Single exposure to testosterone in adulthood rapidly induces regularity in the growth hormone release process

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Am J Physiol Endocrinol Metab 278: E933–E940, 2000.—The neonatal gonadal steroid milieu is known to be important in imprinting the striking sexual dimorphism of growth hormone (GH) secretion; however, the influence of the sex steroids on GH control in adult life and their mechanism/site of action are largely unknown. In the present study, we tested the hypothesis that testosterone (T) serves the gender-specific regularity of the GH release process in adulthood. The approximate entropy statistic (ApEn) was used to quantify the degree of regularity of GH release patterns over time. Eighteen hours after a single subcutaneous injection of 1 mg T, both sham-operated and ovariectomized (OVX) female adult rats displayed plasma GH profiles that were strikingly similar to the regular male-like ultradian rhythm of GH secretion. The highest ApEn values, denoting greater disorderliness of GH secretion, were observed in the ovary-intact group, and T injection significantly (P < 0.001) reduced this irregularity whether or not the ovaries were present. Serial intravenous injections of GH-releasing hormone (GHRH) caused a similar increase in plasma GH levels in sham-operated females independently of time of administration. In contrast, female rats administered T exhibited a male-like intermittent pattern of GH responsiveness to GHRH, the latter known to be due to the cyclic release of endogenous somatostatin. These results demonstrate that acute exposure to T during adult life can rapidly and profoundly “masculinize” GH pulse-generating circuits in the female rat. Our findings suggest that the enhanced orderliness characteristic of the GH release process in males, compared with females, is regulated by T. We postulate that this T-induced regularity is mediated at the level of the hypothalamus by inducing regularity in somatostatin secretion, which in turn governs overall GH periodicity.

The sexual dimorphism of growth hormone (GH) secretion and postpubertal growth has been investigated intensively in both the rodent (23) and human (16). For example, in the rat, there is a striking gender difference in the pattern of GH secretion and rate of somatic growth (10, 16, 23). Whereas males exhibit high-amplitude GH secretory bursts at very regular 3.3-h intervals separated by low or undetectable plasma GH nadir levels (45), females show more frequent, seemingly irregular lower-amplitude GH pulses and an elevated GH baseline concentration (7, 11, 41). These distinct sex differences in the temporal patterns of GH release are of biological significance, because they evoke remarkable male-female differences in body growth (22, 40), liver enzyme gene expression (29, 49), and GH intracellular signaling pathways (50). Thus an understanding of the physiological mechanisms that govern the differential orderliness of the GH release process is important for a better comprehension of GH-regulated growth and metabolism.

There is considerable evidence that the gender-related differences in GH secretion and growth rate are attributable, at least in part, to the influence of gonadal steroids during the neonatal period (24–27). In general, testosterone (T) appears to play an important “organizational” role in generating the high-amplitude GH pulses and rapid growth rate typical of the male rat, whereas estradiol (E2) in early development is responsible for the elevated basal GH level observed in the female. Although it is well documented that the neonatal gonadal steroid milieu is an important determinant of the adult pattern of GH secretion and body growth, the influence of the sex steroids on GH control in adult life and their mechanism/site of action are largely unknown.

We recently demonstrated that the effects of the gonadal steroids are not limited to the critical period of neonatal imprinting; indeed, short-term exposure of adult male rats to E2 had profound effects on both the GH secretory pattern and the rate of somatic growth (35). There are, however, conflicting reports regarding the impact of T in adulthood. Gonadectomy of adult male rats was shown to suppress GH pulse amplitude, in one study (35), whereas other studies failed to demonstrate a significant effect of either prepubertal (24, 25) or adult (3, 15) orchidectomy. Moreover, although T treatment of adult rats was reported to enhance the GH response to GH-releasing hormone (GHRH)
in vivo (51), T has been reported to decrease (18), increase (12, 20), or have no effect (13, 51) on basal and/or GHRH-induced GH release from pituitaries in vitro.

The primary control of GH secretion from the pituitary gland is exerted through two hypothalamic hormones, somatostatin (SRIF) and GHRH, whose sexually dimorphic signaling patterns at the level of the pituitary appear to generate the sex-specific patterns of GH secretion (8, 34, 39, 44). Various gender-related differences in GH control by SRIF and GHRH have been described. A growing body of evidence suggests that the effects of the sex steroids are mediated at the level of the hypothalamus via alterations in the expression of both SRIF (6, 52) and GHRH (53) genes. Despite the foregoing evidence favoring a hypothalamic, rather than pituitary, site of action for T, this inference is still somewhat controversial (31).

In the present study, we tested the hypothesis that neuroendocrine actions of T can subserve the gender-specific regularity of the GH release process in adulthood. To accomplish this, we examined the effects of acute (single-dose) exposure to T on both spontaneous and GHRH-stimulated GH secretion in free-moving, intact, and ovariectomized adult female rats. Parameters of GH pulsatility were assessed by Cluster analysis, and the approximate entropy statistic was used to quantify the degree of serial orderliness or regularity of the GH release process over time.

**MATERIALS AND METHODS**

Animals and experimental procedure. Adult female Sprague-Dawley rats (210–225 g) were obtained from Charles River Canada (St. Constant, QC, Canada). The animals were either bilaterally ovariectomized (OVX) or sham-operated under ether anesthesia and were then housed in groups of three or four for 2 wk on a 12:12-h light-dark cycle (lights on between 0600 and 1800) in a temperature (22 ± 1°C)- and humidity-controlled room. Purina rat chow (Ralston-Purina, St. Louis, MO) and tap water were constantly available, and body weight was monitored daily. Subsequently, chronic intracardiac venous canulas were implanted under pentobarbital sodium (25–30 mg/kg ip) anesthesia by use of a previously described method (45). After surgery, the rats were placed directly in isolation test chambers with food and water available ad libitum until their body weights returned to preoperative levels (within 5–7 days).

In the first experiment, we examined spontaneous GH secretory patterns in both sham-operated and OVX females. On the test day, food was removed 1.5–2 h before sampling, and blood samples (0.35 ml) were drawn every 15 min for 6-h periods between 1000 and 1600. All blood samples were immediately centrifuged, and the plasma was separated and stored at −20°C for subsequent assay of GH. To prevent hemodynamic disturbance, the red blood cells were resuspended in normal saline and returned to the animal after removal of each blood sample.

In the second experiment, we assessed the effects of acute T treatment on GH secretory dynamics in both sham-operated and OVX females bearing chronic intracardiac canulas. The rats were administered subcutaneously 1 mg T (Sigma, St. Louis, MO) dissolved in 0.2 ml sesame oil, or sesame oil (0.2 ml) alone, at 1600, and spontaneous 6-h plasma GH profiles were obtained on the next day from 1000 to 1600, i.e., 18–24 h after the injections.

The third experiment was designed to assess the effects of OVX and T treatment on GH responsiveness to GHRH. To this end, both OVX and T-treated (18–24 h after sc injection of 1 mg T) rats were administered GHRH [rat GRF-(1–29)NH2; 1 μg/0.3 ml iv] at two different times (1100 and 1300) during the 6-h sampling period, and their responses were compared with those of respective controls. The GHRH peptide (lot CH-23–25, 31:10–16, kindly provided by Dr. P. Brazeau, Notre-Dame Hospital, Montreal, QC, Canada) was diluted in normal saline immediately before use. To document the rapidity of the response, an additional blood sample was obtained 5 min after each injection of the peptide.

Additional plasma samples were obtained at the end of the sampling period from both OVX and T-treated animals, as well as from two other groups of normal female and male rats, for subsequent measurement of circulating plasma E2 and T levels.

All animal-based procedures were approved by the McGill University Animal Care Committee.

Hormone assays. Plasma GH concentrations were determined in duplicate by double-antibody RIA with materials supplied by the National Institute of Diabetes and Digestive and Kidney Diseases Hormone Distribution Program (Bethesda, MD). The mean sample plasma GH values are reported in terms of the rat GH reference preparation rGH-RP-2. The standard curve was linear between 0.62 and 160 ng/ml. The least detectable concentration of plasma GH under the conditions used was 1.2 ng/ml; all samples with values above 160 ng/ml were reassayed at dilutions ranging from 1:2 to 1:10. The intra- and interassay coefficients of variation were 6.3 and 7.5%, respectively, for duplicate samples of pooled plasma exhibiting a mean GH concentration of 14.8 ng/ml and were 8.6 and 12.1%, respectively, for duplicate samples of pooled plasma having a mean GH concentration of 102.7 ng/ml.

Plasma E2 and T concentrations were measured with commercial kits for E2 and T (Radio System Laboratories, Carson, CA). All samples of a given hormone were measured in a single assay.

Statistical analysis. The plasma GH profiles of individual rats in all groups were analyzed using the Cluster analysis program as a model-free technique for hormone pulse detection (48). Briefly, a pooled-variance t-statistic of 2 was selected to maintain a maximal false-positive rate of ≤1% by using test cluster sizes of 2 consecutive data points each in the prepeak nadir, peak, and postpeak nadir. ANOVA for repeated measures and the Tukey honestly significant difference (HSD) post hoc test or Student’s two-tailed t-tests for unpaired and paired data, as appropriate, were used for statistical comparisons between and within experimental groups. The results are expressed as means ± SE. P < 0.05 was considered significant.

Approximate entropy. A sensitive metric of relative disorderliness of hormone concentration profiles, termed approximate entropy (ApEn), was utilized to quantify objectively the serial regularity or orderliness of GH release patterns over 6 h (38). This statistic is a finite positive nonzero real number developed for any single entire hormone pulse profile as an ensemble estimate of the “point-by-point” subpattern reproducibility within the data. As such, ApEn provides a scale-invariant and model-independent quantitation of relative disorderliness, wherein higher ApEn values denote greater relative disorderliness or reduced regularity of the release process (38). Technically, ApEn designates the negative logarithm of the probability that a given pattern of successive hormone measurements is repeated upon next incremental comparison within a tolerance r for a data window length m.
The parameter \( r \) is typically set at 20% of the individual within-series standard deviation to normalize ApEn for unequal mean serum hormone concentrations. For series of lengths < 200, \( m \) is typically given as unity. This choice of \( m \) and \( r \) yields high statistical replicability (37). Thus ApEn is a family of statistics conditional on \( m \) and \( r \). ApEn is relatively insensitive to occasional outliers within the data and to experimental variability (noise) smaller in magnitude than \( r \). Monte Carlo simulations (300 runs/series) were used to estimate the SD of ApEn for each series based on the within-sample variance of the GH assay (above).

**RESULTS**

Effects of OVX on somatic growth. As shown in Fig. 1, OVX accelerated somatic growth. There was a twofold increase in mean rate of body weight gain in the OVX rats compared with that in sham-operated females \([5.5 \pm 0.9 \text{ (SE)}] \text{ vs. } 2.6 \pm 0.5 \text{ g/day; } P < 0.001\). A significant difference between groups was noted at 8 days post-OVX and thereafter.

Effects of OVX on spontaneous GH secretory profiles. Sham-operated female rats exhibited the characteristic female pattern of GH secretion, with frequent irregular GH pulses of variable amplitude separated by an elevated baseline GH concentration (Fig. 2A). At 3–4 wk after OVX, basal plasma GH levels were markedly reduced (Fig. 2B).

Cluster analysis of serum GH concentration profiles (see Fig. 4) showed that OVX resulted in a two- to threefold decrease in plasma GH nadir levels \([3.4 \pm 0.7 \text{ vs. } 8.4 \pm 0.8 \text{ ng/ml in sham controls; } P < 0.001\). However, neither the GH peak amplitude \([66 \pm 4.5 \text{ vs. } 76 \pm 24 \text{ ng/ml}] \) nor the GH peak frequency \([3.3 \pm 0.2 \text{ vs. } 3.4 \pm 0.2 \text{ peaks/6 h}] \) was significantly altered compared with sham-operated controls.

Effects of acute T treatment on GH secretory dynamics in sham-operated and OVX females. Eighteen hours after a single subcutaneous T injection, there was a striking alteration in the irregular female GH secretory pattern in both sham-operated and OVX rats compared with their respective oil-treated controls (Fig. 3). Both T-treated groups displayed a regular male-like ultradian rhythm of GH secretion, with higher-amplitude GH pulses occurring at precise intervals separated by prolonged periods of low or undetectable plasma GH levels (Fig. 3, C and D). Cluster analysis (Fig. 4) showed that T treatment of OVX rats resulted in a significant decrease in both the GH peak frequency \((P < 0.01)\) and GH nadir \((P < 0.02)\) and a significant augmentation of GH peak amplitude \((P < 0.01)\) as well as a prolongation of GH interpeak interval \((P < 0.01)\) compared with OVX rats administered oil.

ApEn. ApEn was calculated as an objective statistical measure of the serial regularity or orderliness of the GH release process over time. Because the GH time series are relatively short (25 samples), we calculated ApEn for fixed \( m = 1 \) and \( r = 0.2 \) times the within-series SD, and we also estimated (see MATERIALS AND METHODS) the standard deviation of each ApEn value for any given GH series. Individual ApEn values and their Monte Carlo-estimated SD values for each animal in the four treatment groups are given in Fig. 5. Higher ApEn values denote greater relative disorderliness of the release process.

The highest mean ApEn values were observed in the ovary-intact (Sham+Oil) group \((\text{mean } \pm \text{ SE: } 0.98 \pm 0.03)\). Administration of T to both Sham and OVX groups significantly reduced the mean ApEn value to \(0.64 \pm 0.09\) and \(0.47 \pm 0.04\), respectively \((P < 0.001 \text{ by ANOVA and the Tukey HSD post hoc test})\). ApEn values for the OVX+Oil group were intermediate between...
Effects of OVX and T treatment on GH responsiveness to GHRH injection. Figure 6 illustrates the effects of OVX and T treatment on GH responsiveness to GHRH in individual rats representative of the four experimental conditions. In sham-operated control females (Fig. 6A), the intravenous administration of 1 µg rGRF-(1–29)NH₂ at 1100 and 1300 caused a similar increase in plasma GH levels at both time points. In contrast, rats subjected to OVX or T treatment exhibited an intermittent pattern of GH responsiveness to GHRH independent of the time of administration (Fig. 6, B, C, and D); the sequences of the large-to-small or small-to-large responses were not consistent within these groups. In both OVX- and T-treated animals there was a marked difference in the integrated (5- and 15-min postinjection) GH response to GHRH observed at 1100 vs. 1300; the ratio of the large to small GH response in each of these three groups was significantly higher than that observed in sham-operated controls (Table 1).

Plasma E₂ and T levels. The mean plasma E₂ level in OVX rats (n = 6) was significantly decreased compared with that of a group of normal female rats (n = 5) killed at various times in the estrous cycle (33 ± 0.9 vs. 83 ± 25 pg/ml; P < 0.02). Plasma T levels observed at 24 h after T injection in five Sham + T-treated females (147 ± 31 ng/dl) were similar to those seen in a group (n = 6) of normal adult male rats (141 ± 26 ng/dl).

DISCUSSION

In the present study we have shown that withdrawal of ovarian hormones in adult life markedly reduces the elevated GH baseline characteristic of the female rat. This finding, along with our earlier demonstration of a rapid E₂-induced elevation of GH nadir levels in adult males (35), provides further support for the notion of a major role for E₂ in maintaining higher interpulse GH secretion in the adult female compared with the male
rat. Although it was previously shown that neonatal OVX resulted in an increased GH pulse amplitude in adulthood (24), our study failed to demonstrate an effect of adult OVX on either GH peak amplitude or GH peak frequency. These results are supportive of the hypothesis that neonatally secreted testicular androgen is a prerequisite for imprinting the high GH pulse amplitude observed in postpubertal males (25), and that it is the presence of T during the neonatal period, rather than the absence of E2 in the adult, that is the important determinant of adult GH pulse height.

The rate of body weight gain was enhanced twofold in OVX females compared with sham-operated controls, which is consistent with an inhibitory role attributed to E2 on body growth (9). It has been proposed that the sex difference in the GH secretory pattern of the rat may account, at least in part, for the striking sex difference in somatic growth of this species (22, 40). Our findings are congruent with this view and further suggest that it is the period of low or no plasma GH between the GH secretory episodes, and hence the pattern of GH release, rather than the magnitude of individual GH pulses per se, that is key to the male's characteristically rapid rate of somatic growth. A similar conclusion has been reached in the case of GH-regulated hepatic proteins; e.g., Waxman et al. (49) showed that it is neither the total GH exposure nor the GH pulse amplitude, but rather the interval between pulses, that governs the sexually dimorphic expression of hepatic cytochrome P-450.

The results of our experiments examining the impact of T on the GH release process clearly indicate that acute exposure to T in adulthood rapidly and profoundly alters the irregular female pattern of GH secretion. We demonstrate here for the first time that T (at physiological levels similar to those observed in normal males) can induce a rapid "masculinization" of GH pulse-generating circuits of the female in adulthood. Eighteen hours after a single subcutaneous injection of T, both sham-operated and OVX female rats displayed plasma GH profiles that were strikingly similar to the male-like ultradian rhythm of GH secretion (45), characterized by regular GH secretory bursts occurring at 3- to 4-h intervals separated by prolonged periods of typically undetectable plasma GH levels (see Fig. 3). In particular, OVX female rats administered T exhibited a significant increase in both GH peak amplitude and interpeak interval, concomitant with a decrease in GH peak frequency and GH nadir, compared

Table 1. Effects of OVX and T treatment on GHRH-induced GH release

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>n</th>
<th>Higher Integrated GH Response, ng/ml</th>
<th>Lower Integrated GH Response, ng/ml</th>
<th>Ratio of Large to Small GH Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>8</td>
<td>789 ± 74.8</td>
<td>609 ± 78.3</td>
<td>1.26 ± 0.1</td>
</tr>
<tr>
<td>OVX</td>
<td>6</td>
<td>707 ± 99.5</td>
<td>392 ± 94.5*</td>
<td>2.35 ± 0.5†</td>
</tr>
<tr>
<td>SHAM + T</td>
<td>8</td>
<td>583 ± 61.3</td>
<td>372 ± 51.4*</td>
<td>1.66 ± 0.1†</td>
</tr>
<tr>
<td>OVX + T</td>
<td>5</td>
<td>867 ± 173.0</td>
<td>390 ± 167.0*</td>
<td>3.50 ± 1.3†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, nos. of rats/group. GH, growth hormone; OVX, ovariectomy; T, testosterone; GHRH, GH-releasing hormone. *P < 0.05 or less vs. higher response within each group; †P < 0.05 or less vs. sham-operated control group.
with corresponding parameters in oil-treated OVX controls. These findings are concordant with a previous observation that adult female rats given subcutaneous T implants exhibit a sex reversal of their GH secretory pattern after 1 wk of treatment (32).

We used ApEn to quantify the degree of serial regularity or orderliness of the GH plasma profiles in the four treatment groups. Previous studies have shown that ApEn distinguishes male and female GH time series (14, 36) and discriminates between tumoral and physiological hormone release profiles in the case of GH, adrenocorticotropic hormone, prolactin, and aldosterone (17, 43, 47). Higher ApEn values denote greater disorderliness or reduced regularity of release. The highest ApEn values were observed in the ovary-intact group. T injection significantly reduced this irregularity, whether or not the ovaries were present. The OVX GH time series were less orderly than those of OVX+T but relatively more regular than that of Sham+Oil. Thus OVX reduces the irregularity of GH release seen in the intact female, and this irregularity is even further reduced by T injection, whether or not the ovaries are present. These findings strongly suggest that T has a major role in determining gender differences in the regularity of GH secretion and provide support for the notion that the enhanced orderliness of the GH release process in males, compared with females, is regulated by T.

The mechanism(s) by which T masculinizes the orderliness of GH secretion was evaluated by sequential GHRH injections. Assessment of GH responsiveness to GHRH stimulation revealed that neither OVX nor T treatment significantly altered the magnitude of the GH response to GHRH, in agreement with earlier findings of Wehrenberg et al. (51). These data differ from those of Shulman et al. (42), who found greater GH responses to GHRH in OVX rats; however, this difference likely reflects the use of pentobarbital-anesthetized animals in the latter study, as distinguished from the conscious rats used here. Of further importance, whereas sham-operated females exhibited similar GH release after serial GHRH injections, consistent with that previously reported for the female rat (8, 34), OVX and T-treated groups showed an intermittent pattern of responsiveness to GHRH that is characteristic of the male rat (34, 44). This male-like partial refractoriness to successive GHRH stimuli observed in OVX animals fits well with our previous report demonstrating that E2 during adult life can feminize the male pattern of GHRH-stimulated GH secretion (35).

We (34, 44) and others (8) have hypothesized that the sexual dimorphism of GH secretion in the rat is primarily due to a gender difference in the mode of hypothalamic SRIF signaling to pituitary somatotropes, with females exhibiting tonic, rather than episodic, SRIF secretion compared with males. Furthermore, we have demonstrated that the variable responsiveness to GHRH in the male rat is due to antagonism of GH secretion by the cyclic release of endogenous SRIF (44). Thus the present results in females suggest that the removal of ovarian hormones and/or the administration of T alters the postulated continuous pattern of hypothalamic SRIF secretion, converting it to a cyclical mode of release. This phasic release of SRIF likely plays a key role in determining the pulsatility and periodicity of GH secretion (46). The absence of full male-like synchronization of the GH responses to GHRH (44) indicates that, despite the dramatic influence of T on the pattern of spontaneous GH secretion in adulthood, it is not sufficient to initiate the light-dark entrainment of GH pulses that characterizes the GH secretory profile of the adult male rat (45). On the other hand, we cannot discount the possibility that T during the neonatal period plays a role in entraining the GH secretory episodes, because adult circadian hormone rhythms have been attributable to the organizational effects of early gonadal hormone exposure (30).

Although reports of a direct effect of T on pituitary somatotropes are limited and conflicting (12, 13, 18, 20, 51), there is compelling evidence for one or more hypothalamic site(s) of T's actions on GH secretion. Clear sex differences exist in the mRNA (1, 6, 31) and peptide (28) content of SRIF and GHRH neurons, as well as in the expression of SRIF receptor subtypes (54), in the hypothalamus. In particular, gonadal steroids appear to be intimately involved in the regulation of hypothalamic SRIF. Female and male rats subjected to OVX or gonadectomy exhibit a decrease in SRIF mRNA levels in the periventricular region of the hypothalamus (6, 52). T, administered either neonatally (5) or in adulthood (6, 52), stimulates expression of the SRIF gene in the periventricular nucleus. This response may represent a direct effect of T on SRIF transcript accumulation, because androgen receptors are expressed by SRIF neurons in a sexually dimorphic manner (19, 21). Furthermore, the effects of T are not dependent on its aromatization to E2, because dihydrotestosterone, a nonaromatizable androgen, but not E2, was capable of inducing an increase in SRIF mRNA (2, 55). Consistent with this concept, androgen-resistant (testicular feminized) rats, which lack functional androgen receptors, display GH secretory profiles resembling those of intact females (33). Finally, the present interpretation of GH neuroregulation recognizes that the effects of E2 on the GH secretion profile (35) are opposite to those reported here for T. Although some studies have reported that the GHRH gene appears to be stimulated by T through activation of androgen receptors (1, 53), other reports have noted that neither orchidectomy of male rats nor T administration to OVX rats influences hypothalamic GHRH mRNA levels (4, 31). Taken together, these findings suggest that T masculinizes the orderliness of GH secretion by stimulating hypothalamic SRIF synthesis and/or release, putatively by way of an androgen receptor-dependent pathway.

In conclusion, the present experiments demonstrate that acute single-dose exposure to T during adult life can rapidly (within 24 h) and profoundly masculinize GH pulse-generating behavior in the female rat. Our findings further suggest that the enhanced orderliness characteristic of the GH release process in males,
Further investigations are warranted to substantiate secretion, which in turn governs overall GH periodicity. That this T-induced regularity is mediated at the level of growth hormone-releasing hormone (GHRH) secretion, which in turn governs overall GH periodicity.

We thank Martine Lapointe for expert technical assistance. J ulie Temko for skilful preparation of the manuscript, and Drs. Steve Pincus and Martin Straume for technical implementation of ApEn calculations. We are grateful to the National Institute of Diabetes and Digestive and Kidney Diseases Hormone Distribution Program for the continuing supply of rat GH RIA materials.

This work was supported by Grant MT-6837 (to GS Tannenbaum) from the Medical Research Council of Canada and National Institutes of Health Grant AG-14799 (to J Veldhuis). GS Tannenbaum is a Chercheur de Carrière of the Fonds de la Recherche en Santé du Québec. J C Painson was the recipient of a Studentship Award from the Fonds pour la Formation de Chercheurs et l’Aide à la Recherche. Address for reprint requests and other correspondence G. S. Tannenbaum, Neuropeptide Physiology Laboratory, McGill University-Montreal Children's Hospital Research Institute, 2300 Tupper St., Montreal, QC, Canada H3H 1P3 (E-mail: mcta@musica.mcgill.ca).

Received 17 September 1999; accepted in final form 8 December 1999.

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