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Hypothalamic Ghrelin Treatment Modulates NPY- but Not CRH-ergic Activity in Adrenalectomized Rats Subjected to Food Restriction

Evidence of a Novel Hypothalamic Ghrelin Effect

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It has been proposed that ghrelin induces food intake by a mechanism due to the stimulation of hypothalamic NPY-ergic activity. It is recognized that bilateral adrenalectomy (ADX) enhances hypothalamic CRH-ergic function and reduces appetite. Thus, the aim of the present study was to test whether, icv-administered, ghrelin modulates NPY- and CRH-ergic functions after food restriction (FR) and glucocorticoid deprivation. For this purpose, 1 µg ghrelin was administered icv to *ad libitum* (AL) eating and to corticosterone (B)-depleted (ADX) and -replete (sham and ADX+B) male animals habituated, for 15 d, to FR. Food intake, hypothalamic function, and peripheral ghrelin, ACTH, and B concentrations were evaluated 2 h after ghrelin administration. Results indicate that while icv ghrelin treatment stimulated 2-h food intake in AL rats, it failed to do so in sham- and ADX+B-FR animals; moreover, 2-h food intake was inhibited by icv ghrelin treatment in ADX-FR rats. Regarding peripheral hormone levels: (a) basal circulating ghrelin levels, already enhanced (vs AL rats) by FR, significantly increased 2 h after icv ghrelin treatment in AL and sham-FR rats; (b) central ghrelin treatment stimulated ACTH secretion in circulation of AL and glucocorticoid-replete-FR rats; and (c) B circulating levels remained unchanged after ghrelin treatment, although they were in relation to the food intake condition of rats. Finally, hypothalamic NPY mRNA expression was enhanced by FR and, in response to icv ghrelin treatment, it decreased in ADX-FR rats only. ADX-enhanced hypothalamic CRH mRNA levels were reduced by ghrelin icv administration only when animals received B replacement therapy. Our data indicate an inhibitory effect of hypothalamic ghrelin on NPY-ergic activity in FR rats lacking endogenous glucocorticoid.

Key Words: HPA axis; energy balance; leptin; ghrelin-R1a; GC-R.

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Introduction

Ghrelin, a peptide mainly expressed in the stomach *(1)*, is considered, at this time, the most relevant peripheral hunger signal *(2,3)*. Once this endogenous ligand of the growth hormone secretagogue receptor (GHS-R) *(1)* reaches high circulating concentrations, it is sensed at the hypothalamic level, thus modulating food intake *(4–6)*.

In mammals, fasting/starvation-related high plasma ghrelin concentrations *(7,8)* is a characteristic associated with increased circulating glucocorticoid levels *(9)*, while both decrease after food intake *(10–12)*. Moreover, the inverse relationship in the circulating levels of ghrelin and leptin *(2)* clearly indicate that an inappropriate signaling of these two peptides could have physiopathological implications. Thus, the relation between hypothalamo–pituitary–adrenal (HPA) axis function and ghrelin levels seems to be crucial for maintaining homeostasis *(13)*. However, recent studies suggest some controversy on the ability of ghrelin to stimulate food intake in the absence of endogenous glucocorticoid. In fact, while Proulx et al. *(14)* claimed that ghrelin is able to stimulate feeding in the absence of endogenous glucocorticoid, conversely, Tung et al. *(15)* and Tschop et al. *(16)* did not observe any ghrelin stimulatory effect on food intake in *ad libitum* (AL) eating, bilateral adrenalectomized (ADX) rats.

In the present study, we tested the hypothesis of whether hypothalamic ghrelin treatment is able to restore normal food intake in a rat model of ADX-induced anorexia when combined with prolonged food restriction (FR). The rationale of this experimental design is sustained by the fact that ADX is able to reduce leptinemia *(17)*, thus minimizing peripheral

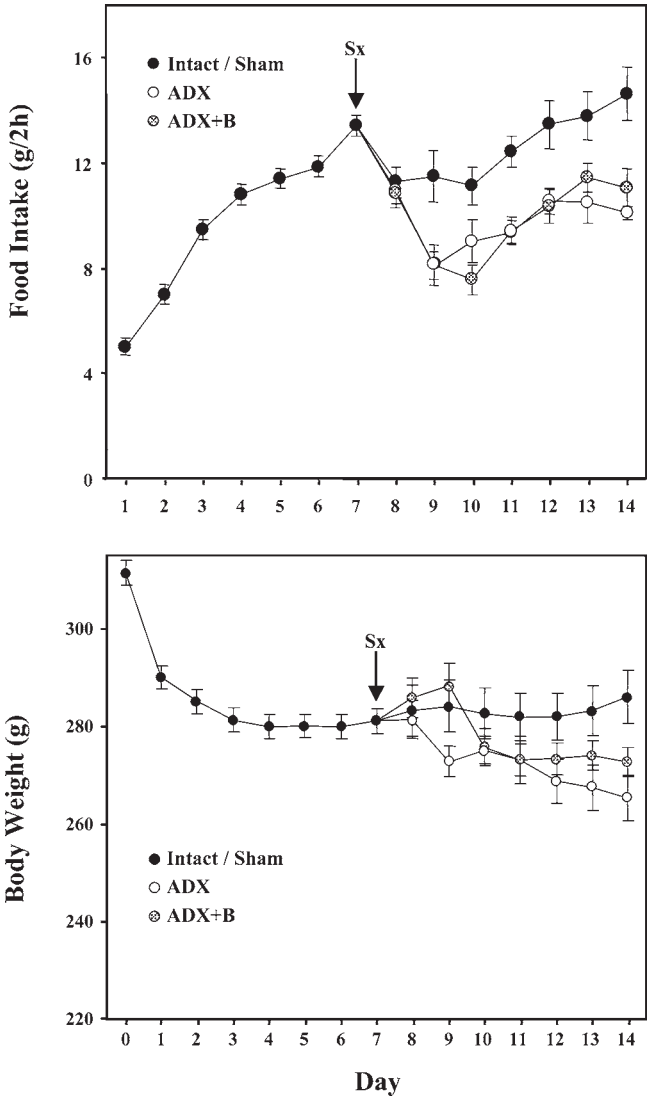

Fig. 1. Daily 2-h food intake (upper) and body weight (lower) values in food-restricted rats before (closed circles represent intact animals between d 1 and 7; n = 18–21 rats) and after (d 8–14; n = 6–7 rats per group) surgeries (Sx; see Table 2). Body weights were recorded daily before 2-h food access. Day zero body weight values (lower) were recorded on the morning of the day when food restriction began.

anorexigenic signal activity, in combination with FR-induced enhanced hypothalamic NPY-ergic function *(18)*. Thus, these manipulations will allow the interpretation of whether neuroendocrine, hypothalamic CRH-mediated stress is a major factor characterizing social anorexia.

Results

Effects of Food Restriction and Surgeries on Rat Body Weight and Peripheral Leptin Levels

Figure 1 (upper panel) shows daily 2-h food intake (between 9:00 and 11:00) of individually housed rats. As depicted, all rats increased the amount of food eaten in 2 h, in a time-dependent fashion, between d 1 and 7 of the food restriction (FR) design (see Materials and Methods). Once subjected to the FR paradigm, rats had already lost body weight 24 h after initiation but remained at a stable level of body weight until d 7 (Fig. 1, lower panel). On d 7, after weighing, rats were subjected to ADX or adrenal sham operation, and implantation of iv and icv catheters. Adrenal gland-operated rats were implanted, at the time of surgery, with a sc placebo (sham and ADX animals) or corticosterone (B) pellet (ADX+B rats). As shown (Fig. 1), surgeries induced a decline in 2-h food intake (upper panel) and, consequently, in BW (lower panel). However, from d 10/11 of the FR paradigm animals stabilized both daily 2-h food intake and BW, although both groups of ADX rats remained anorectic and lighter vs sham rats up to the experimental day (d 15). Rats subjected to our experimental design were characterized by the following morning basal circulating leptin concentrations (in ng/mL; mean ± SEM, n = 6–7 rats per group): 4.45 ± 0.63 in *ad libitum* (AL) rats; 1.31 ± 0.33, in sham-FR rats ($p < 0.05$ vs AL values); 0.24 ± 0.03, in ADX-FR rats ($p < 0.05$ vs AL and sham-FR values); and 0.28 ± 0.07, in ADX+B-FR rats ($p < 0.05$ vs AL and sham-FR values).

Effect of Ghrelin icv Administration on 2-h Food Intake in AL and FR Rats

Figure 2 shows results of individual food intake (expressed in relation to individual BW) in icv-treated AL rats. As depicted, ghrelin icv administration significantly ($p < 0.05$ vs veh-injected rat values) stimulated 2-h food intake. Spontaneous 2-h food intake was several-fold higher ($p < 0.05$) in FR than in AL rats, regardless of group (Fig. 2). As depicted, spontaneous 2-h food intake was significantly lower ($p < 0.05$ vs values in sham-FR rats icv treated with veh) in both ADX rats and ADX animals receiving corticosterone (B) replacement therapy (ADX+B) (Fig. 2). Finally, sham- and ADX+B-FR rats icv-treated with ghrelin ate, in 2 h, a similar amount of food as that observed after icv veh treatment (Fig. 2). Surprisingly, 2-h food intake was significantly reduced ($p < 0.05$ vs veh-treated ADX-FR rats) after icv ghrelin administration in ADX-FR rats (Fig. 2).

Effects of Ghrelin icv Administration and Food Intake on Circulating Hormone Levels in Rats

Basal ghrelin levels in AL rats were not modified 2 h after veh icv administration and food intake; however, they resulted significantly ($p < 0.05$ vs respective basal values) enhanced 2 h after ghrelin icv administration and food intake (Fig. 3). As expected, FR rats have developed basal hyperghrelinemia ($p < 0.05$ vs basal values in AL rats) (Fig. 3). Among FR rats, we found that basal circulating ghrelin concentrations were significantly ($p < 0.05$ vs basal sham values) enhanced by ADX, and this effect was abolished by B replacement therapy (Fig. 3). Basal circulating ghrelin levels declined 2 h after both icv veh treatment and food intake in FR rats ($p < 0.05$ vs respective basal values); how-

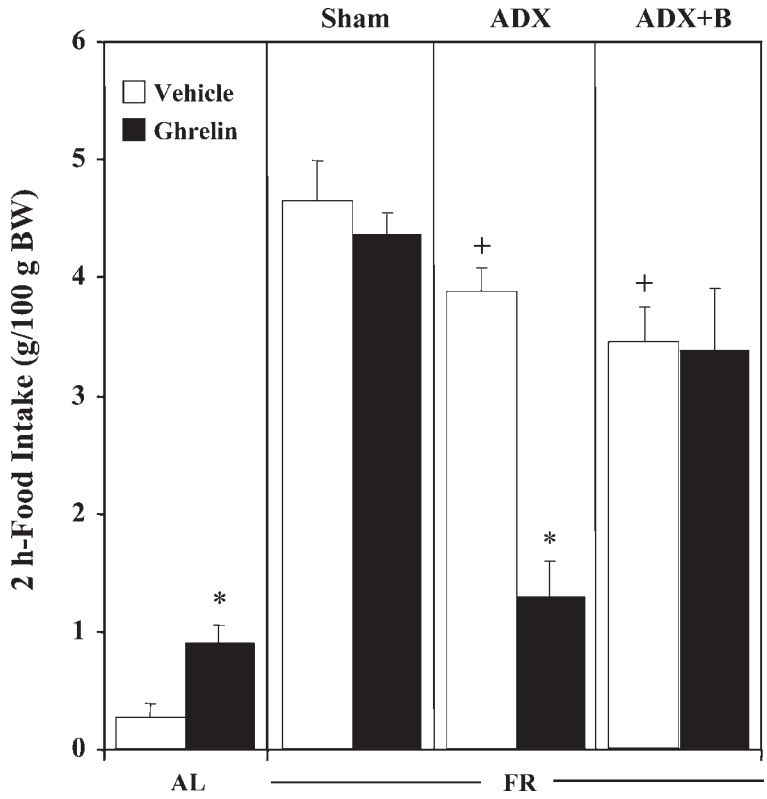

Fig. 2. Food intake 2 h after icv administration of vehicle (□) or ghrelin (■) in *ad libitum* (AL) eating and in, glucocorticoid-replete (sham and ADX+B) and -depleted (ADX), food restricted (FR) rats (n = 6–7 per group/condition). *$p < 0.05$ vs values in icv vehicle-treated rats of the same group. $^{+}p < 0.05$ vs values in icv vehicle-treated sham-FR rats.

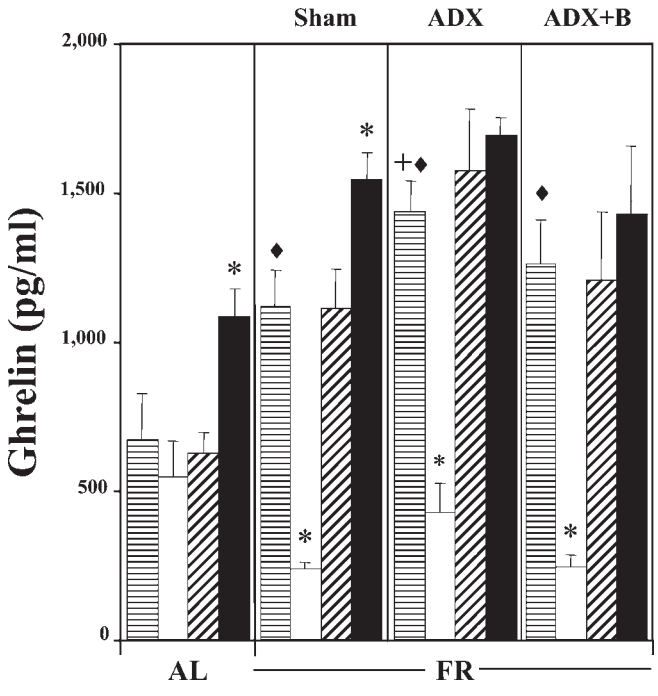

Fig. 3. Circulating ghrelin levels 10 min before ▤ and ▨) and 120 min after icv administration of either vehicle (□) or ghrelin (■) in *ad libitum* (AL) eating and in sham, ADX, and ADX+B food restricted (FR) rats (n = 6–7 per group/condition). *$p < 0.05$ vs respective basal (time –10 min) values in the same group. $^{\blacklozenge}p < 0.05$ vs basal (time –10 min) values in AL rats. $^{+}p < 0.05$ vs basal (time –10 min) values in sham-FR rats.

ever, values in ADX-FR animals remained somewhat higher (albeit not significant) than in sham- and ADX+B-FR rats (Fig. 3). Finally, 2 h after icv ghrelin injection and food intake circulating ghrelin levels were high, regardless of the group examined (Fig. 3).

When AL and sham-FR rats were icv-administered with veh, we found no changes in circulating ACTH levels 2 h after treatment (Fig. 4, upper panel). These observations argue in favor of effective adaptive mechanisms developed in glucocorticoid-replete FR animals (sham- and ADX+B-FR rats) to normalize basal circulating ACTH levels. On the contrary, the enhanced basal ACTH levels ($p < 0.05$ vs basal sham-FR rat values) characterizing ADX-FR rats were significantly ($p < 0.05$ vs respective basal values) decreased 2 h after icv veh treatment and food intake (Fig. 4, upper panel). Interestingly, icv ghrelin administration significantly ($p < 0.05$ vs basal values) increased circulating ACTH concentrations, 2 h after treatment and food intake, only in groups of glucocorticoid-replete rats (Fig. 4, upper panel).

Peripheral B concentrations (Fig. 4, lower panel) in AL rats, were not modified 2 h after icv veh administration and food intake, and icv ghrelin treatment increased, although not significantly, plasma B levels 2 h after events. In sham-FR rats basal B circulating levels were significantly ($p < 0.05$) decreased 2 h after icv veh, but not ghrelin, treatment and food intake (Fig. 4, lower panel). As expected in ADX-FR

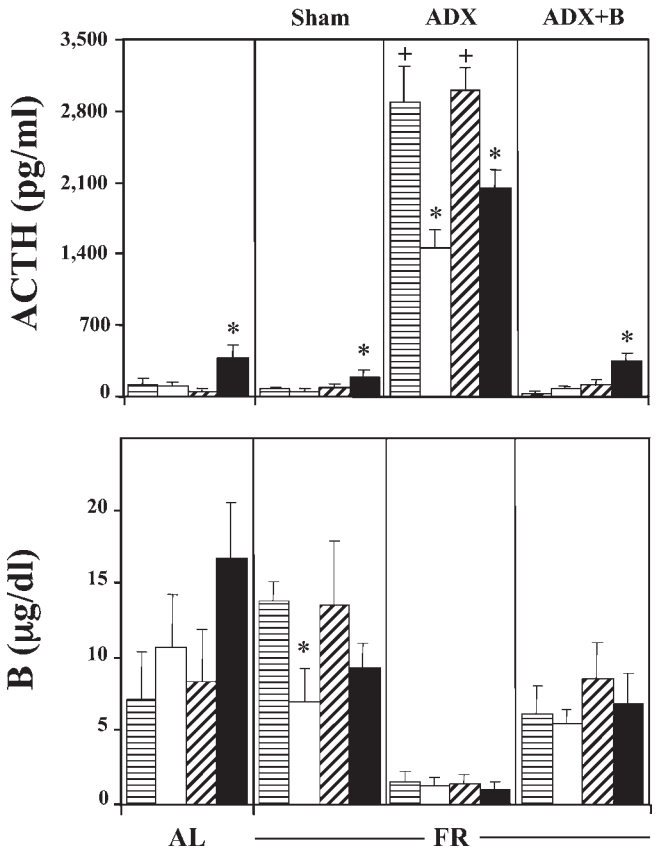

Fig. 4. Circulating ACTH (upper) and corticosterone (lower) levels 10 min before (▤ and ▨) and 120 min after icv administration of either vehicle (□) or ghrelin (■) in *ad libitum* (AL) eating and in sham, ADX, and ADX+B food restricted (FR) rats ($n = 6–7$ per group/condition). *$p < 0.05$ vs respective basal (time –10 min) values in the same group. $^{+}p < 0.05$ vs values, obtained in similar condition, in sham-FR rats.

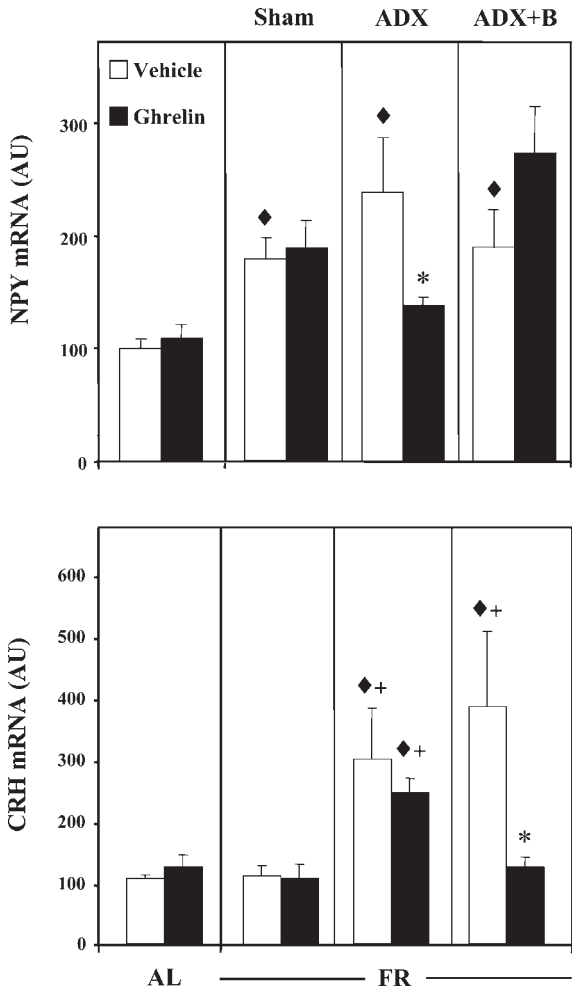

Fig. 5. Hypothalamic NPY (upper) and CRH (lower) mRNA expression (in arbitrary units, AU), 2 h after icv administration of vehicle (□) or ghrelin (■), in *ad libitum* (AL) eating and in sham, ADX, and ADX+B food restricted (FR) rats ($n = 6–7$ per group/condition). *$p < 0.05$ vs vehicle values in the same group. $^{+}p < 0.05$ vs vehicle values in sham-FR rats. $^{\blacklozenge}p < 0.05$ vs vehicle values in AL rats.

rats, the very low B circulating concentrations (at the lower limit of the detection range of the assay) were not modified 2 h after food intake and either icv treatment (Fig. 4, lower panel). Finally, ADX+B-FR rats showed a constant peripheral B level (expected to be in a range of 5–8 μg/dL), regardless of icv treatment and food intake (Fig. 4, lower panel).

Effects of Food Intake, the Glucocorticoid Environment and icv Treatment on Hypothalamic mRNAs Levels in Rats

Two hours after icv ghrelin treatment and food intake in AL rats, we found that basal hypothalamic NPY mRNA levels were unmodified compared to AL veh-treated rats while eating (Fig. 5, upper panel). As could be expected, this hypothalamic parameter, in basal condition, was significantly ($p < 0.05$ vs values in AL rats) enhanced in all groups of FR rats, regardless of treatments (Fig. 5, upper panel). Two hours after icv ghrelin administration and food intake, hypothalamic NPY mRNA levels were unchanged (vs respective icv veh values) in sham- and ADX+B-FR rats (Fig. 5, upper panel). Surprisingly, hypothalamic NPY mRNA levels were significantly reduced ($p < 0.05$ vs veh-values) in ADX-FR rats 2 h after icv ghrelin administration and food intake (Fig. 5, upper panel).

Fig. 5, lower panel, shows the results of hypothalamic CRH mRNA expression in different groups. As depicted, similar levels of this parameter were found in AL and sham-FR rats, regardless of treatment. As expected, they were significantly enhanced ($p < 0.05$ vs AL and sham-FR rats values) in ADX- and ADX+B-FR rats treated with veh (Fig. 5, lower panel). Finally, 2 h after icv ghrelin treatment and food intake, although hypothalamic CRH mRNA remained high in ADX-FR rats, it was reduced ($p < 0.05$ vs basal values) in ADX+B-FR rats (Fig. 5, lower panel).

Basal hypothalamic ghrelin-R1a mRNA expression (Table 1) was high ($p < 0.05$ vs AL-veh values) 2 h after veh administration and food intake in sham- and ADX-FR rats; conversely, ADX+B-FR rats showed significantly ($p < 0.05$)

Table 1
Hypothalamic Glucocorticoid-Receptor (GC-R) mRNA Expression[a]

icv treatment	Vehicle		Ghrelin	
Group	GC-R	Ghrelin-R1a	GC-R	Ghrelin-R1a
AL	102.9 ± 4.3	99.8 ± 8.9	106.1 ± 11.4	119.9 ± 9.7
FR				
Sham	100.9 ± 9.2	142.9 ± 21.7♦	105.9 ± 13.3	138.9 ± 16.2
ADX	114.1 ± 6.7	132.9 ± 22.5♦	121.9 ± 10.5	114.1 ± 9.5
ADX+B	116.2 ± 7.2	68.1 ± 22.4°	121.8 ± 9.1	157.8 ± 11.9*

[a]Two hours after food intake and icv treatment, in *ad libitum* eating (AL) and food restricted (FR), glucocorticoid-depleted (ADX) and -replete (Sham and ADX+B), rats.

Values, expressed in arbitrary units, are the mean ± SEM (n = 5–7 hypothalami per group). ♦$p < 0.05$ vs AL-veh; °$p < 0.05$ vs sham- and ADX-veh values; *$p < 0.05$ vs ADX+B-veh values.

lower hypothalamic ghrelin-R1a mRNA expression than sham- and ADX-FR rats. In ADX+B-FR rats, ghrelin-R1a mRNA expression was significantly ($p < 0.05$) higher 2 h after icv treatment with ghrelin than with veh. Finally, the expression of hypothalamic GC-R mRNA was similar in all groups and conditions studied (Table 1).

Discussion

Ghrelin, the most relevant physiological peripheral hunger signal *(1)*, belongs to a very long list of molecules involved in the control of the circuitry regulating appetite *(19)*. Data in the literature indicate controversy on the effectiveness of adrenal gland ablation in disrupting ghrelin production and actions *(14–16)*. Our data demonstrate that icv ghrelin treatment, although able to stimulate food intake in *ad libitum* eating rats, has no effect on food-restricted rats with normal HPA axis activity. Moreover, after FR, we found a novel ghrelin role in ADX-induced anorectic rats. In fact, hypothalamic ghrelin injection inhibited food intake in ADX-FR rats, even though that these animals still expressed hypothalamic ghrelin-R1a gene.

Interestingly, corticotrope function was stimulated, although to a different degree, by icv ghrelin administration in both AL and glucocorticoid-replete-FR rats. Ghrelin is known to enhance HPA axis function after the stimulation of hypothalamic CRH and vasopressin secretion *(20,21)*, but has no direct effect on adrenal steroid output *(22–24)*. Thus, our data support that a normally glucocorticoid-tuned hypothalamo–corticotrope axis is needed in order to be directly stimulated by ghrelin.

As our data indicate, the ghrelin inhibitory effect on food intake in ADX-FR rats seems to operate via a reduction in hypothalamic NPY-ergic activity, while ADX-induced enhanced CRH-ergic function remains. This probably indicates that the lack of glucocorticoid could have a primary impact, impeding dynamic changes in central CRH-ergic function. Under these circumstances, the direct/indirect effect of ghrelin could result in the exacerbation of the CRH inhibitory function on NPY-ergic activity *(25)*. Moreover, our data agree with previous observations *(26,27)* indicating that hypothalamic treatment of AL rats with CRH antagonist enhanced NPY-induced food intake. This also concords with authors who claim that NPY-stimulated food intake can be either blocked by PVN injection of urocortin *(28)* or enhanced by toxin-impaired hypothalamic CRH function *(29)*. In addition, while increased food intake was found after central injection of GHRP-6 in AL rats, this effect was accompanied, as we now observed after ghrelin, by unchanged hypothalamic NPY mRNA expression *(18)*. Similarly, FR-stimulated hypothalamic NPY mRNA expression remained unmodified after icv GHRP-6 treatment *(18)*. Thus, these data could indicate that GHRP-6–stimulated feeding, in FR rats, did not depend on enhanced hypothalamic NPY-ergic function *(18)*. It must be stressed, however, that our FR paradigm, characterized among others by hypoleptinemia *(17,30,31)*, was able to enhance not only pre- (NPY-ergic) but also post-synaptic hypothalamic orexinergic effects *(32)*. Also, our FR design increased basal circulating ghrelin levels, while hypothalamic ghrelin-R1a mRNA expression remained, and was even higher than in AL rats, thus confirming that FR did not result in hypothalamic downregulation of ghrelin-R1 as an interfering factor for the lack of hypothalamic ghrelin action. Moreover, a recent study *(33)*, although it used a different species, demonstrated that central ghrelin treatment inhibited food intake by enhancing hypothalamic CRH-ergic activity. Furthermore, this effect was fully abrogated by CRH antagonist cotreatment, while hypothalamic NPY-ergic activity remained similar *(33)*.

Some authors reported that, in AL rats, prolonged ghrelin treatment failed to stimulate food intake if animals underwent a previous bilateral ADX *(15,16)*. Conversely, other authors found that the orexigenic activity of ghrelin remained in anorectic ADX rats, but only if animals were not previously subjected to feeding manipulations *(14)*. These discrepancies could be related to differences between experimental designs *(14–16)*. Precisely, we chose our FR design

partly because we were able to modulate signals allowing ghrelin action on food intake, e.g., by developing enhanced hypothalamic NPY-ergic function and decreasing circulating levels of leptin, while anorectic hypothalamic CRH-ergic activity was adapted in rats with intact adrenal function *(34)*. Also, recent results *(35)* reinforce the rationale of our FR paradigm. Because they indicate that after the fasting-induced hypoleptinemia, a condition resembling our FR state, a clear increase in the spike frequency of arcuate NPY/AgRP neurons takes place, an effect fully abolished by ip leptin administration *(35)*. Thus, their *(35)* data and ours confirm that the hypothalamic NPY network is the primary pathway inducing appetite *(36)*.

Our study provides additional evidence of the modulation of hypothalamic CRH-ergic activity exerted by prolonged FR. In fact, after prolonged FR in animals with intact adrenals, hypothalamic CRH *(18,34)* and GC-R mRNAs expressions were normal (e.g., similar to that observed in AL rats). Moreover, the stimulatory effect exerted by ADX on basal hypothalamic CRH mRNA expression was not reversed by the low B levels reached by the steroid replacement therapy applied. To our knowledge, this is the first report demonstrating that in 7-d ADX rats receiving low B replacement therapy, with normal basal circulating levels of ACTH, the hypothalamic CRH-ergic function pathway remains enhanced. This observation could be related to the fact that the median eminence is an important target for rapid establishment of the negative glucocorticoid feedback mechanism on both CRH and vasopressin secretion *(37)*. Thus, low B levels were effective for normalization of corticotrope function. Interestingly, while icv ghrelin treatment did not modify hypothalamic CRH mRNA expression in ADX-FR rats, the low B circulating levels characterizing ADX+B-FR animals were, however, permissive enough to reveal an inhibitory effect of icv ghrelin on CRH-ergic function, thus allowing these rats to eat as much as sham-FR rats did.

Basal ghrelin circulating concentrations were high (vs values in AL rats) in FR rats. The increase in basal ghrelinemia was even more pronounced in ADX-FR (significantly different vs sham-FR values), an effect that was absent in ADX+B-FR animals. These observations argue in favor of a glucocorticoid dependency *(15,16)* of peripheral ghrelin secretion in physiological and under negative balance conditions. Also, the fact that in the absence (ADX-FR) or in the presence of constant low B (ADX+B-FR) peripheral levels, initial and final ghrelinemia were similar probably indicates that intact functionality of the adrenals could modulate the ghrelin metabolic clearance rate. The high basal ghrelin peripheral levels in FR rats significantly decreased 2 h after food intake and icv veh administration. This indicated that acute food intake/satiety is a highly important signal to trigger mechanisms inhibiting ghrelin release. Interestingly, we found significant increases in ghrelin circulating levels in AL and sham-FR rats after icv ghrelin administration. The latter observation probably indicates that a ghrelin brain-to-blood passage has taken place. Data in the literature confirm this statement, and bi-directional passage of ghrelin across the blood–brain barrier (BBB) has been suggested *(38)*. This study also claims that the intact ghrelin molecule (like the one used in our study), rather than des-octanoyl ghrelin, is the active molecular form crossing the BBB, from the central nervous system to the periphery. This supports an important role that hypothalamic ghrelin *(20,21,39)* plays in regulating GH secretion and energy balance *(40)*.

In brief, the present study supports an important role of hypothalamic ghrelin in the mechanisms related to chronic hypothalamic stress-induced anorexia by inhibiting NPY-ergic function in glucocorticoid-deprived animals subjected to FR. Indeed, physiopathological imbalance between hypothalamic NPY- and CRH-ergic activities *(25,27,29)* results in eating disorders. Therefore, this novel hypothalamic ghrelin effect could operate by exacerbating wasting in clinical pictures characterized by anorexia and corticoadrenal dysfunction *(41–43)*.

Materials and Methods

Animals, Surgeries and Experimental Design

Adult male (280–330 g BW) Wistar rats were used in all experiments. Rats were kept in a light- (lights on between 06:00 and 18:00) and temperature- (20–22°C) controlled room, and caged (five rats per cage) for 7 d with free access to Purina Chow Diet and water. The following day (d 1 of different food intake protocols) at 07:00 h rats were weighed, individually housed in plastic cages, and left undisturbed without food availability. At 09:00 h, food (50 g) was provided, and the remaining food withdrawn at 11:00 h. The grams of food eaten in a 2-h period were then calculated. On this basis, two groups of rats emerged, the first one formed by rats eating AL for 15 d, while the second group was subjected to a previously described FR paradigm *(30, 44)*. Briefly, rats have had daily access to food between 09:00 and 11:00 h for a period of 15 d. Daily individual 2-h food intake and BW (registered at 07:00 h) values were recorded from d 1 to d 15 of the experimental design. Rats (AL and FR) were implanted, under ketamine anesthesia, with both icv (coordinates from the point of bregma, in mm, were: 1.0 posterior, 1.8 lateral, and 3.5 deep) and iv catheters *(30)* on d 7 of the food intake protocol. AL rats were then returned to their home cages and continued in similar conditions to those described before surgeries. Additionally, on the same day (d 7) of the paradigm, FR rats were subjected to either adrenal gland sham-operation (sham) or bilateral ADX (performed by the dorsal approach). At the time of surgery, whereas sham- and ADX-FR rats were sc implanted with a placebo pellet, ADX+B animals were sc

Table 2
Brief Summary of the Experimental Design

Groups	
AL	*Ad libitum* eating, between d 1–15.
FR	Eating with daily free food availability for a 2-h period only, between d 1 and 15.
Surgeries	
AL	icv and iv catheters on d 7 of the food intake protocol.
FR	Sham operation of adrenal glands or bilateral adrenalectomy, combined with icv, iv catheters, and sc pellet (placebo or B) implantations on d 7 of the food intake protocol.
Experimental day	
	On d 15, animals were bled before (–10 min; basal) and 2 h after icv treatment, while rats had free access to food. Rats were sacrificed immediately after the last bleeding and hypothalamic tissues were immediately dissected.

implanted with 75 mg B pellets (Innovative Research of America, Toledo, Ohio, USA) (ADX+B-FR) *(31)*. Animals were returned to their cages and continued with the FR paradigm. ADX- and ADX+B-FR rats drank NaCl 0.9% until the end of experimentation. On d 15 of the food intake protocols (AL and FR), animals were iv bled 10 min before (basal) and 120 min after icv injection (performed at 09:00 h) of 2 μL of sterile saline solution either alone (vehicle; veh) or containing ghrelin (Phoenix Pharmaceuticals Inc.; 1 μg). Animals were then sacrificed by decapitation and medial basal hypothalamus (MBH; containing the median eminence) tissues were dissected *(37)* and kept frozen (–80°C) until measurements. The amount of food eaten during the 2-h period after icv treatment was recorded. A summary of our experimental design is depicted in Table 2.

Hormone Measurements

Peripheral ghrelin concentrations were assayed by a specific radioimmunoassay (RIA) with a kit supplied by Phoenix Pharmaceuticals Inc. (cat. no. RK-031-31). The standard curve ranged between 1 and 1280 pg/tube; intra- and interassay coefficients of variation (CVs) were 3–5 and 8–11%, respectively. Circulating ACTH concentrations were measured by a commercial immunoradiometric assay (cat. no. 40-2195, Nichols Institute Diagnostics, San Juan Capistrano, CA, USA) with a standard curve ranging between 5 and 1400 pg/mL and with intra- and interassay CVs of 3–4 and 6–8%, respectively. Plasma concentrations of B were evaluated by a specific RIA described previously *(37)*, the standard curve ranging between 1 and 250 μg/dL, and intra- and interassay CVs were 4–6 and 8–10%, respectively. Peripheral leptin concentrations were determined by a specific RIA kit supplied by Linco Research Inc. (St. Charles, MO, USA; cat. no. RL-83K). The standard curve of this assay ranged between 0.5 and 25 ng/mL, intra- and interassay CVs were 2–5 and 3–6%, respectively.

Hypothalamic RNA Isolation and Analysis

Total RNA was extracted from MBH tissues, using a commercially available reagent (TriPure®, Roche Diagnostics, cat. no. 11667165001). The yield of extraction was assessed by measuring light absorbency at 260 nm, and the quality of RNA by calculating the ratio of the absorbencies measured at 260 and 280 nm. Reverse transcription was performed, starting with 2 μg of total RNA and using random primers and the enzyme SuperScript™ II Reverse Transcriptase (Invitrogen, cat. no. 18064-022). The relative gene expression of NPY, ghrelin-R1a, GC-R, and CRH was assessed by real-time quantitative RT-PCR, using the LigthCycler® technology (Roche Diagnostics, Rotkreuz, Switzerland) with the Quantitect Sybr® Green PCR Kit (Qiagen, Hilden, Germany) as previously described *(45)*. The following primer pairs, designed in our laboratory and synthesized by Microsynth (Windish, Switzerland), were used for the amplification: NPY (F) 5'-TCC GCT CTG CGA CAC TAC AT-3', and (R) 5'-TGC TTT CTC TCA TTA AGA GAT CTG-3' (100 bp; GenBank accession number NM 012614); ghrelin-R1a (F) 5'-ACT GTG CTC TAC AGT CTC ATC GGG A-3', and (R) 5'-CCA CCA CAG CAA GCA TCT TCA CT-3' (118 bp; GenBank accession number NM 032075); GC-R (F) 5'-TTC AGG ATG TCA TTA CGG GGT-3', and (R) 5'-CCT GAA GAC ATT TCC GAT AGC G-3' (167 bp; GenBank accession number Y 12264); CRH (F) 5'-CTG CCA AGG GAG GAG AAG GTA G-3', and (R) 5'-TCA GAA TCG GCT GAG GTT GCT G-3' (188 bp; GenBank accession number NM 031019); β-actin (F) 5'-CGT TGA CAT CCG TAA AGA-3', and (R) 5'-TAG AGC CAC CAA TCC ACA C-3' (176 bp; GenBank accession number NM 031144). For quantification purposes, NPY, ghrelin-R1a, GC-R, and CRH mRNA levels were always reported to the level of β-actin mRNA in the same sample. Different dilutions of the samples were tested in preliminary experiments to ensure that quantification would be performed within the linear part of the amplification. Following this test, all samples were quantified in at least two different runs, to obtain an interassay CV < 10%. Relative expression was then determined using crossing point values and amplification efficiencies of the target gene and the reference gene.

Analysis of Data

Results are expressed as the mean ± SEM. Data for circulating hormone concentrations were analyzed by multiple factor ANOVA, followed by Student–Newman–Keul's test *(46)* for comparison of different mean values. The nonparametric Mann–Whitney test *(46)* was used for analysis of data obtained from hypothalamic mRNA expression.

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