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Relationship between Pituitary and Adipose Tissue after Hypothalamic Denervation in the Female Rat

A Morphometric Immunohistochemical Study

Gisela Camihort[a] César Gómez Dumm[a] Georgina Luna[a] Celia Ferese[a]
Susana Jurado[a] Griselda Moreno[b] Eduardo Spinedi[b] Gloria Cónsole[a]

[a]Department of Cytology, Histology & Embryology B, School of Medicine, National University of La Plata, UNLP and CICBA, and [b]Neuroendocrine Unit, Multidisciplinary Institute on Cell Biology, CONICET and CICBA, La Plata, Argentina

Key Words
Hypothalamic denervation · Pituitary · Adipocytes · Leptin · Triglycerides

Abstract
Neonatal administration of monosodium glutamate (MSG) to rats produces severe lesions in certain hypothalamic nuclei, with repercussions in different neuroendocrine axes, and serves as a model for their study. In addition, adipose tissue, as a target organ, is known to be directly related to several neurondocrine axes. We used 21-day-old female Sprague-Dawley rats that had received a neonatal treatment with MSG (4 mg/g body weight, i.p., from day 2 up to day 10 of age) in addition to control rats (injected with 10% NaCl solution, on a similar schedule). We performed a specific immunohistochemical study on each anterior-pituitary cell population, along with the morphometry of these cells and of the parietal and visceral adipose tissue, and measured the levels of serum leptin and triglycerides. The MSG animals evinced significant changes in volume density (VD), cell density (CD), and cell size (CS) in the corticotropes, thyrotropes, and LH gonadotropes, but not in the somatotropes, lactotropes, and FSH gonadotropes. The modification common to the three cell types was a hyperplasia, but with different results depending on cell size. Furthermore, in the MSG rats significant changes were also observed in the VD, CD, and CS of the adipose tissue, consisting of adipogenesis and decrease of adipocyte size in visceral fat, together with probable lipogenesis as judged by an increase in adipocyte size in the parietal fat. The serum levels of leptin and triglycerides appeared significantly higher in MSG animals. For the first time in this animal model, and at the level of three neuroendocrine axes, our results suggest changes that

Abbreviations used in this paper

ACTH	corticotropin
CD	cell density
CS	cell size
CTR	control
FSH	follicle-stimulating hormone
GH	growth hormone
LH	luteinizing hormone
MSG	monosodium glutamate
PRL	prolactin
RA	reference area
TRH	thyrotropin-releasing hormone
TSH	thyrotropin
VD	volume density

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Gloria Cónsole
Cátedra de Citología, Histología y Embriología B
Facultad de Ciencias Médicas, UNLP, CC 455
La Plata 1900 (Argentina)
Tel. +54 221 4256735, Fax +54 221 4250924, E-Mail gconsole@e-glu.com.ar

correlate hypothalamic damage, cellular pituitary alterations, and the response of the adipose tissue as a target organ for MSG insult.

Introduction

Neonatal treatment of rats with monosodium glutamate (MSG) induces a severe neuronal damage, mainly at the level of the hypothalamic arcuate nucleus activity [Olney, 1969; Burde et al., 1971; Holzwarth-McBride et al., 1976; Nemeroff et al., 1977; Krieger et al., 1979]. This neurotoxin produces a partial denervation that alters several neuroendocrine functions, causing a number of behavioral, endocrine, and morphological abnormalities [Redding et al., 1971; Lamperti and Blaha, 1976; Nemeroff et al., 1977].

In rodents, MSG treatment also alters adiposity, inducing increased body-fat mass and arrested body-weight gain resulting in hypothalamic obesity [Iwase et al., 1998]. Because of these conjoint responses, the administration of MSG provides a useful animal model for the study of different hypothalamus-pituitary axes and could serve to clarify their relationship to adipose-tissue metabolism.

Legradi et al. [1998] have demonstrated that arcuate-nucleus ablation by MSG abolishes the homeostatic responses of the hypothalamus-pituitary-thyroid axis during fasting. This treatment also produces serious abnormalities in the hypothalamus-pituitary-adrenal [Miyabo et al., 1982; Olney, 1979; Magariños et al., 1988; Spinedi et al., 1984; Sasaki et al., 1994] and the FSH-LH-gonadal [Dada et al., 1985] axes. Moreover, the neuroendocrine axes related to growth hormone [Sasaki et al., 1994] output and to lactotrope [Zelena et al., 1998] activities were shown to be affected by MSG administration as well. Nevertheless, despite the existence of numerous studies dealing with this animal model, the morphological changes that this neurotoxin causes in the cell populations within the anterior pituitary [Olubunmi et al., 1984] have not been fully documented.

Furthermore, adipose tissue is now considered not only as a traditional depot for the storage of energy, with lipid being mobilized under the influence of the appropriate hormones, but also as an endocrine organ, capable of regulating metabolism and homeostasis, and closely connected with the nervous system [Rosen and Spiegelman, 2000; Fajas, 2003]. Adipose tissue can also secrete paracrine and endocrine factors, such as leptin, which acts at the hypothalamus as well as at the peripheral tissues [MacDougald and Mandrup, 2002]. Several neuroendocrine axes are known to be directly related to this adipocyte activity [Giovambattista et al., 2000; Huang et al., 1998; Cai and Hyde, 1999; Seoane et al., 2000; Menéndez et al., 2003].

The aim of the current study was to evaluate by means of a morphometric immunohistochemical study the effects of hypothalamic MSG denervation in the rat on the pituitary GH, TSH, ACTH, FSH, LH and PRL cell populations, as well as on the adipose tissue, and to correlate, if possible, the morphological findings with circulating levels of leptin and triglycerides.

Materials and Methods

Animals and Experimental Design

Adult male (300–320 g body weight) and female (240–260 g body weight) Sprague-Dawley rats were housed and mated in a temperature-controlled (22°C) colony having a 07:00 to 19:00 h light period with rat chow and water available ad libitum. Pregnant rats were transferred to individual cages. Beginning on day 2 after parturition, the neonates were injected i.p. with either 4 mg/g body weight monosodium glutamate (MSG; Sigma Chemical, St. Louis, Mo., USA) dissolved in sterile 0.9 % (w/v) NaCl (injected volume on days 2, 4, 6, 8, and 10: 50, 65, 95, 112 and 125 μl, respectively) or with an equivalent volume of the vehicle [littermate controls; CTR; Spinedi et al., 1984]. Rats were weaned and sexed at 21 days of age. MSG-injected animals were screened for effectiveness of treatment by macroscopic observation of optic-nerve degeneration at the time of sacrifice.

CTR (120-day-old) and MSG female animals (10 rats per group) were weighed and killed by decapitation (between 08:30 h and 09:15 h) according to protocols for animal use in agreement with the NIH Guidelines for care and use of experimental animals. All experimentation was approved by our Institutional Animal Care Committee.

Pituitaries as well as visceral (perirenal and periadrenal) and parietal (femoral) fat pads from animals of both groups were immediately dissected, weighed, and processed for routine histological studies. For measurement of the circulating metabolites, trunk blood was collected into plastic tubes containing 0.2 ml EDTA (100 g/l) and immediately centrifuged for further determination of plasma triglyceride and leptin concentrations.

Immunohistochemistry

Stated in brief, pituitaries and adipose tissues from 6 animals of each group were fixed in Bouin's fluid and embedded in paraffin. For pituitaries, serial sections of 4 μm were obtained at different levels of the blocks following a ventral-to-dorsal sequence. The sections were stained with hematoxylin-eosin, immunostained, and then incubated for 1 h at room temperature with the primary antibody, anti-GH, anti-TSH, anti-ACTH, anti-FSH, anti-LH, or anti-PRL (murine; Dako, Calif., USA), diluted 1:100. For adipose tissue, the sections were immunostained with the primary serum, containing leptin-specific antibodies (rabbit polyclonal, Santa Cruz Biotechnology, USA), diluted 1:200. Thoroughly washed sections

were then treated for 30 min with a ready-to-use EnVision reaction system (Dako, Calif., USA). The peroxide-sensitive chromogen was diaminobenzidine. In all instances, the specificity of the primary antiserum was monitored either by observing its ability to block the immunocytochemical reaction after its preabsorption with an excess of the related antigen or by its replacement with normal rabbit serum or phosphate-buffered saline.

Morphometry

Morphometry was performed as reported in detail previously [Cónsole et al., 2002]. Stated in brief, measurements of immunostained pituitary cells and adipocytes were made by means of an image-analysis system (Imaging Technology, Optimas 5.2). The cells and reference area (RA) were analyzed in each field for an average of ten micrographs taken from two levels (e.g. a and b). These measurements were recorded and processed automatically and the following parameters subsequently calculated: volume density (VD = Σ cell area/RA), cell density (CD = number of cells/RA), and cell size (CS, expressed in μm^2). RA represents the total area throughout which the cells were scored. Thus, this area divided into the sum (Σ) of the individual cell areas (A) yielded VD, which parameter represents an estimate of cell mass according to generally accepted criteria. The number of cells (CD) was calculated by dividing the immunostained area of the pituitary or adipose tissue by the mean individual cell area. For this parameter, 100 cells were recorded in each field.

Biochemistry

Plasma-leptin concentrations (n = 10 rats per group) were determined by a specific radioimmunoassay developed in our laboratory and validated for rat leptin [Piermaría et al., 2003]. Stated in brief, synthetic murine leptin (PrePro Tech, Inc.) was used for both labeled peptide and standards as well as for the development of anti-leptin serum. Leptin was labeled with ^{125}I (specific activity 15 Ci/mmol, from Amersham Pharmacia Biotech, UK) by the chloramine-T method and purified from the radioiodination mixture by Sephacryl S-300 chromatography (Sigma) on a 1.5 × 60-cm column equilibrated with 2 g/l BSA, 10 mg/l sodium azide in 50 m*M* sodium phosphate (pH 7.4). The anti-leptin serum was raised in a rabbit by immunization with leptin (PrePro Tech) coupled to BSA. The detection range of the standard curve in the leptin radioimmunoassay was 0.4–50 ng/ml. Unknowns or standards (200 µl) were incubated overnight at room temperature in the presence of 50 µl of anti-leptin rabbit serum (final dilution 1:15,000) and 50 µl (approximately 30,000 cpm) of tracer. For separation of bound and free fractions, 200 µl of normal saline solution containing anti-rabbit γ-globulin followed by 500 µl of polyethylene glycol 6,000 (10% [w/v]) in that saline were added to each sample. After a 30-min incubation at 4°C, the sample was centrifuged for 40 min at 4°C and, the supernatant aspirated, and the bound radioactivity counted. The assay displayed 2 and zero cross-reactivity with human leptin. The within-assay and interassay coefficients of variation were 5–8% and 10–13%, respectively.

Circulating concentrations of triglycerides (n = 10) were determined by an enzymatic assay from Wiener Laboratories (Argentina).

Analysis of Data

Data were expressed as the mean ± SEM. Mean values were compared by ANOVA, followed by subsequent comparisons with the Fisher test. Morphometric data were analyzed by the Least Significant Difference test for multiple comparisons [Zar, 1974].

Results

Body and Fat-Pad Weights in Experimental Animals

When values for body weights (n = 10 rats per group) were analyzed, a significant ($p < 0.05$) difference was found between groups, with the MSG-treated rats (181 ± 7 g) being lighter than the CTR rats (218 ± 6 g). Conversely, analysis of fat-pad weights indicated that both visceral and parietal fat pads in the MSG animals (7.14 ± 0.48 and 2.81 ± 0.22 g, respectively) were significantly ($p < 0.05$) higher than in the CTR rats (3.82 ± 0.23 and 1.04 ± 0.51 g, respectively).

Morphometric Immunohistochemical Studies on Pituitary Cells

Morphometry of the different pituitary-cell populations indicated the following relationships between the respective data for the two experimental groups.

In the somatotropes (fig. 1), lactotropes (fig. 5), and FSH cells (fig. 4), the values for the respective parameters between the two groups were statistically indistinguishable.

In the thyrotropes (fig. 2 and 7A, B), the values for CS, CD, and VD were higher ($p < 0.05$) in the MSG rats than in the CTR animals.

With the corticotropes (fig. 3 and 7C, D), however, although the respective values for VD were not significantly different, the data for CS were lower and for CD higher ($p < 0.05$) in the MSG group than in the CTR group.

In the LH gonadotropes (fig. 4 and 7E, F), the values showed a decrease ($p < 0.05$) in CS along with an increase ($p < 0.05$) in CD and VD in the MSG-treated rats compared to the CTR animals.

Morphometric Studies in Adipose Tissue

The parietal fat showed only unilocular adipocytes, whereas the visceral fat stored lipids in unilocular and multilocular adipocytes with multiple small droplets instead of one large one. Adipocytes from the area corresponding to unilocular visceral fat (62.5%) did not show significant differences in the CS, CD, or VD between the two experimental groups. The cells constituting the area of multilocular visceral fat (37.5%) in the MSG-treated rats, however, exhibited decreases in VD and CS ($p < 0.01$) along with an increase in CD ($p < 0.01$) compared to the CTR animals (fig. 6).

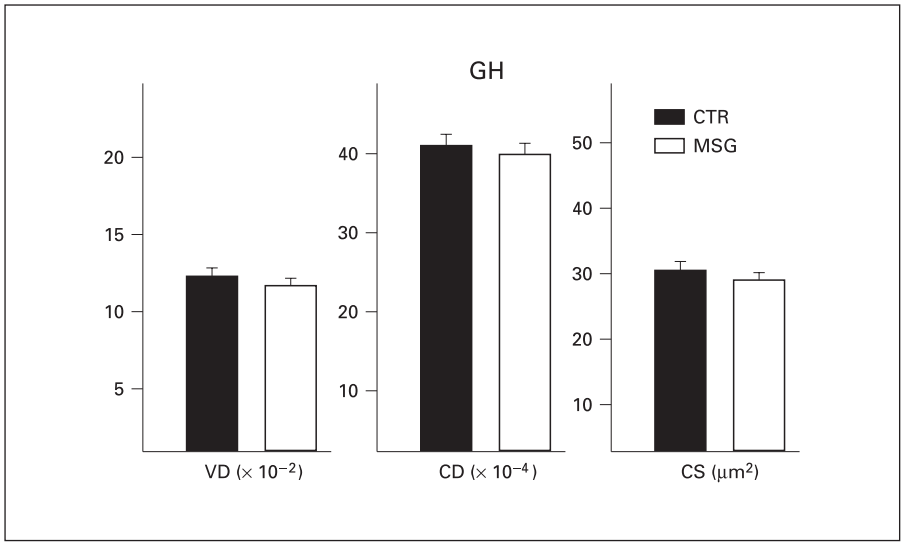


Fig. 1. Volume density (VD), cell density (CD), and cell size (CS) of GH cells of CTR and animals submitted to MSG treatment.

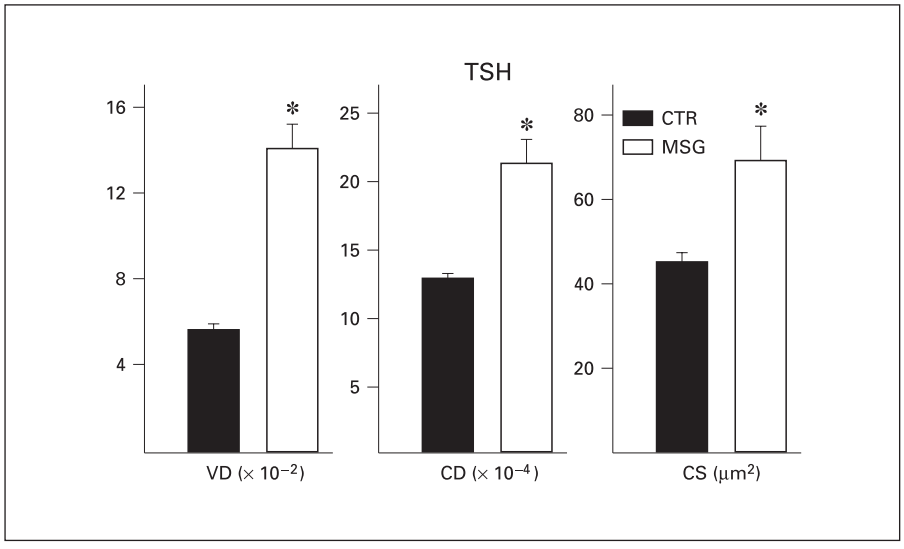


Fig. 2. Volume density (VD), cell density (CD), and cell size (CS) of TSH cells of CTR and animals submitted to MSG treatment. * $p < 0.05$ between MSG-treated and CTR.

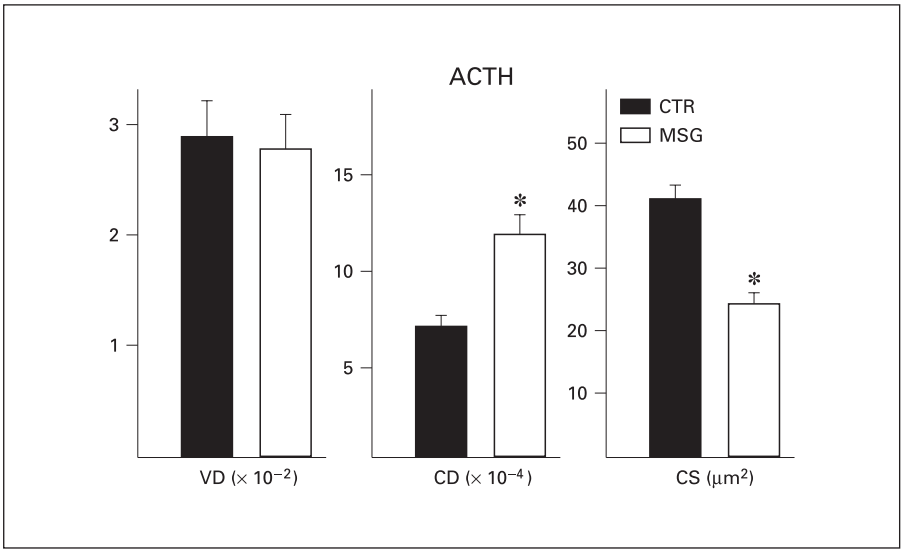


Fig. 3. Volume density (VD), cell density (CD), and cell size (CS) of ACTH cells of CTR and animals submitted to MSG treatment. * $p < 0.05$ between MSG-treated and CTR.

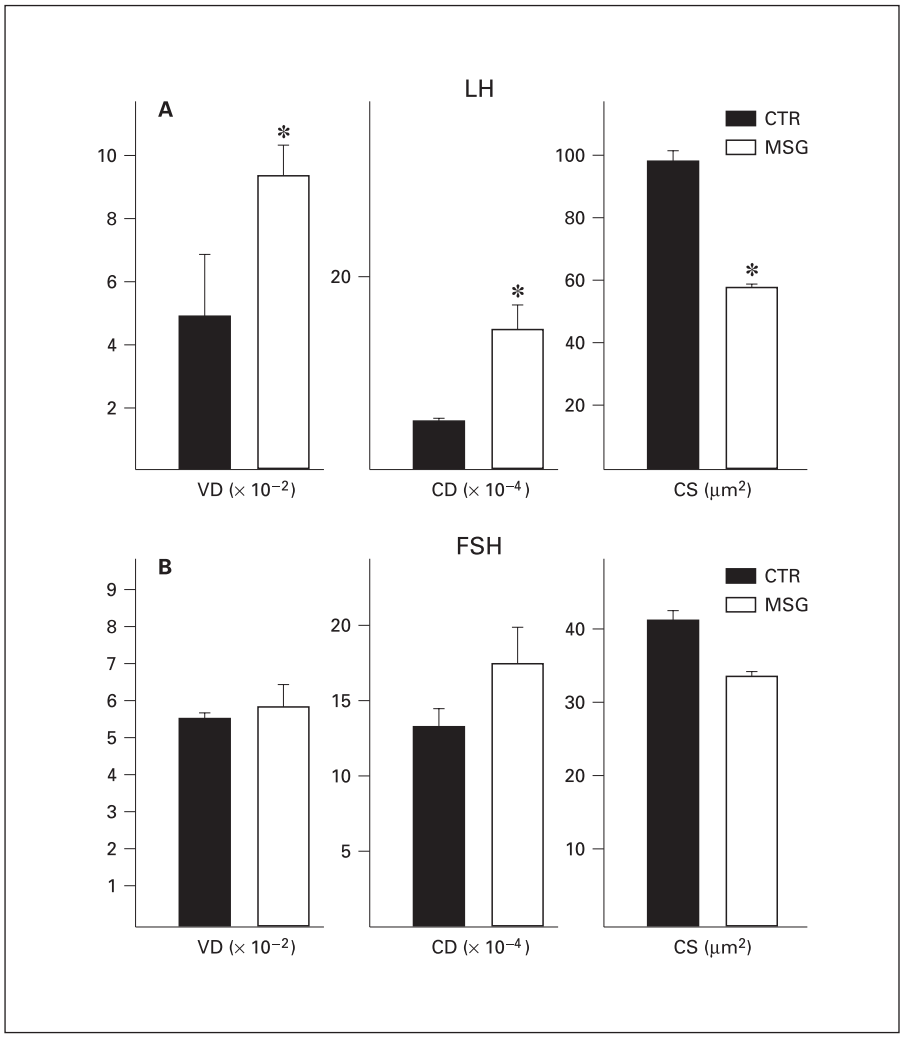


Fig. 4. Volume density (VD), cell density (CD), and cell size (CS) of LH (**A**) and FSH (**B**) cells of CTR and animals submitted to MSG treatment. * $p < 0.05$ between MSG-treated and CTR.

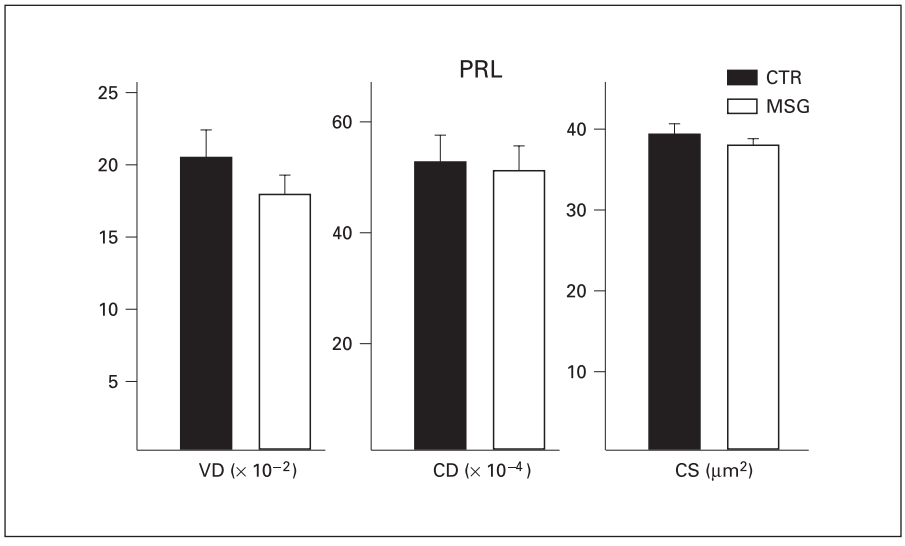


Fig. 5. Volume density (VD), cell density (CD), and cell size (CS) of PRL cells of CTR and animals submitted to MSG treatment.

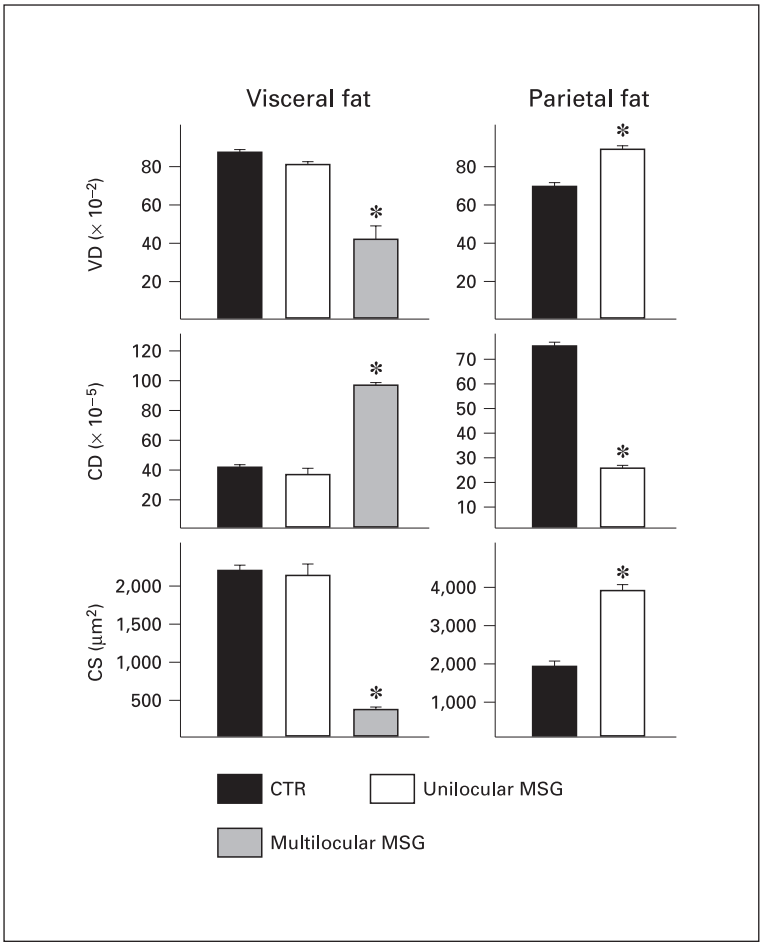


Fig. 6. Volume density (VD), cell density (CD), and cell size (CS) of adipocytes of the CTR group and animals submitted to MSG treatment. * $p < 0.01$ between MSG-treated and CTR.

Adipocytes from the parietal fat with one large droplet were found in the two experimental groups and evinced increments in both CS and VD ($p < 0.001$ and < 0.01, respectively) together with a decrease in CD ($p < 0.001$) in the rats treated with MSG relative to the CTR group.

Figure 7G–J shows both parietal and visceral adipose tissue belonging to MSG and CTR rats.

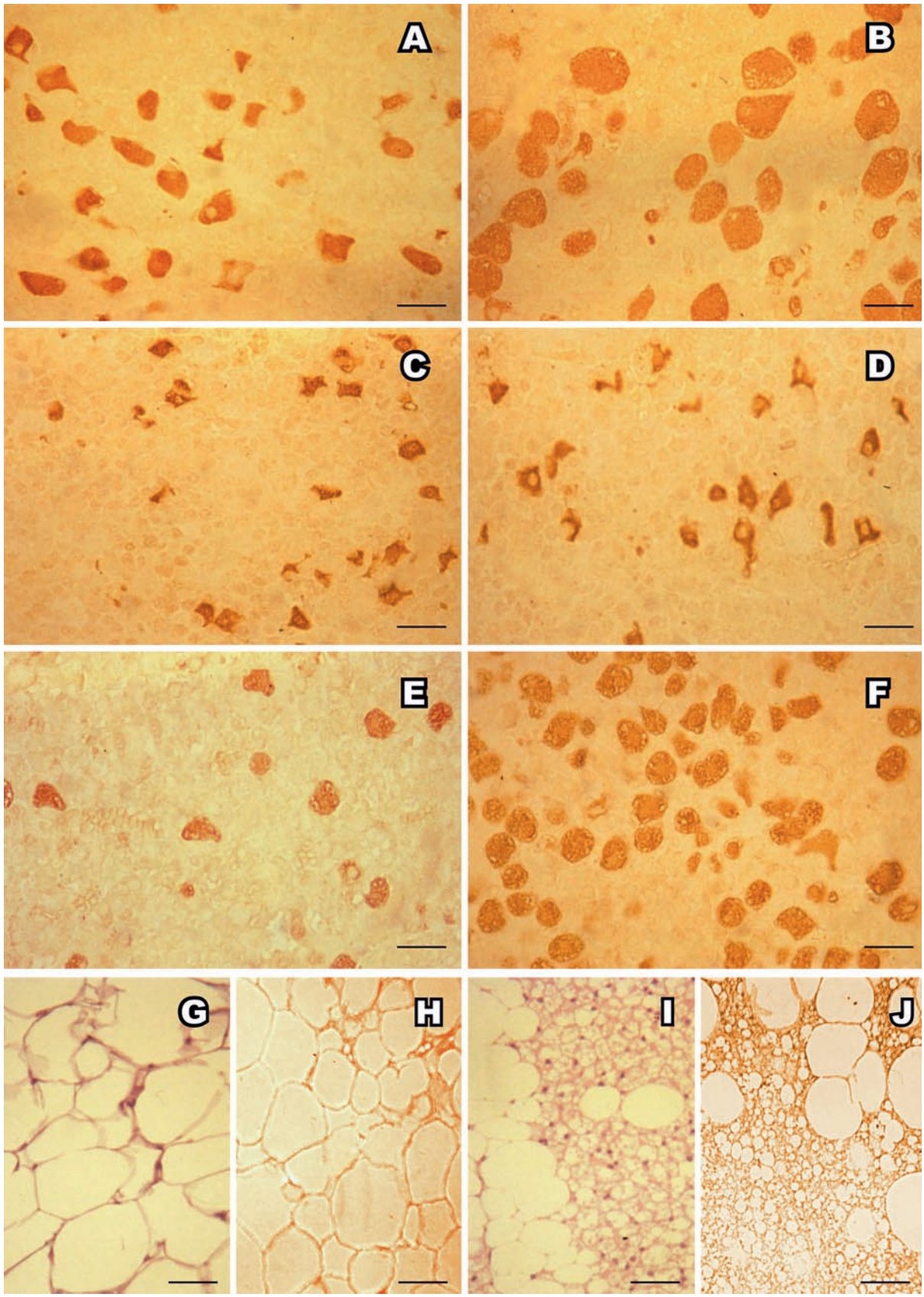
Biochemical Studies

Circulating basal triglyceride (fig. 8) and leptin (fig. 9) levels were higher ($p < 0.05$ and < 0.0001, respectively) in MSG-treated than in CTR rats.

Discussion

Our results showed that neonatal treatment of female rats with MSG induced different changes in the pituitary cell populations, as revealed by the morphological parameters (VD, CD, CS) used. Thus, the corticotropes and the LH gonadotropes appeared diminished in size, while an hypertrophy of thyrotropes was evident. Moreover, a clear hyperplasia of these three cell types was also detected in the experimental animals.

On the basis of this experimental model, Legradi et al. [1998] suggested that during fasting, the arcuate nucleus, where leptin receptors are highly concentrated, is essential for the normal homeostatic response of the hypothalamic-pituitary-thyroid axis and constitutes a critical locus to mediate the action of leptin on pro-TRH gene expression in the paraventricular nucleus. Furthermore, the decreased leptin concentrations would be the primary event responsible for the suppression of that axis in food-deprived rats [Seoane et al., 2000]. In addition, studies performed in organ cultures of human adipose tissue [Menéndez et al., 2003] would indicate that leptin and the thyroid axis maintain a complex and dual relationship, where TSH may contribute to the regulation of



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leptin pulses. The present experiments extended these findings to the area of morphology. Our results revealed that, through the damage to the arcuate nucleus, MSG treatment interrupts the control exerted on the thyroid axis, causing hypertrophy and hyperplasia of the TSH cell population. This observation is in accord with the experiments by Ribeiro et al. [1997] that describe an increase in plasma thyroid hormone in MSG-obese rats in response to fasting. At odds with this finding, however, is the report by Legradi et al. [1998] showing that a similar treatment did not produce any significant effect on plasma thyroid-hormone and TSH levels in fasting rats.

The neurotoxin-induced denervation in these experiments elicited hyperplasia of the ACTH cells, which elements also appeared reduced in size. This alteration is in keeping with other observations in MSG-lesioned rats, where there occurred a pronounced overresponse to secretagogues in vitro, with a resulting enhancement in the release of ACTH from the anterior pituitary [Spinedi et al., 1984]. Our morphological results are also in agreement with the earlier reports that an increment in corticosterone secretion was evoked in MSG-treated rats [Ribeiro et al., 1997; Macho et al., 1999; Magariños et al., 1988].

The present observations showed that in MSG-lesioned rats, LH gonadotropes evinced hyperplasia and appeared diminished in size, although neither FSH gonadotropes nor lactotropes showed any significant difference from the respective elements in the control animals. In male neonatal MSG-treated rats, experiments in vitro demonstrated a marked overresponse by the median eminence with respect to LHRH release after stimulation with high K+ medium [Spinedi et al., 1984]. This finding is consistent with both the hyperplasia and the VD increase of the LH cells that we detected in the MSG-injected animals.

By contrast, Millard et al. [1982] detected no significant changes in the plasma levels of PRL or in the concentration of that hormone in the anterior pituitary in MSG-treated male rats relative to the values in control animals. Moreover, the same neurotoxin, when administered to newborn rats of both sexes, did not induce abnormal responses in plasma-PRL levels after the injection of an excitatory amino acid receptor subtype agonist, suggesting that an intact tuberoinfundibular dopaminergic pathway is not essential for its prolactin-stimulating effect [Zelena et al., 1998]. The findings cited above are in agreement with our current morphological observations on lactotropes, where no changes occurred in MSG-treated rats.

We did not detect any significant morphological change in the GH-cell population of MSG-lesioned rats. Conversely, Sasaki et al. [1994] found a decrease in the number and size of both somatotropes and lactotropes after neonatal administration of MSG to male mice. These differing results could be ascribable to the dissimilar species and sexes used in the experiments. In this regard, Olubunmi et al. [1984] studied the effect of MSG on somatotropes and GH secretion in male and female rats. These authors found no differences in the number and VD of GH cells from female rats, compared to controls, in keeping with our findings; but in the same animals the authors detected a diminished size in those types of cells. Nevertheless, a body of evidence exists regarding the severe lesions that MSG causes in GHRH neurons within the arcuate nucleus [Kovacs et al., 2000; Millard et al., 1982; Zelena et al., 1998]. In spite of sex differences found in rats with respect to somatotropes and GH secretion [Olubunmi et al., 1994], it is currently clear that MSG treatment decreases serum-GH levels and retards linear growth [Kovacs et al., 2000; Olubunmi et al., 1994; Remke et al.,

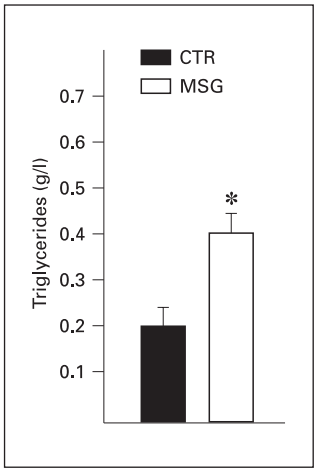

Fig. 8. Effect of hypothalamic denervation on serum triglyceride levels. * $p<0.05$ between MSG-treated and CTR.

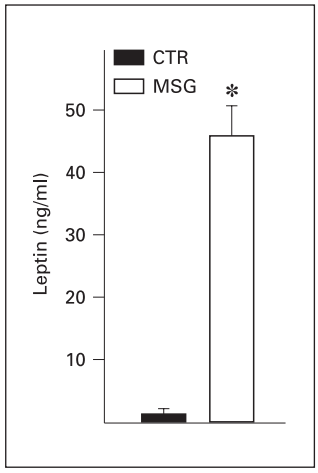

Fig. 9. Effect of hypothalamic denervation on serum leptin levels. * $p < 0.0001$ between MSG-treated and CTR.

Fig. 7. Representative fields of specifically immunostained TSH cells (**A** CTR, **B** MSG); ACTH cells (**C** CTR, **D** MSG); LH cells (**E** CTR, **F** MSG); parietal fat (**G** CTR hematoxylin-eosin, **H** CTR leptin), and visceral fat (**I** MSG hematoxylin-eosin, **J** MSG leptin). EnVision system peroxidase. **A**–**F**: Bar = 25 μm, **G**–**J**: bar = 100 μm.

1988]. These functional changes do not seem to have their morphological counterpart at the level of the anterior pituitary, at least as revealed by the parameters we examined.

The present morphometric studies of adipose tissue indicate that MSG treatment in female rats produced an increment in the VD of parietal fat, ascribable to an enlargement of the individual adipocytes. By contrast, in the visceral fat the neurotoxin induced a higher number of multilocular adipocytes, which elements appeared reduced in size.

Adipose tissue is composed mainly of unilocular adipocytes. During adipogenesis, however, fatty tissue develops multilocular small cells showing multiple lipid inclusions. Adipogenesis is known to occur throughout the lifetime of an organism, in both the prenatal and postnatal periods. It consists of two major events: initial preadipocyte proliferation and subsequent differentiation into mature fat cells [Rosen and Spiegelman, 2000; Fajas, 2003]. In obesity, however, hypertrophy is thought to be the first event that takes place. Adipogenesis is a complex regulated process that is ultimately linked to hyperplasia, the additional response that generates new adipocytes from precursor cells [Fajas, 2003].

Obesity is not a homogeneous condition. Instead, the regional distribution of adipose tissue is an important key to understanding the relationship between obesity and metabolic disturbances [Wajchenberg, 2000]. In MSG-obese rats, differences have been found between the role of visceral and subcutaneous fat tissues [Kim et al., 1995]. Moreover, preadipocytes isolated from different areas of the body have shown dissimilar adipogenic potentials [Rosen and Spiegelman, 2000]. In the MSG-injected rats of the present study, the behavior of the adipose tissue varied depending on the area observed. The visceral fat showed clear signs of adipogenesis, whereas the parietal (subcutaneous) fat evinced an increment in adipose mass at the expense of adipocyte hypertrophy. This latter response is indicative of a higher rate of lipogenesis consistent with increased adipocyte function, observations which are in full agreement with the high circulating levels of triglyceride and leptin characteristic of MSG-treated animals.

We confirmed here the increment in plasma-leptin levels that our group had previously found in MSG-treated female rats [Perelló et al., 2004]. This present finding is, moreover, in keeping with the generally accepted concept that plasma-leptin levels correlate positively with the amount of body adipose tissue and with adipocyte size [Hynes et al., 2003]. Whereas the hypothalamus is considered the principal site of action for leptin, the anterior pituitary would be an additional target organ for the hormone [Cai and Hyde, 1999]. Nevertheless, although it is well known that leptin modulates the secretion of several pituitary hormones, only scant information is available on the reciprocal effect of this gland upon leptin production [Menéndez et al., 2003]. The present results would strongly suggest that the higher leptin secretion in MSG-treated rats is consistent with the observations of increased adipogenesis in the visceral fat and augmented lipogenesis in the parietal fat. In view of the elevated circulating levels of lipids in MSG-lesioned rats, investigators have suggested that this animal model develops, in a MSG-dose-dependent fashion, a shift in carbohydrate metabolism towards lipogenesis, thus leading to hyperlipidemia [Malik and Ahluwalia, 1994]. This fact has been partially attributed to the hyperinsulinemia characteristic of MSG-treated animals [Oida et al., 1984]. It has also been recently reported [Perelló et al., 2004] that the neurotoxic damage of the hypothalamus induces, among other effects, an enhanced basal hypothalamo-pituitary-adrenal axis function. In this way, elevated circulating glucocorticoid levels could be responsible, at least in part, for the development of hyperadiposity-related hyperleptinemia.

In conclusion, MSG-induced hypothalamic denervation in rats resulted in hyperplasia and size changes in pituitary thyrotropes, corticotropes, and LH gonadotropes along with modifications in the visceral and subcutaneous adipose tissues suggesting adipogenesis, lipogenesis, and increased adipocyte functionality (triglyceride production and plasma-leptin secretion). In this model, we provide, for the first time, evidence that there exists a clear correlation between neonatal hypothalamic damage and alterations in both anterior pituitary-cell morphology and adipose-tissue, size, cellularity, and functioning.

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