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Differential Effects of Adrenalectomy on Melanin-Concentrating Hormone and Orexin A

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Removal of glucocorticoids by adrenalectomy (ADX) reduces food intake and body weight in rodents and prevents excessive weight gain in many genetic and dietary models of obesity. Glucocorticoids play a key role to promote positive energy balance in normal and pathological conditions, at least in part, by altering the sensitivity to hypothalamic peptides. The hyperphagia after central neuropeptide Y administration, for example, is attenuated by ADX, and there is evidence that glucocorticoids influence both MCH and orexin A activity. In the present study, feeding responses to third ventricular MCH and orexin A were measured in rats after bilateral ADX or sham surgery. ADX rats were significantly less sensitive to the orexigenic action of third ventricular MCH, whereas orexin A-induced hyperphagia was unaffected. Replacement of corticosterone in the drinking water of ADX rats reversed the effects of ADX on MCH sensitivity. Although we found significant populations of glucocorticoid receptors in the lateral hypothalamus, none were colocalized with either MCH or orexin A-containing cell bodies. Furthermore, whereas ADX significantly reduced hypothalamic MCH and orexin gene expression, this could not be restored by glucocorticoids in the drinking water. Collectively, the present data suggest that glucocorticoids may promote food intake in part by potentiating the orexigenic actions of MCH without affecting the actions of orexin A and that glucocorticoids act indirectly to influence the effects of MCH on food intake. (*Endocrinology* 145: 3404–3412, 2004)

OBESITY IS A MAJOR risk factor for many diseases, including diabetes mellitus, hypertension, cardiovascular disease, and some cancers (1). Among the myriad factors mediating the regulation of food intake and body weight is the glucocorticoid, cortisol (corticosterone in rats) that is secreted from the adrenal cortex. Glucocorticoids are lipophilic molecules that freely cross the blood-brain barrier and interact with receptors distributed throughout the central nervous system, including those nuclei in the hypothalamus that are critical for energy balance regulation (2). Glucocorticoids have long-term effects on food intake and obesity (3), and in rodents, most forms of genetic obesity are associated with increased glucocorticoid levels, whereas a lack of glucocorticoids is linked to hypophagia and reduced body weight (4). The central infusion of glucocorticoids also leads to an increase in food intake and body weight (5). In humans, a change in glucocorticoid status can also alter body weight. For example, individuals with Addison's disease exhibit anorexia and weight loss due to adrenal insufficiency, whereas patients with Cushing's disease and other glucocorticoid excess syndromes have increased food intake and visceral adiposity (reviewed in Ref. 2). In rodents, adrenalectomy (ADX) reverses many forms of obesity, whereas glucocorticoid replacement restores the obese state (4, 6) and glucocorticoid administration to adrenal-intact animals increases food intake and weight gain (4, 7).

In addition to their important metabolic effects in the periphery, glucocorticoids act in several hypothalamic nuclei associated with the control of energy balance. Several studies have elucidated the actions of glucocorticoids on activity of both the paraventricular and arcuate nuclei (ARC) (8). Less explored is whether glucocorticoids also act upon neuropeptide systems within the lateral hypothalamus (LH). Because it has long been known that activity of the LH is associated with increased food intake and weight gain, one reasonable hypothesis about the powerful effects of glucocorticoids to stimulate positive energy balance is that they act in part by stimulating the neuropeptide systems within the LH.

The first of these neuropeptide systems in the LH is melanin-concentrating hormone (MCH), a 19-amino acid polypeptide originally isolated from salmon pituitaries (9). Neuroanatomical studies in rats demonstrate that MCH gene expression is limited to the LH and the zona incerta with extensive fiber projections throughout the brain (10). MCH is produced from prepro-MCH along with two other putative peptides, neuropeptides EI and GE (11). The only known MCH receptor in rats is SLC-1, a G-coupled protein receptor present throughout the brain and periphery (12, 13). Central MCH is involved in many different physiological functions including reproduction, stress, activity, and importantly energy balance (reviewed in Ref. 14). Food restriction increases MCH expression, and central injection of MCH promotes feeding (15). In addition, MCH-deficient mice have reduced body weight and are lean due to hypophagia and an increase

Abbreviations: ADX, Adrenalectomy; AgRP, agouti-related protein; ARC, arcuate nucleus; CORT, corticosterone; GR, glucocorticoid receptor; i3vt, third ventricular; LH, lateral hypothalamus; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; PB, phosphate buffer; PVN, paraventricular nucleus; VMN, ventromedial nucleus.

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in metabolic rate (16). The central administration of MCH also elicits an increase of water intake that is independent of food intake (17).

The second peptide system contained in the LH is the orexins (also known as hypocretins), which originate from prepro-orexin (prepro-hypocretin) (reviewed in Ref. 18). Orexin influences a variety of endocrine, autonomic, and metabolic functions, including the control of food intake, wakefulness, motor activity, metabolic rate, and blood pressure (19). Orexin A is a 33-amino acid peptide that increases food intake and body weight in a dose-dependent manner when injected into the brain (20). Conversely, administration of an orexin receptor antibody reduces food intake in fasted rats (21). Similar to MCH, fasting up-regulates orexin mRNA expression (22). Orexin-containing neurons project widely through the central nervous system and to hypothalamic nuclei important for the control of feeding where orexin receptors are located such as the ventromedial hypothalamus (VMN), LH, ARC, and paraventricular nucleus (PVN) (reviewed in Ref. 18).

Given the profound effects of glucocorticoids on feeding and orexigenic hypothalamic peptides, the goal of the present study was to assess the potential role of glucocorticoids to influence the effects of MCH and orexin A on food intake. We tested the hypothesis that glucocorticoids promote the actions of the peptides in the lateral hypothalamus as part of the complex hypothalamic system that regulates energy balance. As a result, the present study first investigated the effects of third ventricular (i3vt) administration of MCH and orexin A on food intake in both ADX rats and sham-operated controls. Next, we examined the influence of glucocorticoids on the expression of both MCH and orexin mRNA using semiquantitative PCR. Because ADX causes a deficiency of circulating hormones in addition to glucocorticoids, we also examined whether or not glucocorticoid replacement could reverse any of the effects observed after ADX. Finally, we assessed the potential colocalization of MCH and orexin protein with glucocorticoid receptors within the LH to determine whether glucocorticoids may act directly on these neuronal populations.

Materials and Methods

Animals

Male Long-Evans rats weighing between 267 and 329 g were obtained from Harlan (Harlan, Indianapolis, IN) and housed at the University of Cincinnati. Rats were housed individually in clear, plastic cages with *ad libitum* access to pelleted rat chow and water (unless otherwise specified) in a temperature-controlled vivarium on a 12-h light, 12-h dark schedule. After ADX or sham surgery, all animals had access to a 0.9% NaCl solution in addition to their tap water. The research with animals for all studies presented here was conducted in Association for Assessment and Accreditation of Laboratory Animal Care International-approved facilities and was conducted with approval of the Institutional Animal Care and Use Committee.

Procedures

Third ventricular cannulation. Animals were surgically implanted with a 22-gauge (Plastics One, Roanoke, VA) cannula with its tip in the third ventricle as described previously (23). Rats were anesthetized with ketamine (86 mg/kg, ip)/xylazine (12.9 mg/kg). Verification of cannula placement was accomplished by i3vt injection of 10 ng of angiotensin II in 1 μl 0.9% saline. Animals that did not drink 5 ml of water within 60 min after this treatment were not included in the experiments.

Adrenalectomy. A short incision was made in the skin rostral to each kidney. The perirenal fat was located, the prominent adrenal veins were located, and both adrenals were isolated from the surrounding fat capsule and removed. Animals were allowed to recover for at least 10 d before experiments began. Because ADX also removes the mineralocorticoids that are necessary for sodium retention, all ADX animals had access to isotonic (0.9%) saline in addition to tap water. To maintain a balanced design, sham animals also had access to both of these fluids. ADX was verified by measuring plasma corticosterone levels by RIA. Tail blood was sampled after the completion of behavioral testing at 1 h before lights off (100 μl from the tip of the tail) for determination of plasma corticosterone levels. Animals with plasma levels above 1 μl/dl were removed from the study.

Corticosterone RIA. Plasma corticosterone levels were determined by RIA using rabbit antiserum raised against corticosterone-21-hemisuccinate (B3–163; Endocrine Sciences, Tarzana, CA). Assay sensitivity was 0.5 μl/dl, and the intraassay coefficient of variation was between 2 and 5% for all experiments.

Experiment 1: effect of ADX on the feeding response to MCH

Behavioral procedures. Rats (sham, n = 10; ADX, n = 9) were adapted to a schedule in which food, water, and saline were removed from the cages 5 h before the end of the light cycle. On experimental days, 1 h after food and fluid were removed, all animals received an i3vt infusion of 2 μl of either MCH (American Peptide Co., Sunnyvale, CA) dissolved in 0.9% saline or saline alone via a Hamilton syringe. Food was returned immediately after injection, and food and fluid were weighed after 30 min and 2 h. Each animal received three doses of MCH (2.5, 5, 10 μg) or vehicle alone with the order counterbalanced across subjects using a Latin-square procedure. Animals were allowed at least 3 d without injections between experimental days.

Experiment 2: effect of ADX on the response to MCH in corticosterone-replaced rats

ADX animals had their saline drinking fluid replaced with a solution of 0.9% saline, 40 μg/ml corticosterone (CORT), and 0.5% alcohol to keep the corticosterone in solution. Providing ADX rats this corticosterone solution results in plasma corticosterone levels comparable to those of sham-operated controls (24). Rats had access to this solution for 5 d before any behavioral testing. Food intake was subsequently determined after 30 min and 2 h for both groups of rats (sham, n = 10; ADX + CORT, n = 8) after i3vt administration of either 5.0 μg of MCH or saline, with the order of treatments counterbalanced across subjects. Tail blood was sampled after the completion of behavioral testing at 1 h before lights off (100 μl from the tip of the tail) for determination of plasma corticosterone levels.

Experiment 3: effect of ADX on the feeding response to orexin A

Procedures. Surgical procedures and verifications were performed as described for experiment 1. All ADX animals maintained plasma corticosterone levels less than 1 μl/dl.

Behavioral procedures. Rats (sham, n = 10; ADX, n = 9) were adapted to a schedule in which food, water, and saline were removed from the cages 5 h before the end of the light cycle. On experimental days, 1 h after food was removed, all animals received an i3vt infusion of 2 μl of either orexin A (Phoenix Pharmaceuticals, Inc., Mountain View, CA) dissolved in 0.9% saline or saline alone via a Hamilton syringe. Food was returned immediately after injection, and food and fluid were weighed after 1 and 2 h. Each animal received three doses of orexin A (1.5, 3.0, 6.0 nmol) or saline alone, with the order counterbalanced across subjects using a Latin-square procedure. Animals were allowed at least 3 d without injections between experimental days.

Experiment 4: effect of ADX and corticosterone replacement on hypothalamic MCH and orexin expression

Procedures. Half of a group of ADX animals had their 0.9% saline solution replaced with a corticosterone solution for 5 d in the drinking water (see above), so that there were three groups of rats for semiquantitative PCR analysis: sham (n = 13), ADX (n = 7), and ADX + CORT (n = 9). All animals were killed at 1 h before lights out by brief exposure to CO_2 followed by rapid decapitation and removal of fresh brain tissue.

RNA isolation and cDNA synthesis. Brains were rapidly removed after decapitation and placed in RNA Later (Ambion, Austin, TX) and stored at 4 C for 24 h. They were then stored at −80 C until use for gene measurement. Mediobasal hypothalami were dissected and total RNA was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH). All RNA samples were deoxyribonuclease treated using deoxyribonuclease free (Ambion, Austin, TX). The Superscript III first-strand synthesis system (Invitrogen Life Technologies, Carlsbad, CA) was used to synthesize cDNA from 5 μg of total RNA.

Semiquantitative expression of MCH and orexin A mRNA (real-time PCR). Primers were designed to amplify a portion of the rat MCH and orexin genes using Primer3 primer design software (25). Primer sequences from 5′ to 3′ were forward ATATGAGCCTCTCTTAC and reverse GTCGTCTTCTACGTTCCTGA for MCH and forward TTCCTTCTACAAAGGTTCCCT and reverse TAGCAGCAGCAGCAGCGTCA for orexin. The constitutively expressed ribosomal protein L32 was used to obtain relative quantification of each gene. The primer sequences for L32 were forward CATCGTAGAAAGAGCAGCAC and reverse GCACACAAGCCATCTATTCAT. A master mix including cDNA prepared from 5 μg total RNA (see below) using the Superscript III first strand synthesis kit (Invitrogen Life Technologies), 10 μM each primer, and the Bio-Rad SYBR-green supermix was used for all PCRs. All PCRs were performed using the Bio-Rad iCycler (Richmond, CA) at a final volume of 25 μl. The PCR conditions for measurements of L32 and orexin consisted of a two-step reaction with an annealing temperature of 58 C and extension time of 30 sec for 40 cycles. PCR conditions for measurements of MCH were 60 C for 30 sec for 40 cycles. A standard curve for all genes was performed using a dilution series spanning 7 orders of magnitude (dilution factor of 5). The PCR efficiencies for L32, MCH and orexin were all between 95 and 100%. Correlation coefficients for all standard curves were between 0.997 and 1.00. Standard curves were calculated from the slope of the plot threshold cycle *vs.* log starting concentration. Samples were run in triplicate and normalized to the constitutively expressed ribosomal protein L32. Data are expressed as mean ± SEM of MCH and orexin relative to L32 using the **ΔΔ**Ct method (Applied Biosystems, Foster City, CA; User Bulletin No. 2, December 1997).

Experiment 5: visualization of glucocorticoid receptor, MCH, and orexin A

Tissue preparation. Adult male rats (n = 4) were deeply anesthetized using pentobarbital (200 mg/kg) and perfused transcardially with 100 ml of 0.9% NaCl followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.3). The brains were removed and postfixed for 1 h at room temperature in the fixative, then placed in 20% sucrose in 0.1 M PB and stored at 4 C. Coronal sections (35 μm) were cut on a freezing microtome (Richard Allen, Kalamazoo, MI), collected in four parallel series in cryoprotectant solution (30% sucrose, 30% ethylene glycol in 0.1 M PB) (26), and stored at −20 C until further processing.

Immunocytochemistry. All incubations were performed at room temperature with gentle agitation. Free-floating sections were washed extensively with 0.1 M PBS between incubations. Sections were incubated for 10 min with 1% H_2O_2, then blocked for 1 h with incubation solution (PBS containing 0.1% BSA and 0.4% Triton X-100). All primary antibody incubations were performed in the incubation solution, overnight at room temperature. After staining, the sections were washed thoroughly in 0.1 M PB, mounted onto glass slides with 0.3% gelatin in ddH_2O and coverslipped with DPX (Electron Microscopy Sciences, Fort Washington, PA). Immunocytochemical controls included omission of primary antibodies. Sections containing the hypothalamus were incubated overnight with a rabbit polyclonal antibody recognizing glucocorticoid receptor (1:200; PA1–511A; Affinity Bioreagents, Golden, CO) followed by 1-h incubations with biotinylated goat antirabbit IgG (1:600; Vector Laboratories, Burlingame, CA) and avidin-horseradish peroxidase complex (1:1000; ABC Elite Kit, Vector Laboratories, Burlingame, CA). Finally, the sections were incubated for 10 min in 0.02% diaminobenzidine (Sigma-Aldrich, St. Louis, MO) in 0.1 M PB containing 0.012% hydrogen peroxide and 0.08% nickel sulfate, resulting in a blue-black reaction product. Next, sections were incubated overnight with rabbit polyclonal antibody to orexin (1:1000; Phoenix Pharmaceuticals, Belmont, CA) or rabbit anti-MCH (1:150,000; Phoenix Pharmaceuticals), biotinylated goat antirabbit IgG (1:600; Vector Laboratories, Burlingame, CA) and ABC as described above. Finally, the sections were incubated for 10 min in 0.02% diaminobenzidine in 0.1 M PB containing 0.012% hydrogen peroxide, resulting in a reddish-brown reaction product.

Data analysis

Food intake data for experiments 1 and 3 were analyzed using mixed model ANOVA. Experiment 2 was analyzed using a one-way ANOVA, and experiment 5 was analyzed using two-tailed *t* tests. All pair-wise comparisons of mean differences were conducted using Tukey's honestly significant difference *post hoc* comparisons. Differences between group means were considered statistically significant if $P < 0.05$. For experiment 5, MCH and orexin-positive cells were examined for coexpression of glucocorticoid receptor throughout the hypothalamus. Digital images of immunostained sections were captured using a digital camera (Magnafire, Optronics, Goleta, CA) attached to a Leica microscope (Leica Microsystems; Wetzlar, Germany). Images were imported into Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA) to compose the figures. Images were not adjusted or altered in any way, except for occasional adjustment of brightness.

Results

Experiment 1

In this set of rats, the percent body weight change, mean ± SEM (from presurgery to the time the experiments were performed) was 39.9% ± 3.8 in sham, *vs.* 22.3% ± 4.4 in ADX (weight gain was significantly less in ADX than in sham, $P < 0.05$, one-way ANOVA). MCH significantly increased food intake at 5 and 10 μg in sham animals at 30 min and 2 h ($P < 0.05$ in all cases), whereas in ADX animals MCH only increased food intake at the 10 μg dose at 2 h ($P < 0.05$). MCH increased food intake at 5 and 10 μg to a greater extent in sham compared with ADX animals at both 30 min and 2 h ($P < 0.05$) (see Table 1). When assessed as the absolute change in food intake from when the animals were injected with vehicle, food intake was significantly greater in sham compared with ADX animals at 5 and 10 μg at both 30 min and 2 h ($P < 0.05$ in both cases) (see Fig. 1). Total fluid intake (water + 0.9% NaCl solution) was significantly increased by 5 and 10 μg of MCH in sham animals at both 30 min and 2 h, but only at 10 μg in ADX animals at 30 min and 2 h ($P < 0.05$). Ten micrograms of MCH increased fluid intake to a greater

TABLE 1. Mean (±SEM) 30-min and 2-h food intake (g) in rats treated i3vt with MCH

MCH dose (μg)	Sham 30 min	ADX 30 min	Sham 2 h	ADX 2 h
Vehicle	1.03 ± 0.62	0.41 ± 0.53	1.18 ± 0.76	0.61 ± 0.66
2.5	1.81 ± 0.57	1.29 ± 0.58	3.11 ± 0.70	1.48 ± 0.72
5.0	3.83 ± 0.57	1.15 ± 0.58[a]	4.88 ± 0.70	2.15 ± 0.72[a]
10.0	4.93 ± 0.56	1.76 ± 0.53[a]	5.68 ± 0.65	2.72 ± 0.66[a]

[a] Significantly different from sham at the same time point and MCH dose.

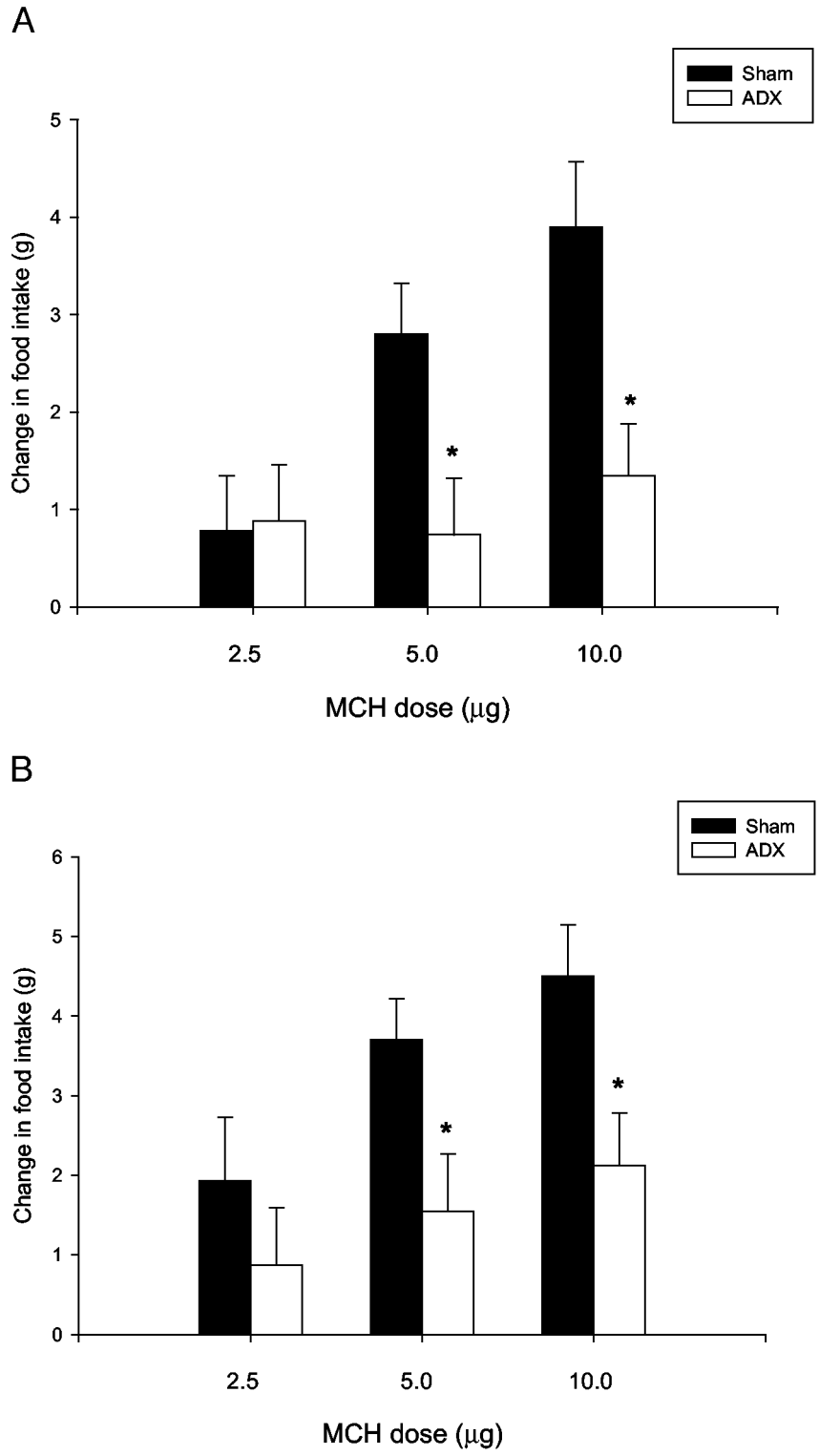

FIG. 1. Mean (±SEM) (A) 30-min and (B) 2-h food intake in rats that received either bilateral ADX surgery or sham surgery and were treated i3vt with MCH or vehicle. Data are represented as absolute change from when animals were injected with vehicle. *, Significantly different from paired sham ($P < 0.05$).

extent in sham animals compared with ADX animals at both time points ($P < 0.05$) (see Table 2).

Experiment 2

Plasma corticosterone concentrations were significantly reduced (to undetectable levels) in ADX compared with sham animals (11.7 ± 1.7 μg/dl) when assessed at 1 h before lights out ($P < 0.05$). ADX + CORT animals had plasma corticosterone concentrations similar to sham animals (8.3 ± 2.8 μg/dl) ($P > 0.05$). Administration of 5 μg MCH significantly increased food intake in both sham and ADX + CORT animals ($P < 0.05$ in both cases). MCH increased food intake

to a similar extent in sham and ADX + CORT animals at 30 min (see Fig. 2) and 2 h (data not shown) ($P > 0.05$ in both cases).

Experiment 3

In this set of rats, the percent body weight change, mean ± SEM (from presurgery to the time the experiments were performed) was 51.6% ± 3.1 in sham, *vs.* 31.5% ± 2.3 in ADX (weight gain was significantly less in ADX than in sham, $P < 0.05$, one-way ANOVA). Orexin A (3 nmol) significantly increased food intake ($P < 0.05$) to the same extent ($P > 0.05$) in both sham and ADX animals at 1 and 2 h (see Table 3). When assessed as the absolute change in food intake from when the animals were injected with vehicle, 1-h and 2-h food intake were similar in both sham and ADX animals at 3.0 nmol (see Fig. 3).

Experiment 4

Hypothalamic mRNA expression of MCH relative to L32 control was significantly reduced in both ADX and ADX + CORT animals compared with sham animals ($P < 0.05$ in both cases) (see Fig. 4A). Hypothalamic expression of orexin relative to L32 control was also significantly reduced in ADX and ADX + CORT animals when compared with sham animals ($P < 0.05$ in both cases) (see Fig. 4B).

TABLE 2. Mean (±SEM) 30-min and 2-h total fluid (water + 0.9% NaCl solution) intake (ml) in rats treated i3vt with MCH

MCH dose (μg)	Sham 30 min	ADX 30 min	Sham 2 h	ADX 2 h
Vehicle	1.01 ± 0.69	1.30 ± 0.59	2.19 ± 0.80	2.68 ± 0.69
2.5	3.14 ± 0.63	1.77 ± 0.65	4.38 ± 0.73	4.95 ± 0.75
5.0	4.33 ± 0.63	3.05 ± 0.64	6.66 ± 0.52	4.90 ± 0.73
10.0	6.44 ± 0.63	3.51 ± 0.59[a]	8.18 ± 0.73	6.28 ± 0.69[a]

[a] Significantly different from sham at the same time point and MCH dose.

Experiment 5

Expression of glucocorticoid receptor (GR), MCH, and orexin was similar to the distributions previously described (10, 18, 27). Although GR was observed in neurons in close vicinity to neurons expressing orexin or MCH, there was no observed colocalization of GR and orexin, or of GR and MCH in the LH (see Fig. 5).

Discussion

The results of the present study suggest that the well-established orexigenic actions of MCH are influenced by glucocorticoid status, whereas orexin A, another lateral hypothalamic orexigenic peptide, is not. More specifically, whereas ADX attenuated the orexigenic actions of MCH, the actions of orexin A were unaffected. The effect of ADX on the sensitivity to MCH was reversed by corticosterone replacement, suggesting that this effect was specific to the glucocorticoid deficiency associated with ADX.

In addition to examining the effect of glucocorticoids on the hyperphagic actions of MCH and orexin A, we examined the actions of glucocorticoids to alter mRNA expression of MCH and orexin. In accord with previous data, ADX reduced hypothalamic MCH mRNA, and orexin mRNA (28, 29). However, these reductions observed in both MCH and orexin were not restored by 5 d of corticosterone replacement in the drinking water despite this regimen achieving glucocorticoid levels comparable to those of sham-operated an-

TABLE 3. Mean (±SEM) 1- and 2-h food intake (g) in rats treated i3vt with orexin A

Orexin A dose (nmol)	Sham 1 h	ADX 1 h	Sham 2 h	ADX 2 h
Vehicle	0.63 ± 0.31	0.34 ± 0.33	0.88 ± 0.38	0.44 ± 0.39
1.5	1.08 ± 0.31	0.34 ± 0.33	2.17 ± 0.36	1.44 ± 0.38
3.0	1.89 ± 0.31	1.53 ± 0.31	2.65 ± 0.38	1.77 ± 0.38
6.0	1.15 ± 0.31	1.10 ± 0.35	2.71 ± 0.36	1.96 ± 0.37

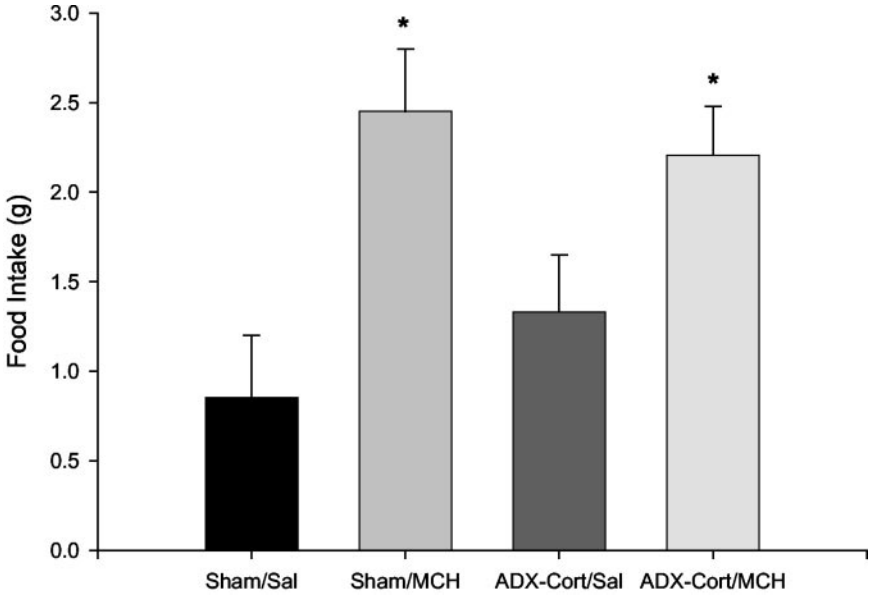

FIG. 2. Mean (±SEM) 30-min food intake in rats that received either sham surgery or ADX surgery and a consecutive 5 d of corticosterone in the drinking water (2.7 mg/ml) and were treated i3vt with 5 μg MCH or vehicle. *, Significantly different from saline-treated rats ($P < 0.05$).

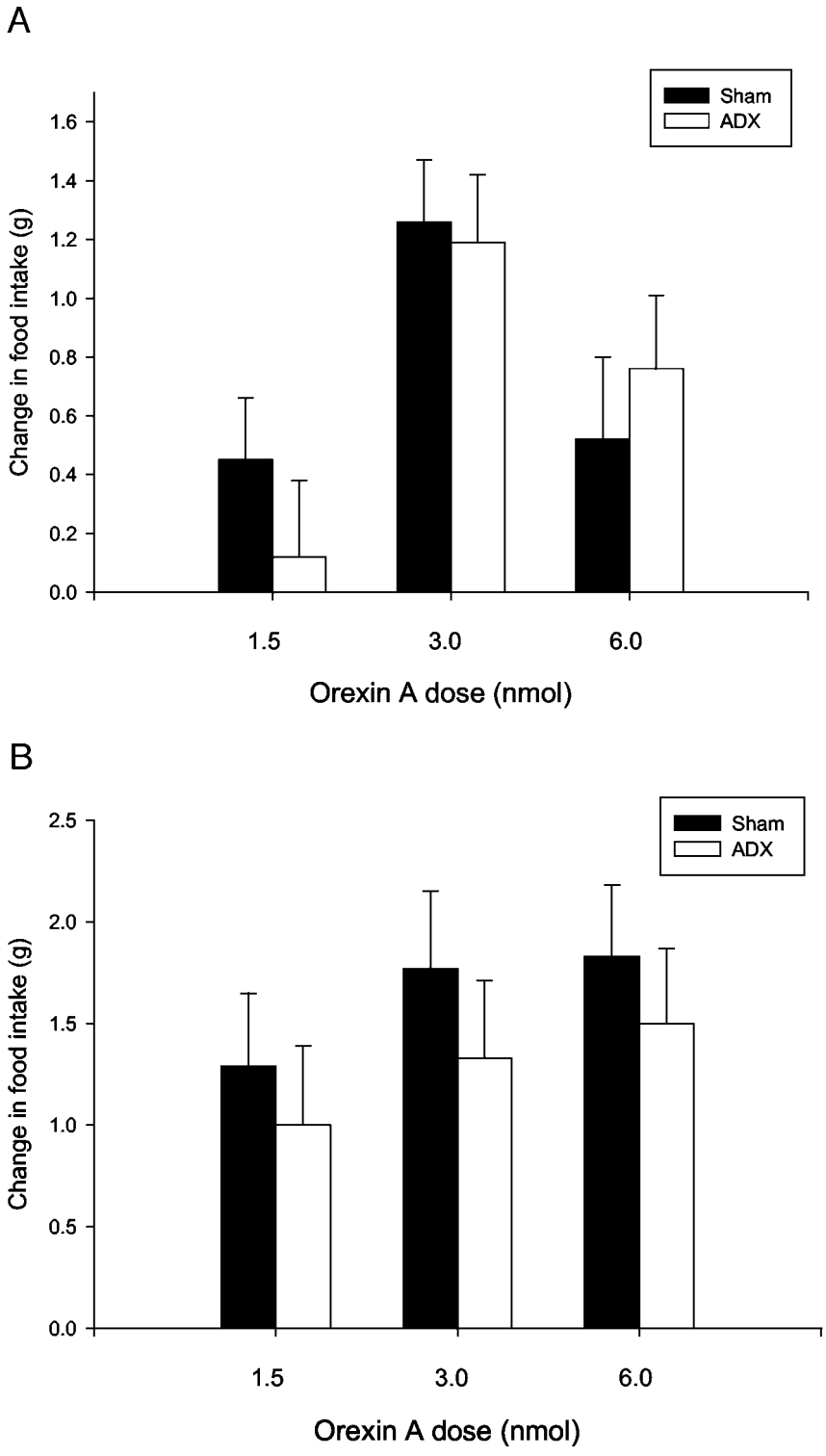

FIG. 3. Mean (±SEM) (A) 1-h and (B) 2-h food intake in rats that received either ADX or sham surgery and were treated i3vt with orexin A or vehicle. Data are represented as absolute change from when animals were injected with vehicle.

imals. These data suggest that the gene expression changes in these lateral hypothalamic peptides are not glucocorticoid dependent but depend on nonglucocorticoid aspects of ADX. Previous reports described that MCH mRNA was increased above that of sham controls in ADX rats treated with dexamethasone (28). Similarly, dexamethasone administration restored orexin mRNA after ADX (29). Although it is commonly used, dexamethasone does not have the same binding properties as glucocorticoids (30), and it makes it much harder to assess whether physiological levels of glucocorticoid replacement are achieved. Further work is necessary to address whether these methodological differences underlie the differences between the current data and reports from other groups. Nevertheless, it is tempting to speculate that

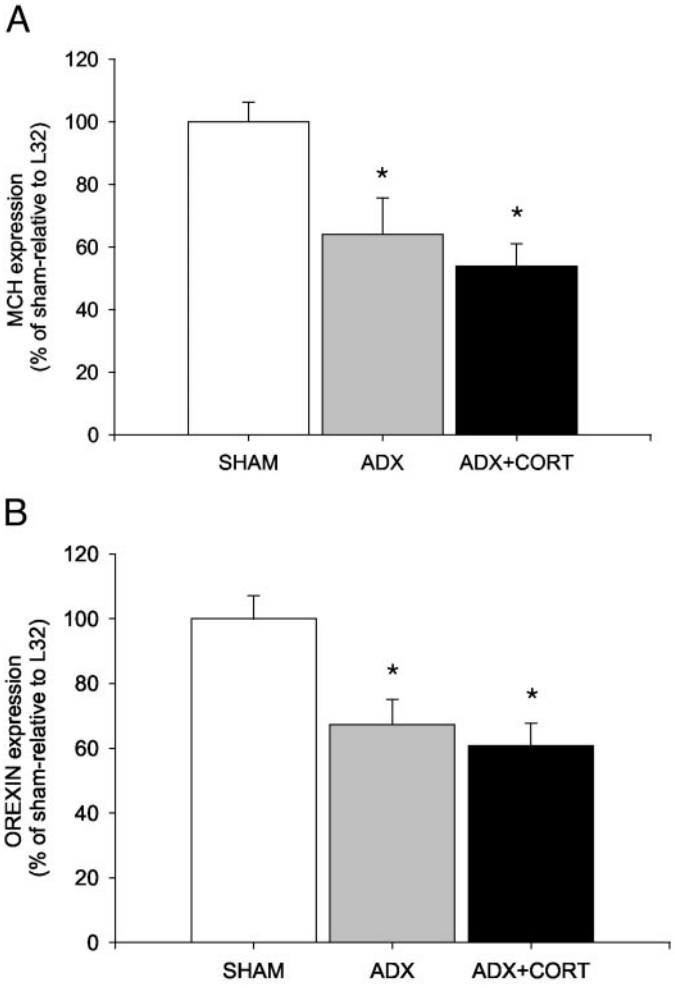

FIG. 4. Hypothalamic expression of (A) MCH and (B) orexin mRNA relative to L32 control; data are represented as a percentage of sham expression. *, Significantly different from sham animals ($P < 0.05$).

mineralocorticoids may have significant effects on the lateral hypothalamus. This is particularly true in the case of MCH because we have reported that MCH has a potent and rapid effect to increase water intake even in the absence of available food (17), making it possible that the osmoregulatory aspects of mineralocorticoids may influence fluid ingestion via actions on MCH.

Glucocorticoid receptors are present in regions of the hypothalamus important for the regulation of food intake and body weight, including the ARC, PVN, and LH (27), suggesting that glucocorticoids may directly influence the activity or responsiveness of numerous neuropeptide systems regulating feeding. Despite the fact that there is a large population of glucocorticoid receptor-positive cells within the LH, there is no evidence that any of them are located on either MCH- or orexin-containing neurons. This is consistent with the data from the gene expression experiments, in which changes in both MCH and orexin expression appeared not to be tied to glucocorticoid action.

Collectively, these data make several interesting points. First, these data provide another dissociation between the MCH and orexin systems in the LH. Although both systems have cell bodies almost exclusively in the LH and act to regulate food intake, they appear to do so by separate neural circuits. These data indicate that the actions of MCH to stimulate food intake are glucocorticoid dependent, whereas the actions of orexin-A are not. In addition, MCH should now be added to the extensive list of regulatory peptides whose effects are altered by glucocorticoids. Thus it appears that glucocorticoids have broad effects on a number of central nervous system circuits that have been linked to the control of food intake.

The present data also allow for some speculation about how glucocorticoids influence the actions of MCH to increase food intake. MCH has projections to many brain regions important in the regulation of energy balance (10). For example, SLC-1 (the first and only identified MCH receptor in rodents) mRNA and immunoreactivity have been observed in the ARC, PVN, LH, and VMN (13). It is possible, then, that glucocorticoids exert their effects on the MCH-food intake system by acting on the target cells for MCH. For example, strong signals for both SLC-1 and GR have been observed in the PVN, making this a potential candidate for the site of action of glucocorticoids to reduce MCH's ability to increase food intake (13, 27).

There are data indicating that the melanocortin system antagonizes the MCH system. Consistent with this, MCH and α-MSH exert opposing and antagonistic effects on food intake (31). Other studies demonstrate that administration of either agouti-related protein (AgRP) or SHU9119 increases MCH mRNA but not orexin mRNA (32). Because ADX increases melanocortin tone in some rodent models (33, 34), it is possible to speculate that this increase in melanocortin tone may antagonize the orexigenic effects of MCH, leading to the present effects of ADX on MCH, but not on orexin.

Another possibility is that MCH exerts its effects via actions in the ARC to alter the release of neuropeptide Y (NPY) and/or AgRP. Glucocorticoid receptors are found on these neurons (35) and the potent orexigenic effects of NPY and AgRP are blocked by glucocorticoid deficiency (34, 36). Recent reports indicate that the ARC is a potent site for MCH to increase food intake and that in hypothalamic explants, the administration of MCH increases the release of NPY, suggesting that MCH may act through the activation of NPY neurons to affect feeding (37). These data lead to the hypothesis that reduced action of MCH during glucocorticoid deficiency could be due to decreased actions of NPY and/or AgRP. The reverse may also be true; that is, it has been proposed that NPY and/or AgRP exert their effects via recruiting MCH neurons in the LH (38). Double-label immunohistochemistry demonstrates that MCH neurons receive innervation from NPY-immunoreactive fibers (39), and data also suggest that MCH neurons may express NPY receptors (40). Such evidence encourages the hypothesis that the effect of ADX to reduce the orexigenic actions of MCH may be responsible for the alteration of NPY actions after ADX. Future research should identify the glucocorticoid receptor populations that are responsible for the ability of glucocorticoids to dramatically alter the effects of a variety of neuropeptides such as MCH, NPY, α-MSH, and AgRP.

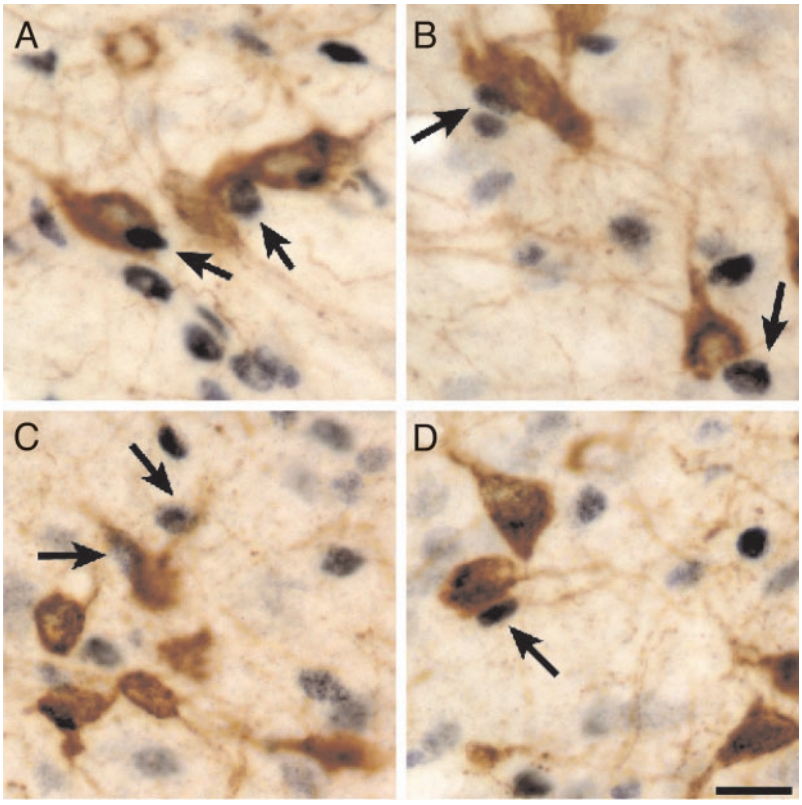

FIG. 5. Lack of colocalization of GR and orexin (A and B) or MCH (C and D). GR was observed in neurons in close vicinity to neurons expressing orexin or MCH (indicated by *arrows*). *Scale bar*, 100 μm.

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