Hypophagia induced by glucocorticoid deficiency is associated with an increased activation of satiety-related responses

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On the other hand, bilateral adrenalectomy (ADX) reduces food intake and body weight gain, and these effects are reversed by glucocorticoid replacement in rats (12). Furthermore, ADX reduces hyperphagia and obesity in different experimental models, whereas glucocorticoid replacement reverses these effects (4, 8, 52). Hypophagia induced by ADX has been associated with a decrease of mRNA expression of hypothalamic neuropeptide Y (NPY), a potent orexigenic neuropeptide (41). ADX also may reduce food intake through the increase of corticotrophin-releasing factor (CRF) mRNA expression in the paraventricular nucleus (PVN), since CRF is a well-known anorexigenic peptide (1, 2). CRF neurons in the PVN receive projections from neurons of the nucleus of the solitary tract (NTS) (6, 35, 45). This brain stem nucleus receives sensory information from gastrointestinal tract and abdominal viscera, as well as taste information from the oral cavity (19, 48), being involved in the satiety pathways.

Satiety signals control meal size and include mechanical and chemical stimulation of the stomach and small intestine, as well as hormones released during feeding (16, 38). Oral, pharyngeal, and esophageal stimulation and gastric distension induce Fos expression in catecholamine-expressing neurons in the NTS (11, 51). Furthermore, duodenal nutrient infusion inhibits food intake and induces neuron activation in the NTS (29), with a positive correlation between the amount of food intake and this neuron activation (54).

Responses associated with satiety also involve hypothalamic pathways, such as PVN and the arcuate nucleus (ARC), as demonstrated by their activation following food intake (39). These nuclei have been implicated in the control of energy homeostasis and express orexigenic and anorexigenic peptides (38). PVN receives projections not only from satiety-related neurons of the NTS but also from ARC neurons (38), indicating that the hypothalamus plays an important role in the integrative responses that control food intake.

Several hormones released during a meal, such as cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1), and bombesin (26), are known to participate in the satiety-associated responses (16). CCK is a meal-related signal released from mucosal endocrine cells in response to nutrients entering the duodenum (13, 27). CCK induces pyloric and gallbladder contraction and inhibits gastric emptying (20, 25). It also inhibits food intake in rodents and humans (14, 18) through an activation of NTS neurons mediated by vagal afferent fibers (10, 22). Furthermore, catecholaminergic neurons in the NTS have been shown to be activated by CCK treatment (31, 37).

To investigate the role of glucocorticoids on satiety-associated responses, we designed the present study to evaluate the effects of adrenalectomy and glucocorticoid replacement on activation of NTS catecholaminergic neurons, CRF neurons in the PVN, and ARC neurons induced by feeding. We also investigated the food intake and the activation of NTS neurons in response to CCK stimulation.
EXPERIMENTAL PROCEDURES

Animals

Male Wistar rats (200–250 g) obtained from the Animal Facility of the Campus of Ribeirao Preto, University of Sao Paulo, were housed in individual cages in temperature-controlled conditions (23 ± 2°C) and a fixed light-dark cycle (lights from 6:00 AM to 6:00 PM). Animals had ad libitum access to pelleted rat chow and fluid, unless otherwise specified. To improve adaptation to the laboratory, rats were handled daily during the 7 days before experiments. All experimental procedures were conducted between 7:00 AM and 12:00 PM and were approved by the Ethical Committee for Animal Use of the School of Medicine of Ribeirao Preto, University of Sao Paulo.

Bilateral ADX and sham surgeries were performed under 2.5% 2,2,2-tribromoethanol (1 ml/100 g body wt ip) anesthesia via dorsal incision. Sham-operated animals underwent similar surgical procedures without removal of the adrenal glands. All ADX animals were given 0.9% saline to drink during the experimental period, and one-half of them also were supplemented with 25 mg/l corticosterone (B) in 0.5% ethanol dissolved in 0.9% saline. To ensure completeness of ADX surgery and the adequacy of glucocorticoid replacement, we determined B plasma levels by radioimmunoassay (5). ADX animals with 0.9% saline showed undetectable B plasma levels. Plasma B levels were measured 7 days after surgery in ad libitum food intake (experiment 1) and also after 16 h of fasting (experiment 2).

Perfusion, Tissue Preparation, and Immunohistochemistry

Animals were anesthetized with an injection of 2.5% tribromoethanol (1 ml/100 g body wt ip). Thereafter, rats were transcardially perfused with 200 ml of cold isotonic saline containing heparin (50 UI/l), followed by 500 ml of cold 4% paraformaldehyde solution in 0.1 M phosphate buffer (PB), pH 7.2. The brain was removed, fixed for 1 h in 4% paraformaldehyde solution, and stored at 4°C in PB containing 30% sucrose. Coronal sections of 30 μm were obtained in a cryostat (Microm) and collected in PB. Sections were first processed for Fos immunoreactivity, with an overnight incubation at room temperature with an anti-Fos antibody raised in rabbit (Ab-5; Oncogene Science, Manhasset, NY), diluted 1:10,000 in 0.1 M PB containing 2% normal goat serum and 0.3% Triton X-100. Free-floating sections were then washed with PB and incubated with biotin-labeled anti-rabbit immunoglobulin (Vector Laboratories, Burlingame, CA; 1:200 dilution in 1.5% normal goat serum-PB), followed by the avidin-biotin-peroxidase complex (Vector; 1:200 dilution in PB), both for 1 h at room temperature. The blue-black labeling of cell nucleus was detected using diaminobenzidine hydrochloride (DAB; Sigma Chemical, St. Louis, MO), diluted 1:10,000 in 0.1 M PB containing 0.01% H2O2. Sections were then washed with PB and incubated with biotin-labeled antibody anti-mouse (Vector; 1:200) or biotinylated goat anti-rabbit IgG (Vector, 1:200), followed by avidin-biotin-peroxidase complex (Vector; 1:200 dilution in PB), both for 1 h at room temperature. TH or CRF cytoplasmic labeling (brown) was counted only if it had a clearly labeled cell body surrounding a nucleus. Sections were unilaterally counted in sections of the NTS, PuMP, PuPo, ARC, and RCA. The visual counting of neurons was performed in two to three sections for Fos, Fos/TH, and Fos/CRF and in four to five sections of each animal for RCA and ARC from five to seven animals of each experimental condition by participants blind to the experimental protocols.

Experimental Protocols

Experiment 1: Effects of ADX and B replacement on food intake, body weight gain, and fluid intake. Rats were housed in metabolic cages and subjected to sham or ADX surgery. One-half of the ADX animals were replaced with B (ADX+B) in their drinking water (25 mg/l B in 0.9% NaCl with 0.5% ethanol). During the 7 days following surgery, food intake, body weight, and fluid intake were monitored daily in the morning.

Experiment 2: Effects of ADX and B replacement on food intake and neuron activation in the NTS, PVN, ARC, and RCA in the fasting-refeeding regimen. Animals were divided into sham, ADX, or ADX+B groups and kept in metabolic cages. Six days after surgery, they were fasted from 4:00 PM until 8:00 AM the next day (16 h of fasting), and fluid intake during this period of fasting was measured. Food was reintroduced, and food intake was determined after 2 h of refeeding. Another set of sham, ADX, and ADX+B rats, kept in individual cages, was fasted for 16 h, as described above, on the sixth day after surgery, and thereafter they were transcardially perfused, with the aid of a computerized system that includes a Leica microscope equipped with a DC 200 Leica digital camera attached to a contrast enhancement device. Fos-immunoreactive neurons, as indicated by black staining, were identified when the nuclear structure demonstrated a clear immunoreactivity compared with the background level. TH or CRF cytoplasmic labeling (brown) was counted only if it had a clearly labeled cell body surrounding a nucleus. Sections were unilaterally counted in sections of the NTS, PuMP, PuPo, ARC, and RCA. The visual counting of neurons was performed in two to three sections for Fos, Fos/TH, and Fos/CRF and in four to five sections of each animal for RCA and ARC from five to seven animals of each experimental condition by participants blind to the experimental protocols.

Fig. 1. The number of Fos (A)- and Fos/tyrosine hydroxylase (Fos/TH)-immunoreactive (ir) neurons (B) in the nucleus of the solitary tract (NTS) of fasted and refed sham, adrenalectomized (ADX), and ADX plus corticosterone treatment (ADX+B) animals (n = 5–6 rats/group). Data are means ± SE. *P < 0.01.

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before or 2 h after refeeding, for brain tissue collection and immunohistochemistry studies.

Experiment 3: Effects of ADX and corticosterone replacement on food intake and catecholaminergic neuron activation in the NTS after CCK administration. Animals were divided into sham, ADX, or ADX+B groups and kept in individual cages. Six days after surgery, animals were fasted for 16 h, as described above, and on the seventh day after surgery, they were injected with CCK (Bachem; 3.5 µg/kg ip) or vehicle (0.9% NaCl). Soon after the injections, all animals had access to food, and food intake was determined during 2 h. Another set of sham, ADX, and ADX+B rats, fasted for 16 h, was subjected to the same treatment with CCK or vehicle, and 2 h later they were transcardially perfused for brain stem tissue collection and immunohistochemistry studies.

Statistical Analysis

Data are means ± SE. The Kruskal-Wallis test (experiments 1 and 2), followed by Dunn’s post test, and two-way ANOVA, followed by Bonferroni’s post test (experiments 2 and 3), were used when appropriate. Differences were considered significant at P < 0.05.

RESULTS

Experiment 1: Effects of ADX and B Replacement on Food Intake, Body Weight Gain, and Fluid Intake

The ADX group showed reduced (P < 0.001) mean daily food intake (19.3 ± 0.7 g) compared with the sham (24.9 ± 0.7 g) and ADX+B groups (23.3 ± 0.7 g). The ADX group (61.0 ± 5.2 g) showed a lower (P < 0.05) body weight gain than the sham (79.5 ± 3.9 g) and ADX+B groups (71.3 ± 4.8 g), with no difference in mean daily food intake and body weight gain between the sham and ADX+B groups. There was no difference in mean daily fluid intake among the sham (53.2 ± 4.2 ml), ADX (62.6 ± 3.3 ml), and ADX+B groups (59.8 ± 5.8 ml). There was no difference in plasma corticosterone levels between the sham (2.0 ± 0.5 µg/dl) and ADX+B groups (1.5 ± 0.2 µg/dl), whose values were higher than those obtained in the ADX group (0.4 ± 0.0 µg/dl).

Experiment 2: Effects of ADX and B Replacement on Food Intake and Neuron Activation in the NTS, PVN, ARC, and RCA in the Fasting-Refeeding Regimen

ADX animals showed a lower (P < 0.001) food intake compared with sham animals (5.7 ± 0.3 vs. 8.7 ± 0.4 g). B replacement reversed (P < 0.001) this anorexigenic effect, as observed in the ADX+B group (7.9 ± 0.3 g). During 16 h of fasting, fluid intake of the ADX and ADX+B groups was higher (P < 0.05) than that of the sham group. After 16 h of fasting, there was no difference in plasma corticosterone levels between the sham (1.7 ± 0.5 µg/dl) and ADX+B groups (1.8 ± 0.3 µg/dl), whose values were higher than those obtained in the ADX group (0.4 ± 0.0 µg/dl). There was no

Fig. 2. Representative photomicrographs (×40 magnification) of coronal sections showing Fos/TH immunoreactivity in the NTS of fasted and refeed sham, ADX, and ADX+B animals. Each inset depicts the area where the photomicrograph was taken at ×20 magnification. Scale bar, 100 µm.
difference in plasma corticosterone levels in the ADX+B group obtained after ad libitum food intake and those values obtained after fasting for 16 h.

Figure 1 shows the number of Fos- and Fos/TH-immunoreactive neurons in the NTS of sham, ADX, and ADX+B animals in fasting conditions and 2 h after refeeding. There was an interaction between group (sham, ADX, or ADX+B) and feeding status (fasted or refed) on the number of Fos ($F_{5,40} = 7.7; P = 0.002$) and Fos/TH-immunoreactive neurons ($F_{5,40} = 6.2; P = 0.005$) in the NTS. We observed an effect of group (Fos: $F_{5,40} = 9.3; P < 0.001$; Fos/TH: $F_{5,40} = 6.5; P = 0.004$) and feeding status (Fos: $F_{5,40} = 314.6; P < 0.001$; Fos/TH: $F_{5,40} = 227.0; P < 0.001$) on the number Fos- and Fos/TH-immunoreactive neurons in the NTS. Compared with sham and ADX+B animals, refeeding increased ($P < 0.05$) the number of Fos-immunoreactive neurons compared with the sham and ADX+B groups. Representative photomicrographs of Fos expression and Fos/CRF double labeling in the NTS are shown in Fig. 2.

The number of Fos- and Fos/CRF-immunoreactive neurons in the PaMP and PaPo of sham, ADX, and ADX+B animals before or 2 h after refeeding is shown in Fig. 3. There was no interaction between group and feeding status on the number of Fos-immunoreactive neurons in the PaPo. In the PaPo, refeeding increased ($P < 0.05$) the number of Fos-immunoreactive neurons in the three experimental groups, with a higher value ($P < 0.05$) in the ADX group compared with the sham and ADX+B groups. Representative photomicrographs of Fos expression in the PaPo subdivision are shown in Fig. 4.

Figure 5 shows the number of Fos-immunoreactive neurons in the RCA and ARC of sham, ADX, and ADX+B animals in fasting conditions and 2 h after refeeding. Fasted ADX animals showed a higher number of Fos-immunoreactive neurons in the ARC compared with fasted sham and ADX+B groups. There was no interaction between group and feeding state on the number of Fos-immunoreactive neurons in the RCA. We observed an effect of group (Fos: $F_{5,31} = 11.2; P < 0.001$) and feeding state ($F_{5,31} = 28.1; P < 0.001$) on the number Fos-immunoreactive neurons in the ARC. In this nucleus, compared with fasted conditions, the three experimental groups showed an increase ($P < 0.05$) in the number of Fos-immunoreactive neurons after refeeding, with higher values ($P < 0.05$) in the ADX group.

There was no interaction between group and feeding status on the number of Fos-immunoreactive neurons in the ARC. We observed an effect of group (Fos: $F_{5,33} = 10.9; P < 0.001$) and feeding status ($F_{5,33} = 9.5; P = 0.005$) on the number of Fos-immunoreactive neurons in the RCA. Refeeding increased ($P < 0.05$) the number of Fos-immunoreactive neurons in the RCA only in the ADX group. Representative photomicrographs of Fos expression in the ARC are shown in Fig. 6.
Experiment 3: Effects of ADX and Corticosterone Replacement on Food Intake and Catecholaminergic Neuron Activation in the NTS After CCK Administration

Figure 7 shows the food intake of sham, ADX, and ADX+B animals in response to vehicle or CCK injection. There was no interaction between group (sham, ADX, or ADX+B) and treatment (vehicle or CCK) on food intake. We observed an effect of group ($F_{5,79} = 12.4; P < 0.001$) and treatment ($F_{5,79} = 17.8; P < 0.001$) on food intake. We observed that in vehicle-treated animals, the ADX group showed lower ($P < 0.01$) food intake compared with the sham and ADX+B groups. CCK treatment reduced ($P < 0.01$) food intake in sham and ADX+B animals compared with their respective vehicle groups. However, in ADX animals, CCK did not alter food intake compared with vehicle treatment.

Figure 7 shows the number of Fos- and Fos/TH-immunoreactive neurons in the NTS of sham, ADX, and ADX+B animals treated with vehicle or CCK. There was no interaction between group and treatment on the number of Fos- and Fos/TH-immunoreactive neurons in the NTS. We observed an effect of treatment on the number of Fos ($F_{5,36} = 85.4; P < 0.001$) and Fos/TH-immunoreactive neurons ($F_{5,36} = 40.1; P < 0.001$) in the NTS, with no effect of group. Compared with vehicle-treated animals, CCK increased ($P < 0.01$) the number of Fos- and Fos/TH-immunoreactive neurons in the NTS, with no difference among the three groups.

DISCUSSION

The present study investigated the effects of adrenalectomy and glucocorticoid replacement on activation of NTS, PVN, RCA, and ARC neurons induced by feeding. We observed that adrenalectomy reduced food intake and body weight gain, with an increased activation of NTS, PVN, RCA, and ARC neurons after refeeding.

To investigate the effect of glucocorticoid withdrawal, we used ADX rats with and without corticosterone replacement in drinking fluid. Jacobson et al. (17) using corticosterone pellet in ADX rats showed constant corticosterone plasma levels, whereas replacement in the drinking water resulted in an increase in plasma corticosterone after lights-off, corresponding to the feeding period. They also demonstrated that ADX rats with pellet corticosterone replacement showed an increased ACTH response to restraint, whereas rats with replacement in the drinking water showed a similar response, compared with sham animals. These data suggested that maintenance of circadian variation of corticosterone plasma levels in ADX rats, achieved by replacement in the drinking water as used in the present study, would benefit the control of hypo-
The hypothalamic neuronal responses to feeding was assessed using a fasting and refeeding regimen. After fasting for 16 h, the hypothalamic-pituitary-adrenal axis activity. The brain stem and thalamic-pituitary-adrenal axis activity. The brain stem and hypothalamic neuronal responses to feeding was assessed using a fasting and refeeding regimen. After fasting for 16 h, the ADX+B group showed an increased fluid intake, which allowed similar plasma corticosterone levels compared with the sham and also the ADX+B group at ad libitum feeding, confirming the adequacy of corticosterone replacement.

We observed that refeeding induced an increase of Fos- and Fos/TH-immunoreactive neurons in the NTS in control (sham) animals, as previously demonstrated in the literature (32, 54). Mechanical and chemical stimulation of the stomach and small intestine, as well as humoral signals released during food intake, limit the size of a meal (16). Catecholaminergic neurons in the NTS receive direct synapses from vagal afferents of the gastrointestinal tract, showing that these NTS neurons participate in the short-term control of food intake (38, 42).

We also observed that refeeding increased the activation of CRF neurons in the PVN, as well as that of RCA and ARC neurons. These results are in agreement with data previously described, which show that refeeding activates ARC and PVN neurons, indicating that these nuclei also are implicated in the responses associated with satiety (39). Activation of parvocellular PVN in response to food intake is likely to involve vagal innervation, since vagotomy abolishes the activation of this region induced by feeding (47). It is well known that NTS catecholaminergic neurons project to the paravascular subdivision of the PVN; therefore, ascending projections from NTS presumably could activate CRF neurons in the PVN (21). On the other hand, activation of ARC following a meal has been associated with direct actions of meal-related signals, such as GLP-1 and peptide YY (3, 23).

It is well established that glucocorticoid deficiency causes hypophagia; however, there is no report on the effects of ADX in the satiety. The present study is the first to demonstrate that in the absence of glucocorticoid, the responses associated with satiety are augmented, as shown by the increased Fos and Fos/TH expression within the NTS after refeeding in ADX animals. NTS is known as the site that relays satiety signals, and it is implicated in the short-term control of food intake (16). Interestingly, glucocorticoid withdrawal induced a further activation of catecholaminergic and noncatecholaminergic neurons of the NTS after refeeding. Glucocorticoid receptors have been identified in the NTS catecholaminergic neurons (15, 24), reinforcing the suggestion that glucocorticoids can modulate neuron activation in the NTS during the short-term control of food intake. In humans, patients with chronic adrenal insufficiency present anorexia, weight loss, general malaise, and nausea. Nausea itself could affect satiety; thus, in ADX rats, although difficult to determine, it is conceivable that nausea also could contribute to the higher responses associated with satiety.

We observed that CCK reduced food intake in the sham and ADX+B groups, confirming the anorexigenic effect of this peptide, as first described by Gibbs et al. (14). CCK induced Fos and Fos/TH expression in the NTS in the sham, ADX, and ADX+B groups. These findings agree with data from the literature, showing that CCK activates catecholaminergic and noncatecholaminergic neurons in the brain stem of intact rats (10, 37). CCK induced an activation of NTS neurons, but, interestingly, it did not reduce food intake in the ADX group. Therefore, the unchanged food intake in ADX animals following CCK administration was dissociated from the increased Fos expression in NTS neurons. These data differ from those of the refeeding protocol (experiment 2), in which lower food intake was associated with higher NTS activation in the ADX group. A similar increase of Fos expression in the NTS in ADX rats, compared with sham rats, also was described by Raboin et al. (30) using a higher dose of CCK (40 μg/kg ip). During food intake, multiple signals besides CCK, including a combination of oral sensory and/or gastric distension signals, mediate responses associated with satiety (16, 26). Therefore, we believe that CCK alone cannot further increase the anorexigenic effect induced by glucocorticoid deficiency.

The present study also suggests that the increase of satiety-associated responses induced by glucocorticoid deficiency involves hypothalamic pathways, as demonstrated by the increased activation of RCA, ARC, and CRF neurons in the PVN in response to food intake. ADX animals showed higher activation of CRF parvocellular neurons in the PVN after refeeding, mostly located in the posterior subdivision of this nucleus. Activation of these neurons, known to participate in the control of sympathetic activity and the regulation of food intake (34, 36, 44), may contribute to the hypophagia in ADX rats. In addition, CRF receptors were identified in NTS neurons, reinforcing the modulation of NTS activity by CRF parvocellular neurons (40, 49). Thus the augmented activation of descending CRF neurons from the PVN may contribute to the higher activation of NTS neurons observed in ADX animals. On the other hand, ascending projections
from NTS to the parvocellular subdivision of the PVN (6, 21, 35) may contribute to the increased activation of CRF neurons in the PVN after refeeding in ADX animals.

We also observed that glucocorticoid deficiency induced an activation of ARC neurons, and this effect was further increased by refeeding, demonstrating that ARC neurons are responsive to satiety signals and that this effect can be modulated by glucocorticoids. It was previously shown that ARC neurons participate in the regulation of food intake during the early phase of refeeding (39). NPY in the ARC is known to increase food intake; thus we can predict that NPY neurons would not be activated in the ADX group. In fact, previous studies demonstrated that adrenalectomy decreases NPY mRNA expression in the ARC (41, 50). α-melanocyte-stimulating hormone, derived from proopiomelanocortin (POMC) cleavage in the ARC, has well-established anorexigenic effects (38); thus we can speculate that the phenotype of NPY neurons in the ARC could be POMC neurons. In addition, direct projections from RCA to the NTS have been demonstrated (9, 43). The increased activation of ARC and RCA neurons after refeeding in ADX animals can modulate the activity of CRF neurons in the PVN, since it is known that ARC neurons project to the parvocellular PVN (36), which in turn might modulate NTS neuron activity through the descending pathway mentioned above. Moreover, reciprocal innervations between ARC and NTS (7, 43, 53) could be involved in the responses associated with satiety and might account for the lower food intake in ADX rats.

In summary, our results show that glucocorticoid withdrawal reduces food intake, which is associated with a higher activation of NTS, PVN, RCA, and ARC neurons in response to feeding. Together, these data suggest that in the absence of glucocorticoids, meal-related signals elicited during feeding lead to an augmented activation of satiety-associated responses through brain stem and hypothalamic pathways, which could contribute to the hypophagia in ADX animals.

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Fig. 7. Food intake after 2 h of refeeding (A) (n = 10–15 rats/group) and the number of Fos (B) and Fos/TH-ir neurons (C) in the NTS (n = 6 rats/group) of sham, ADX, and ADX+B animals treated with vehicle or cholecystokinin (CCK; 3.5 μg/kg). Data are means ± SE. *P < 0.05.

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