Effects of Immunoneutralization of Substance P on Hypothalamic Neurotransmitters in Normal Mice and in Transgenic Mice Expressing Bovine Growth Hormone (44269)

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Abstract. **It is well known that transgenic mice expressing bovine growth hormone have altered neuroendocrine functions. Substance P was shown to influence the secretion of gonadotropins. In this investigation, the effect of a single injection of an antiserum to substance P was investigated in intact and castrated transgenic (MT-bGH) mice and in their normal litter mates. In the median eminence, the administration of antisubstance P serum resulted in a decreased dihydroxyphenyl acetic acid/dopamine index in intact and castrated normal mice but was without effect in transgenics. The homovanillic/dopamine index was decreased in normal mice (intact or castrated) but unchanged in transgenics. Norepinephrine and epinephrine were increased in normal mice (intact and castrated) treated with the anti-SP serum, but in transgenic mice, the anti-SP serum induced significant changes of norepinephrine only in intact animals, with no modifications in epinephrine levels. In the whole hypothalamus (minus the median eminence), the injection of antisubstance P serum resulted in an increased dihydroxyphenyl acetic acid/dopamine index in castrated, but not in intact, normal mice. In transgenic mice, this index was increased in intact but decreased in castrated animals. The homovanillic/dopamine index was decreased in normal intact mice treated with the antiserum but increased in intact transgenic mice. Norepinephrine and epinephrine were decreased by the antiserum treatment in normal intact mice but were unchanged in transgenics, except for norepinephrine in castrated transgenics, in which it was found increased. The administration of the antiserum did not affect plasma LH, FSH, or prolactin in normal mice but it reduced LH levels in intact transgenic mice. These results indicate that the response to the treatment with the antiserum to substance P shows considerable alterations in transgenic mice as compared with their litter-mate, normal controls, producing divergent effects on hypothalamic catecholamine metabolism. The present findings confirm that transgenic mice overexpressing the bGH gene have marked neuroendocrine alterations as compared with their normal litter mates.** [P.S.E.B.M. 1998, Vol. 218]

Growth hormone (GH) influences a wide array of physiological functions, including reproduction. Studies in transgenic mice overexpressing bovine or human GH (1–4) and in GH-deficient animals (5, 6) provided evidence that GH can alter plasma levels of gonadotropins and prolactin (PRL) as well as the susceptibility of their release to normal regulatory influences. Thus chronic elevation of GH levels in transgenic mice is typically associated with severely attenuated gonadotropin responses to castration (1, 2, 4) and with reduced PRL responses to pharmacological blockade of catecholamine synthesis (4, 7). We also observed previously that transgenic animals expressing the hGH gene failed to show the response of hypothalamic neurokinin A to estradiol injections such as shown in normal

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mice (8). Moreover, transgenic males expressing bovine (b) GH failed to respond to immunoneutralization of neuropeptide Y by elevation of plasma follicle-stimulating hormone (FSH) and PRL levels, thus differing from the responses observed in their normal siblings (9). It was against this background that we decided to compare the effects of immunoneutralization of substance P (SP) on several indices of hypothalamic and adenohypophyseal function in bGH transgenic versus normal mice, and to examine the interaction of gonadectomy and immunoneutralization of SP in these animals. Substance P can modulate the release of several adenohypophyseal hormones, including gonadotropins and PRL (10–12), and there is evidence that it can act at both the pituitary and the hypothalamic levels (12). Both the pituitary and the hypothalamus are potential targets for direct and insulin-like growth factor-I (IGF-I)–mediated actions of GH (13–15). Expression of GH in transgenic mice is associated with alterations in noradrenergic and dopaminergic transmission in the hypothalamus (2–4) and with failure of the median eminence turnover of norepinephrine (NE) to increase in response to orchidectomy (2, 4).

Materials and Methods

Animals. Transgenic mice expressing bGH under control of mouse metallothionein-I (MT) promoter were derived from animals kindly provided by Dr. Thomas Wagner and June Yun (7, 16, 17) and were propagated by mating transgenic males to normal females (C57 BL/6 × C3H F—1 hybrids purchased from the Jackson Laboratory, Bar Harbor, ME). This breeding system produced both hemizygous transgenic animals and their nontransgenic siblings, which served as normal controls. Animals were housed under controlled conditions of photoperiod (12:12) and temperature 22 ± 2°C) with constant access to standard pelleted food and tap water. Mice were weaned at the age of 21 days, maintained in groups of three to five animals of the same gender per cage, and used for the experiments when they were between 2.5 and 3.5 months old. Gonadectomies and sham surgery were carried out *via* midventral incision under ether anesthesia. All experiments have been performed according to the principles and procedures outlined in the *NIH Guide for the Care and Use of the Laboratory Animals* and according to a protocol approved by an institutional committee.

Antiserum and Treatment. Antiserum (As) to SP was produced in our laboratory by immunizing a rabbit with SP bound to bovine serum albumin using the glutaraldehyde method. Details of the production and characterization of this As have already been published (18). This As binds 125I-SP at a level of 30% when used at a final dilution of 1:840,000.

The antiserum to SP or normal rabbit serum (NRS; Sigma Chemical Co., St. Louis, MO) were administered undiluted as a single ip dose, 0.15 ml/animal, approximately 25 hr before sacrificing the animals. Castrated and sham-castrated mice were injected with As or NRS immediately after surgery, using 7–9 normal and 10–13 transgenic animals per group. The injection of the antiserum immediately after castration was given to investigate the effect of the blockade of endogenous SP on the early repercussions of the gonadal removal. It had previously been reported that castration resulted in changes of gonadotropin secretion as early as 12–24 hr after surgery (19). The animals were sacrificed by decapitation, and blood was collected from the trunk into tubes containing 30 μl of 6% EDTA to prevent clotting. After centrifugation, plasma was aspirated and kept frozen at −20°C until assayed. The median eminence and the rest of the hypothalamus were dissected free, immediately frozen on dry ice, and kept at −70°C thereafter. Just before the determinations, the tissues were submerged in 2 *N* perchloric acid, sonicated, and then centrifuged at 11,000 rpm for 20 min at 4°C. The supernatants were then subjected to HPLC analysis.

Measurements of Catecholamines and Hormones. The concentrations of NE, dopamine (DA), dihydroxyphenyl acetic acid (DOPAC) and homovanillic acid (HVA) in the supernatants from the median eminence and the remainder of the hypothalamus were measured using high performance liquid chromatography with electrochemical detection (HPLC-ED). Separation of catecholamines or metabolites was achieved by a reverse phase column (Nucleosil 5 C18 100A, 150 × 4.6 mm, Phenomenex, Torrance, CA) using a mobile phase composed of 0.1 *M* acetate-0.1 *M* citrate buffer containing ethylenediaminetetraacetic acid (disodium salt, 200 mg/l), and sodium octyl sulphate (200 mg/l). At this point, pH was adjusted (pH 4), and afterwards methanol was added (10% v/v). The flow rate was set a 1 ml/min. Catecholamine detection was performed with a coulometric detector (Coulochem 5100A, Esa Inc., Chelmsford, MA). Fixed potentials versus $H2/H^+$ reference electrodes were: conditioning electrode −0.40 V, preoxidation electrode +0.10 V, and working electrode +0.35 V. Catecholamines and their metabolites were evaluated from the chromatographic peak heights using external standards (NE, DA, and DOPAC from Sigma Chemical Co., St. Louis, MO, and HVA from Merck, Darmstadt, Germany). This method was described previously (20).

Plasma levels of LH and FSH were measured by radioimmunoassay (RIA) using reagents generously provided by the NIDDKD (Rockville, MD), as described previously (1). Mouse plasma prolactin levels were determined by a specific homologous RIA using reagents generously provided by Dr. A. F. Parlow as previously described (21).

Statistical Analysis of the Results. The results were analyzed by means of an analysis of variance (ANOVA), followed by a Student Neuman Keuls' test. The values were considered statistically significant when $P < 0.05$.

Results

The ratio of DOPAC to DA content (DOPAC/DA Index) in the median eminence was significantly lower in transgenic than in normal mice (Fig. 1), and was not altered

by castration in either of the groups (Fig. 1A and B). Administration of As to SP (SP-As) significantly reduced the DOPAC/DA index in normal intact and castrated mice (Fig. 1A) and castrated mice (Fig. 1B). In contrast, injection of the same dose of SP-As failed to alter the DOPAC/DA index in transgenic animals (Fig. 1A and B).

Similarly, the HVA/DA index in the median eminence was also significantly lower in transgenic than in normal mice (Fig. 2A). Treatment with SP-As significantly reduced the HVA/DA index in intact normal (Fig. 2A) and castrated normal mice (Fig. 2B). In transgenic animals, the effect of SP-As was numerically lower and significant only in the intact group (Fig. 2A and B).

Norepinephrine content in the median eminence did not differ between normal and transgenic mice (Fig. 3A and B) and was reduced by castration only in the normal animals (Fig. 3B). Administration of SP-As was followed by a small but statistically significant increase in NE content in intact normal and intact transgenic mice (Fig. 3A) and a major increase in this parameter in gonadectomized normal mice (Fig. 3B). In contrast, in gonadectomized transgenics, SP-As did not affect the median eminence NE content (Fig. 3B).

The content of epinephrine in the median eminence did not differ between normal and transgenic mice, regardless of their gonadal status (Fig. 4A and B). Castration reduced epinephrine content only in normal animals (Fig. 4A). Administration of SP-As was followed by a marked and highly significant ($P < 0.001$) increase in epinephrine content in normal males (both intact and castrated), but failed to influence this parameter in transgenic animals (Fig. 4A and B).

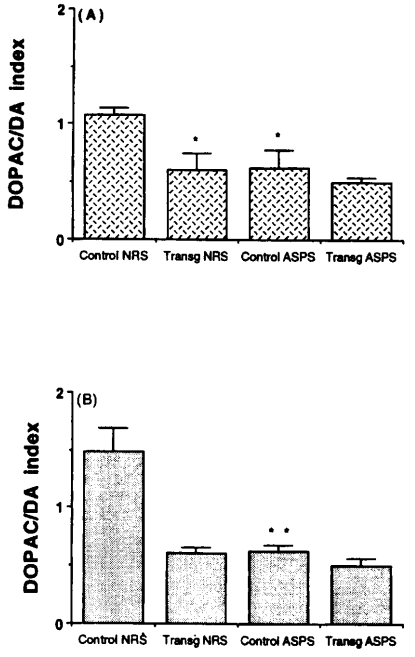


Figure 1. Ratio of DOPAC to DA contents (DOPAC/DA ratio) in the median eminence of (A) intact and (B) gonadectomized transgenic (overexpressing b-GH) and normal mice. Values are expressed as mean ± SEM. $^{*}P < 0.05$ vs control NRS. $^{**}P < 0.01$ vs control NRS.

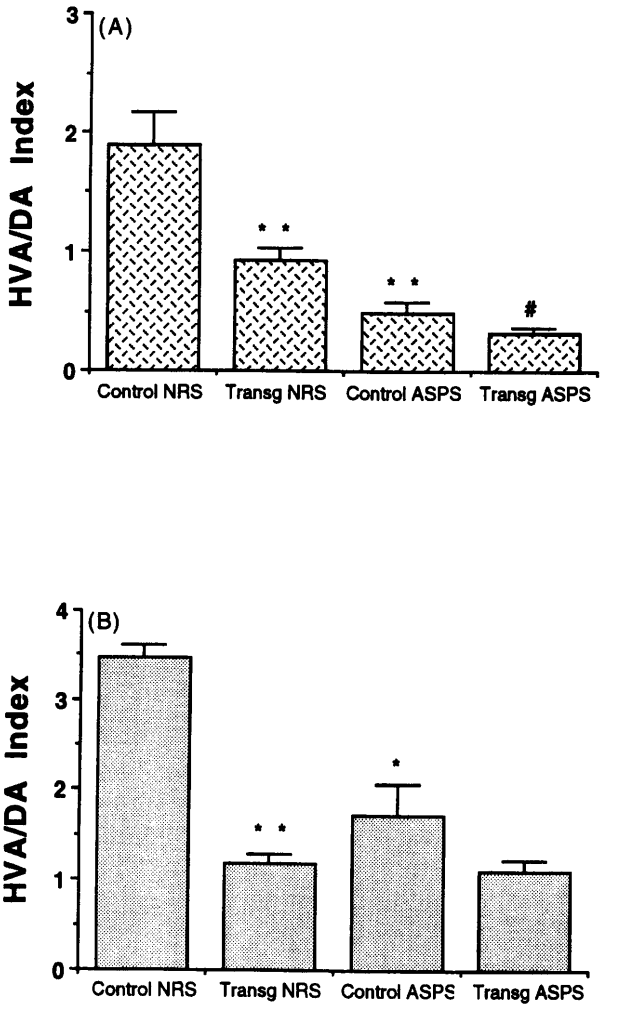
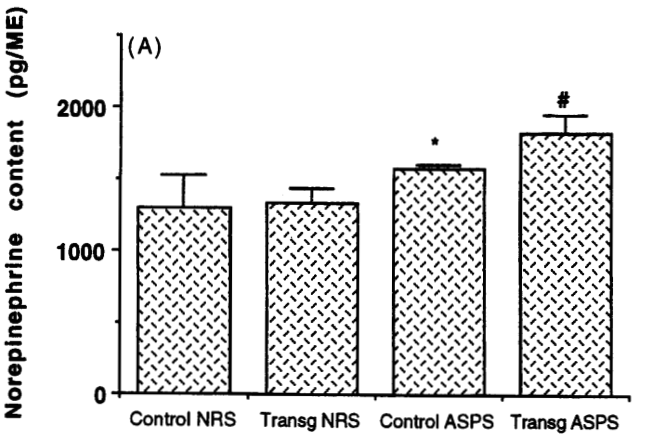


Figure 2. Ratio of HVA to DA contents (HVA/DA ratio) in the median eminence of (A) intact and (B) gonadectomized transgenic (overexpressing b-GH) and normal mice. Values are expressed as means ± SEM. $^{*}P < 0.05$ vs control NRS. $^{**}P < 0.01$ vs control NRS. $^{\#}P < 0.05$ vs trang NRS.

In the hypothalamus minus the median eminence (hereafter referred to as "hypothalamus"), the DOPAC/DA index was lower in intact transgenic than in intact normal animals (Fig. 5A vs B). Gonadectomy reduced the DOPAC/DA index only in normal males (Fig. 5A) and thus eliminated the difference between transgenic and normal animals. Administration of SP-As did not affect hypothalamic DOPAC/DA index in intact normal males but increased it in intact transgenic and castrated normal animals (Fig. 5B), and reduced it in castrated transgenic mice (Fig. 5B).

The hypothalamic HVA/DA index was lower in intact transgenic than in intact normal mice (Fig. 6A vs B), and was increased after castration only in transgenic animals (Fig. 6B). Administration of SP-As produced divergent results in normal and transgenic mice. It was followed by a reduction in the HVA/DA index in intact normal mice (Fig. 6A) and by an increase in the HVA/DA index in intact



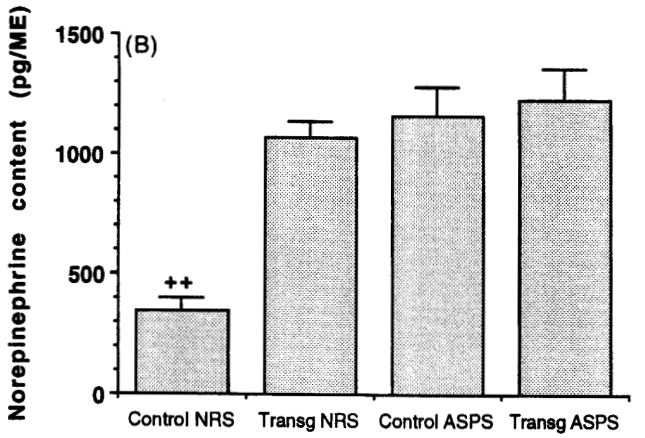
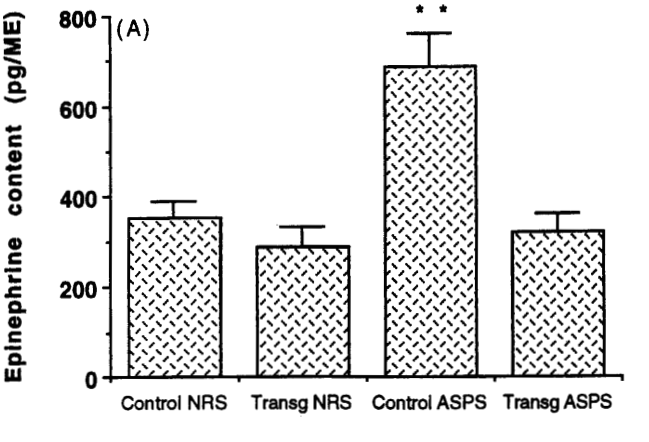


Figure 3. Norepinephrine content in the median eminence of (A) intact and (B) gonadectomized transgenic (overexpressing b-GH) and normal mice. Values are expressed as means ± SEM. $^{*}P < 0.05$ vs control NRS. $^{\#}P < 0.05$ vs trang NRS. $^{++}P < 0.01$ castrated vs intact normal.



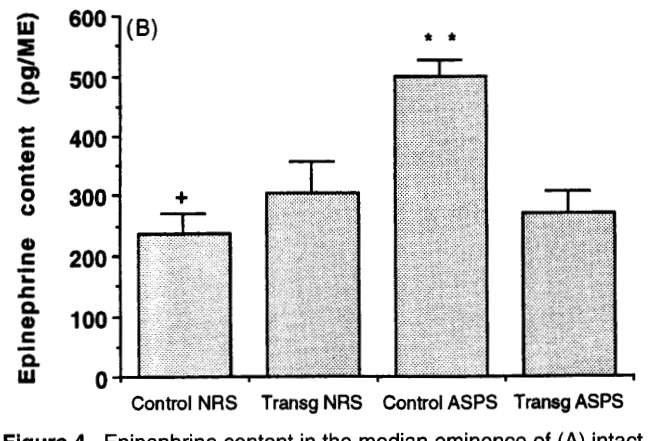


Figure 4. Epinephrine content in the median eminence of (A) intact and (B) gonadectomized transgenic (overexpressing b-GH) and normal mice. Values are expressed as means ± SEM. $^{**}P < 0.01$ vs control NRS. $^{+}P < 0.05$ castrated vs intact normal.

transgenic (Fig. 6A) and castrated normal mice whereas castrated transgenic mice were not affected (Fig. 6B).

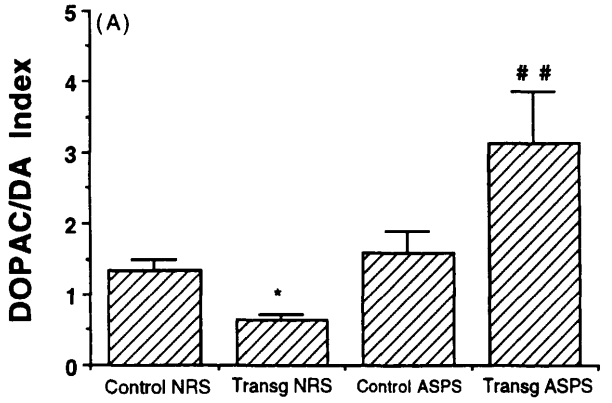
The hypothalamic NE content was considerably lower in intact transgenic than in intact normal mice (Fig. 7A vs B). Castration reduced NE content only in the normal animals (Fig. 7B). Treatment with SP-As produced a marked depletion of NE in the hypothalamus of normal intact mice (Fig. 7A) whereas it had no effect in intact transgenic animals. In contrast to these findings, administration of SP-As to gonadectomized males significantly increased hypothalamic NE content in both normal and transgenic animals (Fig. 7B).

The effects of bGH gene expression, gonadectomy, and SP-As administration to intact animals on the hypothalamic epinephrine content were similar to the corresponding effects on NE, namely, the epinephrine content was reduced in intact transgenic versus normal mice (Fig. 8A vs B), and reduced by castration or AP-As administration only in normal males (Fig. 8B). However, in contrast to its stimulatory effect on hypothalamic NE content, administration of SP-As did not affect the epinephrine content in castrated normal or castrated transgenic mice (Fig. 8B).

Plasma LH levels were higher in intact transgenic than in intact normal mice whereas plasma levels of FSH and PRL did not differ between these two groups (Table I). Castration led to the expected increase in plasma LH and FSH levels, but these changes were proportionally smaller in transgenic than in normal mice. Administration of SP-As did not affect plasma hormone levels except for a significant reduction in plasma LH levels in intact transgenic males (Table I).

Discussion

Results of the present study indicate that the endogenous blockade of SP led to a number of divergent effects on the hypothalamic neurotransmitter metabolism in MT-bGH transgenic as compared with normal mice. Evidence collected by different authors indicates that SP may have a role in the control of secretion or release of most of the



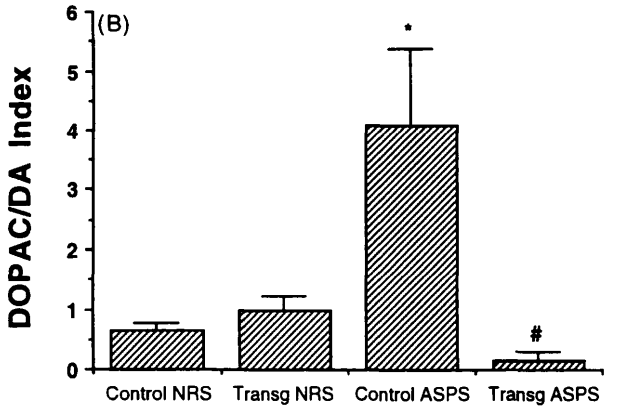
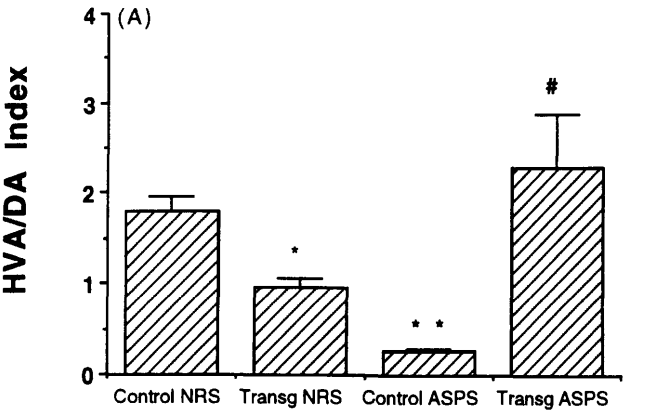


Figure 5. Ratio of DOPAC to DA contents (DOPAC/DA ratio) in the hypothalamus of (A) intact and (B) gonadectomized transgenic (overexpressing b-GH) and normal mice. Values are expressed as mean ± SEM. $^{*}P < 0.05$ vs control NRS. $^{\#}P < 0.05$ vs trang NRS. $^{\#\#}P < 0.01$ vs transg NRS.



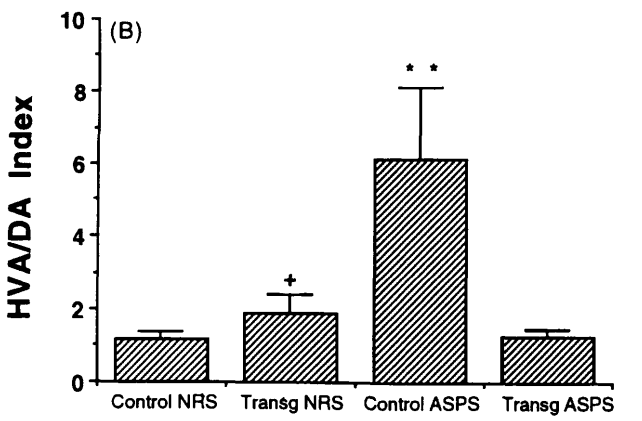
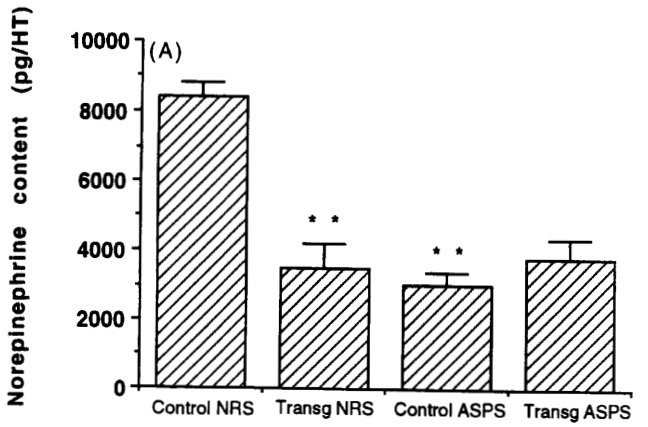


Figure 6. Ratio of HVA to DA contents (HVA/DA ratio) in the hypothalamus of (A) intact and (B) gonadectomized transgenic (overexpressing b-GH) and normal mice. Values are expressed as means ± SEM. $^{*}P < 0.05$ vs control NRS. $^{**}P < 0.01$ vs control NRS. $^{\#}P < 0.05$ vs trang NRS. $^{+}P < 0.05$ castrated vs intact normal.

anterior pituitary hormones (12). This role of SP seems to be exerted both at the hypothalamic and at the anterior pituitary level (12). Our present investigation shows that the blockade of endogenous SP, obtained by passive immunization, resulted in changes in the catecholamine profile in the hypothalamus of normal mice. Therefore, it is possible that some of the physiological effects induced by SP in the hypothalamus may be mediated through changes in catecholamine synthesis or release. It had been demonstrated already that SP was able to induce significant changes in neurotransmitters, like GABA, in the hypothalamus (22) and dopamine in the striatum (23, 24). In the hippocampus, SP was shown to stimulate acetylcholine release (25). However, the changes induced by the anti-SP serum in transgenic animals show that the endogenous secretion of high amounts of a heterologous GH is able to significantly affect the tachykininergic function in the hypothalamus of these animals.

We have no explanation for the absence of significant changes in plasma hormone levels in SP-As-treated animals. There is considerable evidence for the involvement of SP in the control of gonadotropin and PRL release (12) and the injected dose of SP-As must have interfered with the actions of endogenous SP because it produced a number of significant alterations in hypothalamic neurotransmitters. One of us had previously demonstrated that injection of SP-As in hyperprolactinemic rats resulted in a decrease of serum prolactin and LH levels (10–11). Perhaps a longer period of treatment or a larger number of animals would have been necessary to demonstrate any effect(s) of SP-As on plasma LH, FSH, or PRL levels. It is also conceivable that the effects of SP-As administration on the regions of the hypothalamus lacking blood-brain barrier may have counteracted its action(s) at the pituitary level. It is known that SP induces effects at the hypothalamic level that are different from those that the same peptide induces at the pituitary level (12). Differences in the responses of hypothalamic neurotransmitter function to experimental disturbance in these animals are consistent with previous findings concerning the influence of GH overexpression on the control of



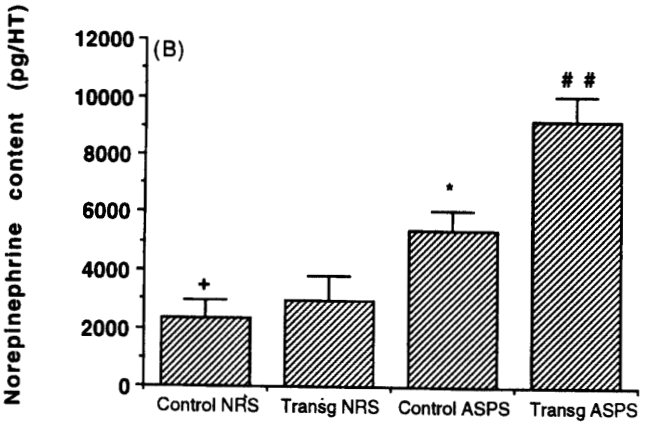
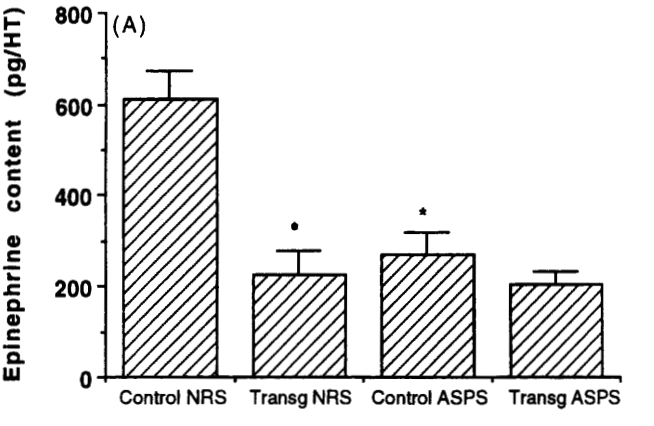


Figure 7. Norepinephrine content in the hypothalamus of (A) intact and (B) gonadectomized transgenic (overexpressing b-GH) and normal mice. Values are expressed as means ± SEM. $^{*}P < 0.05$ vs control NRS. $^{**}P < 0.01$ vs control NRS. $^{\#\#}P < 0.01$ vs trang NRS. $^{+}P < 0.05$ castrated vs intact normal.



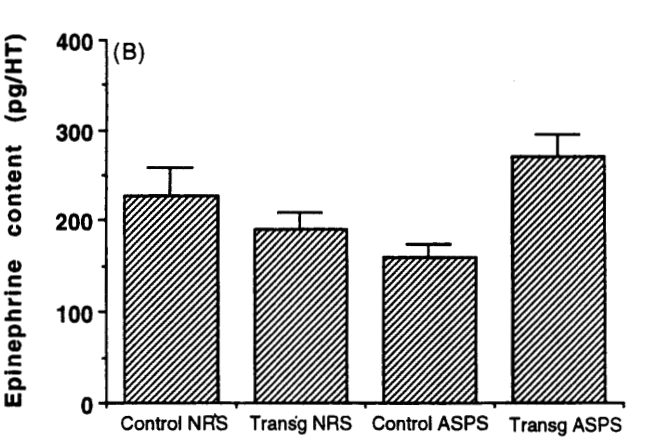


Figure 8. Epinephrine content in the hypothalamus of (A) intact and (B) gonadectomized transgenic (overexpressing b-GH) and normal mice. Values are expressed as means ± SEM. $^{*}P < 0.05$ vs control NRS.

adenohypophyseal hormone release. Thus, we have previously reported differential effects of treatment with antiserum to neuropeptide Y on plasma gonadotropin and PRL levels in transgenic versus normal males from the same line that was used in the present investigation (9). Studies in several lines of transgenic mice expressing bGH or hGH under control of different promoters provided numerous examples of altered responses of plasma LH levels to castration, testosterone replacement, and LHRH administration in transgenic versus normal animals (1–4). Moreover, in MT/hGH transgenic males, NE turnover in the median eminence was reduced after gonadectomy, a response opposite to that recorded in normal males from the same line (2).

Differences in the DOPAC/DA and HVA/DA ratios in the median eminence and in the remaining hypothalamic tissue in transgenic versus normal mice suggest that hypothalamic dopaminergic activity was reduced in transgenic males. Although this conclusion is not consistent with results obtained previously using a different method of estimating dopaminergic transmission (7), it agrees with earlier findings in transgenic females of the same line (7) and with the tendency of transgenic mice overexpressing bGH to exhibit hyperprolactinemia (3, 4, 7). A single dose of SP-As reduced DA activity in the median eminence of normal males but had little effect on their transgenic counterparts. The effects of SP-As on the employed measures of DA activity in the remaining hypothalamus were different in transgenic versus normal mice. Indeed, administration of SP-As altered DOPAC/DA index in castrated males and HVA/DA index in opposite directions in transgenic, as compared to normal males.

The content of NE and epinephrine in the hypothalamic tissue is presumably inversely related to NE activity because it was consistently reduced after gonadectomy of intact males. If this interpretation is correct, transgenic males exhibited increased NE activity in the hypothalamus but not in the median eminence, and failed to respond to castration by the expected increase in NE activity. We have previously reported increased NE turnover in the median eminence and

Table I. Effects of Anti-Substance P Antiserum Administration on Plasma Levels of PRL, LH and FSH in Control and Transgenic Mice Overexpressing b-GH in A) Intact or B) Castrated Adult Mice

A) Intact mice	LH ng/ml	FSH ng/ml	PRL ng/ml
Control normal	0.341 ± 0.136	17.49 ± 1.72	11.87 ± 1.8
Control transgenic	0.920 ± 0.221*	14.13 ± 1.41	8.33 ± 1.4
ASPS normal	0.558 ± 0.186	19.37 ± 1.11	10.75 ± 2.02
ASPS transgenic	0.573 ± 0.117	15.04 ± 1.12*	6.49 ± 1.08
B) Castrated			
Control normal	1.23 ± 0.508	36.72 ± 2.49	21.0 ± 2.8
Control transgenic	1.59 ± 0.28	28.15 ± 2.11*	16.81 ± 2.53
ASPS normal	2.020 ± 0.33	34.53 ± 1.15	14.76 ± 2.65
ASPS transgenic	1.157 ± 0.25	20.16 ± 1.317*	13.86 ± 1.43

* $P < 0.05$ vs control.

in the medial basal hypothalamus in transgenic versus normal males from the same line (7).

As was the case with DA activity, administration of SP-As produced major alterations in hypothalamic tissue content of NE and epinephrine in normal males and had little or no effect on transgenic animals (except for increased hypothalamic NE levels in castrated transgenic males).

In the present study, we have evaluated four different measures of neurotransmitter function in two different brain regions in intact and castrated males, for a total of 16 comparisons between normal and transgenic mice. In 12 of these 16 comparisons, the effects of SP-As differed between two groups, the difference typically involving a significant response in the normals and lack of response in the transgenics. This striking difference in the response of transgenic and normal mice to identical treatment extends our previous observations concerning the responses of the hypothalamic-adenohypophyseal system in MT/bGH and other GH transgenic mice to gonadectomy (2, 4, 21), gonadal steroid feedback (1, 4), LHRH (1, 4), and antiserum to neuropeptide Y (9). The mechanism(s) responsible for these effects of lifelong GH excess in transgenic animals remain to be elucidated. However, there is considerable evidence that both the hypothalamus and the pituitary are potential targets for the actions of GH and GH-induced increases in local or systemic IGF-I levels (13–15).

Hormonal findings in transgenic MT/bGH mice in the present study included elevation in plasma LH levels and no changes in plasma FSH or PRL. These results were not expected and may be related to age-dependent changes in adenohypophyseal hormone release in these animals. We have previously observed normal plasma LH and PRL levels in 11–24-week-old MT/bGH transgenic males (7), and significant suppression of plasma LH with concomitant hyperprolactinemia in 30–35-week-old transgenic males from the same line (9).

In summary, these experiments show that among the neuroendocrine alterations that the high levels of ectopic bGH induce in transgenic mice, the tachykininergic function is one of them. These results also suggest that some of the effects of the altered tachykinin function may be mediated through changes in the catecholamine synthesis or release in the hypothalamus. Thus, catecholamines may, to some extent, be mediators of some tachykinin effects.

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