Food deprivation differentially modulates orexin receptor expression and signaling in rat hypothalamus and adrenal cortex

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Karteris, Emmanouil, Rachel J. Machado, Jing Chen, Sevasti Zervou, Edward W. Hillhouse, and Harpal S. Randeva. Food deprivation differentially modulates orexin receptor expression and signaling in rat hypothalamus and adrenal cortex. Am J Physiol Endocrinol Metab 288: E1089–E1100, 2005. First published January 25, 2005; doi:10.1152/ajpendo.00351.2004.—Although starvation-induced biochemical and metabolic changes are perceived by the hypothalamus, the adrenal gland plays a key role in the integration of metabolic activity and energy balance, implicating feeding as a major synchronizer of rhythms in the hypothalamic-pituitary-adrenal (HPA) axis. Given that orexins are involved in regulating food intake and activating the HPA axis, we hypothesized that food deprivation, an acute challenge to the systems that regulate energy balance, should elicit changes in orexin receptor signaling at the hypothalamic and adrenal levels. Food deprivation induced orexin type 1 (OX1R) and 2 (OX2R) receptors at mRNA and protein levels in the hypothalamus, in addition to a fivefold increase in prepro-orexin mRNA. Cleaved peptides OX1R and OX2R are also elevated at the protein level. Interestingly, adrenal OX1R and OX2R levels were significantly reduced in food-deprived animals, whereas there was no expression of prepro-orexin in the adrenal gland in either state. Food deprivation exerted a differential effect on OX-R protein coupling. In the hypothalamus of food deprived rats compared with controls, a significant increase in coupling of orexin receptors to Gq, Gs, and G11 was demonstrated, whereas coupling to G11 was relatively less. However, in the adrenal cortex of the food-deprived animal, there was decreased coupling of orexin receptors to Gq, Gs, and G11 and increased coupling to G11. Subsequent second-messenger studies (cAMP/IP3) have supported these findings. Our data indicate that food deprivation has differential effects on orexin receptor expression and their signaling characteristics at the hypothalamic and adrenocortical levels. These findings suggest orexins as potential metabolic regulators within the HPA axis both centrally and peripherally.

STARVATION-INDUCED BIOCHEMICAL and metabolic changes are perceived by the hypothalamus, which in turn coordinates behavioral, autonomic, and neuroendocrine responses to these stimuli (1). Studies in rodents have shown that food deprivation induces marked ACTH and corticosterone responses, implicating feeding as a major synchronizer of rhythms in the hypothalamic-pituitary-adrenal (HPA) axis (2, 3). Besides nutritional states and neuropeptides, such as corticotropin-releasing hormone and neuropeptide Y (NPY), known to regulate both the HPA system and feeding behavior, several “signals” are known to regulate the HPA system. Hypoglycemia is a potent activator of the HPA axis, reflecting the strong functional relationship between the hypothalamic feeding centers and the HPA axis (4), and leptin, whose concentrations are governed by nutritional status, has an inhibitory effect on plasma corticosterone in rats (5).

More recently, orexin A (OR-A) and orexin B (OR-B), produced by neurons localized in the lateral and dorsal hypothalamic area and perifornical hypothalamus (6), have been implicated in the central regulation of feeding and energy homeostasis (7). Both OR-A (a 33-residue peptide) and OR-B (a 28-residue peptide) are proteolytically cleaved from a common precursor, propre-orexin, and share a 46% amino acid sequence homology. Besides playing a role in the regulation of feeding and energy homeostasis, orexins have been reported to exert divergent physiological actions (8–12). Orexins orchestrate their actions by binding and activating two types of G protein-coupled receptors, orexin-1 receptor (OX1R) and orexin-2 receptor (OX2R), which display 64% homology in their amino acid sequence (6). The OX1R preferentially binds OR-A, whereas OX2R binds both OR-A and OR-B, apparently with similar affinity (6).

In the hypothalamus, levels of prepro-orexin messenger RNA are regulated by several nutritional states and signals. Fasting (6) and insulin-induced hypoglycemia (13, 14) increase prepro-orexin mRNA in the rat lateral hypothalamic area, whereas leptin has the converse effect (15). Interestingly, fasting and leptin also regulate the hypothalamic expression of orexin receptors (15, 16). These findings, and the observation that intracerebroventricular administration of OR-A or OR-B stimulates food consumption in rats (6), suggests a physiological role for these neuropeptides as mediators in the regulation of feeding, particularly in response to energy deprivation.

Although the hypothalamus is considered the cornerstone for maintenance of energy homeostasis, the adrenal gland plays a role in the integration of metabolic activity and energy balance. For example, studies in food-deprived rats have suggested that the HPA axis is integral to a larger hypothalamic system that mediates energy flow (2) and that, in these animals, catabolic activity quickly predominates, reinforced by elevated corticosterone not driven by hypothalamic control (ACTH), implicating adrenal activity as a metabolic regulator (3). In addition to their hypothalamic effects, “nutritional signals” such as leptin and NPY have a direct action at the adrenal level, via their receptors, including the modulation of corticosteroid secretion (17, 18). Of interest, rat adrenals express both orexin receptors (19), and orexins stimulate corticosterone secretion of rat

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adrenocortical cells through the activation of the adenyl cyclase-dependent signaling cascade (20).

The expression of orexin receptors in both rat and human adrenals is well documented. In the rat, both receptors are expressed in the adrenal cortex. Interestingly, the highest amount of OX2R mRNA was found in the adrenal gland of male rats, which was four times higher than brain OX2R mRNA levels (19). In situ hybridization revealed that OX2R mRNA is localized primarily in the zona glomerulosa and zona reticularis, whereas there was no expression at the adrenal medulla (19).

In view of these findings and the observation that changes in orexin levels are closely related to nutritional status rather than to the state of hunger or satiety (21), we hypothesized that activation of orexin receptors at the adrenal level may explain the ACTH-independent rise in corticosterone seen in “starved” rats. We sought to investigate, in both the hypothalamus and adrenal gland, 1) whether levels of orexins and their receptors OX1R and OX2R differ in fed and food-deprived rats, by assessing their expression at mRNA and protein level and 2) to elucidate whether nutritional status elicits functional changes of OX1R and OX2R, by studying G protein coupling and the subsequent activation of second messenger pathways.

MATERIALS AND METHODS

Animal Preparation

The University of Warwick, UK, ethics committee on use and care of animals approved all procedures described. Adult male (initially 250 g) Wistar rats were housed in groups of two (n = 6 for each group) in environmentally controlled conditions (22 ± 2°C, humidity 40–60%) under a 12:12-h light-dark schedule (lights on 0600). Rats were allowed unrestricted access to standard laboratory pellet rodent diet (13.1 kcal/g CRM; Biosure, Cambridge, UK) and access to tap water before being subjected to the study. After a week of habituation to these conditions, rats were randomly distributed into two groups. The first group was allowed to eat freely/ad libitum. The second group to these conditions, rats were allowed unrestricted access to standard laboratory pellet rodent diet (13.1 kcal/g CRM; Biosure, Cambridge, UK) and access to tap water before being subjected to the study. After a week of habituation to these conditions, rats were randomly distributed into two groups. The first group was allowed to eat freely/ad libitum. The second group was food deprived for 24 h, beginning at the onset of the dark cycle. The second group was allowed unrestricted access to standard laboratory pellet rodent diet (13.1 kcal/g CRM; Biosure, Cambridge, UK) and access to tap water before being subjected to the study. After a week of habituation to these conditions, rats were randomly distributed into two groups. The first group was allowed to eat freely/ad libitum. The second group was food deprived for 24 h, beginning at the onset of the dark cycle.

Hypothalamic and Adrenal Dissection

To isolate the hypothalamus, animals were decapitated and their brains removed rapidly. The hypothalami, defined by the posterior margin of the optic chiasm and the anterior margin of the mamillary bodies to the depth of 2–3 mm, was dissected out. Adrenal cortex fractions were freed from adipose tissue and further separated from inner adrenomedullary tissue by pressure between glass plates. Upon removal, both tissues were immediately snap-frozen in liquid nitrogen. Samples were then stored at −70°C until further use.

Assay of Plasma Corticosterone Concentration

Plasma corticosterone levels between fed (n = 6 from each group; i.e., CO2/cervical dislocation) and food-deprived rats (n = 6 from each group) were measured by radioimmunoassay (Amersham Life Sciences, Buckinghamshire, UK). The range for this radioimmunoassay-specific for corticosterone is between 0.78 and 200 ng/ml.

Total RNA Extraction and cDNA Synthesis

Total RNA was prepared from individual samples using an RNase reverse transcriptase (GIBCO-BRL; Paisley, UK) according to the manufacturer’s recommendation.

Real-Time RT-PCR

Quantitative PCR was performed on a Roche Light Cycler system (Roche Molecular Biochemicals, Manheim, Germany). PCR reactions were carried out in a reaction mixture consisting of 5.0 µl of reaction buffer and 2.0 mM MgCl2 (Biogene, Kimbolton, UK), 1.0 µl of each primer (1 ng/µl), 2.5 µl of cDNA, and 0.5 µl of Light Cycler SYBR Gold (Biogene).

Protocol conditions consisted of denaturation of 95°C for 15 s, followed by 40 cycles of 94°C for 1 s, 58°C for 5 s, and 72°C for 12 s, followed by melting curve analysis. For analysis, quantitative amounts of genes of interest were standardized against the housekeeping gene β-actin. As a negative control for all the reactions, preparations lacking RNA or reverse transcriptase were used in place of the cDNA. For the quantitative analysis, cDNAs from six fed and six food-deprived rats were used. Serial dilutions of hypothalamic and adrenal cDNAs provided the template on which a line of best fit was plotted and used as a standard curve to demonstrate accuracy and reproducibility of analysis. Quantification data were analyzed using the Light Cycler analysis software. The RNA levels were expressed as a ratio using the “delta-delta method” for comparing relative expression results between treatments in real-time PCR (22).

The resultant PCR products were sequenced in an automated DNA sequencer, and the sequence data were analyzed using BLAST nucleic acid database searches from the National Center for Biotechnology Information.

Preparation of Hypothalamic and Adrenocortical Membranes

Rat hypothalami and adrenal cortex were obtained from food-deprived and control male Wistar rats as described above. Tissues were homogenized in Dulbecco’s phosphate-buffered saline containing 10 mM MgCl2, 2 mM EGTA, 1.5 g/l bovine serum albumin (BSA; wt/vol), 0.15 mM bacitracin, and 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.2 (extraction buffer) at 22°C. The homogenate was centrifuged at 800 g for 30 min at 4°C. The pellet was discarded and the supernatant spun at 45,000 g for 60 min at 4°C. The resultant pellet was washed, resuspended in extraction buffer, and spun at 45,000 g for a further 60 min at 4°C. The final pellet was resuspended in 5 ml of extraction buffer by use of a homogenizer. The protein concentration of the membrane suspension was determined using the bicinchoninic acid method, with BSA as a standard (23).

Western Blotting

Hypothalamic (n = 6 from each group, i.e., fed and food deprived; 100 µg) and adrenocortical membranes (n = 6 from each group; 100 µg) were centrifuged at 15,000 g for 15 min at 4°C. The supernatant was then discarded and the resultant pellets solubilized with Laemmli buffer [5 M urea, 0.17 M sodium dodecyl sulfate (SDS)], 0.4 M dithiothreitol (DTT), and 50 mM Tris-HCl, pH 8.0], mixed and placed in a boiling-water bath for 5 min and allowed to cool at room temperature.

Samples were separated on a 12% SDS-polyacrylamide gel, and the proteins were electrophoretically transferred to a nitrocellulose membrane at 250 mA for 1 h in a transfer buffer containing 20 mM Tris, 150 mM glycine, and 20% methanol. The filter was then blocked in PBS containing 0.1% Tween-20 and 5% milk powder (wt/vol), for 2 h at room temperature. After three washes with PBS-0.1% Tween, the nitrocellulose membranes were incubated with primary antibody for the OX1R and OX2R (Santa Cruz Biotechnology, Santa Cruz, CA). The primary antisera were used at a 1:11,000 dilution in PBS-0.1% Tween for 1 h at room temperature. The filters were washed thoroughly for 30 min with PBS-0.1% Tween, before incubation with the secondary anti-rabbit horseradish peroxidase-conjugated immuno-
globulin (1:2,000) for 1 h at room temperature and further washing for 30 min with PBS-0.1% Tween. Antibody complexes were visualized as previously described (24). To ensure specificity, we also performed preabsorption of both OX1R and OX2R with their blocking peptides (Santa Cruz Biotechnology) prior to Western blotting. For the detection of OR-A and OR-B, similar procedures were followed using specific non-cross-reactive antibodies (Phoenix Peptides, Belmont, MA) and total hypothalamic and adrenal lysate. To ensure that the same protein amount was loaded in all of the samples used for Western blotting, we used antibodies against the housekeeping gene β-actin (Santa Cruz Biotechnology).

Treatment of Membranes with Pertussis and Cholera Toxins

Both pertussis (50 μg/ml) and cholera (150 μg/ml) toxin were preactivated in 0.05 M Tris buffer, pH 7.5, containing 20 mM DTT and 50 mM glycine for 45 min at 37°C in a final volume of 50 μl and cooled on ice for 20 min. Hypothalamic and adrenal membranes (n = 3 from each group; 100 μg) were incubated in 20 mM Tris, pH 7.5, containing 1 mM EDTA, 1 mM DTT, 1 mM ATP, 1 mM GTP, 5 mM MgCl2, 10 mM thymidine, 10 μM NAD, and 5 μCi [32P]NAD together with the preactivated toxins. All reactions were carried out at 37°C for 30 min, and the incubations were terminated with 0.7 ml of ice-cold 20 mM Tris buffer, pH 7.5, containing 1 mM EDTA. Control samples were prepared by incubating membranes in the same medium but in the absence of any toxin. After termination, samples were centrifuged at 13,000 rpm for 20 min, and the pellets were washed and resuspended three times. The resultant pellets were resuspended in 100 μl of 1% SDS and 320 μl of buffer containing [1% (vol/vol) Triton X-100, 1% deoxycholate, 0.5% (wt/vol) SDS, 150 mM NaCl, 10 mM Tris·HCl, pH 7.4, 1 mM DTT, 1 mM EDTA, 0.2 mM PMSF, and 10 μg/ml aprotinin]. Resuspended samples were centrifuged at 11,000 rpm for 10 min at room temperature, and the resulting supernatants were equally aliquoted (200 μl). Into each of these aliquots 10 μl of G, and G, antisera (New England Nuclear-DuPont, Boston, MA) were added and left for continuous agitation for 2 h, followed by addition of 60 μl of protein-Sepharose A per tube and further agitation overnight at 4°C. The samples were then centrifuged at 12,000 rpm for 10 min, and the pellets were solubilized with Laemmli buffer, mixed, and placed in a boiling-water bath for 5 min before cooling to room temperature. Each sample was loaded onto an SDS-12% polyacrylamide gel, and after electrophoresis the gels were dried and autoradiographed using Kodak X-ray film to assess the extent of ADP ribosylation.


GTP-azidoanilide (GTP-AA) was synthesized using a method previously described (23). Hypothalamic and adrenocortical membranes (n = 3 from each group; 150 μg) were incubated for 3 min at 30°C with OR-A (100 nM) in buffer A (50 mM HEPES, 30 mM KCl, 10 mM MgCl2, 1 mM benzamidine, 0.1 mM EDTA), followed by the addition of 5 μM GDP and 6 μCi of GTP-AA. After incubation for 3 min at 30°C in a darkened room, membranes were placed on ice and collected by centrifugation at 15,000 g for 15 min at 4°C. The supernatant was carefully removed, and the membrane pellet was resuspended in 120 μl of modified buffer A (1.6 mg DTT in 5 ml buffer A). Samples were vortexed and irradiated for 5 min at 4°C with an ultraviolet light (254 nm) from a distance of 5 cm to cross-link the GTP-AA to the G proteins. Immunoprecipitation using 10 μl of undiluted G protein antiserum (New England Nuclear-DuPont) to the α-subunit was then carried out as previously described (24). Samples were subjected to gel electrophoresis using discontinuous SDS-PAGE slab gels (10% running, 5% stacking). The gels were stained with Coomassie blue, dried using a slab gel dryer, and exposed to Fuji X-ray film at −70°C for 2–5 days with intensifying screens.

**cAMP and Inositol Triphosphate Second Messenger Studies**

**cAMP.** For cAMP studies, using a commercially available kit (New England Nuclear-DuPont), hypothalamic and adrenocortical membrane suspensions (n = 3 from each group; 100 μg) were incubated with increasing concentrations of OR-A, and the amount of cAMP in the incubate was determined by radioimmunoassay, as previously described (25). Standard cAMP concentrations, covering the range 0.138–100 pmol/ml, were used for determination of the standard curve of the radioimmunoassay. The intersay coefficient of variation was 8%. CAMP assay buffer (without any membrane preparations) was used as the negative control.

**Inositol triphosphate.** For the inositol triphosphate (IP3) assay (Amersham Pharmacia Biotech, Little Chalfont, UK), hypothalamic and adrenocortical membranes were incubated with increasing concentrations of OR-A, followed by the addition of 200 μl of IP3 generation buffer, as previously described (24). Membranes were incubated for 3 min at 37°C, and the reaction was terminated by the addition of 1 M ice-cold trichloroacetic acid followed by extraction of inositol phosphates and neutralization. IP3 levels were estimated by radioimmunoassay on the basis of the displacement of [3H]IP3 from a specific bovine adrenocortical IP3-binding proteins. The intersay coefficient of variation was 8.7%.

**Statistical Analysis**

Data are shown as means ± SD of each measurement. For the real-time PCR measurements, photoaffinity labeling, and Western immunoblotting, results were evaluated between groups by using two-tailed Student’s t-test, with significance determined at the level of P < 0.05. For Western immunoblotting and photoaffinity labeling experiments, the densities were measured using a scanning densitometer coupled to scanning software ImageQuant; (Molecular Dynamics, Amersham Pharmacia, Little Chalfont, UK). For the second messenger measurements, a one-way analysis of variance was used, followed by Dunnett’s test, to compare each treatment dose.

**RESULTS**

**Effect of Food Deprivation on Corticosterone Level**

Plasma corticosterone levels were almost twofold higher (P < 0.01) in the food-deprived rats (51 ± 2.9 ng/ml) compared with the fed ones (28 ± 2.7 ng/ml) as they were measured by radioimmunoassay (Fig. 1A). Given that all rats were killed by CO2 inhalation, corticosterone levels of rats killed by cervical dislocation were also measured. After the two subgroups were compared, it appeared that there were no apparent differences between the two different methods of killing animals, thus suggesting that, in our model, CO2 inhalation does not constitute an additional stress factor (Fig. 1A).

**Effect of Food Deprivation on Peptide and Receptor Expression in Hypothalamus**

**Peptide expression.** Serial dilutions of hypothalamic cDNA provided the template on which a line of best fit was plotted and used as a standard curve to demonstrate accuracy and reproducibility of analysis. Melting curve analysis of the PCR products was presented as fluorescence over time (df/dT) against temperature (T°C). The melting curve analysis showed a single melting maximum of 89.20°C for the prepro-orexin gene, a single melting maximum of 90.30°C for the β-actin gene, thus confirming product specificity (data not shown).

There was a fivefold increase in prepro-orexin message (P < 0.01) in the hypothalami of food-deprived animals (Fig. 1B).
Fig. 1. A: significant upregulation ($P < 0.01$) of corticosterone production in food-deprived rats ($n = 6$) compared with fed ones ($n = 6$). The method of sacrificing did not exert any effects on corticosterone levels of the 2 groups. B: significant increase in prepro-orexin mRNA levels in food-deprived hypothalamus of the rat ($n = 6$). These differences in mRNA expression can be seen from the large difference in amplification efficiency as demonstrated by the delay in amplification (intercept cycle) of the fed-rat sample. C: Western blot analysis of OR-A and OR-B of rat hypothalamic lysates. Lane 1 corresponds to positive control (OR-A or OR-B); lane 2 corresponds to fed hypothalamus; lane 3 corresponds to food-deprived hypothalamic lysates. Quantification of immunocomplexes revealed a significant increase in protein expression of both OR-A and OR-B under food deprivation conditions ($n = 6$). *$P < 0.05$; **$P < 0.01$. 

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compared with controls. These mRNA changes of the prepro-orexin peptide were also confirmed by immunoblotting analysis, where protein expression of both OR-A and OR-B was significantly (P < 0.01 and P < 0.05, respectively) increased under food deprivation (Fig. 1C).

Receptor expression. Compared with controls, both OXR1 and OXR2 mRNA were significantly (P < 0.05) increased in the hypothalamus of the food-deprived rat (Fig. 2A). The increases were similar for both forms of orexin receptors (2.5-fold increase for OX1R, ~2-fold increase for OX2R). Protein expression of OXR1 and OXR2 was confirmed by immunoblotting using specific goat polyclonal antibodies (Fig. 2B). The OXR1 antibody was raised against a peptide mapping at the carboxy terminus of the OXR1 of rat origin, whereas the OXR2 antibody was raised against a peptide mapping at the amino terminus of the OXR2 of human origin and is rat cross-reactive. The detected protein for OX1R has an apparent molecular mass of ~50 kDa, whereas the OX2R was detected as a 40-kDa peptide. The specificity of the response was confirmed by preincubation of OX1R and OX2R antibodies with their blocking peptides (Fig. 2B). Protein expression reflected the mRNA data (Fig. 2A) with significant increase (P < 0.01) of both receptors in food-deprived animals (Fig. 2C) compared with controls.

Effect of Food Deprivation on Orexin Receptor Expression in Adrenal Cortex

Interestingly, findings in the adrenal cortex were in marked contrast to those observed in the hypothalamus. Quantitative analysis of the PCR products from the rat adrenal cortex showed that both OX1R and OX2R levels were significantly (P < 0.05 and P < 0.01, respectively) reduced in food-deprived animals compared with controls (Fig. 3A). The decrease was more profound for OX2R (4-fold), whereas OX1R demonstrated a twofold decrease (Fig. 3A). Similarly, both OX1R and OX2R protein levels were significantly decreased (P < 0.05 and P < 0.01, respectively) in the adrenal cortex of the food-deprived animals compared with controls. Again, the decrease was more pronounced for OX2R. In both groups studied, the protein levels for β-actin remained unaltered (Fig. 3B).

ADP Ribosylation

Cholera toxin treatment. Incubating membranes with cholera toxin resulted in the incorporation of [32P]ADP ribose into two bands of 45 and 47 kDa for both hypothalamic and adrenal membranes. There were no apparent differences in the incorporation of the probe between fed and food-deprived preparations from both tissues (Fig. 4A).

Pertussis toxin treatment. Treatment of hypothalamic and adrenal membranes with pertussis toxin resulted in the incorporation of [32P]ADP ribose into a single band with apparent molecular masses of 41 kDa (Fig. 4B). No incorporation of label was seen in either hypothalamic or adrenal tissues in the absence of the pertussis toxin. Similarly, there were no detectable differences between the fed and food-deprived G i subunits. These data not only confirm the functional integrity of G protein α-subunits but also reveal that the nutritional status does not interfere with the expression of these signaling proteins.

Functional Analysis of G Protein Activation by OR-A: Effects of Food Deprivation

To determine which G proteins are coupled to the orexin receptors in the food-deprived and fed state, hypothalamic and adrenocortical membranes were labeled with GTP-IAA in the presence or absence of OR-A followed by immunoprecipitation with specific Go subunit antibodies (Go, G i, G q, G o).

Optimal labeling of Go subunits with GTP-IAA requires receptor activation of heterotrimeric G proteins with release of bound GDP. The binding of GTP-IAA is dependent on GDP concentration, GTP affinity of the Go subunit, and agonist incubation time (26). Therefore, the conditions for labeling Go subunits were established empirically. We have demonstrated that optimum labeling was obtained in the presence of 5 μM of GDP (data not shown). OR-A-induced labeling with GTP-IAA was time dependent, with an optimal incubation time of 3 min (data not shown). Using the Goα subunit as a paradigm, we were able to demonstrate that the coupling of orexin receptors upon challenge with OR-A was dose dependent, with maximal activation at a concentration of 100 nM (Fig. 4C).

The specificity of the immunoprecipitating properties of Goα antibody were assessed by comparing the migration positions of orexin-induced GTP-IAA photoaffinity labeled G proteins with those ADP ribosylated using [32P]NAD with cholera toxin to demonstrate that the same protein band was radiolabeled and immunoprecipitated by the specific antibody. Surprisingly, our photoaffinity [32P]GTP-IAA experiment indicated that the orexin receptors in both hypothalamus and adrenal cortex preferentially activate the 45-kDa form of the Goα protein. This observation requires further investigation.

Hypothalamus. Treatment of hypothalamic membranes with OR-A (100 nM) revealed that orexin receptors coupled to multiple G proteins, including Go, Gi, and Gq, and to a lesser extent G i in the control group (Fig. 5A). In food-deprived rats, however, there was a change in the “profile” of G protein activation. Compared with controls, there was a significant increase in coupling of orexin receptors to Go, Gi, and Gq, whereas there was less coupling of orexin receptors to Gi (Fig. 5A). Quantification of the immunocomplexes is shown in Fig. 5B.

Adrenal cortex. Treatment of adrenocortical membranes with OR-A (100 nM) revealed a similar “promiscuity” in the G protein profile of adrenal orexin receptors. In the control group (fed rats) OR-A increased the labeling of Go, Gi, and Gq, but not of Gi (Fig. 5A). As in the hypothalamus, food deprivation modulated the G protein activation profile. Interestingly, in the food-deprived animal, OR-A decreased the coupling of orexin receptors to Gi and Gq, and increased that of G i, whereas there was no coupling toward Gq (Fig. 5A). Quantification of the immunocomplexes is shown in Fig. 5C.

Functional Analysis of Intracellular Second Messenger Generation by OR-A: Effects of Food Deprivation

In addition to G protein data, we dissected further the signaling characteristics of orexin receptors in the hypothalamus and adrenal cortex by measuring the second messengers cAMP and IP3 in both controls and food-deprived animals upon stimulation by different concentrations of OR-A.

Hypothalamus. To test the OR-A ability to activate hypothalamic adenylyl cyclase, we determined the effect of OR-A on cAMP production. When hypothalamic membranes from
Fig. 2. A: significant upregulation of OX1R and OX2R mRNA levels in food-deprived hypothalamus (n = 6) of the rat compared with fed ones (n = 6), as assessed by real-time PCR. B: Western blot analysis of membrane protein extracts from rat adrenal (lane 3) and hypothalamus (lane 4) demonstrate that the antibody against orexin-1 receptor (OX1R) recognized a band with an apparent molecular mass of 50 kDa. Similarly, when the specific OX2R antibody was used, it recognized a single band with an apparent molecular mass of 40 kDa. Both bands appeared to be specific for orexin receptors, because when antibodies were preabsorbed with their respective blocking peptides (lanes 1 and 2 for rat adrenal and hypothalamus, respectively), there was no apparent immunodetection. C: Western blot analysis of OX1R and OX2R of hypothalamic membranes from fed (n = 6) and food deprived rats (n = 6). Quantification of immunocomplexes revealed a significant increase in protein expression of both orexin receptors under food deprivation conditions, whereas protein levels for the housekeeping gene β-actin appeared to be unaltered. **P < 0.01.
Fig. 3. A: significant up/downregulation of OX1R and OX2R mRNA levels in food-deprived rat adrenal cortex (n = 6) compared with fed adrenals as assessed by real-time PCR. These changes in mRNA expression are shown as differences in amplification efficiency as demonstrated by the delay in amplification (intercept cycle) of food-deprived cDNAs. B: Western blot analysis of OX1R and OX2R of adrenal membranes from fed (n = 6) and food-deprived (n = 6) rats. Quantification of immunocomplexes revealed a significant decrease in protein expression of both orexin receptors under food deprivation conditions, whereas protein levels for the house-keeping gene β-actin appeared to be unaltered. These changes are in agreement with the mRNA data. *P < 0.05; **P < 0.01.
Fed rats were incubated with OR-A (10 pM to 100 nM) for 30 min at 25°C, there was a significant increase in cAMP production. This increase was found to be dose dependent, whereas the maximal response (55% of basal) was observed at a concentration of 100 nM (Fig. 6A). The response was amplified in the food-deprived rat, where 100 nM treatment induced a cAMP response of 95±5% of basal (Fig. 6A).

Similarly, we found that OR-A treatment of hypothalamic membranes induced a rapid IP₃ turnover, in a dose-dependent manner. This OR-A effect has a threshold of 1 nM and a maximum response at 100 nM (30±4% of basal; Fig. 6B). Consistent with our G protein labeling studies, there was a significant (P < 0.01) increase in IP₃ production from hypothalamic of the food-deprived rats compared with controls (70±7% of basal; Fig. 6B).

Adrenal cortex. Similarly, when rat adrenocortical membranes from fed rats were incubated with OR-A (0.01 nM to 100 nM), there was a significant increase in cAMP and IP₃ production (Fig. 6, C and D). These increases appeared to be dose dependent, with a maximum response at 100 nM for cAMP (78±8% of basal) and at 10 nM for IP₃ (50±7% of basal). However, in the food-deprived animals compared with controls, treatment of adrenocortical membranes with OR-A induced a modest response toward cAMP production that was significant only at 100 nM, with no apparent effect toward IP₃ turnover (Fig. 6, C and D). Again, these findings were in keeping with our photoaffinity labeling experiments.

**DISCUSSION**

In the present study, we demonstrate the effects of food deprivation (24 h) on orexins and orexin receptor expression in the rat hypothalamus and adrenal cortex at both mRNA and protein levels. In addition, we present the differential effects of this “stressful” nutritional stimulus on the signaling characteristics of orexin receptors at these tissues.

The hypothalamus plays a major role in the regulation of food intake and energy balance by integrating multiple anorexigenic and orexigenic signals, including those elicited by orexins (21, 27). A similar role for the adrenal gland is possible given that it expresses receptors for leptin, NPY (28), and orexin (19, 29), all regulators of energy homeostasis. In agreement with previous studies (6, 15), we were able to show that the levels of prepro-orexin mRNA are influenced by nutritional status, being upregulated upon fasting in the hypothalamus. Detailed analysis at the protein level confirmed that both cleaved bioactive peptides (OR-A and OR-B) are upregulated under food deprivation. Previous studies in fasted lactating rats demonstrated hypothalamic OR-B levels to be raised 10-fold above those for controls (nonfasted), whereas OR-A showed no change (30). These differences can be due to the rodent strain used, the sex, metabolic status, and the duration of food deprivation.

Food deprivation leads to changes in the activity of the HPA axis within 3 h, with an increase in corticosterone (3). These
findings are of interest given that orexins stimulate the HPA axis when administered centrally (31). Furthermore, orexin neurons are sensitive to nutritional signals such as insulin and glucose, which alter with food deprivation (3), and hypothalamic orexin neurons express leptin receptors. As with prepro-orexin expression, fasting and leptin also regulate hypothalamic expression of orexin receptors (15, 16, 32).

Numerous studies have mapped in detail the expression of orexin and orexin receptors at the hypothalamic level. Trivedi et al. (33) have demonstrated that, within the hypothalamus, OX1R mRNA is most abundant in the ventromedial hypothalamic nucleus, whereas OX2R is predominantly expressed in the paraventricular nucleus. In another study, it has been shown that, after 20 h of fasting, levels of rat OX1R mRNA were significantly increased in the ventromedial hypothalamic nucleus and the medial division of amygdala, whereas levels of OX2R mRNA were augmented in the arcuate nucleus but remained unchanged in the dorsomedial hypothalamic nucleus, paraventricular hypothalamic nucleus, and amygdala following fasting (16). Three different studies (34, 13, 15) indicated that prepro-orexin is upregulated in the hypothalamus upon food deprivation in the lateral hypothalamic area of the rat. In agreement with these studies, we noted that, on fasting, hypothalamic OX1R and OX2R gene expression was induced. Given the detailed analysis these studies provided, we shifted our interest to the expression at the protein level. Here, we provide new evidence that these changes are also mirrored at the protein level, as it was assessed by semiquantitative Western blotting analysis.

Previous studies (3, 4) have shown that food deprivation switches feeding responses to adrenocortical responses and that the time of food presentation appears to be a more potent synchronizer of the phase of plasma corticosteroid levels than is the light-dark cycle (35). These observations are of interest.
given that orexins have circadian-dependent actions and that orexins are known to increase corticosterone production in rats (20) and cortisol in human adrenocortical cells (36). However, in view of our data, we can conclude that adrenal orexin receptors do not influence or mediate the rise in corticosterone levels seen under food deprivation conditions.

It is well documented that food deprivation induces multiple metabolic changes that may directly influence orexin receptor expression in the adrenal gland. For example, there is a negative correlation between testosterone levels and fasting (37). Studies from our laboratory have shown that testosterone levels also decrease following 24 h of food deprivation (unpublished observations). Interestingly, in gonadectomized rats, there was a significant downregulation of adrenal OX2R, an effect that was reversed upon testosterone replacement (38). Although the regulation of adrenal orexin receptors by gonadal steroids is not directly relevant to our findings, it suggests that changes in orexin receptor expression may be a secondary phenomenon following initial food deprivation-induced alterations.

Future studies should concentrate on elucidating the central and peripheral actions of orexins. This can be done by systematically deleting OX1R and/or OX2R in a tissue-specific fashion using the Cre-loxP system. By use of this approach, a model can be generated bearing neuronal or adrenal-specific deletions of orexin receptors.

Our data indicate that orexin signaling may be enhanced in response to energy deficit sensed by the hypothalamus, possibly and predominantly via OX1R, but at the same time decreased in the adrenal cortex. Orexins act through two distinct G protein-coupled receptors, which can transduce intracellular signals by activating heterotrimeric G proteins. It is likely that multiple second messenger systems are involved, as orexins have been shown to increase intracellular calcium influx (6, 39, 40, 41). There is also the suggestion that OX2R is coupled to the inhibitory G_i protein (42). We have shown for the first time that native orexin receptors in the hypothalamus can activate four types of G proteins, namely G_s, G_q, G_o, and G_i, in response to OR-A. Although G_i and G_o are the two major inhibitory signaling pathways in the rat brain, we have previously shown that multiple subtypes of G proteins couple to other G protein-coupled receptor systems (43). Food deprivation altered the G protein coupling profile. Our findings are supported by functional assays of cAMP and IP_3 assays, reflecting their G protein coupling status. Further research is needed to investigate whether the βγ-subunits are implicated in activating second messengers and also to assess the effects of OR-B on G protein signaling.

Although we have demonstrated functional orexin receptors in human fetal and adult adrenal glands (23, 44), this is the first report of G proteins coupling to orexin receptors in the rat adrenal cortex. Interestingly, we note a downregulation of all G proteins that is seen in response to food deprivation, which suggests that orexin signaling may be enhanced in response to energy deficit sensed by the hypothalamus, possibly and predominantly via OX1R.
protein(s), apart from Gαi, in response to OR-A upon food deprivation. Second messenger studies confirmed these find-

ings with minimal response toward cAMP production and none of IP3 in the adrenal cortex of the food-deprived rat. Our findings support the observations that, in rat adrenocortical cells, OR-A stimulates corticosterone release via a cAMP pathway (20).

Despite demonstrating for first time that rat orexin receptors can couple differentially to several G protein α-subunits and activate multiple second messengers, caution should be exercised as to how to interpret these changes. Given the plethora of different cell types that reside within the hypothalamus and the adrenal gland, no distinctions can be made between specific subpopulations from brain regions or adrenal zones from our data. Here, we provide evidence about the signaling characteristics of orexin receptors under food deprivation in total hypothalamic and adrenal preparations.

In conclusion, our study indicates that nutritional changes like food deprivation can exert differential effects on orexin receptor expression and their signaling characteristics at the hypothalamic and adrenocortical levels. However, the significance of our novel findings, in particular the differential expression of G protein activation upon food deprivation, in both the hypothalamus and the adrenal cortex needs further elucidation.

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