Is Glutamate-Induced Reduction in Growth Hormone-Releasing Hormone a Neuroendocrine Model of Aging in the Rat? (43724)

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Abstract. **Decreases in serum growth hormone (GH) associated with aging may be a result of age-related degenerative changes in neurons of the hypothalamus resulting in a decrease of growth hormone–releasing hormone (GHRH). This study tests the utility of glutamate-induced hypothalamic neuronal degeneration in the rat as a neuroendocrine model of aging. Sprague-Dawley female rats received three 4-mg/g monosodium glutamate (MSG) subcutaneous injections during the first 5 days following birth. Animals were anesthetized at 60 days of age and challenged with GHRH. Blood samples were assayed for GH.**

Serum GH levels following GHRH challenge in the MSG-treated group rose more slowly and to a lower peak than in controls ($P < 0.05$). There was no difference in total GH release over the 1-hr interval following challenge. MSG-treatment of animals resulted in lower gross body ($P < 0.05$) and kidney ($P < 0.05$) weights with no difference in ovary or adrenal weights. There were also no differences in pituitary GH or total protein content between the groups. Analysis of femurs in the MSG animals revealed both a lower bone strength ($P < 0.05$) and maximum mid-shaft diameter ($P < 0.05$), but no difference in length, mineral/matrix ratio, or tissue density.

Our data suggest that the degree of neuroendocrine disruption resulting from neonatal MSG administration mimics in part systemic changes commonly observed in aged animals. Hence, definite similarities exist between MSG treatment and the documented aging-related changes in hypothalamic GHRH content and GH regulation in the rat. [P.S.E.B.M. 1994, Vol 206]

It is well known that declines in normal body function begin during maturity and progress rapidly with advancing age. Concomitant with these changes, and often preceding them, are reductions in growth hormone (GH) secretion and circulating insulin-like growth factor (IFG-1) levels (1). Many investigators, notably Everitt *et al.* (2) and Sonntag *et al.* (3), suggest that the age-related decrease in growth hormone is responsible for the observed decreases in physiological processes. Such deficiencies are attributed to an impairment of neuroendocrine systems mediated in part by age-related damage to hypothalamic neurons. Prinz *et al.* (4) and Carlson *et al.* (5) attribute declines in human GH to decreases in or absence of normal nocturnal GH pulses, suggesting that decreased pulse amplitudes may be linked to decreased growth hormone–releasing hormone (GHRH) responsiveness in aging individuals. Additionally, Shibasaki *et al.* reported an age-related decrease in GH responsiveness to GHRH (6). In general, two mechanisms have been proposed to explain age-related changes intrinsic to the hypothalamus and pituitary gland: a re-

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duced production and/or capacity of hypothalamic releasing hormones to stimulate the anterior pituitary and aging-associated increases in somatostatin (7).

Aged rats have been shown to have decreased pituitary GH (7, 8), decreased peak plasma GH (3, 7), and no difference in GH trough levels (3, 7). Older rats also have lower plasma GH response to GHRH challenge (9–12), and indeed hypothalamic neuronal degeneration in aging rats has been demonstrated (13, 14). Additionally a measurable decrease in the release of GHRH in old rats has been documented (15). Hence, with aging, there is significant loss of hypothalamic neurons in the arcuate, medial preoptic area and ventromedial and lateral hypothalamus (16, 17). These degenerative changes have been attributed to free radicals, lipofuscin accumulation (15, 18), and chronic exposure to hormones such as estrogen (15, 16). Thus, it appears that declining hypothalamic GHRH levels are a key element in aging-related declines in GH levels.

Administration of monosodium glutamate (MSG) to neonatal rodents and primates produces permanent lesions in the hypothalamic arcuate neurons that secrete GHRH (19–28), an effect much like that observed in aging animals. Glutamate is an excitatory amino acid neurotransmitter which, in abnormal amounts, plays a role in neurotoxicity of the central nervous system (29). MSG circumvents the blood-brain barrier in neonates and accumulates in specific areas of the brain, selectively destroying neurons in the basal hypothalamic area (30, 31). Lesions extend anteriorly toward the preoptic area and posteriorly to the interpeduncular nucleus (25) and are primarily manifest in the arcuate nucleus, the primary site of GHRH production (15, 32). MSG treatment to neonatal rats was shown to cause virtually a complete loss of cell bodies containing immunoreactivity for GHRH, galanin, dynorphin and the neuropeptides Y and K (21) although tyrosine hydroxylase, glutamic acid decarboxylase, neurotensin, and somatostatin immunoreactive cells were still detected. Apparently most neurons in the ventrolateral and -medial arcuate nucleus are sensitive to the toxic effects of MSG whereas dopamine neurons of the dorsomedial division of the arcuate are not (21).

The objective of this study was to test the utility of glutamate-induced hypothalamic neuronal degeneration in the rat as a neuroendocrine model of aging. Emphasis was placed on short-term systemic manifestations as indexed by hypothalamic changes, gross organ weight, bone parameters, and pituitary response to GHRH challenge. Definite similarities exist between MSG treatment and the documented aging-related changes in hypothalamic GHRH content and GH regulation in the rat, suggesting that MSG may serve as a useful tool in the study of neuroendocrine aging in the rat.

Materials and Methods

Pregnant Sprague-Dawley rats were maintained in a controlled temperature (24°–26°C) and light (06:00 to 18:00 hr daily) regulated room. Rat chow and water were available *ad libitum*. At birth, female neonatal pups from each litter were assigned to MSG-treatment or control groups.

All neonate rats received 4 mg MSG/g body wt subcutaneously or the appropriate saline vehicle on Day 1, 3, and 5 immediately following birth. The MSG solution was prepared in 0.9% buffered saline at a concentration of 400 mg/ml enabling injection volumes of 0.04 ml to 0.1 ml, depending on animal weight. Animals were weighed on Day 1, 3, 5, 7, and 9, and weekly thereafter until euthanasia. At 21 days of age, all rats were weaned and placed six animals per cage with each cage containing equal percentages of MSG and control animals. All animals were monitored daily for onset of puberty (vaginal opening).

At 60 days of age, animals were sequentially anesthetized with sodium pentobarbital (35 mg/kg), intra-atrially catheterized, and challenged with 5 μg rat GHRH purchased from Bachem California (Torrance, CA). Blood samples (0.3 ml) were taken at 0, 15, 30 and 60 min for determination of circulating serum GH. Each animal was then euthanized and immediately perfused through the left ventricle with 0.9% buffered saline. Pituitaries were harvested, homogenized in 0.9% saline, and frozen until later GH and total protein assay. The adrenals, kidneys, and gonads were manually resected, cleaned of connective tissue and stored in 10% paraformaldehyde at room temperature for later analysis. The hypothalamus was placed in 10% paraformaldehyde for 2 hr then placed in cryoprotectant solution (33) until sectioning. Femurs were similarly cleaned and frozen prior to bone strength testing and tissue analysis.

Growth Hormone and Pituitary Total Protein Assay. All blood serum samples and pituitaries were tested for GH concentration with a rat growth hormone (rGH) [^{125}I] radioimmunoassay system (Amersham International plc, UK). The assay has a sensitivity of 1.7 ng/tube, with intra- and interassay coefficients of 3% and 10.5%, respectively. Pituitary total protein measurement was carried out via the Bradford method using serum albumin as a standard (34).

Immunocytochemistry. Brain was blocked to isolate the mediobasal hypothalamus and sections cut at 30 μm in a cryostat. Immunocytochemistry (ICC) localization of GHRH was performed using the avidin-biotin-peroxidase complex (ABC) method (35) (Vector Laboratories, Burlingame, CA). Anti-GHRH was used at a concentration of 1:10,000 and was purchased from Chemicon International Inc. (Temecula, CA). The antiserum was produced in rabbits and showed no sig-

nificant cross-reactivity with other related peptides. Localization of GHRH using ICC was performed by using freely floating tissue sections in phosphate buffer solution (PBS), then incubated in normal goat serum diluted in PBS for 30 min at room temperature. The sections were rinsed twice in PBS, and then incubated with the primary antisera diluted in PBS containing 0.4% Triton-X100 for 15–20 hr at 4°C. Sections were then rinsed 10 times in PBS (10 min for each rinse) and incubated for 1 hr in the biotinylated goat anti-rabbit IgG. The tissue was then rinsed four times in PBS. The tissue was then incubated for 1 hr in avidin-biotinylated horseradish peroxidase complex, again diluted in PBS. The tissue was rinsed twice in PBS and then rinsed twice in Tris-buffered saline (0.05 *M*, pH 7.2). The sections were then transferred to a fresh solution of Tris-buffered saline with 0.4% of 3-3′ diaminobenzidine and 0.04% hydrogen peroxide. Incubation was performed at room temperature for 6–7 min and then rinsed in normal saline, mounted on slides and coverslipped.

Organ Weights and Bone Strength. Each set of organs was blotted dry and weighed to the nearest mg. Organ weight values reflect the total weight of each organ pair. Femur bones were allowed to thaw to room temperature. A digimatic caliper (Mitutoyo Mfg. Co. Ltd., Tokyo, Japan) was used to obtain three-dimensional measurements, including maximum length and maximum and minimum midshaft diameter to the nearest 0.01 mm. A three-point bend test of femur strength was carried out on an Instron Model 1000 universal testing instrument (Instron Corp., Canton, MA) set in the compression mode. The femurs were mounted loosely so that bending was allowed during testing. Force values reflect the minimum perpendicular force required to fracture the bone when applied to the midshaft.

Tissue Density and Mineral/Matrix Ratio Analysis. Femoral bone shafts were randomly selected from six rats in each group, cleaned of marrow and cut into 5-mm segments for tissue density and mineral/matrix ratio via the Archimedes method (36). Bone tissue density was calculated by obtaining multiple segment weights in air, then distilled water: tissue density = (weight in air)/(weight in air − weight in water) (36). Mineral/matrix ratio was then determined by obtaining dehydrated (via ethanol) and ash weights of the segments: mineral/matrix ratio = (ash weight)/(dehydrated weight − ash weight) (36).

Statistical Analysis. Significant differences between groups were determined using two-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls test.

Results

MSG Treatment of neonatal rats resulted in retardation of vaginal opening times, with experimental and control rats averaging 35.1 ± 0.4 and 32.8 ± 0.2 days, respectively ($P < 0.01$). Body weights of the two groups began to show divergence between Day 28 and 35 (Fig. 1). Differences were statistically significant beginning on Day 49 and continuing until euthanasia on Day 60 ($P < 0.05$). Maximum differences in weight were observed at euthanasia (196.3 ± 3.7 vs 176.6 ± 4.1 g in MSG-treated animals).

Fifteen minutes after challenge with GHRH, rat serum GH levels were significantly blunted ($P < 0.05$) in the MSG-treated group (Fig. 2). From 30 to 60 min post-GHRH injection, GH levels in MSG groups declined less rapidly than those of control groups, and at 60 min post-challenge, MSG rats had slightly higher, although not significant, circulating levels (Fig. 2). Based on the area under the GH curve, the total GH release during the 1-hr GHRH challenge was slightly decreased in MSG-treated rats (3.97 ± 0.60 vs 4.77 ± 0.54 μg in controls), but this difference is not significant. No statistical differences in pituitary GH content were found between groups, and similar findings were documented in pituitary total protein content (Table I). Immunocytochemistry for GHRH in the hypothalamus revealed a decreased immunoreactivity in the median eminence, near the lateral recess, and along the ventral hypothalamic border in all 12 animals of the

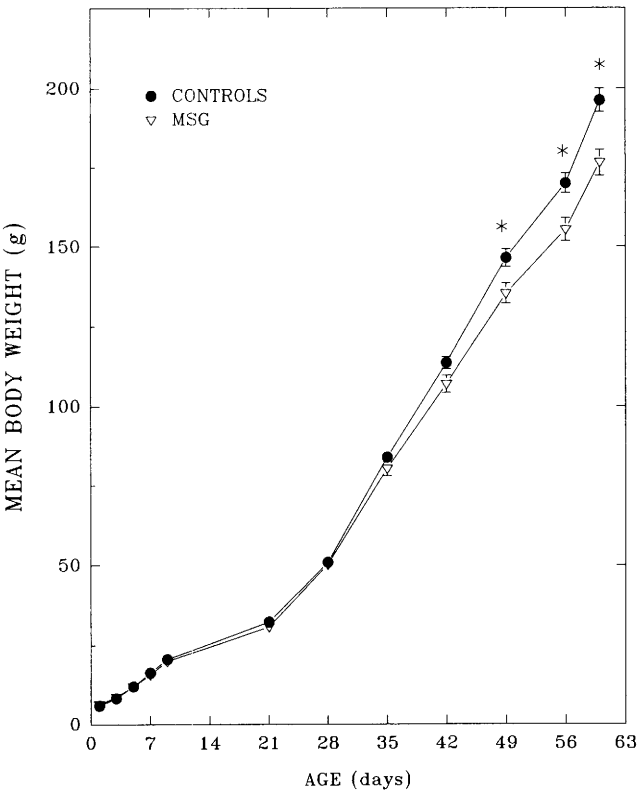

Figure 1. Effect of neonatal subcutaneous administration of MSG on body weight in female Sprague-Dawley rats. Data are presented as the mean ± SE ($n = 16$ for controls and $n = 12$ for MSG-treated groups), $^*P < 0.05$.

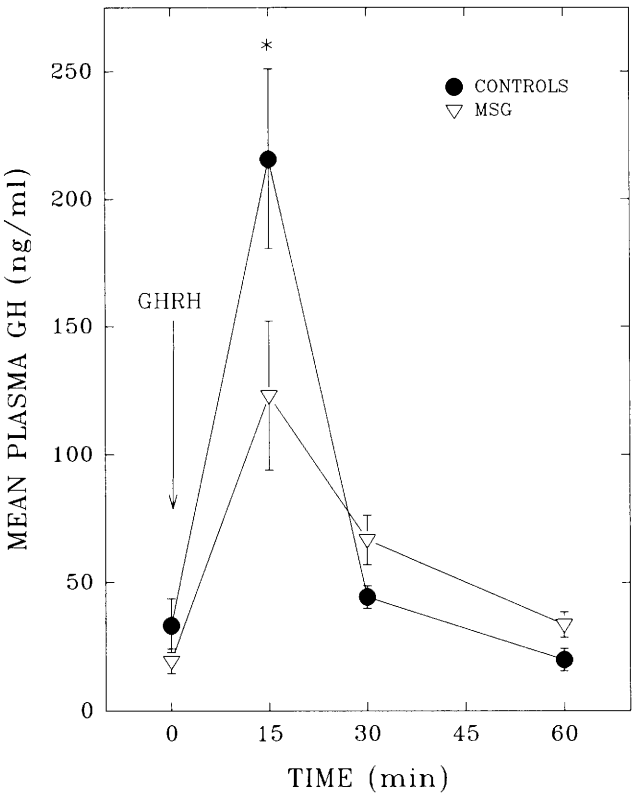

Figure 2. Effect of neonatal subcutaneous administration of MSG on plasma GH levels following GHRH infusion (5 μg) in female Sprague-Dawley rats 60 days of age. GHRH challenge time is denoted by the arrow. Data are presented as the mean ± SE (n = 16 for controls and n = 12 for MSG-treated groups), *P < 0.05.

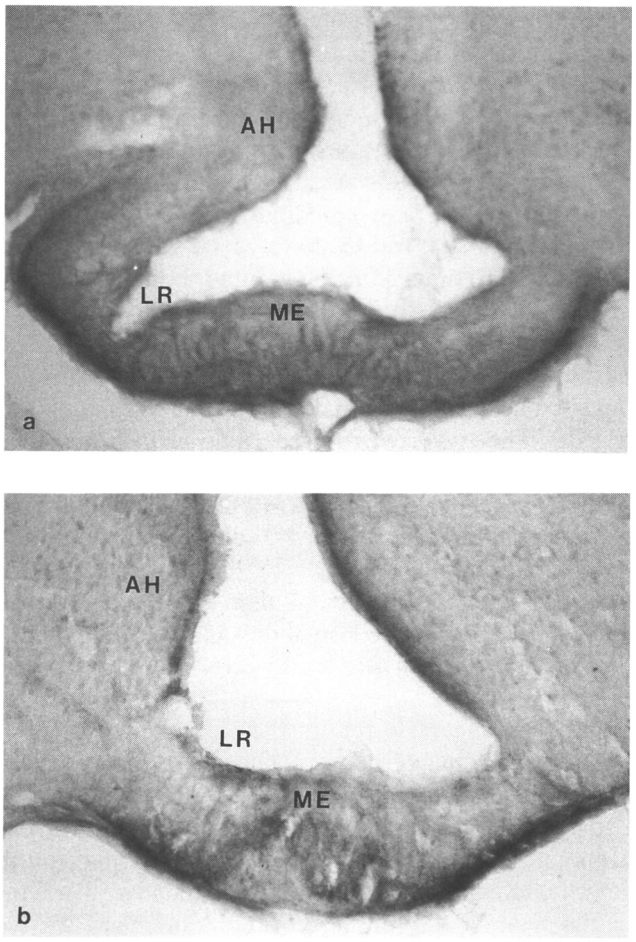

Figure 3. Immunocytochemistry of the mediobasal hypothalamus for growth hormone–releasing hormone (GHRH) in control (a) and MSG-treated (b) female rats. Note loss of immunoreactivity in the median eminence (ME), near the lateral recess (LR), and along the ventral border in the MSG-treated rat hypothalamus. Additionally there appears to be a small decrease of cells in the arcuate nucleus of the hypothalamus (AH).

MSG-treated group (Fig. 3). In addition, the arcuate nucleus from MSG-treated rats was decreased in size (Fig. 3).

Kidneys from MSG-treated rats weighed significantly less compared with the control groups (Table II). There were no significant differences, however, in weight of ovaries or adrenals among the two groups. Adrenal weight, as a function of body weight, was significantly increased in the MSG-treated animals (Table II). Analysis of rat femurs revealed no statistical differences in length and minimum midshaft diameter between control and MSG-treated groups (Table III). MSG treatment did, however, produce a significant decrease in maximum midshaft diameter and minimum fracture force as compared with controls (Table III). Femur matrix/mineral ratio and tissue densities in

Table I. Pituitary Growth Hormone and Total Protein Content in MSG-Treated and Control Female Rats[a]

Group (n)	Pituitary GH[b] (μg)	Pituitary total protein[b] (mg)	Pituitary GH/total protein[b] (μg/mg)
Control (16)	216 ± 26	1.02 ± 0.05	211 ± 25
MSG-treated (12)	192 ± 30	1.06 ± 0.05	178 ± 54

[a] Analysis done on pituitaries from rats 60 days of age.
[b] Values given are the mean ± SE.

Table II. Mean Organ Weights in Control and MSG-Treated Female Rats[a]

Organ[b]	Control (n = 16)	MSG-treated (n = 12)
Kidneys (g)	1.73 ± 0.03	1.56 ± 0.04[c]
Kidneys (mg/g BW)	8.84 ± 0.18	8.89 ± 0.27
Adrenals (g)	0.058 ± 0.007	0.059 ± 0.008
Adrenals (μg/g BW)	297 ± 8	336 ± 10[d]
Ovaries (g)	0.117 ± 0.001	0.120 ± 0.002
Ovaries (μg/g BW)	599 ± 37	678 ± 36

[a] Analysis done on organs from rats 60 days of age.
[b] Values given are the mean ± SE.
[c] $P < 0.05$ vs the control group.
[d] $P < 0.01$ vs the control group.

Table III. Mean Femur Dimensions and Minimum Midshaft Fracture Force in MSG-Treated and Control Female Rats[a]

Group (*n*)	Maximum diameter[b] (mm)[c]	Minimum diameter[b] (mm)[c]	Length (mm)[c]	Minimum fracture force (kg)[c]
Control (16)	3.46 ± 0.03	2.74 ± 0.02	31.02 ± 0.15	7.48 ± 0.15
MSG-treated (12)	3.33 ± 0.05[d]	2.69 ± 0.02	30.83 ± 0.16	6.86 ± 0.18[d]

[a] Analysis done on femurs from rats 60 days of age.
[b] Femur diameters measured at midshaft.
[c] Values given are the mean ± SE.
[d] $P < 0.05$ vs the control group.

MSG-treated rats were not statistically different from the controls (Table IV).

Discussion

The significant delay in puberty onset, or vaginal opening, found with neonatal MSG treatment has been previously reported by our laboratory (32). MSG disruption of the female reproduction system includes irregular vaginal cycles, an absence of compensatory ovarian hypertrophy in the presence of relatively normal fertility, and slightly decreased lutenizing hormone (LH) content and function (37).

GHRH challenge to MSG-treated rats has generated disputed results (20, 22, 38). In this study, neonatally MSG-treated rats had blunted initial responses to GHRH injections but similar total GH release as calculated by the area under the GH curve. These data suggest that chronic GHRH depletion leads to a slower, more prolonged GH response to GHRH of decreased peak amplitude. This finding may be explained by decreased GHRH receptor number, a physical defect in the GHRH receptor, disruption in the molecular receptor signalling mechanism, or possibly interaction of another protein with the GHRH receptor or GHRH molecule; however, definite mechanisms for decreased responsiveness in MSG-treated rats remain to be defined.

Immunocytochemistry of rat hypothalami confirmed a GHRH depletion in the MSG-treated group as well as a decrease in size of the GHRH-producing arcuate nucleus. Neonatal MSG administration to rats has been previously shown to destroy GHRH-producing neurons in the hypothalamus (15, 19, 28, 32).

Table IV. Mean Femur Mineral/Matrix Ratio and Tissue Density in MSG-treated and Control Female Rats[a]

Group (*n*)	Mineral/Matrix Ratio (g/g)[b]	Tissue Density (g/ml)[b]
Control (6)	2.501 ± 0.010	1.769 ± 0.082
MSG-treated (6)	2.518 ± 0.009	1.735 ± 0.053

[a] Analysis done on femurs from rats 60 days of age.
[b] Values given are the mean ± SE.

Previous investigations with rats utilized five subcutaneous injections of MSG on either consecutive or alternating days immediately following birth (19–22, 32, 37, 39–45). In an attempt to reduce the severity of endocrine disruption common to the five-injection procedure yet induce some degree of hypothalamic neuronal damage, the number of injections was curbed to three while the standard dose of 4 mg MSG/g body weight was maintained. Hence, in the three-injection procedure, less systemic disruption was expected.

Anterior pituitary GH content in MSG-treated rats has been disputed (19, 20, 22, 43, 44). Pituitary total protein has been shown to be reduced in MSG-treated rats (20), and several studies have documented a reduction in pituitary weight (20, 22, 37, 40, 41, 43, 45). In addition, the five-injection MSG procedure leads to reduced adrenal and ovary weights (28, 39, 40, 41, 43, 45), while three injections in this study appeared to have little effect. Experimental animals had increased adrenal weight per gram body weight, suggesting MSG treatment leads to a relative increase in adrenal size. Differences in kidney weights are in proportion to body weight and most likely related to the decreased GHRH and resultant decrease in GH. The anabolic effects of GH on protein metabolism in organs such as the liver and kidney have been well documented (2).

While femur length did not change with MSG treatment, maximum midshaft diameter and fracture force decreased. The lack of significant differences in bone mineral density or mineral/matrix ratio most likely reflect the short-term MSG treatment. GH plays an significant role in the production and activation of various growth factors in bone such as platelet-derived growth factor (PDGF) and IGF-1, and is a crucial element in the growth and maintenance of bone in all age groups. Chronic depletion of GHRH and GH is most likely associated with the bone changes encountered since neonatal MSG treatment has a resultant impact on weight-bearing bone size and hence strength in female rats. The absence of compositional changes in bone suggest an overall retardation effect on bone formation with no alteration in mineralization. Concep-

tually, the osteopenia and decrease in osteosynthesis associated with aging may have its origin in neuronal degeneration of the arcuate nucleus of the hypothalamus. The resultant decrease in GHRH and decrease in GH secretion impairs the bone growth–promoting effect of GH.

In comparing our results with documented findings in aging rats, several similarities are identified. Aged rats, similar to MSG-treated rats, have been shown to undergo hypothalamic neuronal degeneration (13, 14, 16, 17), have lower measurable levels of hypothalamic GHRH (15), and exhibit a blunted GH response to exogenous GHRH (9–12). Hence, this evidence suggests that neonatal MSG treatment may serve as a useful tool in delineating aging-related changes in growth hormone regulation in the rat.

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