptides and neurotransmitters are thought to be involved in the response. It is well established that monoamines (11) and corticotropin-releasing hormone (CRH) (13, 14) influence feeding behavior and mediate behavioral and physiological responses to stress (10, 13, 31). Several investigators have attributed stress-induced anorexia to activation of CRH (12) and/or serotonin (5-hydroxytryptamine, 5-HT) pathways (10, 25). Both of these transmitters are elevated in response to stress in a number of brain areas, including those that are involved in the regulation of feeding behavior (18, 26). Intracerebroventricular administration of CRH produces behaviors typical of stress, including depression of appetite (12, 31). Anorexia was observed after injection of CRH into the paraventricular nucleus (PVN), but not into the lateral or ventromedial hypothalamus (13). In addition, it has been shown that pretreatment of animals with the CRH antagonist, α-helical CRH, or anti-CRH antibody blocks the effect of stress (12, 24) and 5-HT (16)-induced anorexia, suggesting that activation of 5-HT promotes CRH release, which, in turn, inhibits food intake. Central injections of 5-HT reduce feeding of fasted animals through the 5-HT1B (11) and/or 5-HT2A/2C (1, 3) receptors. Although it has been shown that serotonergic nerve terminals originate in the raphe nucleus and end with synapses on CRH-containing neurons of the PVN (17) and that 5-HT agonists stimulate the secretion of adrenocorticotropic hormone (ACTH) and corticosterone (19), the interaction between CRH and 5-HT in regulation of food intake during stress remains unclear. A recent study by Bovetto et al. (3) indicated that selective agonism of different subtypes of 5-HT receptor either had no effect on food intake (in the case of the 5-HT1A receptor agonist) or caused hypophagia (5-HT1B and 5-HT2A/2C) in nonstressed rats, but failed to support a role for CRH in the 5-HT anorectic effect. Therefore, although CRH and 5-HT systems are involved in the regulation of food intake of stressed rats, specific mechanisms of action and possible interactions are vague.

An alternative mechanism by which stress may suppress food intake is the CRH inhibition of neuropeptide Y (NPY) release. NPY is a potent stimulator of food intake when injected intracerebroventricularly (30) and blockade of CRH by α-helical CRH in the PVN significantly potentiates the feeding effect of NPY injected into the same locus (9). Additionally, intracerebroventricular inhibition of 5-HT1B/C receptors increases food intake and the level of NPY in the arcuate
MATERIALS AND METHODS

Adult, male Sprague-Dawley rats (n = 90), weighing 380–400 g, were obtained from Harlan Sprague Dawley (Houston, TX). They were individually housed in stainless steel cages in a humidity- and temperature-controlled room (22 ± 0°C, 65–75%) on a 12:12-h light-dark cycle with lights on at 0700. The rats were maintained on standard rat chow (Purina Rodent Chow 5001; Purina Mills, St. Louis, MO) and tap water ad libitum. They were adapted to home cages and handling for 1 wk before the start of the experiment. All animal protocols were approved by the Pennington Biomedical Research Center Institutional Animal Use and Care Committee.

Because of the large number of animals involved, the study was divided into three experiments. The first two experiments had the same design but differed in the time of day that stress was applied. Each experiment was divided into three periods: baseline (7 days), day of restraint stress, and recovery period (11 days after stress). Food intakes, water intakes, and body weights were measured daily at 0800. On the day of stress there were multiple food and water intake measurements. In each experiment rats were divided into control and restraint groups (n = 16 rats each) matched for average body weight on the day of stress.

Experiment 1

On the day of stress rats were moved from their home cages to another room at 1600. Sixteen control rats were placed in regular shoe-box cages without any food or water, and 16 restrained rats were placed in Perspex restraining tubes (Plas Labs, Lansing, MI). After 3 h all animals were returned to their home cages with free access to food and water. Food and water intake were recorded at 0800–1600 before stress (no data during stress time 1600–1900), 1900–2100, 2100–2300, 2300–0100, 0100–0400, 0400–0800. During the recovery period food intake and body weights were recorded daily at 0800.

At the end of the 11-day recovery period the control and restrained groups were switched, and animals that had previously been controls were restrained for 3 h in the same conditions as used previously. Rats were killed in counterbalanced order between 1940 and 2015 (35 min) on the same day as they were stressed for measurements of central neurotransmitters and peripheral hormone concentrations. This time was chosen on the basis of results from the first part of the experiment that showed a large restraint-induced reduction of food intake during first 2 h of the dark cycle. In each group of control or restrained rats, brains from six animals were used for measurement of NPY content in the PVN. Brains from the remaining four animals were used for determination of hypothalamic NPY mRNA expression, and brains from the remaining four animals were used for in situ hybridization of arginine vasopressin (AVP) and NPY mRNA of hypothalamus. After rats were decapitated, blood was collected from 12 rats in each group, excluding rats used for in situ hybridization, and insulin and corticosterone were measured by radioimmunoassay (RIA; corticosterone RIA, ICN Biomedicals, Costa Mesa, CA; rat insulin RIA kit, Linco Research, St. Charles, MO). Serum glucose was measured using a Biochemistry ANALYZER YSI 2700 SELECT (Yellow Springs Instruments, Yellow Springs, OH).

Experiment 2

The rats were stressed in the morning from 0800 to 1100. Food and water intakes were recorded after 0800–1100 stress at 1100–1500, 1500–1900, 1900–2100, 2100–2300, 2300–0100, 0100–0400, and 0400–0800. The recovery period, second restraint, and collection of samples were performed exactly as described in experiment 1.

Experiment 3

Results from the previous experiments showed that restraint applied either in the morning or the afternoon suppressed food intake at the beginning of dark cycle. However, there was no correlation of central NPY concentrations with this behavior (see RESULTS). Therefore, this experiment was designed to evaluate the involvement of monoamines in the suppression of food intake during first 2 h of the dark cycle and whether restraint caused a significant elevation in sympathetic activity at this time point. This experiment included four groups of six rats. After adaptation to handling and a 7-day baseline period, one group of rats was restrained for 3 h from 0800 to 1100 and a second group was restrained from 1600 to 1900. The remaining two groups were controls for the two different time points. All rats were killed at the same time between 1915 and 2030. Hypothalamic and brain stem were analyzed for monoamine content and BAT expression of uncoupling protein (UCP) mRNA, and epididymal fat leptin mRNA expression was measured. Serum glucose, corticosterone, and insulin were assayed as described for experiments 1 and 2.

NPY Radioimmunoassay from PVN Punches

Microdissection of the PVN was performed using the procedure described by Palkovits (21). Cuts of 15 µm were made until the PVN level was reached, and then a single section of ~300 µm was taken for punch. Two sections of 15 µm, one before and one after that section, served as histo-
Northern Blot Analysis

Tissue preparation. Brains were quickly removed and hypothalamic blocks were isolated by vertical coronal cuts through the optical chiasm (tangential to the olfactory tubercles) and immediately posterior to the mammillary tubercles, vertically along the perihypothalamic sulci, and then horizontally immediately below the anterior commissure. The blocks were snap frozen in liquid nitrogen and stored at −70°C until processed. Intracapsular BAT was dissected and snap frozen until processed. Total RNA was extracted from individual hypothalami or fat pads using TRIzol Reagent (GIBCO BRL, Grand Island, NY) and separated by electrophoresis in a 1.2% agarose, 1.2% formaldehyde gel in 0.01 M 3-(N-morpho- lino)propanesulfonic acid running buffer. After capillary transfer to nylon membrane (Hybond N, Amersham, Arlington Heights, IL), RNA was ultraviolet crosslinked to the membrane (GeneLinker Bio-Rad, Laboratories, Hercules, CA).

cDNA probe preparation. The NPY cDNA probe (379 base pair bp) was obtained by reverse transcription polymerase chain reaction (PCR) using rat hypothalamic RNA. Primers were designed based on the complete NPY cDNA sequences. Primer pairs were 5'-CGCCATGATGCTAGGTAAC-3' and 5'-CAGACTGTTTTCACAGGATG-3'. PCR was carried out under the following conditions: 30 cycles of 94, 57, and 72°C for 1 min each followed by a final 10-min extension at 72°C. The PCR product (379 bp) was cloned into pCR 2.1 vector (Invitrogen, San Diego, CA) using the TA Cloning method. The Dr. Daniel Ricquier (24); (InVitrogen, San Diego, CA) using the TA Cloning method.

Membranes for NPY hybridization were prehybridized for 2 h at 42°C in 20 mM sodium phosphate buffer (pH 6.6) containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 1× Denhardt's solution, 1% sodium dodecyl sulfate (SDS), and 0.05 mg/ml salmon sperm DNA and then hybridized for 15 h at 42°C in the hybridization buffer plus 10% dextran sulfate with labeled probe (specific activity 1.5–2.0×10⁶ cpm/mg). Membranes were washed twice in 2× SSC-0.1% SDS at room temperature and once with 0.2× SSC-0.1% SDS for 15 min at 65°C and exposed to a phosphomager plate (Molecular Dynamics, Sunnyvale, CA) overnight. Images were made and stored using image-analysis software (ImageQuant Program, version 3.3, Molecular Dynamics). The membranes were stripped, prehybridized in RapidHyb buffer (Amersham) for 1 h at 42°C and hybridized in the same buffer for 2 h at 42°C with 285 oligoprobe. Membranes were washed twice with 5× SSC-0.1% SDS and once with 0.1× SSC-0.1% SDS for 15 min at 42°C and exposed to a phosphomager plate overnight. Relative levels of NPY mRNA and 28S rRNA were determined by densitometry analysis software (ImageQuant Program, version 3.3, Molecular Dynamics) and expressed as the ratio of NPY mRNA to 28S rRNA. Hybridization for leptin mRNA was carried out as described previously (8).

NPY and AVP mRNA In Situ Hybridization

Brains were rapidly removed and coronal blocks containing hypothalamus were dissected and immersed in prechilled isopentane at −40°C. Sequential coronal sections, 12 µm, were cut in a cryostat at −18°C. Coronal sections at the level of the magnocellular part of PVN (1.7–1.9 anterior to bregma) for AVP mRNA and of the arcuate nuclei (3.2–3.4 anterior to bregma) for NPY mRNA were collected. Hybridization and analysis. After thawing to room temperature, sections were fixed in 4% paraformaldehyde, acetylated, dehydrated in ethanol, delipidated in chloroform, partially rehydrated, dried, and then covered with 100 ml of hybridization buffer containing 50% formamide (Amresco, Solon, OH) and 10 pmol/ml digoxigenin (DIG) end-labeled oligonucleo- probe (Boehringer Mannheim Biochemicals, Indianapolis, IN) (18-mer, oligonucleotide probe, 345 bases corresponding to 1624–1669 bases of NPY gene nucleotide sequence, were used to detect NPY. An oligoprobe to AVP mRNA, specific to a sequence from the glycopeptide region (from 994 to 1041 nucleotides of vasopressin gene) was used to detect vasopressin mRNA. Oligonucleotides were obtained from GeneLab (Louisiana State University School of Veterinary Medicine). Slides were covered with paraffin and incubated for 20 h at 37°C in humidified chambers. After hybridization, sections were washed twice in 1× SSC at 55°C and once in 0.15 M NaCl-0.1 M tris(hydroxymethyl)aminomethane (Tris)-HCl buffer (pH 7.5). After blocking in 2% normal sheep serum in the same buffer, slides were incubated in anti-DIG sheep-derivative antibody conjugated with alkaline phosphatase (Boehringer Mannheim Biochemicals) for 48 h at 5°C. After consecutive washes in 0.15 M NaCl-0.1 M Tris buffer (pH 7.5) and in 0.1 M NaCl-0.05 M MgCl₂-0.1 M Tris-HCl buffer (pH 9.5), sections were incubated in the same buffer containing 340 mg/ml nitroblue tetrazolium and 170 mg/ml 5-bromo-4-chloro-3-indolyl phosphate for 24 h in a dark moist chamber at room temperature. Analysis of the slides was performed under light Zeiss microscope, and photomages were taken at ×40 and ×20 objectives.

High-Performance Liquid Chromatography Analysis of Monoamines

Hypothalami and brain stems were dissected, weighed, snap frozen in liquid nitrogen, and stored at −70°C. Frozen tissue samples were homogenized in 1.0 ml cold 0.1 M perchloric acid, 0.01 M EDTA. Each sample was sonicated for 30 s and centrifuged at 10,000 g for 2 min. The supernatant was separated by high-performance liquid chromatography (HPLC) (ESA, Chelmsford, MA) using a Catecholamine HR-80 column (ESA) with a mobile phase of 75 mM sodium phosphate, pH 3.0, 1.4 mM sodium octyl sulfonate, 0.01 M EDTA, and 10% acetonitrile at a flow rate of 1 ml/min. Norepinephrine (NE), 3-methoxy-4-hydroxyphenylethyl- eneglycol, dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), 5-HT, and 5-hydroxyindoleacetic acid (5-HIAA) were detected electrochemically (5011 High Sensitivity Analytic Cell; ESA). The electrode potentials of the analytic cell were set at −0.04 V for electrode 1 and +0.34 V for electrode 2. The guard cell was set at +0.65 V.
Statistical Analysis

The three response variables, daily food intake, water intake, and body weight, were analyzed separately as repeated measures over time to assess possible effect of restraint and timing of restraint. Similar models were used for hourly food and water intake on the day of stress, but with measurements repeated hourly instead of daily.

Alternative univariate analyses were performed, replacing the repeated food-intake measurement with its "area under the curve," which is equivalent to a weighted total of food intake for each rat, with the first and last measurement receiving one-half the weight of each of the other. This total was analyzed to assess differences due to restraint and experiment (timing of the restraint).

In the analysis of body weight, adjustments for possible effects of different baseline weights were incorporated in two ways: by including baseline weight as a covariate and by analyzing weight change with respect to baseline weight. Baseline values were weights of rats on the morning before the stress.

The postmortem measurements of glucose, corticosterone, and insulin were analyzed for effects of restraint and timing of restraint in a multivariate (3 variants) analysis of variance model. The hypothalamus measurements were analyzed similarly in a multivariate analysis of variance model, as were the brain stem measurements.

The SAS System version 6.12 was used for computations. Data are presented as means ± SE.

RESULTS

Experiment 1: Restraint Immediately Before Onset of Dark Period

Results from measurements of food and water intake are shown in Fig. 1. There was no difference between the control and restrained groups during the baseline period. Three hours of restraint significantly decreased 21-h food intake (−15.8%) on the day of stress (Fig. 1A), but there was no effect of restraint on food intake during the recovery period. Cumulative food intake for the 10-day recovery period, including the day immediately after stress, was not significantly different between control and restrained rats (251.1 ± 5.2 vs. 236.9 ± 5.4 g). There were no acute (day after stress) or chronic (recovery period) effects of restraint on water intake. Restrained animals lost ~8 g of body weight and remained lighter than controls during the recovery period (Fig. 2A). There was no statistically significant interaction between day and restraint [F(10,276) = 0.68, P = 0.74], and body weight gain of the two groups appeared to be almost parallel. Repeated-measurements analysis revealed a significant effect of restraint [F(1,29) = 27.53, P = 0.0001] on body weight over the recovery period.

Figure 3 shows food and water intakes measured at intervals during 21 h on the day of experiment. Three hours of restraint had a significant effect on the food intake in intervals during this day [effect of group F(1,30) = 10.45, P = 0.003] and on the time course of food intake in the two groups [group × time interaction F(5,150) = 4.28, P = 0.001]. There was a significant, 40%, reduction in food intake of restrained rats during the first 2 h after stress, which corresponded to the first 2 h of the dark cycle. Water intake was not altered at any time by restraint.

Serum glucose, insulin, and corticosterone concentrations are shown in Table 1. Glucose was significantly
lower in control than restrained rats, but insulin and corticosterone concentrations were not different between groups. PVN NPY protein content was 36% lower in restrained than control rats (Fig. 4A), but this difference was not statistically significant (P = 0.27). Neither Northern blot analysis nor in situ hybridization revealed any difference in NPY mRNA expression in whole hypothalamus, determined by Northern blot,
Table 1. Serum insulin, corticosterone, and glucose for rats in experiments 1 and 2

<table>
<thead>
<tr>
<th></th>
<th>Glucose, mg/dl</th>
<th>Corticosterone, ng/ml</th>
<th>Insulin, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>125 ± 2</td>
<td>68 ± 9</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Restraint 1</td>
<td>133 ± 2*</td>
<td>80 ± 20</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Control 2</td>
<td>129 ± 2</td>
<td>102 ± 14</td>
<td>3.7 ± 0.4†</td>
</tr>
<tr>
<td>Restraint 2</td>
<td>128 ± 3</td>
<td>104 ± 16</td>
<td>3.5 ± 0.3†</td>
</tr>
</tbody>
</table>

Data are means ± SE for groups of 14 animals. *Significant difference between glucose levels in control and stressed groups in experiment 1 (stress ended 40 min before sample collection; P < 0.01); †significant difference in level of insulin between control and stressed groups in experiment 2 compared with equivalent group of animals in experiment 1 (P < 0.001).

or in the arcuate nucleus, determined by in situ hybridization (Fig. 4).

Experiment 2: Restraint Applied at Start of Light Period

Baseline body weight gain is shown in Fig. 2 and food and water intakes are shown in Fig. 1. Three hours of restraint in the morning significantly reduced food intake, by 11.5%, during the 21 h after stress (Fig. 1C). There were no acute (day after stress) or chronic (recovery period) effects of restraint on water intake of rats (Fig. 1D). Cumulative food intake for the recovery period, including the day immediately after stress, was not significantly different between groups (228.2 ± 5.2 g for controls and 220.4 ± 5.2 g for restrained). Body weights (Fig. 2C) of rats restrained in the morning were altered in the same manner as in experiment 1 and there was a significant effect of restraint on body weight during the recovery period [F(1,28) = 20.13, P = 0.0001].

Food and water intakes measured at intervals during the dark cycle immediately after restraint stress are shown in Fig. 3. There was a statistically significant effect of restraint on food intake in intervals during this day [effect of group F(1,30) = 4.8, P = 0.036]. Food intake was suppressed by 35% (P = 0.01) in restrained rats during the first 4 h after stress, then both groups ate the same amount of food (~4 g) from 1500 to 1900. However, there was a second significant reduction of food intake in restrained rats during the first 2 h of the dark cycle from 1900 to 2100 (19% less, P = 0.035). Food intake was significantly higher (42%, P = 0.025) in the restraint group during the last 4 h of the dark period from 0400 to 0800 but 21-h food intake for this day remained significantly lower in restrained than control rats. There was no significant effect of restraint on water intake of the rats at any time during the 21 h after stress.

As shown in Table 1, there were no differences in glucose, insulin, or corticosterone concentrations. Corticosterone level of controls in experiment 2 were higher than those of controls in experiment 1. Although hypothalamic NPY mRNA expression, measured by Northern or in situ hybridization, did not reveal any differences between groups, PVN NPY protein was significantly elevated in restrained rats compared with their controls (40%; P < 0.05) (see Fig. 4).

Comparison of Two Experiments

Statistical analysis showed that during the baseline period there were no differences in the mean body weights, growth rate [effect of experiment F(1,60) = 0.001, P = 0.98], food intakes [effect of experiment F(1,60) = 0.02, P = 0.89], or water intakes (see Figs. 1 and 2).
and 2) of rats in experiments 1 and 2. There were no effects of time of day of stress on the daily food intakes of rats either on the day after stress or during the recovery period, indicating that in both experiments restrained rats decreased their food intake by the same amount on the day immediately after stress, compared with controls, and that in both cases there was no chronic effect of restraint on feeding. However, cumulative food intake calculated for the recovery period, including the day after the stress, revealed a significant difference between experiments \( F(1,59) = 14.01, P = 0.0004 \) and significant differences between controls of experiment 1 and 2 (251.1 ± 5.2 vs. 228.3 ± 5.2 g/10 day; \( P = 0.003 \)) and between restrained rats of experiments 1 and 2 (236.8 ± 5.4 vs. 220.4 ± 5.2 g/10 day; \( P = 0.03 \)). Weight loss was greater in rats restrained at the start of the light period than in those restrained at the end of the light period, and repeated-measurements analysis revealed a significant difference between experiments \( F(1,58) = 5.72, P = 0.02 \). As can be seen in Fig. 2, the body weight of rats restrained in the morning returned to baseline by day 5 of the recovery period, compared with day 2 in experiment 1, and even the values for controls were lower in experiment 2 than in experiment 1.

As shown in Fig. 1, food consumption during the day after stress was significantly lower for restrained rats than controls in both experiments, although there was no difference between daily food intakes of restrained rats in experiments 1 and 2, as described above. However, distribution of this reduction in food intake during the day was different between the two experiments, such that in experiment 1 all of the reduction in 21-h intake occurred during the first 2 h of the dark cycle. In experiment 2 feeding behavior was suppressed twice, namely during the 4 h after stress and during the first 2 h of the dark cycle. Although measurements of corticosterone and glucose levels during the first 2 h of the dark cycle did not reveal differences between experiments, the concentration of NPY in the PVN of rats restrained in the morning was higher than in the other three groups of rats (Fig. 4). Also there was an elevation of serum insulin in rats from experiment 2 compared with experiment 1, but no differences between control and restrained groups within each experiment.

Restraint had no effect on water intake in either experiment and AVP mRNA expression was not different in the supraoptic nuclei or PVN of the hypothalamus (Fig. 5).

**Experiment 3**

This experiment was designed to determine whether monoamines or sympathetic regulation was involved...
in the restraint-induced suppression of food intake. Monoamine concentrations in specific brain areas are shown in Table 2. Dopamine turnover (DOPAC/dopamine ratio) in the brain stem of rats restrained in the evening was significantly lower (18%) than in their controls, due to an increased dopamine content. In contrast, increased (28%) dopamine turnover in the hypothalamus of the same animals was due to an increased amount of the dopamine metabolite, DOPAC (19.4%). There were nonsignificant elevations of NE (2.18 ± 0.11 vs. 1.87 ± 0.06 ng/mg of tissue) and 5-HIAA (0.763 ± 0.0.049 vs. 0.667 ± 0.025 ng/mg of tissue) but not of 5-HT in the hypothalami of rats restrained in evening compared with their controls. Concentrations of monoamines and their metabolites in the hypothalamus and brain stem of animals restrained in the morning were not different compared with their controls (Table 2).

There was no effect of stress on UCP mRNA expression in interscapular brown fat pads or on leptin expression in epididymal fat (Fig. 6). Serum corticosterone, glucose, and insulin concentrations are shown in Table 3. There was a significant difference between experiments in insulin concentration, but glucose and corticosterone were not different among the four experimental groups.

**DISCUSSION**

These experiments clearly demonstrate a dissociation between restraint-induced inhibition of food intake and body weight loss. Food intake of restrained rats during the 21 h after stress was reduced by the same amount in experiments 1 and 2, but weight loss was greater in rats restrained at the start of the light cycle. Therefore, the increased weight loss in animals stressed in the morning has to be attributed to an elevation of metabolic rate. We made few measures related to energy expenditure in this experiment and it is unclear which metabolic pathway was primarily responsible for the difference in weight loss. Casual observation suggested that rats restrained in the morning, but not those restrained in the afternoon, had a high body surface temperature at the end of stress, indicating increased heat loss due to vasodilation or increased heat production. The end-point measurements of UCP mRNA expression suggested that metabolic activation was not mediated through the sympathetic outflow in BAT, at least during the first hour after stress for rats restrained in the evening and 9 h after the stress for rats restrained in the morning. However, direct measurement of BAT activation is needed to determine its role in promoting weight loss of restrained animals.

The relationship between the stage of the dark-light cycle and stress response has been attributed to circulating concentrations of corticosterone or hypothalamic-pituitary-adrenal (HPA) activity. Our findings are consistent with reports by Dallman's group (4) that responsivity of the HPA axis to stress is highest in the morning, when HPA activity is at its lowest. The stress response is independent of peripheral basal level of corticosterone concentration, as it is maintained in adrenalectomized animals (4), and the corticosterone response is lower in the morning than in the evening (32). In this experiment there was no correlation between serum corticosterone, measured 1 or 9 h after stress, and weight loss and no difference in corticosterone between groups; however, a role for corticosterone as a trigger factor in a cascade of events that results in weight loss cannot be excluded on the basis of a single end-point measure. In contrast to food-deprived animals, which rapidly recover body weight after the end

Table 2. Concentrations of monoamines and their metabolites in the hypothalamus and brain stem of rats in experiment 3

<table>
<thead>
<tr>
<th></th>
<th>Control 1 (Afternoon)</th>
<th>Restraint 1 (Afternoon)</th>
<th>Control 2 (Morning)</th>
<th>Restraint 2 (Morning)</th>
</tr>
</thead>
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<tr>
<td><strong>Hypothalamus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>NE</td>
<td>1.87 ± 0.07</td>
<td>2.18 ± 0.11</td>
<td>2.10 ± 0.05</td>
<td>2.09 ± 0.05</td>
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<tr>
<td>MHPG</td>
<td>0.11 ± 0.01</td>
<td>0.12 ± 0.021</td>
<td>0.11 ± 0.01</td>
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<tr>
<td>MHPG/NE</td>
<td>0.05 ± 0.01</td>
<td>0.05 ± 0.01</td>
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</tr>
<tr>
<td>5-HT</td>
<td>0.64 ± 0.01</td>
<td>0.62 ± 0.01</td>
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<tr>
<td>5-HIAA</td>
<td>0.67 ± 0.03</td>
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<tr>
<td>5-HIAA/5-HT</td>
<td>1.04 ± 0.03</td>
<td>1.21 ± 0.05</td>
<td>1.03 ± 0.02</td>
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<tr>
<td>Dopamine</td>
<td>0.363 ± 0.011</td>
<td>0.350 ± 0.023</td>
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<td>DOPAC</td>
<td>0.039 ± 0.001</td>
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<td>DOPAC/dopamine</td>
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<td><strong>Brain stem</strong></td>
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<tr>
<td>NE</td>
<td>0.72 ± 0.01</td>
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<td>5-HT</td>
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</tr>
<tr>
<td>5-HIAA</td>
<td>0.59 ± 0.01</td>
<td>0.59 ± 0.01</td>
<td>0.59 ± 0.01</td>
<td>0.60 ± 0.02</td>
</tr>
<tr>
<td>5-HIAA/5-HT</td>
<td>0.44 ± 0.01</td>
<td>0.47 ± 0.01</td>
<td>0.43 ± 0.01</td>
<td>0.44 ± 0.01</td>
</tr>
<tr>
<td>Dopamine</td>
<td>0.060 ± 0.002</td>
<td>0.070 ± 0.002</td>
<td>0.073 ± 0.003</td>
<td>0.076 ± 0.003</td>
</tr>
<tr>
<td>DOPAC</td>
<td>0.029 ± 0.0003</td>
<td>0.028 ± 0.0004</td>
<td>0.029 ± 0.001</td>
<td>0.031 ± 0.001</td>
</tr>
<tr>
<td>DOPAC/dopamine</td>
<td>0.491 ± 0.013</td>
<td>0.405 ± 0.009*</td>
<td>0.403 ± 0.01</td>
<td>0.422 ± 0.02</td>
</tr>
</tbody>
</table>

Data are means ± SE for group of 6 animals. NE, norepinephrine; MHPG, 3-methoxy-4-hydroxyphenylethylene glycol; 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid; DOPAC, 3,4-dihydroxyphenylacetic acid. *Significant difference between control and restraint groups in experiment 1 (P < 0.05) determined by t-test considering model for this experiment only.
of restriction, restrained rats failed to return to control body weight, suggesting that regulatory mechanisms that correct weight loss caused by food restriction and by restraint stress are different.

Although 3 h of restraint produced an acute reduction in food intake, regardless of the time of day that stress was applied, the diurnal pattern of feeding was different in experiments 1 and 2. When rats were restrained at the end of the light period all of the depression in 21-h food intake occurred during the first 2 h of the dark cycle, whereas, in rats restrained in the morning, food intake was decreased during the first 4 h after stress and during the first 2 h of the dark cycle, 9 h after the stress had ended. Measurements of hormones and neurotransmitters considered likely mediators of the reduction in food intake were made to determine their importance in the delayed response to restraint stress. The concentration of NPY in the PVN is considered an index of hunger (5, 23), with elevated levels of NPY stimulating feeding. The results from this experiment are not consistent with NPY independently driving food intake, as all rats were killed at a time when the restraint-induced suppression of food intake was present, but PVN NPY protein was not significantly different from controls in rats restrained immediately before the dark cycle and was significantly increased in animals restrained in the morning. Because NPY mRNA expression was not increased, NPY release from the PVN may have been inhibited by other restraint-induced factors or receptor, or postreceptor, events were blocked by other transmitters. Either of these conditions would result in a reduced food intake in the presence of elevated PVN NPY concentrations. Although it has been shown that corticosterone stimulates NPY (15) and insulin suppresses NPY (27), there was no correlation between serum corticosterone or insulin and hypothalamic NPY concentration in this experiment. CRH is believed to be involved in suppression of NPY function because central injection of CRH decreases NPY mRNA expression (2). Although we did not measure CRH concentration in this study, expression of NPY was not altered. Moreover, CRH is involved in the acute stress response (20) and rats in this experiment that had elevated levels of NPY had been stressed 9 h before the tissue was collected. Recently Smith et al. (28) reported that leptin, a cytokine released from adipose tissue, modified the sensitivity of mice to exogenous NPY. In this experiment there was no effect of stress on adipose leptin expression in experiment 1 or 2, suggesting that it was not responsible for a change in sensitivity to NPY under the conditions described here, although measurements of hypothalamic leptin concentration are needed to confirm this conclusion.

The results from experiment 3 indicate that monoaminergic systems were activated immediately after restraint, but that this activation was not maintained in rats that had been restrained 9 h earlier. These results are consistent with those of others (26) who have shown that hypothalamic 5-HT concentration increases to a maximum 40 min after initiation of immobilization but then decreases toward the preimmobilization levels even while the stress continues. The nonsignificant elevation of 5-HIAA in the hypothalamical

| Table 3. Serum insulin, corticosterone, and glucose of rats in experiment 3 |
|-----------------------------|----------------|----------------|----------------|
|                             | Glucose, mg/dl | Corticosterone, ng/ml | Insulin, ng/ml |
| Control 1 (afternoon)       | 120 ± 3        | 77 ± 23          | 2.2 ± 0.8      |
| Restraint 1 (afternoon)     | 126 ± 2        | 25 ± 3           | 1.1 ± 0.1      |
| Control 2 (morning)         | 127 ± 3        | 56 ± 10          | 4.0 ± 0.9*     |
| Restraint 2 (morning)       | 128 ± 3        | 26 ± 14          | 3.0 ± 0.5      |

Data are means ± SE for 6 animals. *Significant difference between insulin levels of controls for rats restrained in the morning and rats restrained in the afternoon.
us of rats restrained immediately before the start of
the dark period was also consistent with observations
that 5-HIAA concentrations increase throughout a pe-
riod of immobilization (26) and that hypothalamic
5-HIAA, but not 5-HT, is elevated immediately after a
single 2-h restraint stress (10). In experiment 3, dopa-
mine concentrations were increased in the brain stem
and dopamine metabolism was increased in the hypo-
thalamus of rats that had been restrained in the
afternoon. It has been shown that dopamine induces
feeding behavior and interruption of ascending dopa-
mine axons by lateral hypothalamic lesions produces
aphagia and adipsia (22). Thus an increased level
of dopamine turnover would be expected to increase food
intake and it is possible that, in this experiment,
dopamine returned food intake to control levels after
the first 2 h of the dark period. However, similar
differences were not present in rats restrained in the
morning although the intake of these rats would return
to control levels at the same time as that of rats
stressed at the end of the dark period, making it
unlikely that dopamine determined the duration of
restraint-induced inhibition of food intake.

Apart from the acute, 21 h, inhibition of food intake,
we did not find any effect of restraint on intake during
the recovery period. However, both restrained and
controls rats from experiment 1 had lower cumulative
food intakes than corresponding groups in experiment
2, although intakes during the control period were not
different between experiments. These results suggest
that, although control rats were not restrained, they
were stressed by exposure to a novel environment when
they were moved into the same room as restrained rats
for the 3-h stress period. This difference in intake and
the difference in body weight between control groups
from the two experiments emphasizes that the time of
the light-dark cycle at which stress is applied is a
critical determinant of the chronic effect of stress on
food intake and body weight loss. In contrast to food
intake, there were no acute or chronic effects of 3 h of
restraint stress on fluid intake. These data and the
results from in situ hybridization for AVP mRNA are
consistent with published results that acute restraint
stress, in contrast to osmotic stress, does not increase
AVP mRNA expression in the rats (18).

Measurements performed in the current study fo-
cused primarily on the acute response to restraint
stress and do not explain physiological responses ob-
served during the recovery period. We found that 3 h of
restraint had no effect on water intake, decreased food
intake during the day after stress, and induced body
weight loss. However, stress applied at the beginning of
the light cycle caused a greater weight loss and reduc-
tion in cumulative intake during the recovery period
than stress applied at the end of the light period,
although there was no difference in the degree of
suppression of intake during the day immediately after
stress. Because we did not find any obvious relation-
ship between the concentrations of neurotransmitters
or hormones measured in these experiments and food
intake of restrained rats during the start of the dark
period, it has to be concluded that restraint activates
mechanisms and neurotransmitters that were not mea-
sured here. Further studies are needed to determine
the effect of restraint stress on energy expenditure and
to define the roles of other candidate signals, such as
urocortin (29), a CRF2 receptor agonist, cytokines,
and melanocorticotropin, in mediating the delayed suppres-
sion of feeding that occurs in rats exposed to restraint
stress.

Perspectives

It is well established that acute and chronic stress
inhibit food intake and cause weight loss. Experiments
described here show a dissociation between suppres-
sion of food intake and body weight loss, indicating that
weight loss after restraint stress is not exclusively due
to hypophagia and that there may be a long-term re-
setting of the level at which body weight is defended.
The composition of this weight loss remains to deter-
mined. We found a significant suppression of food
intake at the start of the dark period irrespective of the
time of day that restraint was applied. The central
mechanisms mediating this response are unclear, and
the pathways investigated in this study did not show
any direct evidence of involvement of sympathetic
nervous system based on UCP mRNA expression, NPY,
or monoamines, although others have shown all these
pathways to be modulated by stress and to influence
energy balance. It has to be concluded that pathways
other than those investigated here were involved in the
response and potential candidates include cytokines
and urocortin.

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