

Analysis of angiotensin II- and ACTH-driven mineralocorticoid functions and omental adiposity in a non-genetic, hyperadipose female rat phenotype

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Abstract The hypothalamic damage induced by neonatal treatment with monosodium L-glutamate (MSG) induces several metabolic abnormalities, resulting in a rat hyperleptinemic–hyperadipose phenotype. This study was conducted to explore the impact of the neonatal MSG treatment, in the adult (120 days old) female rat on: (a) the in vivo and in vitro mineralocorticoid responses to ACTH and angiotensin II (AII); (b) the effect of leptin on ACTH- and AII-stimulated mineralocorticoid secretions by isolated corticoadrenal cells; and (c) abdominal adiposity characteristics. Our data indicate that, compared with age-matched controls, MSG rats displayed: (1) enhanced and reduced mineralocorticoid responses to ACTH and AII treatments, respectively, effects observed in both in vivo and in vitro conditions; (2) adrenal refractoriness to the inhibitory effect of exogenous leptin on ACTH-stimulated aldosterone output by isolated adrenocortical cells; and (3) distorted omental adiposity morphology and function. This study supports that the adult hyperleptinemic MSG female rat is characterized by enhanced ACTH-driven mineralocorticoid function, impaired adrenal leptin sensitivity, and disrupted abdominal adiposity function. MSG rats could counteract undesirable effects of glucocorticoid excess, by

developing a reduced AII-driven mineralocorticoid function. Thus, chronic hyperleptinemia could play a protective role against ACTH-mediated allostatic loads in the adrenal leptin resistant, MSG female rat phenotype.

Keywords Hypothalamic obesity · Hypophagia · Mineralocorticoid · Omental adiposity · Glucocorticoid · Leptin · Insulin

Introduction

Localized hypothalamic lesion in rats is a recognized tool for the study of neuroendocrine dysfunctions. Monosodium L-glutamate (MSG) administration in neonatal rats is a treatment that mainly damages the hypothalamic arcuate nucleus (ARC) [1–3]. As a result, the alteration of several neuroendocrine functions takes place [4–6]. Changes in neuronal functions within a brain area intimately involved in the regulation of the pituitary activity modify the hypothalamo-pituitary-adrenal (HPA) axis function [7–10] among others.

We have previously reported [11] that MSG-treated female rats did develop hyperadiposity, hypophagia, arrest in body weight gain, enhanced in vivo and in vitro ACTH-induced corticosterone secretion, hyperplasia of adrenal zona fasciculata cells, and adrenal leptin resistance to ACTH stimulation on glucocorticoid secretion. The latter mechanism being dependent on a reduction in the adrenal expression of the gene encoding the long-isoform (*ob-Rb*) of the leptin receptor [11], the functional relevant receptor variant for leptin. Moreover, we have earlier addressed that hyperadiposity [12], adrenal leptin resistance [13], and impaired peripheral insulin sensitivity [14] could be overridden by bilateral adrenal enucleation in MSG rats. These

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observations clearly indicate that adrenal leptin resistance seems to be responsible, at least in part, for the enhanced corticoadrenal function in MSG rats [11]. Thus supporting the establishment of vicious circle between the adipose tissue and HPA axis activities [15, 16], and contributing to maintain this hyperadipose rat phenotype.

In this study, we extended our studies on the HPA axis function in the hyperleptinemic female rat model, by examining the adrenal mineralocorticoid activity, and the abdominal fat morphology and functionality, as other components involved in the physiopathological mechanisms established in the adult female MSG rat.

Results

Impact of MSG treatment on in vivo adrenal mineralocorticoid function

Figure 1 shows the results of plasmatic concentrations of aldosterone before (sample time zero) and after (15 and 60 min) i.v. bolus injection of either 0.5- μ g ACTH (upper panel) or 1- μ g angiotensin II (AII; lower panel) in both experimental groups. As showed, despite similar time zero values, aldosterone plasma levels after ACTH i.v. bolus injection induced significantly ($P < 0.05$) higher plasma aldosterone levels in MSG than in control (C) rats on times 15 and 60 min after treatment. Moreover, and contrary to that observed in C rats, peripheral aldosterone concentrations did not recover the respective baseline in MSG animals (Fig. 1, upper panel).

Conversely, mineralocorticoid response to AII i.v. bolus administration was somewhat decreased in MSG rats. In fact, on time 15 min values after AII i.v. treatment, plasma aldosterone concentrations were significantly ($P < 0.05$) lower in MSG than in C rats (Fig. 1, lower panel). On time 60 min post-AII, peripheral steroid levels recovered respective basal values, being similar in both groups (Fig. 1, lower panel).

Effect of MSG treatment on in vitro adrenal mineralocortical function

Figure 2 shows the results of mineralocorticoid secretion by isolated adrenal cells, from C and MSG-treated rats, incubated in vitro with several concentrations of either ACTH (upper panel) or AII (lower panel). Although no differences between groups were noticed in spontaneous and 2.2 pM ACTH-stimulated mineralocorticoid secretion, adrenocortical cells from MSG rats were hyper-responsive ($P < 0.05$ vs. C cells) to higher ACTH concentrations (Fig. 2, upper panel).

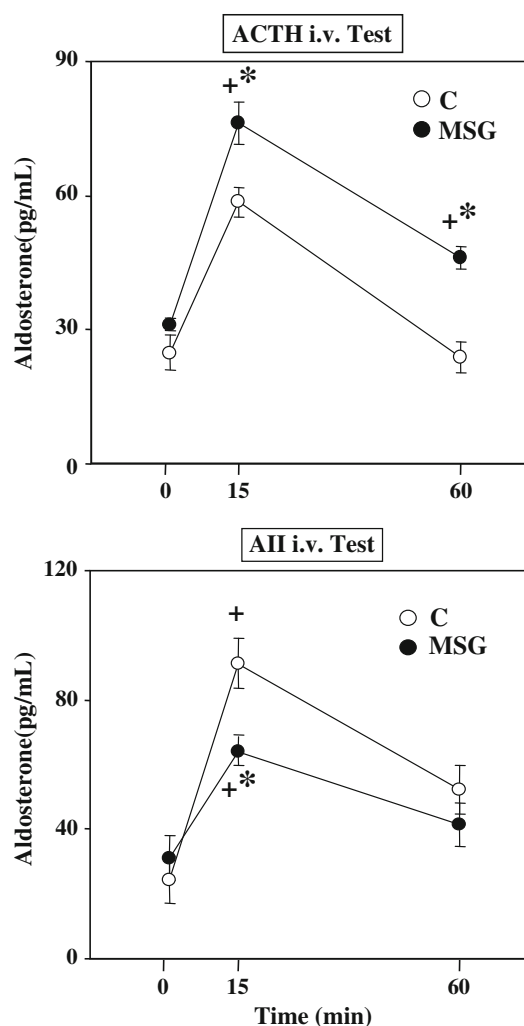


Fig. 1 Basal plasma aldosterone levels (samples time zero) and ACTH (0.5 μ g)- and AII (1 μ g)-stimulated mineralocorticoid (upper and lower panels, respectively) release in the circulation of C and MSG female rats. Values are the mean $\pm$ SEM ($n = 6$ –7 rats per group). + $P < 0.05$ vs. time zero values in the same group. * $P < 0.05$ vs. C values on the same time

Incubation of dispersed adrenal cells in the presence of AII (1–100 nM) indicated that AII was able to enhance aldosterone release by C dispersed adrenocortical cells in a concentration-related fashion (Fig. 2, lower panel). Conversely, only the highest AII concentration (100 nM) was effective to significantly ($P < 0.05$ vs. the respective baseline) increase aldosterone secretion by incubated adrenal cells from MSG rats (Fig. 2, lower panel).

In vitro effect of exogenous leptin on adrenal mineralocorticoid functionality

In order to determine whether exogenous leptin possess any in vitro effect on basal and ACTH-stimulated isolated

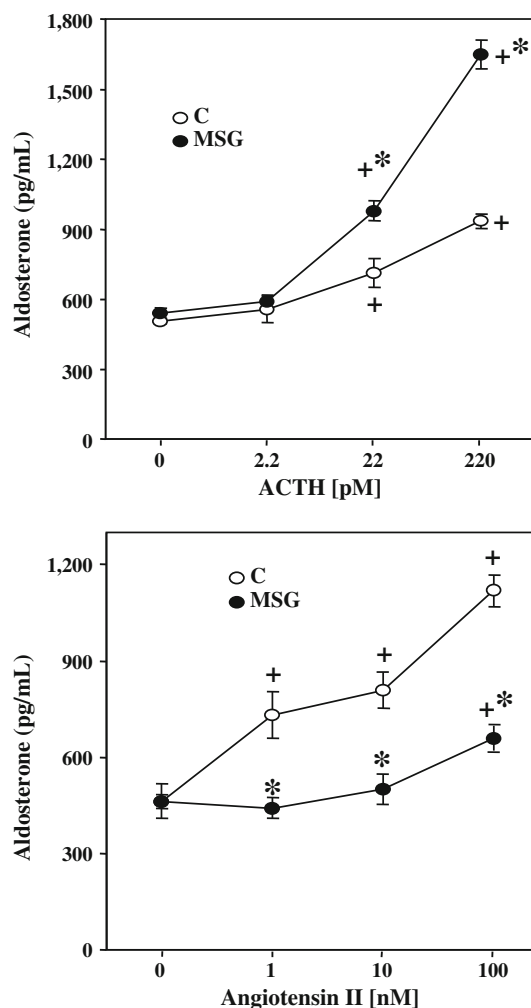


Fig. 2 ACTH (0–220 pM) and AII (0–100 nM) effects on mineralocorticoid (*upper* and *lower* panels, respectively) secretion by isolated total adrenal gland cells from adult C and MSG female. Values are the mean $\pm$ SEM ($n = 3$ –4 experiments, with 6 flasks per point per experiment). + $P < 0.05$ vs. concentration zero values in the same group. * $P < 0.05$ vs. C values in similar condition

adrenal cells, additional experiments were performed with cells from both groups stimulated with 22 pM ACTH. The results indicated that co-incubation of C adrenal cells with 22 pM ACTH and graded leptin concentrations (0.1–10 nM) significantly ($P < 0.05$ or less) reduced, in a concentration-related fashion, ACTH-elicited aldosterone release (Fig. 3), being this effect observed with 1 nM or greater leptin concentration. Conversely, adrenocortical cells from MSG rats were refractory to any inhibitory effect of leptin (0.1–10 nM) on 22 pM ACTH-elicited aldosterone secretion (Fig. 3). Additionally, exogenous leptin (0.1–10 nM) did not modify the AII effect on mineralocorticoid function, regardless of the cell group examined (data not show).

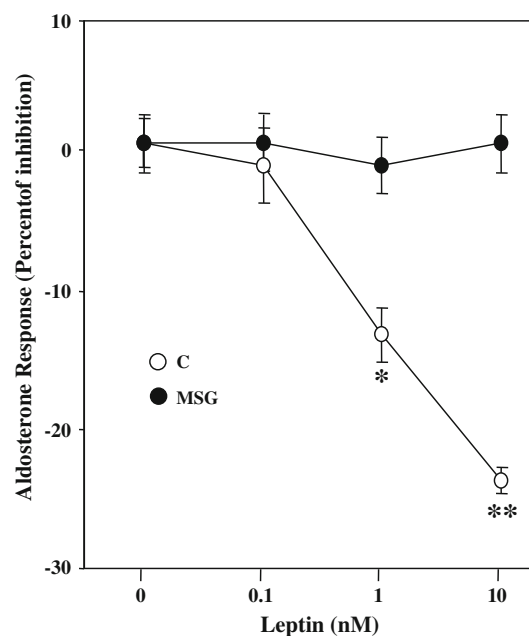


Fig. 3 Effects of exogenous leptin (0–10 nM) on 22 pM ACTH-stimulated aldosterone release by incubated total adrenal cells, from adult C and MSG female rats. Values are the mean $\pm$ SEM ($n = 3$ –4 experiments, with 6 flasks per point per experiment). * $P < 0.05$ vs. C values obtained in the absence of leptin (Leptin 0 nM). ** $P < 0.05$ vs. C values obtained in the presence of 1 nM Leptin

Histological and functional characteristics of omental (OM) adiposity in rats

Figure 4 shows histological observations of OM fat tissue from C (panels A and B) and MSG (panels C and D) animals, either hematoxylin-eosin stained (panels A and C) or immuno-labeled for leptin (panels B and D). Analysis of OM fatness indicates that the unilocular adipocytes (representing $62.6 \pm 2.7\%$ from the total OM fat) from MSG rats displayed a significant ($P < 0.05$) decrease in volume density (Fig. 5, panel A), versus that observed in C cells, without changes in both cell density (Fig. 5, panel B) and cell size (Fig. 5, panel C). Interestingly, OM fat pad from MSG rats presented a large number of multilocular adipocytes ($37.4 \pm 6.4\%$ from the total OM fat), characterized by: a low volume density (Fig. 5, panel D), grouped in high cell density (Fig. 5, panel E) and of a low cell size (Fig. 5, panel F). Conversely, OM fat from C rats displayed incipient signs of adipogenesis (Fig. 4, panels A and B).

Finally, Table 1 shows the results of leptin release into the medium by isolated OM adipocytes (from C and MSG rats) incubated in the absence (basal) or presence of dexamethasone (DXM; 25–50 nM). Basal leptin release by MSG cells was significantly ($P < 0.05$) greater than that spontaneously secreted by C cells (only unilocular adipocytes). As depicted, C cells significantly ($P < 0.05$ vs.

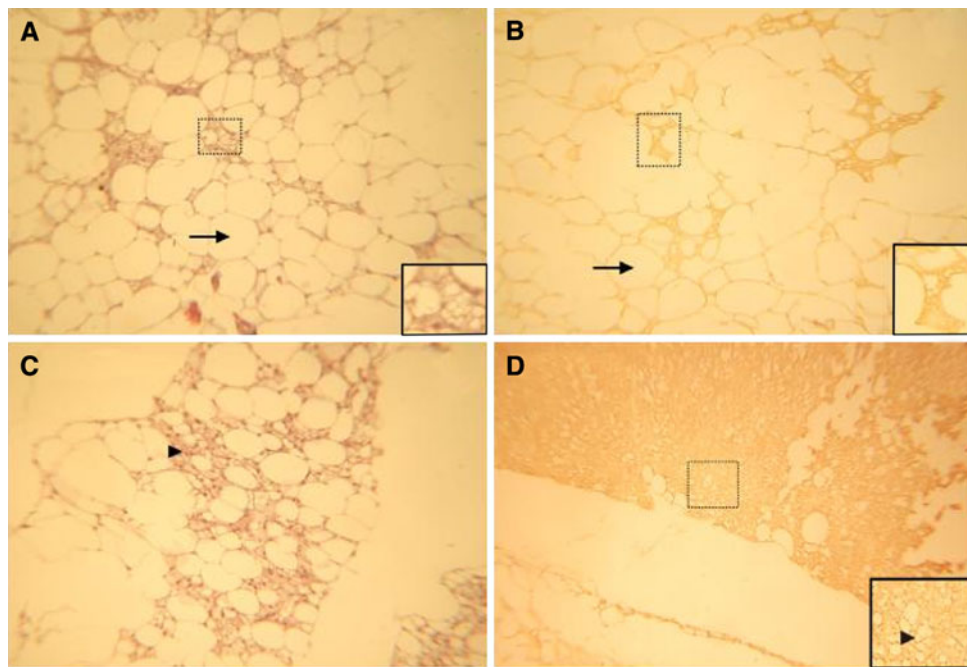


Fig. 4 Representative fields of omental (OM) adipose tissue from Control (**a** and **b**) and MSG (**c** and **d**) rats, stained with hematoxylin-eosin (**a** and **c**, respectively) and immuno-labeled for leptin (**b** and **d**, respectively), showing cytoplasmic rims of unilocular and multilocular adipocytes. Additionally, OM fat from MSG rats is characterized by very active adipogenic process expressed in greater areas of small

multilocular adipocytes with increased expression of leptin. Conversely, OM adipocytes from Control rats only show incipient areas of adipogenesis (see insets of amplified areas in **a** and **b**). Arrows unilocular adipocytes, arrowheads multilocular adipocytes (magnification: $\times 200$, insets: $\times 400$)

basal values) responded, in a concentration-related fashion, to DXM stimulation. Conversely, only the highest (50 nM) DXM concentration assayed was able to significantly ($P < 0.05$) enhance leptin release over the baseline in MSG adipocytes.

Discussion

Our study supports that the early hypothalamic damage induced an ACTH-driven mineralocorticoid hyperfunction and a decreased adrenal sensitivity to the AII-driven mineralocorticoid function. These adrenal failures were accompanied by a lack of leptin inhibitory effect on ACTH-driven mineralocorticoid output in vitro. Additionally, the adult MSG-damaged female rat displayed enhanced omental adipose tissue mass, with distorted morphological and functional adipocyte characteristics.

Importantly to stress is the fact that previously described [11] abnormalities characterizing this rat model were present in the MSG-treated female rats, such as: reduced hypothalamic NPY and adrenal *ob-Rb* genes expression, hypophagia, decreased BW gain, and increased circulating levels of insulin, leptin and the free fraction of glucocorticoid, among others.

Of relevance is the fact that the fail in adrenocortical function was assessed in both in vivo and in vitro conditions. Although we have previously reported [11] that in the adult female MSG rat enhanced in vivo and in vitro adrenal glucocorticoid responses to ACTH stimulation take place, we now add a new dimension to this adrenal dysfunction because an increased ACTH-driven mineralocorticoid secretion also operates in this phenotype. Similarly to that occurred for glucocorticoid secretion in this model [11], ACTH-dependent mineralocorticoid secretion in vitro also resulted not modified by exogenous leptin. Conversely, exogenous leptin did not modify in vitro the AII-driven mineralocorticoid nor glucocorticoid (unpublished data) function, regardless of the cell group studied.

The in vitro impaired adrenal sensitivity to leptin in the MSG rat could be as a consequence of the chronic hyperleptinemia, installed shortly (at least on age 30 days) after the hypothalamic damage and lasting up to adulthood [11]. As earlier [11] and currently observed, this fact could be related in part to the low adrenal expression of the *ob-Rb* gene characterizing this model [11], as occurred after in vitro exogenous leptin treatment in normal rat adrenals [17]. However, it could be questioned whether post-receptor mechanisms signaling leptin activity could be altered in the MSG-damaged rat model, thus resulting in a

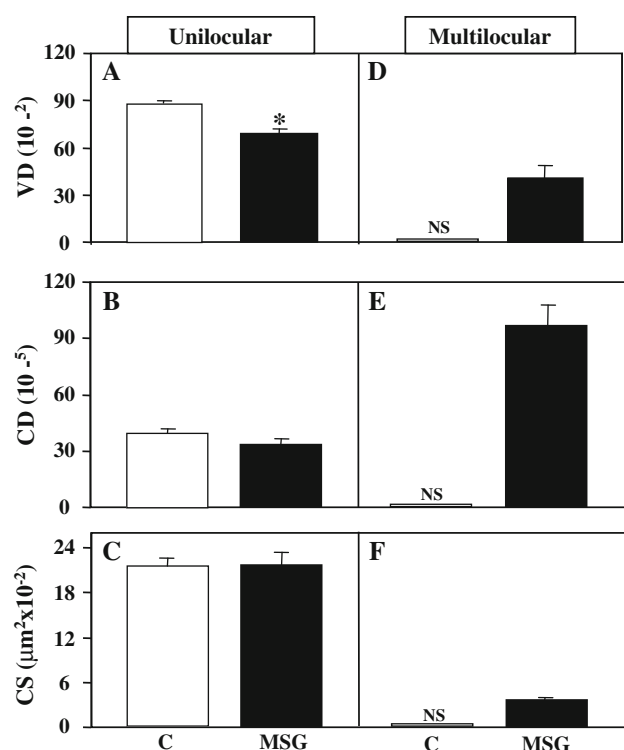


Fig. 5 Morphometric analysis of adipocytes, from C and MSG omental fat pads. *VD* volume density, *CD* cell density, *CS* cell size, *NS* not scored. Values are the mean $\pm$ SEM ($n = 5$ rats per group). * $P < 0.05$ or less vs. the respective C values

Table 1 In vitro spontaneous (Basal) and dexamethasone (DXM)-stimulated leptin release by isolated omental adipocytes from C and MSG rats

	C	MSG
Basal	2.05 $\pm$ 0.18	3.58 $\pm$ 0.36*
DXM		
25 nM	3.69 $\pm$ 0.23 [#]	3.82 $\pm$ 0.34
50 nM	5.41 $\pm$ 0.31 ^{#a}	5.78 $\pm$ 0.42 ^{#a}

Medium leptin concentrations are expressed in ng/ml (means $\pm$ SEM; $n = 5$ experiments, each condition was run in five replicates per experiment)

* $P < 0.05$ vs. respective C values

[#] $P < 0.05$ vs. respective Basal values

^a $P < 0.05$ vs. 25 nM DXM values in the same group

lack/reduced leptin effect on adrenal cells. Nevertheless, we have strong evidences indicating that leptin receptor down-regulation rather than post-receptor mechanisms is the crucial point explaining the lack of leptin effects in MSG rats. In fact, the adrenal leptin resistance present in MSG rats could be reversed on day 21 after adrenal enucleation, a time-point at which MSG rats display normal circulating levels of both glucocorticoid and leptin [13]. Conversely, when MSG rats were examined on day 35 after

adrenal enucleation, full adrenal regeneration appeared and the peripheral levels of glucocorticoid and leptin are as high as their sham-operated counterparts, and the adrenal leptin insensitivity resulted reinstalled [13]. Moreover, prolonged (e.g., long-time food restriction) but not rapid (e.g., 5 days after food withdrawn) reduction in the peripheral concentrations of leptin in MSG rats did result in adrenal cells fully sensitive to the exogenous leptin inhibitory effect on ACTH-stimulated glucocorticoid output [18]. These findings strongly support that post-*ob-Rb* signaling mechanisms remain intact in the MSG rat, and further suggest that transient adrenal down-regulation of the functional leptin receptor appears to be the main explanation for their adrenal leptin insensitivity. In addition, the leptin-resistance characterizing MSG animals seems to be not only at the peripheral level. Indeed, while intact hypophagic MSG rats are insensitivity to i.c.v. leptin injection-inhibited food intake, 7-day bilaterally adrenalectomized MSG rats became sensitive to i.c.v. leptin-inhibition of food intake (unpublished data from our laboratory); importantly to remark is that a very low leptinemia characterizes these 7-day adrenalectomized MSG rats [12].

Our study shows for the first time that leptin did not modify the effect of AII on mineralocorticoid and glucocorticoid secretions by normal adrenocortical cells. Thus indicating that the leptin intracellular signaling system at the adrenal level seems to be mainly due to inhibition of StAR activity [16] and probably without affecting the Ca^{+2} messenger system [19]. An observation strongly supported by data indicating that in the rat, in vivo administration of AII stimulates adrenal aldosterone synthase [20], but not STAR [21] mRNA expression.

Although previous reports exist on the inhibitory effects of leptin on ACTH-stimulated mineralocorticoid production by human adrenal cells in culture [22], and on the in vivo early post-natal HPA axis hyporesponsive period [23], we presently addressed that the inhibitory effect of leptin on normal rat adrenal function has an important impact not only on ACTH-driven glucocorticoid output, but also on ACTH-dependent mineralocorticoid secretion. Our observation adds a new dimension to the regulatory role of leptin in the adrenocortical function. It is known that enhanced glucocorticoid levels are able to produce biochemical changes resulting in obesity, insulin-resistance, dyslipemia, and hypertension [24, 25]. Thus, leptin could lead to control mineralocorticoid release partly avoiding hypertension, namely during the early development of non-aleptinemic, obese phenotypes.

It is known that acutely infused leptin in rats activates the sympathetic nervous system without altering arterial pressure [26]. Conversely, chronic leptin treatment enhances arterial pressure, a process accompanied by

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 h) and temperature-controlled (22°C) room. Rat chow and water were available ad libitum. Pregnant rats were transferred to individual cages. Beginning on post-natal day 2, newborn pups were injected i.p. with either 4 mg/g BW MSG (Sigma Chemical CO., St. Louis, MO) dissolved in sterile physiological (0.9%) NaCl or hypertonic (10%) NaCl (litter-mate controls; C) once every 2 days up to day 10 of age [36]. Rats were weaned and sexed at 21 days of age; daily body weight and food intake of individual female rats were recorded between days 30 and 150 of age. All experiments were performed with 120-day-old animals at the trough time of the day [37] and in non-fasting condition. MSG-injected rats were screened for effectiveness of treatment by macroscopic observation of degeneration of the optic nerves at the time of sacrifice. In each experiment, C and MSG female rats were members of the same litters; however, when accumulating experiments, each different experiment was performed with (C and MSG) animals from different litters. As previously determined [14], MSG rats had an abnormal estrous cycle, the microscopic observations of their daily vaginal smears showing a constant diestrous stage; thus we used rats from C litters for experimentation after ascertaining by screening that they were at the diestrous stage of their estrous cycle. Our Animal Care Committee approved experiments. Animals were killed by decapitation, accordingly to protocols for animal use, in agreement with NIH Guidelines for care and use of experimental animals.

Adult female MSG rat phenotype

After the rat was killed, the MSG phenotype was assessed by the determination of the concentration of various metabolic and hormonal parameters in plasma: ACTH, arginine-vasopressin, aldosterone, free corticosterone, insulin and leptin concentrations. The medial basal hypothalamus (MBH) was dissected as previously described (see below) and kept frozen (−80°C) until total RNA extraction. Adrenal glands (AG) were dissected, free of adipose tissue, and either used for cell isolation or kept frozen (−80°C) until total RNA isolation. The MSG rat phenotype was characterized, as described in published data from our laboratories [11], by a significant ($P < 0.05$ vs. C rats) reduction in MBH NPY and *ob-Rb* mRNAs, AG *ob-Rb* mRNA abundance (Table 2). MSG

Table 2 Neuropeptide Y (NPY) and long-isoform of leptin receptor (*ob-Rb*) mRNAs expression in the medial basal hypothalamus (MBH) and the adrenal glands (AG), from 120-day-old control (C) and MSG rats (values expressed in arbitrary units; mean $\pm$ SEM, $n = 4$ –6 tissues per group)

	C	MSG
MBH NPY mRNA	0.91 $\pm$ 0.08	0.39 $\pm$ 0.07*
MBH <i>ob-Rb</i> mRNA	0.79 $\pm$ 0.04	0.59 $\pm$ 0.05*
AG <i>ob-Rb</i> mRNA	1.27 $\pm$ 0.11	0.61 $\pm$ 0.18*
ACTH (pg/ml)	29.09 $\pm$ 2.61	30.98 $\pm$ 3.54
Arginine-vasopressin (ng/ml)	1.71 $\pm$ 0.33	1.63 $\pm$ 0.26
Free corticosterone (ng/dl)	46.11 $\pm$ 9.07	95.77 $\pm$ 12.05*
Aldosterone (pg/ml)	37.10 $\pm$ 9.06	40.06 $\pm$ 7.45
Leptin (ng/ml)	3.94 $\pm$ 0.72	33.52 $\pm$ 6.34*
Insulin (ng/ml)	0.71 $\pm$ 0.12	1.02 $\pm$ 0.08*
Omental fat mass (g)	8.61 $\pm$ 0.92	13.17 $\pm$ 1.62*

The peripheral concentrations of several metabolites and the omental fat pad mass are shown in both experimental groups (mean $\pm$ SEM, $n = 10$ –12 rats per group)

* $P < 0.05$ vs. C values

rats resulted already hypophagic ($P < 0.05$ vs. C rats) on age 32 days, and this condition remained even up to 5 months of age (Fig. 7, upper panel). Average of BW in MSG rats was significantly ($P < 0.05$) lower than those in age-matched C animals on age 60 days and older (Fig. 7, lower panel). Plasmatic concentrations of several hormones indicated that ACTH, aldosterone, and arginine-vasopressin were similar in both experimental groups (Table 2). However, MSG rats displayed significantly ($P < 0.05$) higher plasma-free corticosterone, leptin and insulin concentrations than C animals (Table 2). The former characteristic seems to be directly related to a significantly higher total fatness [11], including the OM adiposity (Table 3), despite being lighter than C animals (Fig. 7, lower panel).

Experimental designs

Experiment 1: in vivo HPA axis exploratory tests

Adult C and MSG animals (118 days old; 7–8 rats per group) were implanted with and indwelling i.v. catheter (under light ketamine anesthesia) and allowed to recover in individual plastic cages; they were provided with food and water ad libitum. On age 120 days, animals were bleed before (time zero) and, 15 and 60 min after i.v. injection of either ACTH (0.5 μ g/rat; Sigma Chem. CO, St. Louis) or angiotensin II (AII, 1 μ g/rat; Sigma) [38]. Plasma samples were stored (−20°C) until further determination of aldosterone concentrations.

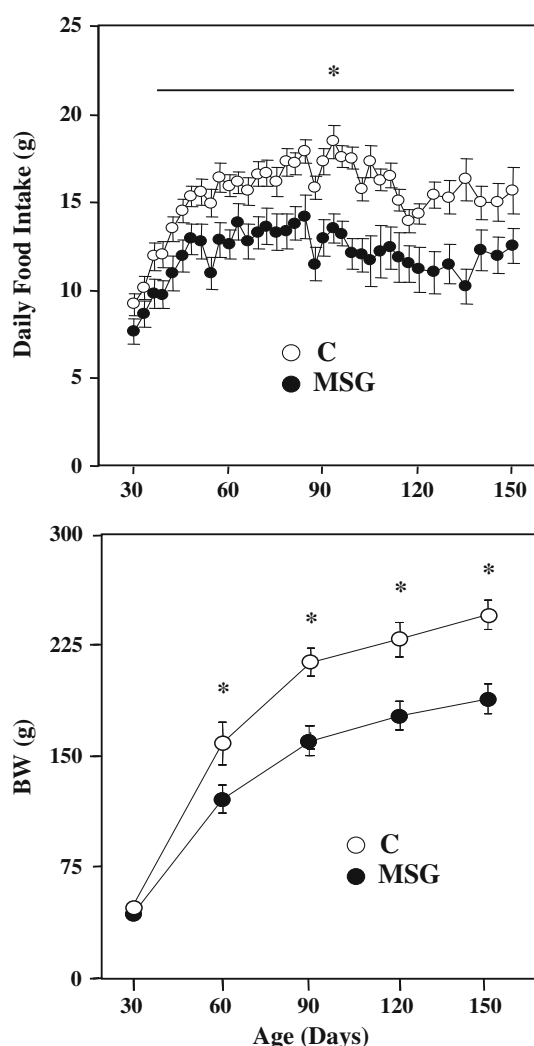


Fig. 7 Daily food intake (*upper panel*) and body weight (BW) in adult C and MSG female rats throughout ages 30 and 150 days. Values are the mean $\pm$ SEM ($n = 25$ –30 rats per group). * $P < 0.05$ vs. between values from C and MSG rats of similar age

Table 3 Primers employed (designed for a high homology region of different genes; GBAN: GenBank Accession Number)

<i>ob</i> -Rb (375 bp; GBAN: AF287268):	
Sense, 5'-ATG AAG TGG CTT AGA ATC CCT TGG-3'	
Antisense, 5'-ATA TCA CTG ATT CTG CAT GCT-3'	
NPY (430 bp; GBAN: NM012614):	
Sense, 5'-CCC GCC ATG ATG CTA GGT AAC-3'	
Antisense, 5'-ACA AGG GAA ATG GGT CGG AAT-3'	
ACTB (764 bp; GBAN: NM031144):	
Sense, 5'-TTG TCA CCA ACT GGG ACG ATA TGG-3'	
Antisense, 5'-GAT CTT GAT CTT CAT GGT GCT AGG-3'	

ob-Rb: long-isoform of leptin receptor; NPY: neuropeptide Y; ACTB: β -actin

Experiment 2: *in vitro* studies on adrenocortical cell function

This method has been extensively described in a previous study [39]. Briefly, C and MSG ($n = 6$ –7 animals per group per experiment) rats were killed, on day 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 Balanced Salt Solution (EBSS) containing 0.3% collagenase (type 1, Sigma; 1 ml of solution per each gland) and gently shaken at 37°C in siliconized glass Erlenmeyer flasks under 95% air–5% CO₂ atmosphere in a Dubnoff metabolic incubator. At the end of this period, remaining tissue fragments were repeatedly passed through a siliconized Pasteur pipette, and the dissociated tissue was filtered through a layer of nylon cloth (30 μ m) to remove cell clumps. The cell suspension was transferred into a conical plastic tube and centrifuged at 100 $\times$ 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. The cell pellet was resuspended in an appropriate volume of fresh incubation medium to obtain, approximately, 100,000 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 (final concentrations ranging between 2.2 and 220 pM) or AII (1–100 nM) and in the presence or the absence of recombinant murine leptin (added in a volume of 10 μ l, final concentrations ranging between 0.1 and 10 nM; PrePro Tech Inc., Rocky Hill, NJ, USA). Cells were then incubated for 2 h at 37°C in metabolic conditions. At the end of incubation, tubes were centrifuged 10 min, 100 $\times$ g, at room temperature, and the supernatants were separated from the cell pellets and kept frozen (–20°C) until measurement of medium aldosterone concentrations. At least 3–4 experiments were performed with 6 flasks per point per experiment.

Experiment 3: omental adiposity characteristics and functionality

Immediately after animals were killed, OM fat pads were rapidly dissected, weighted, and used for either histological or functional studies. Briefly, adipose tissues (from five rats per group) were fixed in Bouin's fluid and embedded in paraffin. Sections of 4 μ m were obtained, at different levels of the blocks, and stained with hematoxylin-eosin. Fat tissue sections were incubated for 1 h at room temperature with the primary serum anti-leptin (rabbit polyclonal, Santa Cruz Biotechnology Inc, USA), 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 either pre-absorption of the antibody with an excess of leptin or replacement of the specific antibody by, similarly diluted, normal rabbit serum. Morphometric studies were performed as earlier reported in detail [11, 14]. Briefly, measurements of adipocyte parameters were made by means of an image analysis system (Imaging Technology, Optimas 5.2). The cells and reference area (RA: total area throughout which the adipocytes were scored) were analyzed in each field for an average of 10 micrographs taken from two different levels (e.g., a and b). These measurements were recorded and processed automatically, and the following parameters were then calculated: volume density ($VD = \text{sum of adipocyte area}/RA$), cell density ($CD = \text{number of adipocytes}/RA$), and cellular size (CS ; expressed in μm^2). As RA represents the total area where cells were scored, the division of the sum of the individual cell areas by the RA yielded VD, a parameter representing an estimate of cell mass according to generally accepted criteria [40]. The number of cells (CD) was calculated by dividing the immunostained area of the adipose tissue by the mean individual cell area. For this parameter, no less than 100 adipocytes were recorded in each field.

For studies on adipocyte function, OM fat pads from C and MSG rats ($n = 5\text{--}7$ rats per experiment) were used for isolation of adipocytes as previously described in detail [14]. Briefly, pre-weighed OM fat pads were slightly minced and placed in KREBS-MOPS medium (Sigma) containing 1% BSA and 0.1% collagenase (Sigma, type 1) in a ratio of 3 ml of solution per gram of fat tissue. Tissues were gently shaken at 37°C for 50 min in a 95% air–5% CO_2 atmosphere inside a metabolic incubator. At the end of this period, cell suspensions were filtered through one layer of nylon cloth (100 μm), transferred to conical polypropylene tubes, and centrifuged at $100\times g$ for 20 min at room temperature. Cells were then washed three times with medium alone to eliminate the stromavascular fraction and collagenase. Adipocytes were then counted and diluted with DMEM (Sigma)–1% BSA medium, pH 7.4, to the necessary volume to obtain approximately 4×10^5 adipocytes per 1.4 ml of medium. This volume was distributed into polypropylene tubes containing 0.1 ml of medium either alone (basal) or with different concentrations of DXM (Sidus Lab., Argentina; final concentrations 25–50 nM) [14]. At least 6 replicates per experimental condition were run in each experiment. Tubes were incubated by shaking at 37°C for 2 h in a 95% air–5% CO_2 atmosphere. At the end of incubation, the supernatant was separated from adipocytes and kept frozen (-20°C) until measurement of medium leptin concentration.

Hormones determinations

Circulating ACTH concentrations were measured by a previously described immunoradiometric assay [41] with a standard curve ranging between 15 and 3,000 pg/ml and with intra- and inter-assay coefficients of variation (CVs) of 2–3 and 6–8%, respectively. Arginine-vasopressin [41] and leptin [42] concentrations, in plasma and medium samples, were determined by specific RIAs from our laboratory. In the arginine-vasopressin assay, the standard curve ranged between 1 and 2,000 pg/ml, with CVs intra- and inter-assay of 4–6 and 8–10%, respectively, and in the leptin RIA, the standard curve ranged between 0.4 and 50 ng/ml, with CVs intra- and inter-assay of 5–8 and 10–12%, respectively. Plasma and medium concentrations of aldosterone were determined by a commercial kit (Diagnostic System Lab., Inc., Webster, Texas) validated for rat in our laboratory; the standard curve ranged between 20 and 1,600 pg/ml, with CVs intra- and inter-assay of 4–8 and 8–11%, respectively. Finally, plasma total corticosterone [41] and corticosteroid binding globulin [43] levels were measured as reported elsewhere; then, plasma concentrations of free corticosterone were calculated as previously described in detail [11].

RNA extraction and RT-PCR analysis

Total RNA was isolated from hypothalami and adrenal glands, for further semi-quantification of NPY and *ob-Rb* mRNAs expression. Hypothalamic tissues were dissected (limits: posterior border of the optic chiasm, anterior border of the mamillary bodies, and lateral hypothalamic border; 3 mm deep, approximately) [41]. Adrenal glands were dissected free of adipose tissue and squeezed to exclude the medulla. Tissue RNA extraction was performed by the single-step, acid guanidinium isothiocyanate–phenol–chloroform procedure (Trizol; Invitrogen, Life Tech., USA; catalog number 15596-026). The yield and quality of extracted RNA were assessed by 260/280 nm optical density ratio and electrophoresis, under denaturing conditions, on 2% agarose gel. One microgram of total RNA was incubated with 0.2 mM dNTPs, 1 mM MgSO_4 , 1 μM of specific primers (Table 3), 1 μM β -actin (ACTB) primers (Table 3), 0.1 U/ μl AMV reverse transcriptase (5 U/ μl), 0.1 U/ml 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 (1 cycle); 94°C —2 min 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); 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). 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).

Analysis of data

Data were expressed as the mean $\pm$ SEM. Mean values were compared by two-way ANOVA, followed by post-hoc comparisons with the Fisher's test. The non-parametric Mann–Whitney test was used for analysis of data from tissue mRNA expression. Morphometric data were analyzed by the Least Significant Difference test for multiple comparisons [44].

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