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# Nature of Changes in Adrenocortical Function in Chronic Hyperleptinemic Female Rats

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**Neonatal treatment of rats with monosodium L-glutamate, which destroys hypothalamic arcuate nucleus neuronal bodies, induces several metabolic abnormalities; as a result, rats develop a phenotype of pseudoobesity. This study was designed to explore, in the monosodium L-glutamate–treated female rat, the influence of chronic hyperleptinemia on adrenal cortex functionality. For this purpose, we evaluated in control and hypothalamic-damaged rats: (a) in vivo and in vitro adrenocortical function, (b) adrenal leptin receptor immunodistribution and mRNA expression, and (c) whether the inhibitory effect of leptin on adrenal function remains. Our results indicate that, compared to normal counterparts, pseudoobese animals displayed (1) hyperadiposity, despite being hypophagic and of lower body weight, (2) in vivo and in vitro enhanced adrenocortical response to ACTH stimulation, (3) an in vitro adrenal fasciculata-reticularis cell hyper-sensitivity to ACTH stimulus, (4) hyperplasia of their adrenal zona fasciculata cells, and (5) adrenal fasciculata-reticularis cell refractoriness to the inhibitory effect of leptin on ACTH-stimulated glucocorticoid production due, at least in part, to decreased adrenal leptin receptor expression. These data further support that increased hypothalamo–pituitary–adrenal axis function, in the adult neurotoxin-lesioned female rat, is mainly dependent on the development of both hyperplasia of adrenal zona fasciculata and adrenal gland refractoriness to leptin inhibitory effect. Our study supports that adrenal leptin resistance could be responsible, at least in part, for enhanced glucocorticoid circulating levels in this phenotype of obesity.**

**Key Words:** ARC damage; hypothalamic obesity; positive energetic balance; Ob-Rb; adiposity; adrenal gland; glucocorticoid; leptin.

## Introduction

Hypothalamic lesion induced in rats is a commonly recognized tool for studies on neuroendocrine dysfunctions. Monosodium L-glutamate (MSG) administration in neonatal rats is a treatment that mainly damages hypothalamic arcuate nucleus (ARC) activity *(1–3)* and alters several neuroendocrine functions *(4,5)*. It is accepted that a number of morphological, behavioral, and endocrine abnormalities are developed after treatment with this neurotoxin *(2–4)*. The early loss of catecholaminergic and peptidergic hypothalamic neurons induced by MSG, namely, those of the ARC *(1,5,6)*, has been considered responsible for these neuroendocrine abnormalities. Changes in neuronal function, in an area of the brain so intimately involved in the regulation of the pituitary activity, modifies hypothalamo–pituitary–adrenal (HPA) axis activity *(7–10)*, among others. It is also recognized that MSG treatment alters adiposity in rodents, inducing increased body fat mass and arrest in body weight gain *(11)*. A classical physiological property, such as the normal function of the hypothalamo–pituitary axis, is altered in MSG-treated rats and mice. In fact, a development of tissue hypersensitivity to specific stimuli *(12)* appears after MSG-induced, partial, hypothalamic denervation. It has been previously observed *(13,14)* that the median eminence (ME) and the anterior pituitary (AP) of MSG-treated rats are hyperresponsive to different stimuli (e.g., high potassium solution, AP hormone–releasing hormones) when studied in vitro. Thus, these observations could help in understanding some of the altered neuroendocrine responses characterizing this animal model *(15)*.

Regarding changes in HPA axis function of MSG rats, they have been partially attributed to altered activity of hypothalamic hypophysiotropic neuronal systems *(16–18)*. As a consequence MSG rats develop, among others, adrenal cortex hyperfunction *(7)*. Adipocyte-derived leptin plays an important role in the relationship between HPA axis and adipose tissue functions *(19)*. In fact, plasma leptin levels act as a pivotal signal for the control of energy metabolism *(20)* and physiologically inhibit ACTH-induced glucocorticoid secretion *(21)*. In the present study we investigated whether changes in adrenocortical function, in the adult

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**Table 1**
Basal Circulating Levels
of Several Parameters, and Body Weight
and Fat Mass Values in 120-d-old CTR and MSG Female Rats

| Parameter | CTR | MSG |
|---|---|---|
| ACTH (pg/mL) | 29.09 ± 2.61 | 38.93 ± 3.55 |
| B (μg/dL) | 11.09 ± 1.47 | 11.62 ± 1.95 |
| CBG (μ*M*) | 1.08 ± 0.12 | 0.59 ± 0.06* |
| CBG $K_d$ (n*M*) | 3.04 ± 0.35 | 2.56 ± 0.41 |
| FB (μg/dL) | 0.046 ± 0.009 | 0.095 ± 0.014* |
| Leptin (ng/mL) | 3.94 ± 0.72 | 43.52 ± 8.34* |
| Body weight (g) | 223.4 ± 7.48 | 174.6 ± 10.8* |
| Food Intake (g/24 h) | 16.54 ± 0.68 | 12.41 ± 0.89* |
| Fat pad mass (g) | 12.73 ± 1.19 | 23.26 ± 1.76* |

*$p < 0.05$ vs CTR values.
[a]B: corticosterone; CBG: corticosteroid binding globulin; $K_d$: dissociation constant; FB: free corticosterone. Fat pad mass: sum of individual retroperitoneal plus parametrial fat pads in grams.
Values are the mean ± SEM, $n$ = 10–12 rats per group.

MSG female rat, could be related to chronic exposure of high leptin circulating levels.

## Results

### *Basal Hormonal Profile and Body Weight Characteristics of the Adult Female MSG Rat*

Circulating concentrations of several hormones, determined in 120-d-old female rats, at the trough time of the day *(22)* and in non-fasting condition, indicated that although basal plasma ACTH and total corticosterone (B) concentrations were statistically similar in both groups, conversely, MSG rats displayed significantly ($p < 0.01$) lower corticosteroid binding globulin (CBG) concentrations, with similar $K_d$ values, thus resulting in higher ($p < 0.02$) free (F) B plasma levels (Table 1). In addition, MSG rats displayed higher ($p < 0.001$) circulating levels of leptin than CTR animals, a characteristic directly related to a significantly ($p < 0.02$ vs CTR rats) higher total adiposity (retroperitoneal plus omental fat pads), despite significantly ($p < 0.05$) lower daily food intake and body weight than their normal counterparts (Table 1).

### *Effect of MSG Treatment on, In Vivo and In Vitro, Adrenocortical Function*

Figure 1A shows the results of an in vivo 1-h-ACTH test performed in CTR and MSG rats implanted with a chronic iv catheter. As depicted, iv administration of 0.5 μg ACTH induced a rapid (15 min after administration) and similar enhancement in plasma circulating levels of B in both experimental groups; however, the resiliency of the adrenal response to ACTH was significantly delayed in MSG rats. In fact, in CTR rats, plasma B levels returned to the respective baseline at 60 min post-ACTH; conversely, in MSG animals, circulating B concentrations were still significantly

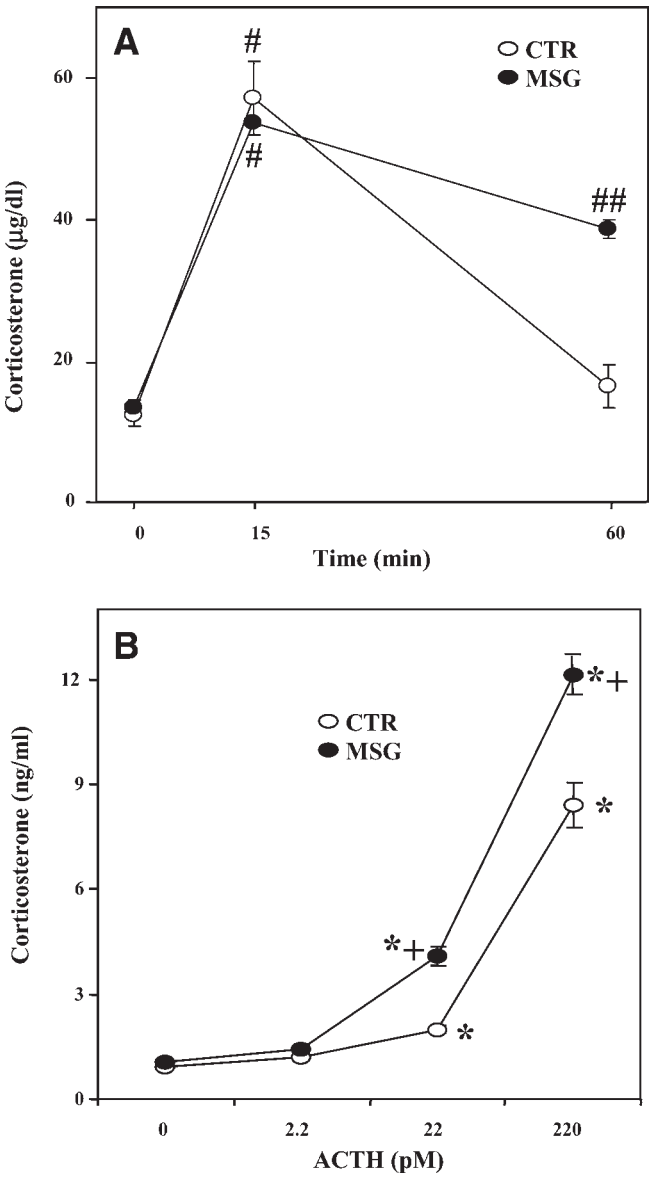


**Fig. 1.** In vivo and in vitro adrenocortical responses in 120-d-old control (CTR) and, neonatally treated, monosodium L-glutamate (MSG) female rats. Panel A shows the results of circulating levels of corticosterone (μg/dL of plasma) before and different times after ACTH administration (0.5 μg; i.v.). Panel B shows the results of corticosterone release, into the incubation medium (in ng/mL of medium), by total adrenal gland cells from, CTR and MSG, female rats incubated in vitro, in the absence (basal release) or presence of several concentrations of ACTH. Values are the mean ± SEM ($n$ = 6 rats per group, for in vivo experimentation, and $n$ = 3–4 experiments, with 5–6 flasks per point per experiment, for in vitro experiments). #$p < 0.05$ vs respective time zero values. ##$p < 0.05$ vs respective time zero values and 60 min CTR values. *$p < 0.05$ vs Basal (ACTH 0 p*M*) values in the same cell-group. +$p < 0.05$ vs CTR-values in similar condition.

($p < 0.001$) higher than both its respective baseline and time-matched CTR values.

Figure 1B shows the results of glucocorticoid secretion by isolated total adrenal cells, from CTR and MSG female rats, incubated in vitro. Although no significant group differ-

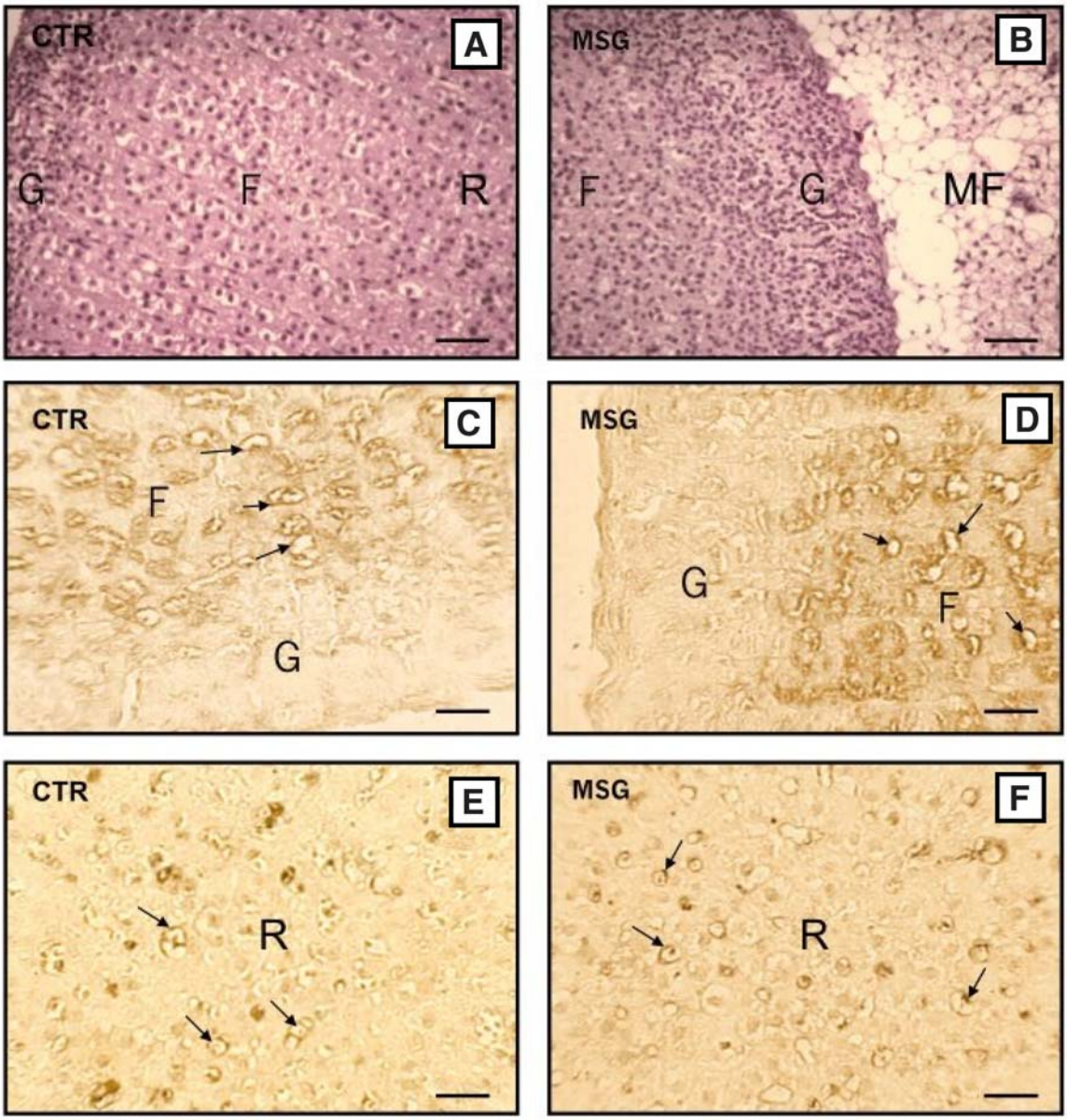


**Fig. 2.** Representative fields of adrenal tissues, from CTR and MSG rats, either stained with hematoxylin-eosin (panels A and B; micrographs taken at ×10 objective) or specifically immnunostained for Ob-Rb (panels C–F; micrographs taken at objective ×40). Arrows indicate cells immunostained for Ob-Rb. Abbreviations; G: zona glomerulosa; F: zona fasciculata; R: zona reticularis; MF: multilocular fat. Scale bars: 1 cm = 67 μm (panels A and B) and 25 μm (panels C–F).

ences were found in spontaneous and 2.2 pM ACTH-elicited glucocorticoid release, whereas total adrenocortical cells from MSG rats released a significantly ($p < 0.05$) higher amount of B, into the medium, than CTR cells after stimulation with both 22 and 220 p*M* ACTH.

### *Morphometric Analysis and Immunostaining for Ob-Rb in Adrenal Glands, and In Vitro Response of Fasciculate–Reticularis Enriched Cells to ACTH Stimulation*

Because of the adrenal hyperfunction found in MSG rats, when analyzed in both in vivo and in vitro conditions, classical histology of adrenal glands, from both groups, was performed. Morphometric analysis of hematoxylin-eosin–stained adrenal glands (Fig. 2, panels A and B) indicated that neither volume density (VD) nor cell size (CS) values were different between groups, regardless of the adrenal zone (data not shown). However, when cell density (CD) values were recorded in the zona fasciculata, a significantly ($p < 0.05$) higher CD value was found in MSG than in CTR adrenal glands, accompanied by a slight decrease in CS (Table 2). Afterwards, the distribution of Ob-Rb in, hematoxylin-eosin–stained areas, adrenal gland cells was determined. Figure 2 (panels C–F) shows Ob-Rb immunostained adrenal cells in both groups studied. As depicted, Ob-Rb

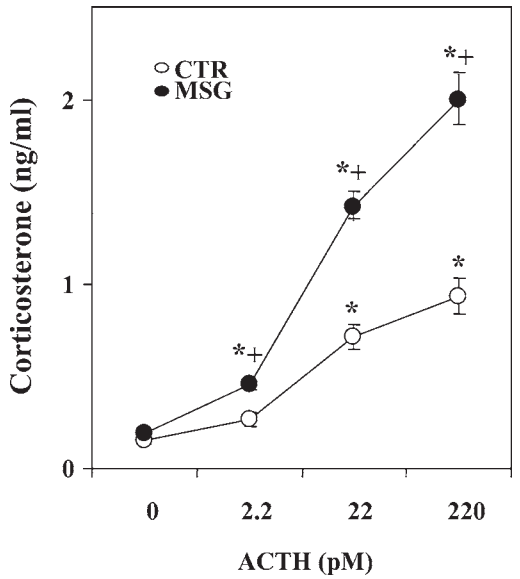


**Fig. 3.** Basal and ACTH-stimulated glucocorticoid (B) release by fasciculata-reticularis–enriched adrenal gland cells from 120-d-old control (CTR) and, neonatally treated, monosodium L-glutamate (MSG) female rats incubated in vitro. Values are the mean ± SEM ($n$ = 3 experiments, with six flasks per point per experiment). *$p < 0.05$ vs Basal (ACTH 0 pM) values in the same cell-group. $^{+}p < 0.05$ vs CTR-values in similar condition.

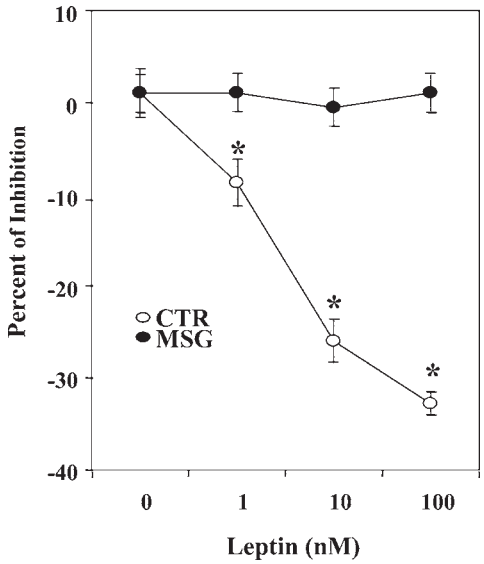


**Fig. 4.** Effects of recombinant murine leptin (0–100 n*M*) on 22 p*M* ACTH-induced corticosterone release by incubated fasciculata-reticularis–enriched adrenal cells, from 120-d-old control (CTR) and monosodium L-glutamate (MSG) female rats, incubated in vitro. Values are the mean ± SEM ($n$ = 3–4 experiments, with six flasks per point per experiment). *$p < 0.05$ vs leptin 0 n*M* values in CTR cells.

**Table 2**
Morphometric Analysis of Zona Fasciculata Adrenal Cells from CTR and MSG Rats[a]

| Group | VD ($\times 10^{-2}$) | CD ($\times 10^{-4}$) | CS ($\mu m^2$) |
|---|---|---|---|
| CTR | 63.8 ± 6.2 | 14.9 ± 0.9 | 426.7 ± 35.5 |
| MSG | 69.3 ± 4.3 | 21.2 ± 1.1* | 332.7 ± 20.9 |

[a]Values are the mean ± SEM ($n$ = 6 rats per group).
*$p < 0.05$ vs CTR values.

positive cells were found in both fasciculata and reticularis cells, regardless of the group examined.

Because of these observations, additional experiments using fasciculata-reticularis–enriched cells from CTR and MSG adrenal glands were performed. These results are depicted in Fig. 3. They indicated that, although no group differences in spontaneous B output, ACTH (2.2–220 p*M*)-stimulated B secretion was significantly higher by MSG than CTR cells, regardless of the ACTH concentration. Thus indicating that the adrenal hyperresponse developed in vivo and in vitro (total adrenal cells) by MSG rats is, at least in part, due to enhanced fasciculata-reticularis cells's sensitivity to ACTH stimulation.

### *Impact of Chronic Hyperleptinemia on the In Vitro Leptin Effect on ACTH-Stimulated Glucocorticoid Release by Fasciculata-Reticularis–Enriched Adrenal Cells*

In order to determine whether exogenous leptin possess any in vitro effect on ACTH-stimulated B secretion by CTR and MSG fasciculata-reticularis–enriched adrenal cells, additional experiments were performed with these cell populations. The results indicated that in CTR cells, 22 p*M* ACTH-stimulated B secretion was significantly ($p < 0.05$) reduced by graded concentrations of recombinant murine leptin (1–100 n*M*) (Fig. 4); in fact, physiological (1 n*M*) and greater concentrations of leptin were able to reduce, in a concentration-dependent fashion, 22 p*M* ACTH-elicited B secretion. Conversely, leptin (1–100 n*M*) was not able to modify ACTH-stimulated B output by fasciculata-rerticularis MSG cells (Fig. 4).

### *Adrenal Ob-Rb mRNA Expression in Normal and Hyperleptinemic Rats*

Additional experimentation was performed in order to determine whether adrenal expression of Ob-Rb was affected by chronic hyperleptinemia in MSG-damaged female rats. For this purpose, adrenal glands, from CTR and MSG, were submitted to semi-quantification of Ob-Rb mRNA expression. As depicted in Fig. 5, adrenal Ob-Rb mRNA expression was significantly ($p < 0.05$) lower in chronic hyperleptinemic (MSG) than in normal (CTR) rats.

## Discussion

Although it has already been recognized that MSG-damaged rats develop, among others, a clear enhancement in HPA axis function *(7–10,14)*, no clear evidence has been provided by the international literature regarding the adrenal mechanisms altered. In fact, although previously reported

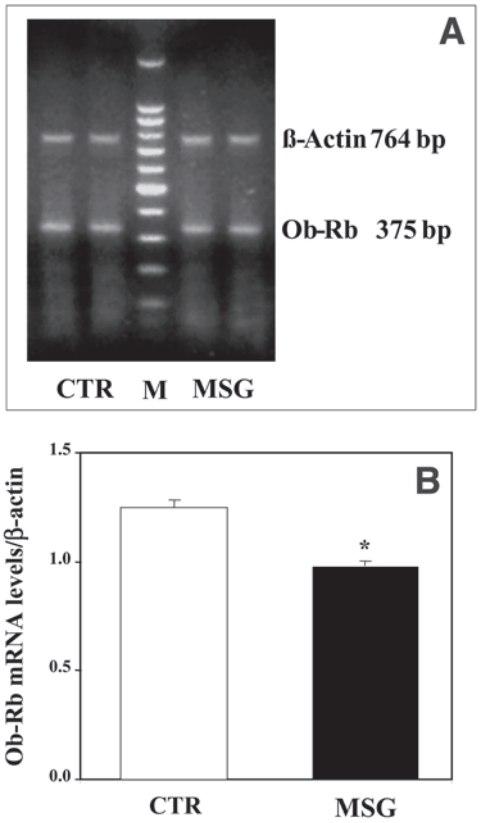


**Fig. 5.** RT-PCR expression analysis of adrenal Ob-Rb mRNA (one representative experiment, two individuals per group) (M: molecular marker, 100-bp ladder) (panel A). Adrenal Ob-Rb mRNA expression levels, relative to β-actin mRNA, in both experimental groups (panel B). Values are mean ± SEM, $n = 3$ experiments (two adrenals per group in each experiment). *$p < 0.05$ vs CTR values.

that an adrenal hyperplasia takes place *(10)*, these are the first data showing decreased adrenal Ob-Rb mRNA expression in this model. Moreover, our data strongly indicate that adrenal hyperfunction in MSG rats is mainly due to changes in fasciculata-reticularis cell function.

Our study suggests that the hypothalamic damage, due to MSG treatment, induced clear changes in HPA axis function. Although this animal model has extensively been revisited, our data strongly support that alterations in HPA axis function of MSG rats seem to be mainly associated with an adrenal gland resistance to the inhibitory effect of leptin on ACTH-driven glucocorticoid release. This abnormality seems to be dependent on, at least in part, a down-regulation in adrenal Ob-Rb mRNA expression after long-term exposure to high circulating leptin levels. In fact, the hyperleptinemia is already detected 5 d after the last MSG injection (not shown).

The neurotoxic damage of the ARC alters NPY-ergic activity *(11)*, resulting in changes in, among others, eating behavior. In fact, we found that our MSG rats displayed optic nerve degeneration, stunted growth, and a large increase in fat mass, despite reduced body weight and daily food intake, than their normal counterparts. As mentioned, the MSG rat model is characterized by ablation ARC neurons *(4,23)*; consequently, a decrease in hypothalamic NPY-ergic activity *(11)* and in neuronal leptin receptor (Ob-Rb) *(24)* density is expected. The later supports the development of an altered function of the neural circuitry involved in the control of energy balance *(25)*. In fact, we found that MSG rats showed decreased appetite and arrest in body weight gain associated with a paradoxical increased body fat mass and hyperleptinemia.

Our study supports that the adrenal resistance to leptin found in the MSG rat could be a consequence of the chronic hyperleptinemia, which is installed shortly after the hypothalamic damage. Although we did not find any significant change in basal circulating levels of ACTH, an observation in full agreement with earlier reported data *(7)*, conversely, circulating free B concentrations were significantly higher in MSG than in normal rats. Increased plasma total glucocorticoid concentrations in MSG rats of both sexes have been observed *(7,10)* and we presently only found group differences in the concentrations of biologically active glucocorticoid in circulation. This fact could indicate that an imbalance between adrenal glucocorticoid and liver CBG productions could take place in MSG-damaged female rats. It could be suggested that enhanced adrenal glucocorticoid production seems to be developed as an adaptive mechanism to counteract a transient enhancement in corticotrop activity. In fact, MSG rats displayed adrenal, zona fasciculata, hyperplasia. It has also been found that reduced corticosterone clearance rate *(8,9)* and increased fasciculata to glomerolosa width ratio *(10)* characterize MSG animals. Altogether, these alterations could also contribute to enhance glucocorticoids in circulation. These data fully agree with our present in vivo results. We found that after the iv administration of ACTH in MSG rats, we found a delayed resiliency *(26)* of circulating glucocorticoid concentrations to challenge.

It is known that high circulating leptin levels, after exogenous leptin administration in vivo, induce both decreased HPA axis response (27), and down-regulation of hypothalamic *(28)* and adrenal *(29)* Ob-Rb mRNA expression. Moreover, a deleterious effect of adrenal hyperstimulation, by ACTH, on adrenal Ob-Rb expression *(29)* could be an additional factor for abrogation of leptin effects on adrenal cortex function. We presently demonstrated that chronic hyperleptinemic MSG animals did not respond to the in vitro inhibitory effect of leptin on ACTH-stimulated B output, even when supraphysiological leptin concentrations were employed. This adrenal refractoriness to leptin in MSG rats could be dependent on the reduced adrenal Ob-Rb expression noted in these animals. However, whether other steps, after leptin binding to adrenal Ob-Rb, in the leptin signaling system *(30,31)* could be affected remains to be determined.

Interestingly, the altered corticoadrenal function (hyperresponse and refractoriness to leptin effect) in MSG rats can be transiently corrected by bilateral adrenal enucleation. AE-MSG rats are characterized by normalized plasma leptin levels, thus, new, regenerating, adrenal cells are not exposed for a long time to high circulating leptin levels are fully sensitive to the leptin inhibitory effect on glucocorticoid production *(32)*.

In summary, our data clearly point out that increased adrenocortical sensitivity to ACTH stimulation, in MSG rats, could be due to (a) early hyperplasia of adrenal zona fasciculata and (b) long-term lack of inhibitory effect of leptin on ACTH-elicited glucocorticoid production.

## Materials and Methods

### *Animals and Treatment*

Adult male (300–330 g BW) and female (240–280 g BW) Sprague-Dawley rats were allowed to mate in colony cages in a light (lights on from 07:00 to 19:00)- and temperature (22°C)-controlled room. Rat chow and water were available *ad libitum*. Pregnant rats were transferred to individual cages. Beginning on d 2 after parturition, newborn pups were injected ip with either 4 mg/g BW MSG (Sigma Chemical CO., St. Louis, MO) dissolved in sterile 0.9% NaCl or 10% NaCl (litter-mate controls; CTR) once every 2 d up to d 10 of age *(14)*. Rats were weaned and sexed at 21 d of age; daily body weight and food intake of individual female rats were recorded up to the experimental day (120 d of age). MSG-injected animals were screened for effectiveness of treatment by macroscopic observation of degeneration of the optic nerves and the reduced ($p < 0.05$ vs CTR values) hypothalamic NPY mRNA expression at the time of sacrifice (Fig. 6). In each experiment performed (either in vivo or in vitro), CTR and MSG animals were members of the same litters; however, when accumulating experiments, each different experiment was performed with (CTR and MSG) animals from new litters. Our Animal Care Committee approved the experiments. Animals were killed by decapitation, according to protocols for animal use, in agreement with NIH Guidelines for care and use of experimental animals.

### *In Vivo Experiments*

One hundred and twenty day old CTR and MSG animals (10–12 rats per group) were weighed and rapidly killed by decapitation (at 09:00). Trunk blood, collected into plastic tubes containing 0.2 mL EDTA 10%, was immediately centrifuged for further determination of plasma concentrations of ACTH, total corticosterone (B), corticosteroid binding globulin (CBG), free B (FB), and leptin. Total, retroperitoneal (RF) plus omental (OF), fat pads from animals of both groups ($n = 5$ rats per group) were immediately dissected and weighed. In addition, dissected adrenal glands (AG), free of adipose tissue, were either submitted

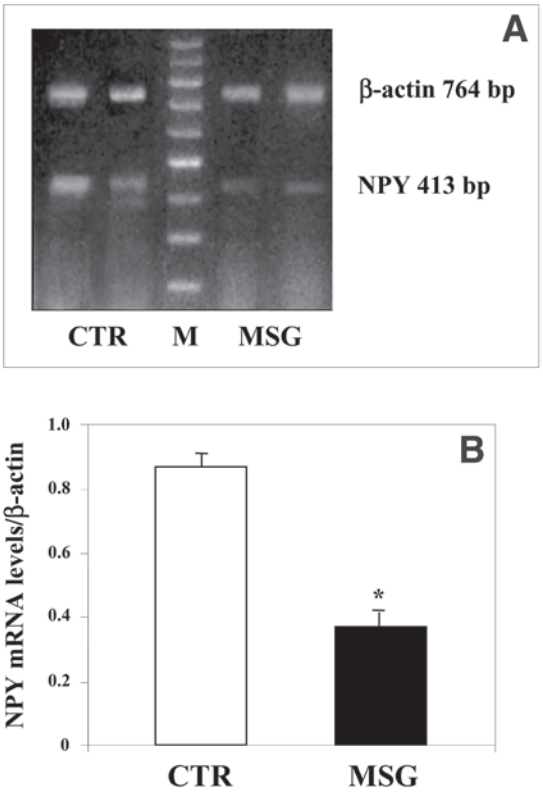


**Fig. 6.** RT-PCR expression analysis of hypothalamic NPY mRNA (one representative experiment, two individuals per group) (M: molecular marker, 100-bp ladder) (panel A). Hypothalamic NPY mRNA expression levels, relative to β-actin mRNA, in both experimental groups (panel B). Values are mean ± SEM, $n = 5$ experiments (two hypothalami per group in each experiment). *$p < 0.05$ vs CTR values.

to RNA extraction or to histological studies. Additional rats ($n = 6$–8), from both experimental groups, were implanted (under light ketamine anesthesia) with an iv catheter and submitted, 1 wk later (at 120 d of age), to an iv ACTH test *(33)* ($ACTH_{1–39}$, Sigma Chemical Co.; 0.5 μg per rat in 100 μL of sterile saline solution). For this purpose, rats were bleed before (sample time zero) and at 15 and 60 min after ACTH iv administration. The blood volume taken at each time interval was 300 μL; this volume was immediately replaced by blood cells resuspended in sterile saline solution containing 0.1 g% BSA, except for the time-zero sample time at which this volume was replaced by a same volume of vehicle only. Plasma samples were kept at −80°C until B concentrations were measured.

### *In Vitro Experiments*

Adrenal cell function in CTR and MSG rats. This method has been extensively described in a previous study *(34)*. Briefly, CTR and MSG rats were killed (at 09:00), on d 120 of age, and their adrenal glands were dissected, free of adipose tissue. Each adrenal gland was cut into four pieces with a fine dissecting knife and placed in an Earle's bal-

anced salt solution (EBSS) containing 0.3% collagenase (type 1, Sigma; 1 mL of solution per each gland) and gently shaken (for 30 min) at 37°C in siliconized glass Erlenmeyer flasks under 95% air–5% $CO_2$ atmosphere in a Dubnoff metabolic incubator. At the end of this period, the remaining tissue fragments were repeatedly passed through a siliconized Pasteur pipet, and the dissociated tissue was filtered through a layer of Nylon cloth (30 μm) to remove cell clumps. The cell suspension was transferred into a conical plastic tube and centrifuged at 100*g*, for 10 min, at room temperature. The cell pellet was washed with 10 mL of fresh incubation medium (EBSS containing 0.2% BSA, 20 mg/L ascorbic acid, 100 IU/mL aprotinin and antibiotics; pH 7.4) and centrifuged as described above. Additional adrenal glands were used in order to obtain enriched population of fasciculata-reticularis cells *(34)*. For this purpose adrenals were transferred into a Petri dish containing 0.3% collagenase in EBSS, the capsule of each adrenal was incised and the gland squeezed firmly within a cellulose tissue to retain the capsule layer; then tissue fragments were repeatedly passed through a siliconized Pasteur pipet and processed as described for isolated total adrenal cells. Cell pellets were resuspended in an appropriate volume of fresh incubation medium in order to obtain, approximately, either 100,000 total adrenal cells or 500,000 fasciculata-reticularis–enriched cells per 0.9 mL of medium. This volume was distributed into polystyrene test tubes together with 0.1 mL of medium alone or containing ACTH (2.2–220 p*M*). Additionally, the effect of recombinant murine leptin (PrePro Tech Inc., Rocky Hill, NJ, USA; added in a volume of 10 μL, final concentrations ranging between 1 and 100 n*M*) was evaluated on 22 p*M* ACTH-induced B secretion. Cells were then incubated for 2 h at 37°C in metabolic conditions. At the end of incubation, tubes were centrifuged 10 min, 100*g*, at room temperature and the supernatants were separated from the cell pellets and kept frozen (–20°C) until measurement of medium B concentration. At least three or four experiments were performed with six flasks per point per experiment.

### *Histological Studies*

Adrenal glands, from animals of both groups ($n = 6$ rats per group), were used for histological studies. Briefly, adrenal tissues were fixed in Bouin's fluid and embedded in paraffin. Sections of 4 μm were obtained, at different levels of the blocks, and stained with hematoxylin-eosin. Immunostaining of adrenal tissue sections were performed by incubation, for 1 h at room temperature, with the primary serum anti-leptin receptor (Ob-Rb) (rabbit polyclonal, Santa Cruz Biotechnology Inc, USA; cat. no. sc 8325), diluted 1:200. Thoroughly washed sections were treated, for 30 min, with a ready-to-use EnVision reaction system (Dako, CA, USA). Diaminobenzidine was used as the peroxide-sensitive chromogen. The specificity of the primary antiserum was monitored by the ability to block the immunocytochemical reaction by replacement of the specific antibody by, similarly diluted, normal rabbit serum. Morphometric studies were performed on hematoxylin–eosin stained cells as earlier reported in detail *(35)*. Briefly, measurements of cell parameters were made by means of an image analysis system (Imaging Technology, Optimas 5.2). Adrenal cells and reference area (RA; area scanned in which cells were scored) were analyzed in each field, for an average of 10 micrographs taken from two levels (e.g., a and b). These measurements were recorded and processed automatically, and the following parameters were afterwards calculated: volume density (VD = Σ cell area/RA), cell density (CD = number of cells/RA), and cellular size (CS; expressed in $\mu m^2$). RA represents the adrenal area scanned, in which cells were scored. Then, with the sum (Σ) of the individual areas (A) of cells, referred to as RA, we obtained VD, which indicates cell mass according to a usually accepted concept. The number of cells (CD) was calculated dividing the immunostained area of the cell population by the mean individual cell area. For this parameter, 100 cells were recorded in each field.

### *Tissue RNA Extraction and RT-PCR Analysis*

Total RNA was isolated from adrenals and hypothalami, for semiquantification of Ob-Rb and NPY mRNAs expressions, respectively. Hypothalamic tissues were dissected as previously reported *(36)*; limits: posterior border of the optic chiasm, anterior border of the mamillary bodies, and lateral hypothalamic border, 3 mm deep (approx). Adrenal glands were dissected free of adipose tissue and squeezed to exclude the medulla. Tissue RNA extraction was performed by using a modification of the single-step, acid guanidinium isothiocyanate-phenol-chloroform extraction method described by Chomzynski et al. *(37)* (Trizol; Invitrogen, Life Technologies, USA; cat. no. 15596-026). The yield and quality of extracted RNA were assessed by 260/280 nm optical density ratio and electrophoresis, under denaturating conditions, on 2% agarose gel. One microgram of total RNA was incubated with 0.2 m*M* dNTPs, 1 m*M* $MgSO_4$, 1 μ*M* of specific primers, 1 μ*M* β-actin primers, 0.1 U/μL AMV reverse transcriptase (5 U/μL), 0.1 U/μL Tfl DNA polymerase (5 U/μL); final volume of 25 μL. Amplifications were done in a thermal cycler (Perkin-Elmer) in the following conditions: 48°C-45 min for reverse transcription step (one cycle); 94°C-2min for AMV reverse transcriptase inactivation and RNA/cDNA/primers denaturation (1 cycle); 94°C-30 s for denaturation; 54°C-1 min for annealing (temperature was the same for both specific primers, Ob-Rb and NPY); 68°C-2 min for extension (40 cycles); 68°C-7 min for final extension (1 cycle), and 4°C for soak (Promega Access RT-PCR System No. A1250). Primers were designed for a high homology region of the Ob-Rb gene: (F) 5'-ATG AAG TGG CTT AGA ATC CCT TGG -3', and (R) 5'- ATA TCA CTG ATT CTG CAT GCT-

3' (375 bp) (GenBank accession number: AF287268). NPY gene: (F) 5'- CCC GCC ATG ATG CTA GGT AAC -3', and (R) 5'-ACA AGG GAA ATG GGT CGG AAT -3' (430 bp) (GenBank accession number: NM012614). In this semi-quantitative technique, the second set of primers was specific for the β-actin gene, having the following sequences: (F) 5'-TTG TCA CCA ACT GGG ACG ATA TGG-3', and (R) 5'-GAT CTT GAT CTT CAT GGT GCT AGG-3' (764 bp) (GenBank accession number: NM031144). Controls without reverse transcriptase were systematically performed to detect cDNA contamination. The amplified products were analyzed on 2% agarose gel and visualized by ethidium bromide UV transillumination in a Digital Imaging System (Kodak Digital Science, Electrophoresis Documentation and Analysis 120 System).

### *Hormones and CBG Determinations*

Circulating ACTH concentrations were measured by a previously described immunoradiometric assay *(36)* with a standard curve ranging between 15 and 3,000 pg/mL and with intra- and interassay coefficients of variation (CVs) of, respectively, 2–3 and 6–8%. Plasma and medium concentrations of B were evaluated by a specific radioimmunoassay (RIA) earlier reported *(36)*, the standard curve ranged between 1 and 250 μg/dL, and intra- and interassay CVs of 4–6 and 8–10%, respectively. Leptin circulating levels were determined by a specific RIA from our laboratory *(38)*; the standard curve ranged between 0.2 and 25 ng/mL, with CVs intra- and interassay of 5–8 and 10–12%, respectively. Finally, plasma CBG concentrations were measured as earlier reported *(39)*. Briefly, 50 μL steroid-stripped serum sample, appropriately diluted in assay buffer, was incubated overnight (4°C) with graded concentrations of cold B (200 μL) in the presence of $^{3}$H-B (New England Nuclear; approx 10,000 cpm in 50 μL of assay buffer); the separation of bound and free hormone fractions was achieved by the addition of 0.1 mL charcoal (1 g%)–dextran T 70 (0.1 g%). Bound radioactivity was determined in a Tracor Analytic Scintillation System. Analysis of binding data for further determination of the concentration and affinity of CBG, and circulating FB levels was performed as previously reported *(39)*.

### *Analysis of Data*

Data were expressed as the mean ± SEM. Data of hormonal circulating and medium concentrations were analyzed by ANOVA followed by Fisher's test *(40)* for comparison of different mean values. The nonparametric Mann–Whitney test *(40)* was used for analysis of data from adrenal gland Ob-Rb mRNA expression. Morphometric data were analyzed by the least significant difference test for multiple comparisons *(40)*.

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