Role of Neuromedin B in the *In Vitro* Thyrotropin Release in Response to Thyrotropin-Releasing Hormone from Anterior Pituitaries of Eu-, Hypo-, and Hyperthyroid Rats (43980)

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Abstract. **A role of neuromedin B (NB), a bombesin-like peptide, as an inhibitory paracrine/autocrine regulator of thyrotropin secretion has been suggested. We previously reported (10) that basal thyroid-stimulating hormone (TSH) release *in vitro* was decreased by NB and increased in the presence of a highly potent antiserum against NB (aNB). In these experiments, we studied the effects of NB (10^{-11}–10^{-7} *M*) and antiserum against NB (aNB, 1:2000 dilution) on basal TSH release and the response to thyrotropin-releasing hormone (TRH) (0.5×10^{-8} *M*) from incubated anterior pituitaries from eu-, hypo-, and hyperthyroid rats. As expected, in euthyroid rats NB decreased basal and TRH-stimulated TSH release, but only at the highest concentration tested (10^{-7} *M*). Incubation of the pituitaries from euthyroid rats with the antiserum against NB increased basal TSH release above that from glands of normal rabbit serum–incubated controls, as anticipated based on the concept that NB inhibits TSH release from the pituitary glands of euthyroid animals. The antiserum did not augment the response to TRH, suggesting that NB released in this situation, although suppressing basal release, had no effort on the stimulated release induced by TRH.**

Glands from hypothyroid rats had a slightly lower basal TSH release and decreased response to TRH than glands from euthyroid rats. They responded with a decrease in basal TSH release at a much lower concentration of NB (10^{-9} *M*) than pituitaries from euthyroid animals. Surprisingly, pituitaries from hypothyroid rats showed a paradoxical increased release of TSH in response to the lowest concentration of NB (10^{-11} *M*), which decreased with increasing concentrations and was not distinguishable from control release in the presence of TRH at the highest concentration of NB (10^{-7} *M*). We hypothesize that the increased responsiveness to the inhibition of basal TSH release by NB in the hypothyroid pituitaries may be related to an upregulation of NB receptors in this situation, in which the release of NB is diminished because of loss of feedback *via* thyroid hormones. The view that NB secretion was reduced in the hypothyroid situation was supported by the fact that there was no

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change in TSH release or the response to TRH following treatment with aNB in these animals.

Remarkably, in the glands from the hyperthyroid rats, although basal TSH secretion was significantly lower than that from euthyroid pituitaries and response to TRH was also decreased, NB (10^{-11}–10^{-7} *M*) instead of decreasing TSH release augmented it significantly. Also, the response to TRH was significantly augmented but only at the lowest concentration of NB tested (10^{-11} *M*). That NB was probably being secreted *in vitro* from the hyperthyroid pituitaries was indicated by an increased basal TSH release as well as a higher TSH medium concentration after TRH in the presence of the aNB. These results support the concept that the glands from the hyperthyroid animals secrete more NB because of positive feedback of thyroid hormones directly on the thyrotropes to increase NB synthesis and release which downregulates NB receptors on the gland. This downregulation of receptors in some manner reverses the inhibitory action of NB on basal and TRH-stimulated TSH release. In conclusion, the results provide further evidence for an important role of NB as an autocrine regulator of TSH release, which is modulated by increased release of NB induced by thyroid hormones.

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Neuromedin B (NB), a bombesin-like peptide, first isolated from porcine spinal cord (1), has been detected in several rat and human tissues, such as gut, pancreas, esophagus, and various brain regions, including the hypothalamus (2–5). However, the highest tissue NB concentration in the rat was found in the pituitary gland (4–7) as a result of local synthesis (8).

Our previous studies suggested a physiological role of NB in pituitary thyroid-stimulating hormone (TSH) secretion. NB decreased plasma TSH when injected into rats, either into the third cerebral ventricle or intravenously. The physiological nature of this effect was suggested by the increase in plasma TSH observed after the administration of a highly specific antiserum against NB (9). The inhibitory action of the peptide on TSH release was also observed on incubation of the peptide with anterior pituitaries, which indicates that NB acts at the pituitary level (9, 10). Furthermore, the incubation of pituitary glands with an antiserum against NB induced an increase in basal TSH release (10), which suggests that the peptide has a physiological role in autocrine regulation of TSH secretion.

Additionally, we previously reported (10) that altered thyroid states modified the responsiveness of the anterior pituitaries to NB and NB antiserum, both *in vivo* and *in vitro*. This could suggest that thyroid hormones modulate the action of NB on basal TSH secretion. In this paper, we report the influence of thyroid function on the effects of NB on thyrotropin-releasing hormone (TRH)–stimulated TSH release *in vitro*.

Materials and Methods

Animals. Male Sprague-Dawley rats ranging in weight from 220 to 250 g were purchased from Simonsen Laboratories (Gilroy, CA). The animals were housed in group cages in a room with controlled lighting (on from 0500 to 1900 hr) and temperature (22°–24°C). They had free access to rat chow and water.

Hypothyroidism was induced by giving the animals 0.05% propylthiouracil in the drinking water for 3 weeks. Control animals drank tap water. Hyperthyroidism was induced by giving the rats subcutaneous injections of thyroxine (T_4, 10 μg/100 g body weight, daily for 5 days). The control animals were given daily injections of saline. As previously demonstrated (10), these treatments produced the expected changes in plasma TSH, T_4, and triiodothyronine (T_3) concentrations.

***In Vitro* Experiments with NB.** After the treatment period described above, the eu-, and hypo-, and hyperthyroid animals were sacrificed by decapitation in separate experiments. Their trunk blood was collected, serum separated, and stored at −20°C until assayed. The pituitaries were quickly dissected out; the anterior was separated from the posterior pituitary and transected by a longitudinal midline cut. Each hemi–anterior pituitary was immediately transferred to a tube containing 1 ml of Krebs-Ringer bicarbonate medium, pH 7.4, and incubated at 37°C in an atmosphere of 95% O_2/5% CO_2 in a Dubnoff metabolic shaker (50 cycles/min). After a 30-min preincubation period, the medium was removed and the hemipituitaries were resuspended in 1 ml of medium alone (control) or medium containing NB (Peninsula Lab., Belmont, CA) to a final concentration of 10^{-11}, 10^{-9}, or 10^{-7} *M*. Each incubation tube contained one hemipituitary and 8–10 tubes were used for each group. At the end of a 1-hr incubation, an aliquot (50 μl) was removed for measurement of basal TSH, and then TRH (10 μl) was added to final concentration of 0.5×10^{-7} *M*. The incubation was continued for 30 min,

after which another aliquot was removed for the measurement of TSH.

***In Vitro* Experiments with the Antiserum against NB.** The same protocol was used for these experiments except that the incubation was performed in the presence of a highly specific antiserum against NB (2) or normal rabbit serum (NRS) both at a final dilution of 1:2000.

Radioimmunoassay. TSH was measured in the incubation medium with kits supplied by the National Institute of Diabetes and Digestive and Kidney Diseases and expressed in terms of the reference preparation (RP1) provided. Triiodothyronine and thyroxine were measured by radioimmunoassay as described before (11).

Statistics. Data were expressed as mean ± SEM. Analysis of variance followed by the Student-Newman-Keuls multiple comparison test was employed for the statistical analysis of all data. Some data was also tested by the Student's t test (see Results). $P < 0.05$ was taken as the level of significance.

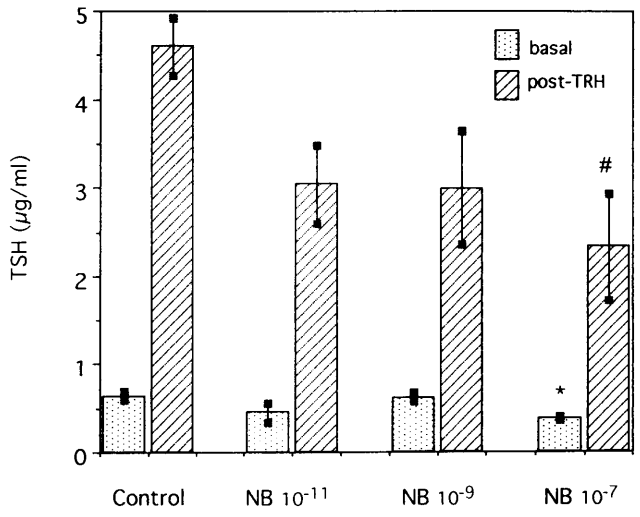

Figure 1. Basal and TRH-stimulated TSH release from isolated rat hemipituitary glands in the presence of neuromedin B (NB). Data are reported as means ± SEM. Glands were incubated in the absence (control) or presence of 10^{-11}–10^{-7} *M* NB. Medium TSH was measured before (basal) and after TRH (0.5×10^{-7} *M*). *$P < 0.05$% versus basal control; #$P < 0.05$% versus post-TRH control.[2]

Results

Euthyroid Rats. As we had shown before (9), TSH release from normal rat pituitary glands decreased in the presence of NB. The decrease was statistically significant ($P < 0.02$ at the concentration of 10^{-7} *M*) (Fig. 1). The amount of TSH released after TRH decreased in a dose-related manner with increasing NB concentrations, although the decrease reached statistical significance only at 10^{-7} *M* (Fig. 1) by analysis of variance. The decrement was significant ($P < 0.05$) by Student's t test with 10^{-9} and 10^{-11} *M* NB.

Hypothyroid Rats. The pituitaries from hypothyroid rats had a reduced basal TSH release and response to TRH than those of pituitaries from euthyroid animals (Fig. 2). The concentration of NB required for inhibition of TSH release was much lower (10^{-9} *M*) in the case of pituitaries from hypothyroid rats than in the case of pituitaries from euthyroid animals (10^{-7} *M*). Surprisingly, medium TSH values instead of being decreased by NB after TRH as in euthyroid rats were significantly increased, but only in the group incubated with the lowest concentration of NB (10^{-11} *M*) (Fig. 2).

Hyperthyroid Rats. Basal TSH release and the response to TRH from these pituitaries were lower than from glands of euthyroid rats. Basal TSH release from pituitaries of hyperthyroid rats were paradoxically increased at the three concentrations (10^{-11}—10^{-7} *M*) of NB tested (Fig. 3). Post-TRH TSH concentrations were also significantly higher in the presence of only the lowest NB concentration tested (10^{-11} *M*) (Fig. 3). The increment in TSH release from this dose of NB (10^{-11} *M*) was also significantly increased.

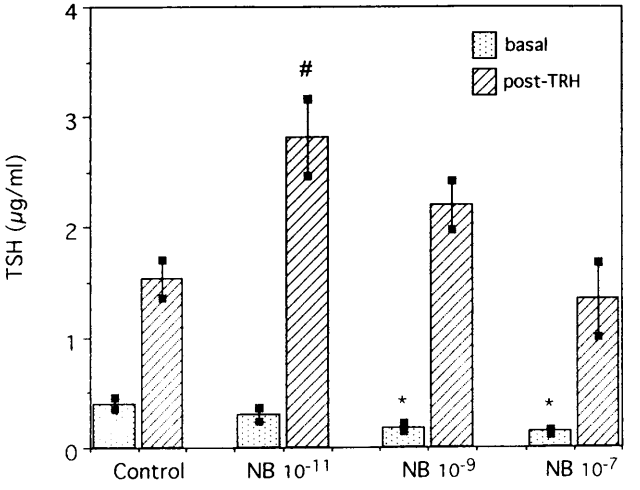

Figure 2. Basal and TRH-stimulated TSH release from isolated hemipituitary glands of hypothyroid rats in the presence of NB. Data are reported as means ± SEM. Glands were incubated in the absence (control) or presence of 10^{-11}–10^{-7} *M* neuromedin B (NB). Medium TSH was measured before (basal) and after TRH (0.5×10^{-7} *M*). *$P < 0.05$% versus basal control; #$P < 0.05$% versus post-TRH control.[2]

Studies with Antiserum against NB *Euthyroid Rats.* The presence of the antiserum against NB (aNB) resulted in a higher basal TSH release than that

[2]Data in the absence of TRH have been previously reported (10).

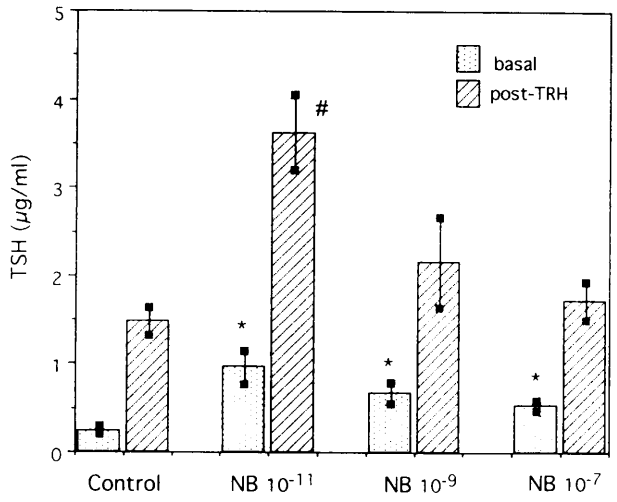

Figure 3. Basal and TRH-stimulated TSH release from isolated hemipituitary glands of hyperthyroid rats in the presence of NB. Data are reported as means ± SEM. Glands were incubated in the absence (control) or presence of 10^{-11}–10^{-7} *M* NB. Medium TSH was measured before (basal) and after TSH (0.5×10^{-7} *M*). $^{*}P < 0.05\%$; $^{\#}P < 0.05\%$ post-TRH control.[2]

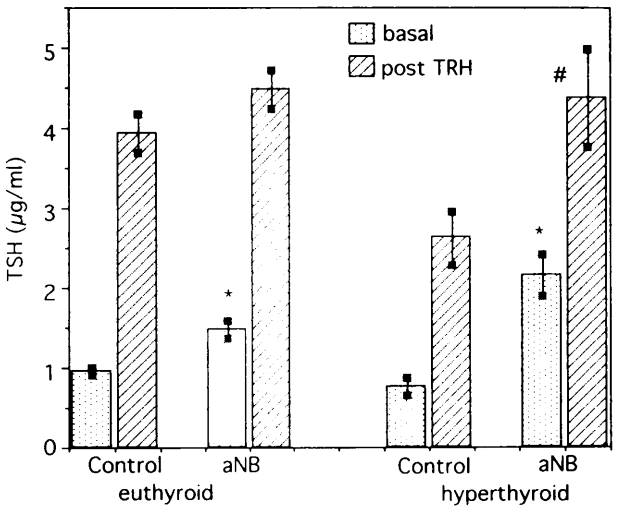

Figure 4. Basal and TRH-stimulated TSH release from isolated rat hemipituitary glands in the presence of the antiserum against NB. Data are reported as means ± SEM for glands obtained, in separate experiments, from euthyroid or hyperthyroid rats. Hyperthyroidism was produced by thyroxine treatment (10.0 μg/100 g body wt for 7 days). After 1-hr incubation in the presence of normal rabbit serum (control) or antiserum against NB (aNB), both at 1:2000 dilution, TRH (0.5×10^{-7} *M*) was added to medium and the incubation proceeded for 30 min. $^{*}P < 0.05\%$ versus basal control; $^{\#}P < 0.05\%$ versus post-TRH control.[2]

observed in the control group in the presence of NRS (Fig. 4). Medium TSH concentrations were increased to similar values after the addition of TRH in both NRS- and aNB-incubated groups (Fig. 4).

Hypothyroid Rats. In contrast to euthyroid pituitaries, the antiserum did not change the basal secretion compared with that of controls incubated with NRS (NRS-control: 0.79 ± 0.12 versus aNB: 0.88 ± 0.12 μg/ml). The response to TRH also was not modified significantly by incubation with antiserum expressed as the concentration of TSH after TRH (NRS-control: 3.12 ± 0.42 versus aNB: 2.97 ± 0.31 μg/ml).

Hyperthyroid Rats. Incubation of pituitaries from hyperthyroid animals with the antiserum against NB resulted in increased basal TSH release, as well as higher absolute TSH medium concentrations after TRH incubation (Fig. 4). However, the increment in TSH released by TRH was unmodified.

Discussion

The basal release of TSH was slightly decreased below that of control animals in both the hypothyroid and hyperthyroid rats, and the response to TRH was also reduced. Since *in vivo* the release of TSH is increased in hypothyroidism and decreased in hyperthyroidism and these changes are accompanied by greater and lesser responses to TRH respectively, at first sight it is difficult to explain the different results obtained *in vitro*. We believe that these are accounted for in the case of the hypothyroid glands by the fact that these glands have low pituitary content of TSH compared with normal pituitaries and would have little TSH stored to be released, so release would be dependent almost exclusively on newly synthesized TSH. The medium (Krebs-Ringer bicarbonate buffer) contains only glucose and no amino acids. Therefore, TSH synthesis is diminished and consequently release is less. This would also account for the lower responsiveness to TRH. In other experiments incubating hypothyroid glands with medium 199 (which contains amino acids) instead of Krebs, the glands had a greater basal TSH release and TRH responsiveness.

In the case of the pituitary glands from hyperthyroid animals, their TSH synthesis and release has been chronically suppressed by high levels of thyroid hormone. Therefore, they also have decreased storage and would release less TSH under resting and stimulated conditions, particularly in as much as the inhibitory action of thyroid hormones on TSH release would still be evident *in vitro*.

Considerable data from several studies suggest that NB, a bombesin-like peptide, is related to the regulation of TSH secretion. Various authors (4–7) agreed that, although it is present in several rat tissues, including the hypothalamus, the pituitary gland has the highest concentration of immunoreactive NB. The presence of the peptide in the pituitary gland is the result of local synthesis, as shown by the abundant NB messenger ribonucleic acid (mRNA) found in this tis-

[2]Data in the absence of TRH have been previously reported (10).

sue (8, 12). NB and TSH immunoreactivities have been shown to coexist in the same cell, which suggests that NB is produced in the thyrotropes (7). The functional role of NB in the regulation of the secretion of pituitary hormones was first addressed by Rettori *et al.* (9), who found a reduction in plasma TSH after a single injection of NB, either administered intravenously or into the third cerebral ventricle. It was also shown that the peptide acted directly on the pituitary since isolated glands incubated with NB secreted less TSH, in a dose-dependent manner. These results were confirmed by Tajima *et al.* (13), who showed that NB decreased the basal release of TSH from perifused rat pituitaries.

In the present study, we evaluated a possible interference of NB with TSH secretion induced by TRH, and the effect of different thyroid states on NB-induced altered responsiveness to TRH. NB decreased the basal as well as TRH-stimulated TSH release from isolated pituitaries of normal rats. The effect was significant only at the highest concentration tested (10^{-7} *M*). These results are in contrast to those reported earlier by Rettori *et al.* (9), who found that the amount of TSH released by TRH was not changed by NB. The differing results could be related to the fact that here NB was incubated with the glands for 60 min before TRH was added, while in the previous work (9) both peptides were added together. Tajima *et al.* (13), who also preincubated rat pituitaries with NB, reported that NB at 10^{-7} and 10^{-5} *M* decreased TRH-stimulated TSH release. It is possible that time is required for NB to diffuse into the tissue to occupy its receptors in order for it to modify the amount of TSH released in response to TRH.

Comparing the results of normal with those of hypo- and hyperthyroid glands, it is clear that the thyroid status of the animals modified the pituitary responsiveness to NB. As we had shown before (10), pituitaries from hypothyroid animals were more sensitive to the inhibitory action of exogenous NB on basal TSH release, since this effect was observed at a 100-fold lower concentration of NB (10^{-9} *M*) than in the euthyroid group. It has been reported that hypothyroid rats had diminished synthesis of NB, as shown by decreased pituitary NB mRNA (11), as well as reduced pituitary NB concentration (8, 11), and, although not demonstrated directly yet, NB release is probably reduced. Reduced NB release in hypothyroidism is supported by the failure of aNB to increase TSH release *in vitro* from pituitaries of hypothyroid rats. Since we hypothesized before (10) that NB inhibits TSH release by an autocrine action on its postulated receptors on the thyrotropes and that thyroid hormones would stimulate synthesis and release of NB as a mechanism for their negative feedback on TSH release (10), in their absence in hypothyroidism, the reduced NB release may bring about up regulation of NB receptors and increased sensitivity to the TSH release-inhibiting action of NB.

Pituitaries from hyperthyroid rats incubated with NB showed an apparently paradoxical increase in both basal TSH release and in responsiveness to TRH demonstrable at 10^{-11} *M* and both inversely correlated to the concentration of NB (Fig. 3). The reason for the altered responsiveness in hyperthyroid glands is not known. It has been shown that hyperthyroid rats had an increment in pituitary NB content and a slight increase in the NB mRNA abundance (8, 11) when compared with those of euthyroid animals. Furthermore, increased release of NB from glands of hyperthyroid rats was indicated by the marked increase in basal and TRH-stimulated TSH release in the presence of aNB. We speculate that hyperthyroid glands secrete more NB because of positive feedback of thyroid hormones directly in the thyrotropes to increase NB synthesis and release, which downregulates NB receptors on the gland. This downregulation of receptors in some manner reverses the inhibitory action of NB on basal and TRH-stimulated TSH release.

We may be able to explain the paradoxical hyperresponsiveness to TRH of both hypo- and hyperthyroid pituitaries in the following way. We speculate that with increasing thyroid hormone levels, the release of NB from the pituitaries increases in proportion to the thyroid hormone concentration, whereas at the same time the number of NB receptors decreases in inverse proportion to the concentration of thyroid hormones. We then postulate that the response to TRH is related to the ratio of the bound to total NB receptors, such that at low thyroid hormone levels in the hypothyroid animals with high receptor density and low NB release, the response to TRH is augmented. It then declines to reach a minimum at euthyroid hormone concentrations as the bound to total ratio increases. With high thyroid hormone levels in the hyperthyroid rats resulting in downregulation of NB receptors, and an increased ratio of bound to total NB receptors, the response to TRH is again enhanced to produce a U-shaped curve of responsiveness to TRH as observed here. Obviously, further work is required to substantiate this hypothesis by measuring the effect of various thyroid hormone levels on NB receptor density and NB release. This is the subject of further experiments.

In conclusion, altered thyroid states modified NB effects on basal and TRH-stimulated TSH release *in vitro*. Although exogenous NB inhibited basal and TRH-stimulated TSH release only at relatively high concentrations (10^{-7} *M*) from normal pituitaries, it inhibited basal release and increased the responsiveness

to TRH in hypothyroidism, at a much lower concentrations (10^{-9} and 10^{-11} *M*, respectively). In hyperthyroidism, there was a paradoxical increase in both basal and TRH-stimulated TSH release observable at 10^{-11} *M* NB, which decreased with increased concentrations of the peptide. Incubation of the glands with aNB revealed that NB within the pituitary was inhibiting TSH release from gland of both euthyroid and hyperthyroid but not hypothyroid rats.

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