Thyrotropin Secretagogues Reduce Rat Pituitary Neuromedin B, a Local Thyrotropin Release Inhibitor

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Neuromedin B (NB), a bombesin-like peptide, highly concentrated in rat pituitary gland, has been shown to act as an autocrine/paracrine inhibitor of thyrotropin (TSH) release. Here it is shown that a single injection of thyrotropin-releasing hormone (TRH, 1.5 μg/animal, ip), the most important stimulator of thyrotropin secretion, induced approximately 35%–45% decrease in pituitary NB content in rats, as well as an important decrease in NB mRNA at 15 and 30 min ($P < 0.05$). Acute cold exposure, which induced higher serum TSH with a peak at 30 min, was associated with progressive decrease in pituitary NB, starting at 15 min although only reaching statistical significance after 2 hr ($P < 0.05$). Although not involved in the early peak, the decrease in NB may be contributing to maintenance of higher serum TSH in cold-exposed animals compared with those at room temperature. Fed rats, 2 hr after being subcutaneously injected with mouse recombinant leptin (8 μg /100 g body wt), showed a ×2 increase in serum TSH and 38% reduction in pituitary NB ($P < 0.05$). In conclusion, TRH and leptin rapidly decreased pituitary NB and it is first proposed that the reduction of the inhibitory tonus of NB on TSH release will ultimately contribute to the amplification of TSH secretion elicited by TSH secretagogues. Exp Biol Med 228:1083–1088, 2003

Key words: thyrotropin; neuromedin B; leptin; TRH; cold; autocrine regulation

Neuromedin B (NB) is a mammalian peptide structurally and functionally related to the amphibian peptide, bombesin. NBs, like other members of the bombesin family, have a wide spectrum of biological activity (1). NB was first isolated from porcine spinal cord (2) and it has been detected in several rat and human tissues, mainly the central nervous system, pituitary gland, and gastrointestinal tract (3, 4). However, in the rat, the highest NB concentration was found in the pituitary gland (3, 5), probably as a result of local synthesis, as demonstrated by the presence of relative abundance of NB mRNA (6). Within the rat anterior pituitary gland, the peptide was localized by dual immunocytochemistry in thyrotropes, which suggests that NB and thyrotropin (TSH) are produced by the same cell type (5). NB has an inhibitory action on TSH secretion both *in vitro* and *in vivo* at physiological doses (7, 8). Moreover, immunoneutralization of NB induced an increase in TSH release not only when the antiserum was injected into the third cerebral ventricle of normal rats but also when it was present in the incubation medium of rat isolated pituitaries (7, 9). Collectively, the data suggest that NB acts as a constitutively autocrine regulator that maintains an inhibitory tonus on TSH release. In addition, several pieces of evidence suggest that NB expression is increased by thyroid hormones and the peptide may participate in thyroid-hormone suppression of TSH release (5, 6, 9–12). Hormone analogs, such as the long-acting somatostatin analog octreotide that inhibits TSH secretion, or situations in which TSH release is impaired, such as experimental fasting and diabetes mellitus were associated with increment in pituitary NB (13, 14). Therefore, we had postulated that the increase in NB induced by TSH inhibitors contributes to their action on TSH release.

Thyrotropin-releasing hormone (TRH) is the most relevant physiological stimulator of TSH release and therefore we reasoned that if NB is a tonic inhibitor of physiological

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relevance, TRH might diminish its content in the gland. In addition, during acute cold exposure there is activation of the hypothalamic-pituitary-thyroid axis (15) with an increase in TRH and TSH release. Also, leptin, the satiety factor produced mainly in the adipose tissue, has been shown to stimulate directly or indirectly TRH production and release (16–19). Recently, we have shown that leptin acutely increased serum TSH after being injected into normal rats (20).

Therefore, the goal of the present study was to investigate whether TSH secretagogues such as TRH, leptin, and cold exposure are able to decrease pituitary NB.

Materials and Methods

Animals. Adult male Wistar rats, weighing 250–300 g, were maintained in a room with controlled lighting (12-hr light/dark cycle, lights on at 7 AM) and temperature (23°–26°C). The animals had free access to food and tap water. Our institution's animal care committee approved all treatments, and all the procedures were in compliance with the International Guiding Principles for Biomedical Research Involving Animals from the Council for International Organizations of Medical Sciences (Geneva, Switzerland).

Acute Effect of TRH Administration on Pituitary NB Expression. Rats received a single intraperitoneal injection of saline (control) or TRH (Sigma, St. Louis, MO) 1.5 μg/animal, 15 or 30 min before sacrifice. Animals were sacrificed by decapitation, and their anterior pituitaries were taken and immediately frozen in liquid nitrogen until processed to measure NB content or NB mRNA. Four to five animals per group were used in two independent experiments for NB content and in three independent trials for mRNA evaluation.

Effect of Acute Cold Exposure on Pituitary NB Expression. Rats, housed in individual cages, were placed in a 4°C room 15, 30, 60, or 120 min before they were sacrificed by decapitation in the cold room. A group of animals was kept at room temperature and used as the control group. Their anterior pituitaries were taken and immediately frozen in liquid nitrogen until processed to measure NB content. Four animals per group were used in three independent experiments.

Effect of Acute Leptin Administration on Pituitary NB Expression. Rats were divided into three groups that received a single subcutaneous injection of 8 or 16 μg/100 g body wt of mouse recombinant leptin (National Pituitary Hormone Program, NIH, Torrance, CA) or 0.2 ml saline vehicle (control group). The rats were sacrificed by decapitation 30 or 120 min after the injection. Anterior pituitaries were immediately frozen in liquid nitrogen until processed to measure NB content. Three to four animals per group were used in three independent experiments.

Quantification of Anterior Pituitary NB. NB was measured as described before (11). Each anterior pituitary was homogenized in 500 μl of 500 m*M* acetic acid. NB was extracted by boiling (100°C) homogenates for 15 min. The extracts were centrifuged at 1600*g*, 4°C for 15 min, and the supernatants were stored at −20°C until assayed. NB concentrations were measured by radioimmunoassay (RIA) using a highly specific antiserum as previously described (21). N-terminal-tirosylated NB-10 (American Peptides, Sunnyvale, CA) was labeled with ^{125}I (Amersham, Buckinghamshire, England) by conventional chloramine-T oxidation method (11). Labeled NB was purified using carboxymethyl-cellulose-52 (microgranular, preswollen, Sigma, St. Louis, MO) cation-exchange chromatography. The samples were assayed in duplicate. All samples from each experiment were measured within the same assay. Within-assay variation was 5.3% and the coefficient of variation between assays was 8.6%. The minimum detectable level was 8 fmol/100 μl.

NB values were expressed as concentration corrected for the total protein in the gland (fmol per mg protein). Pituitary protein content was determined by the Bradford method (22).

Ribonuclease Protection Assay. Total pituitary RNA was extracted from four pooled anterior pituitaries, using a commercial kit (Quickprep, Amersham). Samples of total RNA were stored in liquid nitrogen until assayed. The full-length rat NB cDNA (6) cloned in pGem-2 (Promega, Madison, WI) (kindly supplied by Dr. James Battey, National Institutes of Health) was linearized with *Eco*RI, digested with proteinase K, and extracted once with phenol/chloroform. Rat NB cDNA and rat β-actin cDNA (Ambion, Austin, TX) were used as templates for antisense RNA synthesis. The templates were transcribed *in vitro* with T7 RNA polymerase and α^{32}P-UTP 800 Ci/mmol (Amersham) using the protocol described by the manufacturer (Maxiscript kit, Ambion). The antisense RNA was separated from unincorporated nucleotides on a quick spin, Rnase-free, Sephadex G-50 column (5-prime-3-prime, Inc., Boulder, CO).

Both probes were evaluated with 10 μg of yeast tRNA in the presence of RNase A and T1 following the ribonuclease protection assay kit (RPA II, Ambion) protocol. Radiolabeled antisense NB (2×10^5 cpm/sample) and β-actin (1×10^4 cpm/sample) probes were mixed with 40 μg of total pituitary RNA. The β-actin probe was used to control for loading. Samples were hybridized for 18 hr at 42°C. Then, RNase A and T1 were added. Analysis of protect fragments was made in 8 *M* urea/5% polyacrylamide gels, which were dried and exposed to Kodak XAR-5 film in cassettes with intensifying screens at −70°C. Bands corresponding to protected fragments were evaluated by densitometry (NIH Image, the National Institutes of Health).

Quantification of Serum TSH, T4, and T3. In the experiments described previously, trunk blood samples were centrifuged, and serum was stored at −20°C for T4, T3, and TSH determinations by specific RIAs. TSH was determined with reagents supplied by the National Institute of Diabetes, Digestive and Kidney Diseases (Torrance, CA), as previously described (11), and was expressed in terms of the reference preparation (RP3). Within-assay variation was

7.9%, and the coefficient of variation between assays was 6.7%. Minimum assay detection was 0.52 ng/ml.

Thyroxin and triiodothyronine were also measured by RIA using specific antiserum (Sigma, St. Louis, MO). For the T4 assay, within-assay variation was 8.9% and the coefficient of variation between assays was 7.2%. For T3, within-assay variation was 10.6% and the coefficient of variation between assays was 9.2%. The limit of detection was 0.49 μg/dl for T4 and 0.64 ng/dl for T3.

Statistical Analysis. Data are reported as mean ± SEM. One-way analysis of variance (ANOVA) followed by Student-Newman-Keuls multiple comparisons test was employed for assessment of significance of all data except for serum TSH and for leptin experiment. Serum TSH was analyzed by ANOVA only after logarithmic transformation (23), and for the leptin experiment two-way ANOVA test was used. Differences were considered to be significant at $P < 0.05$.

Results

Acute Effect of TRH Administration on Pituitary NB. Within 15 min after a single injection of TRH (1.5 μg/rat) into normal rats, pituitary NB content was significantly ($P < 0.05$) decreased (Fig. 1A). Low levels were maintained at 30 min ($P < 0.05$). The same profile was seen for NB mRNA abundance. There is no statistical difference in NB mRNA levels between 15 and 30 min (Fig. 1D). The groups sacrificed 15 and 30 min after TRH injection showed high serum TSH concentrations (Fig. 1B), and the peak was reached at 15 min, and as expected, no change in serum T4 and T3 was observed (Fig. 1C).

Effect of Acute Cold Exposure on Pituitary NB Content. Cold exposure induced a progressive decrease in pituitary NB content (Fig. 2A), starting at 15 min and reaching statistical significance after 2 hr ($P < 0.05$). Serum TSH was elevated by cold exposure, reaching a peak at 30 min (Fig. 2B). Serum T3 was higher in the cold-exposed animals at 2 hr, whereas serum T4 did not change (Fig. 2C).

Acute Effect of Leptin Administration. The administration of 8 μg/100 g body wt leptin induced an approximately ×2 increase in serum TSH in rats sacrificed 2 hr after a single injection (C = 1.35 ± 0.26, 30 min = 0.87 ± 0.11, 120 min = 2.76 ± 0.39* ng/ml, *$P < 0.05$) as well as a 35% decrease in pituitary NB content ($P < 0.05$; Fig. 3). No significant effect was observed earlier, at 30 min. The higher dose of leptin (16 μg/100 g body wt) was not able to significantly change either serum TSH (30 min = 0.99 ± 0.19, 120 min = 1.48 ± 0.19 ng/ml) and pituitary NB (Fig. 3).

Discussion

It had been previously reported that pituitary NB content increased after 12 days' treatment of normal rats with TRH (5). However, that result can be attributed to the hyperthyroidism induced by the chronic TRH treatment. Using an acute protocol and making the measurements shortly after TRH administration, we were able to avoid changes in serum thyroid hormones, and therefore, for the first time, we demonstrated the direct effect of TRH, which is a marked reduction in NB content and mRNA. Although there is no evidence, it cannot be ruled out that TSH itself, rather than TRH directly, would be modulating NB. Previously (11), it

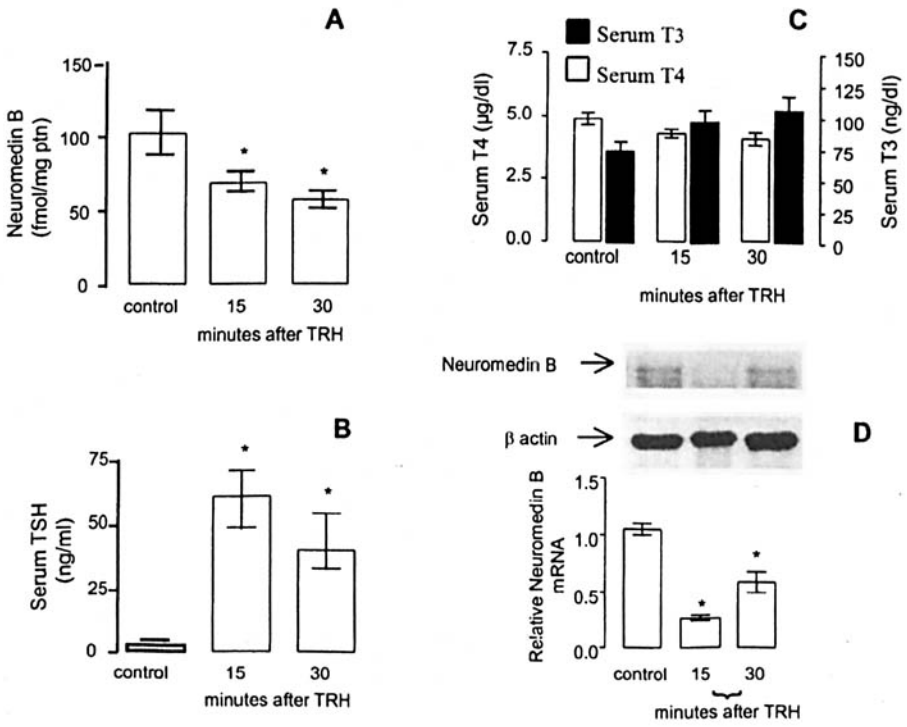

Figure 1. Pituitary NB content (A), serum TSH (B), T4 and T3 (C), and NB mRNA (D) of rats that received injections once, intraperitoneally, with TRH (1.5 μg/rat) 15 or 30 min before sacrifice. Data in panel D represent the ratio of arbitrary densitometer units of NB mRNA to β-actin mRNA. Data are reported as mean ± SEM for 8–10 animals per group (A–C) and for 12 animals per group (D). *$P \leq 0.05$ versus control (vehicle injected).

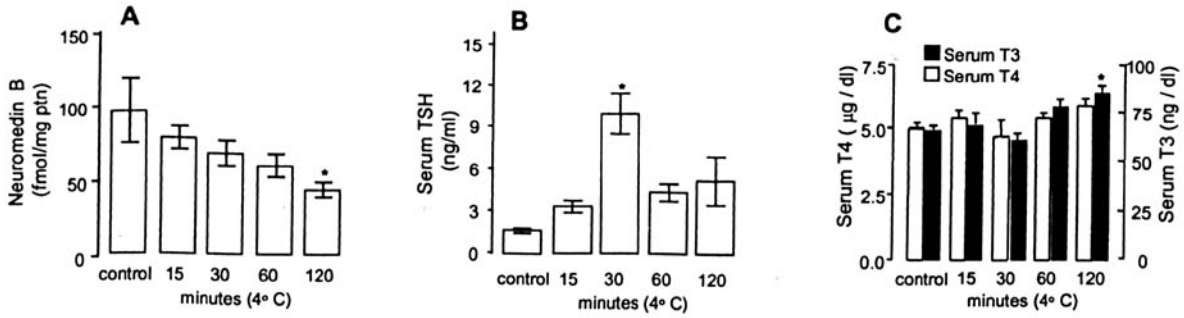

Figure 2. Pituitary NB content (A), serum TSH (B), and T4 and T3 (C) of normal rats placed in a 4°C room 15, 30, 60, or 120 min before sacrifice. Data are reported as mean ± SEM for 9 animals per group. $^*P < 0.05$ (or less) versus control (room temperature).

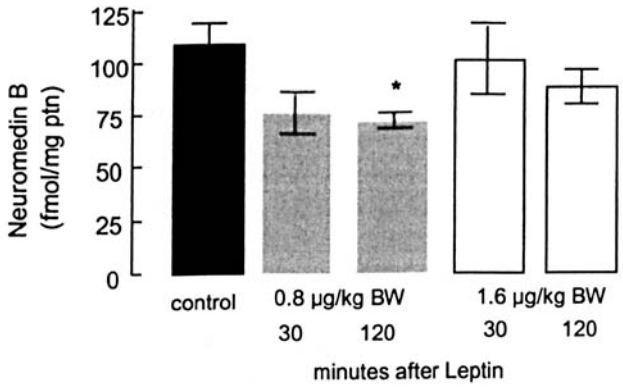

Figure 3. Pituitary NB content after a single injection of leptin (8 µg/100 g body wt [BW], gray bars; or 16 µg/100 g BW, open bars) after 30 min and 2 hr. Data represent means ± SEM for 8 animals per group. * $P < 0.05$ versus control (vehicle injected).

has been demonstrated that thyroxine injected into rats induced an increase in pituitary NB that preceded the reduction in serum TSH, which argues against the possibility that TSH modulates directly NB.

The decrease in pituitary NB mRNA after TRH administration is remarkably fast, which may suggest increased degradation of NB mRNA. The ability of TRH to degrade mRNA was previously demonstrated for the TRH receptor mRNA transfected rat pituitary GH3 cells (24). It has been proposed that TRH may increase RNAse activity and induce destabilization of TRH receptor mRNA (25).

The mechanism explaining the reduced pituitary NB content cannot be elucidated by the present data. The early and marked decrease in NB mRNA suggests that TRH is acting in the direction of reducing the peptide synthesis. However, at such short times other mechanisms related to release or degradation are likely to be mainly responsible for the decrease in peptide content. Regardless of the mechanism, it is possible that the negative regulation of pituitary NB by TRH will ultimately lead to amplification or prolongation of TRH action on TSH release.

The fact that pituitary NB is a target of the main regulators of TSH secretion, thyroid hormones, as previously demonstrated (5, 11), and TRH, as demonstrated here, highlights the potential physiological importance of the local TSH-inhibitory action of the peptide.

In acute cold exposure experiments we observed a gradual, progressive, time-dependent decrease in NB pituitary content. However, the early peak of serum TSH at 30 min occurred when the decrease in NB content was not statistically significant, which suggests that at this time point NB modulation by cold-related factors, including TRH, is not a major mechanism leading to TSH secretion. However, the hypothesis that at 2 hr the decreased pituitary NB content may contribute to the sustained and higher rates of TSH secretion observed during acute cold exposure would be physiologically coherent. This hypothesis must be further tested.

The mechanisms leading to the reduction of pituitary NB during cold exposure cannot be elucidated by the present study. The early activation of the thyrotropic axis during acute cold exposure has been attributed to increase in the release of hypothalamic TRH (26–29). However, although a precise definition of the time course of TRH release after animals were exposed to low temperature is difficult to define from the different studies, the peak release of TRH is reported to be earlier than 2 hr when NB is significantly reduced. Considering that the decrease of NB seems to start earlier, it remains possible that it represents a latter effect of TRH on the pituitary. In addition, the amount of TRH released during cold exposure in relation to the amount reaching the pituitary after TRH injection is another variable that may influence the results. Therefore, it remains to be established whether the effect of TRH on NB is physiological or pharmacological.

Surprisingly, after 2 hr of cold exposure low levels of pituitary NB coexist with a slight, but significant, increase in serum T3 (Fig. 2). This suggests that at this time point, the direct inhibitory effect of cold-related factors overrode the thyroid hormone–stimulatory effect on NB expression (11). Previous studies had reported that in cold-exposed animals over a 24-hr period, there was also a late rise in serum TSH (24 hr) by unknown mechanisms, in the presence of a 2-fold increment in T3 (15, 19). The persistence of decreased pituitary NB during cold exposure might contribute to this late increase in TSH release.

It is of interest that both NB and TRH are involved in the modulation of central control of body temperature (29,

30), having opposite effects: TRH stimulates thermogenesis (29), whereas NB has hypothermic effects when centrally administered to rodents (8, 30, 31). This would be physiologically coherent with their antagonism at TSH release (5, 6, 11), because thyroid hormones are essential for cold-induced thermogenesis.

Leptin stimulated TSH release and was also able to decrease pituitary NB content. As leptin has a direct effect increasing TRH synthesis (16), we do not know whether leptin is decreasing NB directly, or is acting through TRH increase, or both. A direct pituitary effect seems unlikely because we had previously shown that leptin inhibits TSH release from incubated pituitaries (20). A double dose of leptin that did not change serum TSH was also not able to change NB. The lack of effect of the higher dose of leptin may be consequent to secondary actions of leptin induced by the higher but not the lower dose. Other authors studying the adrenal-pituitary axis (32), employing the same leptin doses we used here, observed similar lack of effect of the higher dose, and the authors also did not have an explanation for that phenomenon.

In conclusion, the pituitary content of NB is reduced by acute injections of TRH, leptin, and cold exposure, and TRH also reduced NB mRNA. We propose that the decrease in the inhibitory local action of pituitary NB on TSH release is an important mechanism induced by TSH secretagogues, which ultimately may contribute to facilitating their action.

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