Endocrine, *vol. 19, no. 3, 313–317, December 2002* 0969–711X/02/19:313–317/$20.00

Inhibition of Prolactin Secretion from the Male Rat Anterior Pituitary by Cryptic Sequences of Prothyrotropin Releasing Hormone, ProTRH$_{178–199}$ and ProTRH$_{186–199}$

Thomas H. Alexander,[1] Robert J. Handa,[2] and Robert F. McGivern[1]

[1]Department of Psychology, San Diego State University, San Diego, CA 92120; and [2]Department of Biomedical Sciences, Colorado State University, Ft. Collins, CO 80523

Previous studies have shown that intronic peptide sequences in the prohormone for thyrotropin-releasing hormone (TRH) have physiological actions on pituitary hormone secretion. The aim of this investigation was to examine the effect of the cryptic peptides, prothyrotropin-releasing hormone$_{178–199}$ (ProTRH$_{178–199}$) and ProTRH$_{186–199}$, on prolactin (PRL) release from the anterior pituitary. Perifusion studies were performed with anterior pituitaries obtained from individual adult male Sprague–Dawley rats at 70–90 d of age. Perifusate was collected in 5-min fractions for 25 min prior to peptide administration and for 60 min afterward. Pituitaries were perifused with a single 5 min pulse of either 2, 10, or 40 n*M* concentrations (peak pulse) of each peptide or the vehicle. Sixty minutes after peptide administration, a 200 m*M* pulse of potassium chloride was delivered to check tissue viability. Prolactin was measured in the perifusate by radioimmunoassay. Results showed that both peptides induced a significant long-term suppression of prolactin secretion that was still evident at 60 min after peptide exposure. ProTRH$_{186–199}$ was similar to ProTRH$_{178–199}$ in suppressing prolactin release at the 2 and 40 n*M* dose, suggesting that the amino acid sequence necessary for prolactin inhibition is contained within the smaller peptide fragment. These data indicate that a cryptic sequence within the proTRH peptide can have biological activity at the level of the anterior pituitary gland in regulating prolactin secretion.

Key Words: Prolactin; prothyrotropin-releasing hormone; perifusion; anterior pituitary.

Introduction

Prothyrotropin-releasing hormone (ProTRH) is a 255 amino acid prohormone that contains five TRH progenitor and seven or more biologically active cryptic sequences following posttranslational proteolytic cleavage *(1)*. Immunocytochemical studies demonstrated an overlapping, but differential, distribution for TRH and several of the cryptic peptides with ProTRH in brain, suggesting that differential posttranslational processing of ProTRH may occur in the CNS *(2–6)*. Subsequent work showed that cryptic ProTRH sequences found in brain and spinal cord are processed from the prohormone, packaged in secretory granules, subject to axonal transport, and secreted from axon terminals *(7,8)*.

Important posttranslational processing of ProTRH occurs in part through the actions of the prohormone convertases (PCs) PC1 and PC2 *(9,10)*. The differential distribution of these enzymes in the CNS, in combination with their separate endoproteolytic actions at multiple ProTRH sites *(11)*, suggest that ProTRH may serve as a multifunctional biosynthetic precursor similar to other prohormones such as proopiomelanocortin and proenkephalin *(12)*.

The biological importance of TRH is well established as both a regulator of thyroid-stimulating-hormone (TSH) release and a neuromodulator of several neurotransmitter systems, as well as behavior *(13,14)*. A growing body of evidence also indicates that the cryptic peptides may regulate physiological functions at the level of the CNS. Both ProTRH$_{160–169}$ and ProTRH$_{178–199}$, the cryptic sequences flanking the fourth TRH progenitor sequence within ProTRH, have been shown to possess biological actions both in vitro and in vivo. Peripheral administration of ProTRH$_{160–169}$ to rats enhances TSH release by TRH and increases TSHβ gene promoter activity *(15–16)*. In addition, ProTRH$_{160–169}$ potentiates gastric secretion induced by TRH injection into the dorsal motor nucleus of the vagus *(17)*. A receptor for ProTRH$_{160–169}$ has been described in rat brain, pituitary and peripheral tissue, as well as in a neuroblastoma cell line *(18,19)*, but a receptor for ProTRH$_{178–199}$ has yet to be identified. However, the ProTRH$_{178–199}$ peptide is biologically active based on studies showing that it inhibits the secretion of adrenocorticotropin (ACTH), growth hormone, and prolactin *(20–23)*.

The rationale for the present experiments was based on in vivo evidence that ProTRH$_{178–199}$ inhibits prolactin secre-

Received October 3, 2002; Revised November 11, 2002; Accepted November 13, 2002.

Author to whom all correspondence and reprint requests should be addressed: Robert F. McGivern, 6330 Alvarado Rd, 207, San Diego State University, San Diego, CA 92120. E-mail: mcgivern@sunstroke.sdsu.edu

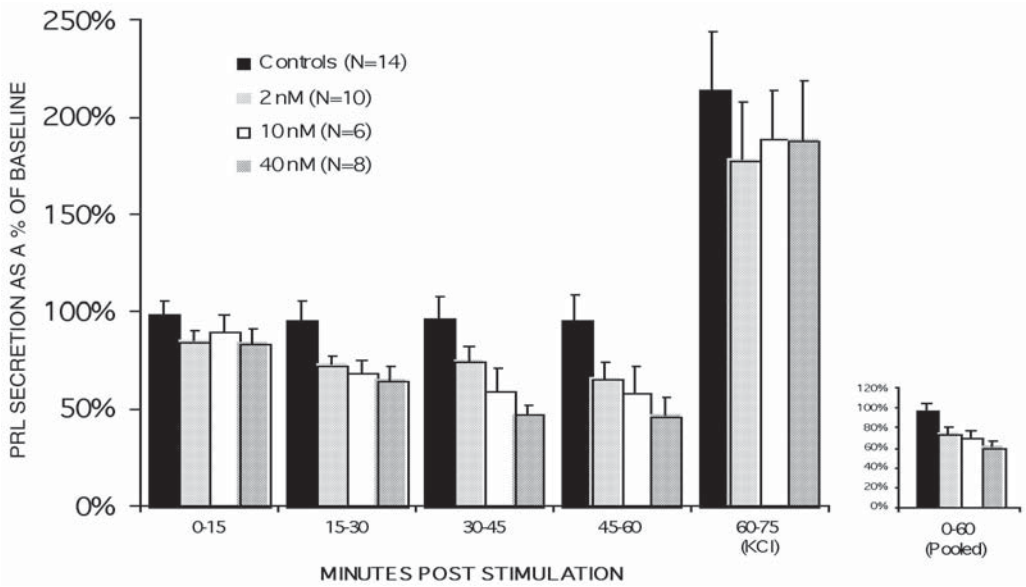

Fig 1. Percentage change in PRL secretion over 60 min from anterior pituitary following administration of a pulse of $ProTRH_{178–199}$. A similar pulse of KCl (200 n*M*) was delivered at the end of 60 min. Each bar represents the mean ± SEM of 15 min averages for 6–14 glands. The mean (±SEM) of all samples collected over the 60 min is shown on the right. $ProTRH_{178–199}$ induced a significant, dose-related suppression of prolactin secretion compared to medium alone.

tion when male rats were administered the peptide intravenously 5 min prior to restraint stress *(23)*. In the present study, we employed an in vitro superfusion system to examine whether the inhibitory actions of $ProTRH_{178–199}$ on prolactin release occurs at the level of the anterior pituitary. In addition, we studied the effects of $ProTRH_{186–199}$ in this system based upon recent evidence that posttranslational processing of $ProTRH_{178–199}$ by PC2 generates this additional bioactive peptide, $ProTRH_{186–199}$ *(24)*.

Results

When compared to the baseline period, the administration of a single pulse of $ProTRH_{178–199}$ (Fig. 1) and $ProTRH_{186–199}$ (Fig. 2) caused significant decreases in prolactin levels within the perifusate that lasted up to 60 min for the highest and lowest doses tested (40 n*M* and 2 n*M*) following the initial onset of the pulse. Similar time effects were observed for the 10 n*M* dose of $ProTRH_{178–199}$. However, the 10 n*M* dose of $ProTRH_{186–199}$, induced a significant suppression of PRL only for the first 30 min following administration. Reasons for this discrepancy are not known. We observed no effect of vehicle or of $ProTRH_{178–185}$ tested at 40 n*M* (Fig. 3). All pituitaries exhibited a KCl response at the end of 60 min.

Because baseline secretory rates differed significantly between individual animals, the posttreatment values were divided by the average baseline value for the 15 min preceding peptide infusion to obtain a percent of baseline secretion value. Data were analyzed using a 4 (dose) × 4 (time) ANOVA with repeated measures over the time factor.

The analysis of the response to $ProTRH_{178-199}$ yielded significant main effects for dose ($F[3,34] = 4.44$; $p < 0.01$) and time ($F[3,102] = 9.78$; $p < 0.0001$) (Fig. 2). A similar analysis of the response to $ProTRH_{186–199}$ yielded a significant main effect of time ($F[3, 81] = 7.25$; $p < 0.001$), a time × dose interaction dose ($F[9,81] = 2.66$; $p < 0.01$), as well as a marginally significant main effect of dose ($F[3,27] = 2.72$; $p = 0.06$; Fig. 3 for $ProTRH_{186–199}$). Representative individual pituitary responses that include the 15-min baseline period as well as the posttreatment response are shown in Fig. 3.

Discussion

In this study we found that both $ProTRH_{178–199}$ and $ProTRH_{186–199}$ inhibit prolactin secretion from perifused anterior pituitary fragments. With the exception of the 10 n*M* dose, the inhibitory effect of each peptide appears to be generally equipotent, suggesting that the biologically active sequence for $ProTRH_{178–199}$ is contained within the amino acid sequence of $ProTRH_{186–199}$. Under the present conditions, the inhibitory effect of these cryptic peptides on prolactin release extended well beyond the exposure period to either peptide. Moreover, the inhibition of release increased during the 60-min sampling period. For the 40 n*M* dose of both peptides, there was only a 20% inhibition observed during the first 15 min, compared to approx a 50% inhibition observed during the last 30 min.

Our results from anterior pituitaries obtained from male rats are in contrast to those from females. Using static expo-

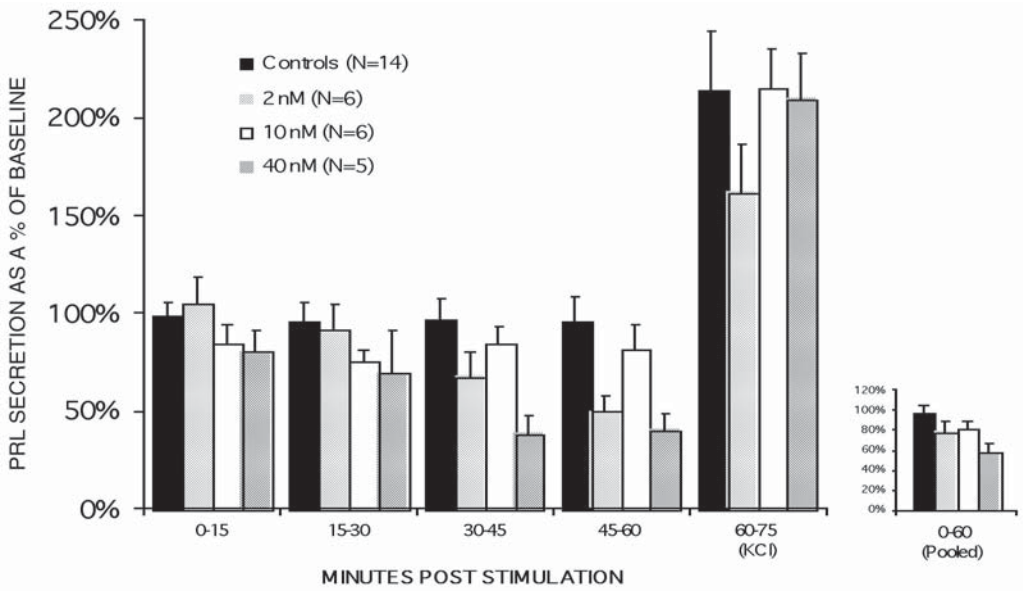

Fig 2. Percentage change in PRL secretion from anterior pituitary for 60 min following administration of a pulse of ProTRH$_{186–199}$. A similar pulse of KCl (200 n*M*) was delivered at the end of 60 min. Each bar represents the mean (±SEM) of 15 min averages for 5–14 glands. Controls are the same as in Fig. 1. ProTRH$_{186–199}$ induced a significant, dose-related suppression of prolactin secretion compared to medium alone.

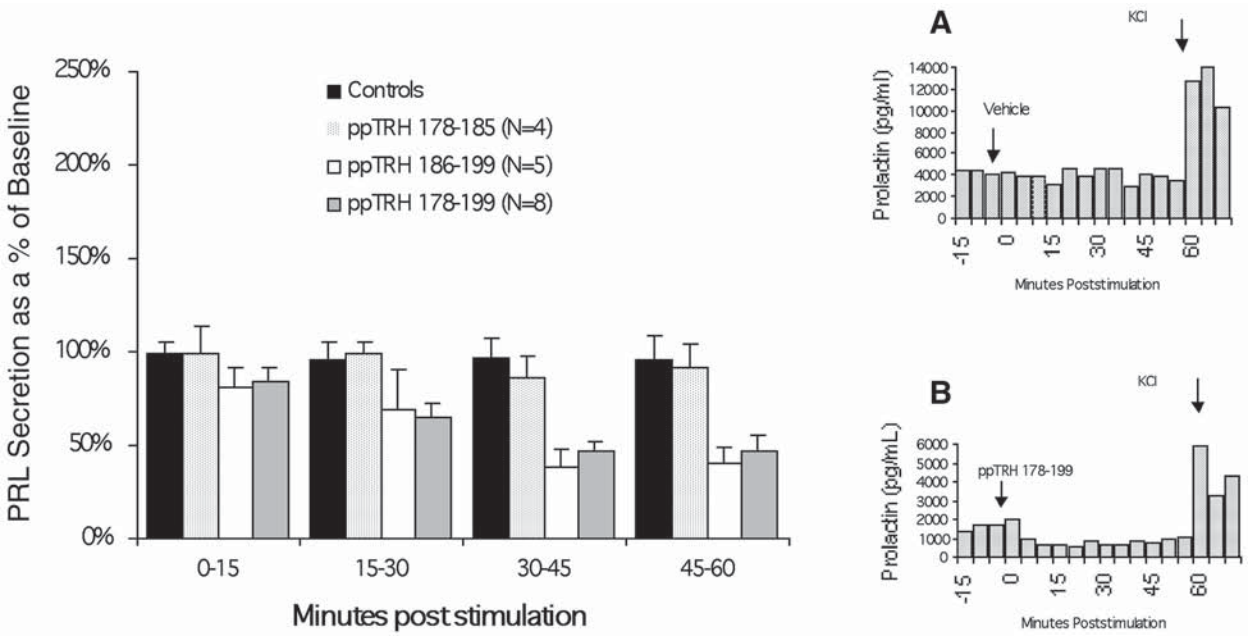

Fig. 3. Comparison of the percentage change in PRL secretion following administration of 40 n*M* ProTRH $_{178–185}$, ProTRH $_{178–199}$, or ProTRH$_{186–199}$. ProTRH$_{178–185}$ produced no significant change in PRL secretion. On the right, data are shown from representative pituitaries administered vehicle (**A**) or 40 n*M* ProTRH$_{178–199}$.

sure of primary cultures of anterior pituitary cells from females, Nillni and co-workers *(24)* reported that both ProTRH$_{178–199}$ and ProTRH$_{186–199}$ released prolactin. Incubation for 30 min with 1–1000 n*M* of either peptide for 30 min induced a dose-related increase that was approx twofold over baseline. Whether these differential results reflect a sex-related effect, differences in duration of peptide exposure, or other unidentified factors remains to be determined. Interestingly, Mitsuma and co-workers *(25)* observed no effect of ProTRH$_{178–199}$ on prolactin secretion using a primary cultures of anterior pituitary cells from males.

Under in vivo conditions, we previously found that the intravenous administration of 200 μg of ProTRH$_{178–199}$ reduced the normal stress-induced rise in prolactin secretion

following restraint stress 5 min later *(23)*. Because stress-induced prolactin release results from a reduction in hypothalamic dopamine (DA) release into the portal blood, an interaction with the tuberoinfundibular dopamine system is suggested *(26)*. However, the present results show that both $ProTRH_{178–199}$ and $ProTRH_{186–199}$ inhibit prolactin release in the absence of hypothalamic inhibitory factors such as DA, or paracrine factors from the posterior pituitary. Therefore, the effects we have observed may be mediated at the level of the lactotroph or other paracrine factors released by another population of anterior pituitary cells.

Peptides produced within the anterior pituitary that inhibit or release prolactin are candidates for future studies. These include the endothelins, calcitonin-like peptides, and activin, all of which have been shown to be inhibitory, and TRH, vasoactive intestinal polypeptide, and angiotensin II, which have been shown to be stimulatory *(27)*. Interactive effects of these peptides with $ProTRH_{178–199}$ and $ProTRH_{186–199}$ could be addressed using primary cultures of lactotrophs or prolactin-secreting cell lines such as GH3 cells. Finally, the possibility that the inhibitory effect of $ProTRH_{178–199}$ is restricted to a subpopulation of lactotrophs must also be considered *(28)*.

Materials and Methods

Animals

Anterior pituitaries were obtained from adult male Sprague–Dawley rats (Harlan, San Diego, CA) weighing 300–450 g. Animals were group housed under a 12/12 light/dark cycle (lights on at 0600) with rat chow and tap water available *ad libitum*. All experiments were conducted between 0900 and 1200 h.

Peptides

$ProTRH_{178–199}$ was purchased from Peninsula Labs (Belmont, CA). $ProTRH_{186–199}$ and $ProTRH_{178–185}$ were synthesized by the macromolecular facility at Colorado State University. The peptides were dissolved in media immediately prior to each experiment.

Perifusion

A six-chamber, two-channel perifusion system was used for all experiments (Endotronics, Minneapolis, MN). Administration of peptide to the six chambers was split to deliver two different dose conditions (three chambers per condition) during each perifusion run. At the start of each experiment, the animals were moved in their home cage to the laboratory and decapitated within 1 min to minimize stress. Anterior pituitaries were immediately isolated from the posterior and intermediate lobes, quartered, and placed in an individual perifusion chamber. Fragments from a single anterior pituitary were used in each of the six chambers.

The pituitary tissue was perifused with minimum essential medium (MEM; Sigma, St. Louis, MO) at a rate of 0.2 mL/min. The media were saturated with 95% O_2/5% CO_2. Temperature of 37ºC and pH of 7.2 were maintained within each chamber. Five-minute fractions were collected following a 2-h equilibration period. Twenty-five minutes of baseline measurements were obtained prior to exposing the cells to a single pulse of one dose of a peptide.

Flow through each of the six perifusion chambers was calibrated to deliver the substance in a peak concentration approx 5 min after onset. Verification was done with a colorimetric analysis of percentage of the initial concentration of a water-soluble dye. We observed 90% clearance within 10 min. Based on these data, the pituitaries were stimulated with doses designed to produce peak concentrations of 2, 10, or 40 n*M* of either peptide dissolved in medium. Controls were perifused in the same fashion with medium alone. One hour after the initiation of the pulse, a pulse of 200 m*M* KCl was delivered to each chamber and 5-min fractions were collected for an additional 15 min.

Radioimmunoassay

Prolactin concentrations in each fraction were measured by RIA using antiserum and standards provided by the NIDDK/National Hormone and Pituitary programs. ^{125}I-labeled rat prolactin was purchased from NEN Life Science Products (Boston, MA). NIH-RP-3 was used as the reference preparation. The minimum detectable level was 300 pg/mL. Inter- and intraassay coefficients of variation were 8.4% and 3.1%, respectively.

Acknowledgments

Steve Aguilar, Keri Gibson, and Dana Koomoa provided technical assistance. Supported in part by NIH AA11600.

References

1. Nillni, E. A. and Sevarino, K. A. (1999). The biology of pro-thyrotropin-releasing hormone-derived peptides. *Endocr. Rev.* **20,** 599–648.
2. Jackson, I. M., Wu, P., and Lechan, R. M. (1985). Immunohistochemical localization in the rat brain of the precursor for thyrotropin-releasing hormone. *Science* **229,** 1097–1099.
3. Lechan, R. M., Wu, P., Jackson, I. M. D., et al. (1986). Thyrotropin-releasing hormone precursor: characterization in brain. *Science* **231,** 159–161.
4. Lechan, R. M., Wu, P., and Jackson, I. M. D. (1987). Immunocytochemical distribution in rat brain of putative peptides derived from thyrotropin-releasing hormone prohormone. *Endocrinology* **121,** 1879–1891.
5. Suzuki, S., Solberg, L. C., Redei, E. E., and Handa, R. J. (2001). Prepro-thyrotropin releasing hormone 178–199 immunoreactivity is altered in the hypothalamus of the Wistar-Kyoto strain of rat. *Brain Res.* **913,** 224–233.
6. Wu, P., Lechan, R. M., and Jackson, I. M. (1987). Identification and characterization of thyrotropin-releasing hormone precursor peptides in rat brain. *Endocrinology* **121,** 108–115.
7. Bulant, M., Delfour, A., Vaudry, H., and Nicholas, P. (1988). Processing of thyrotropin-releasing hormone prohormone (pro-TRH) generates pro-TRH connecting peptides. *J. Biol. Chem.* **263,** 17189–17196.
8. Nillni, E. A., Sevarino, K. A., and Jackson, I. M. (1993). Identification of the thyrotropin-releasing hormone-prohormone and

its posttranslational processing in a transfected AtT20 tumoral cell line. *Endocrinology* **132,** 1260–1270.
9. Nillni, E. A., Luo, L. G., Jackson, I. M., and McMillan, P. (1996). Identification of the thyrotropin-releasing hormone precursor, its processing products, and its coexpression with convertase 1 in primary cultures of hypothalamic neurons: anatomic distribution of PC1 and PC2. *Endocrinology* **137,** 5651–5661.
10. Pu, L. P., Ma, W., Barker, J. L., and Loh, Y. P. (1996). Differential coexpression of genes encoding prothyrotropin-releasing hormone (pro-TRH) and prohormone convertases (PC1 and PC2) in rat brain neurons: implications for differential processing of pro-TRH. *Endocrinology* **137,** 1233–1241.
11. Schaner, P., Todd, R. B., Seidah, N. G., and Nillni, E. A. (1997). Processing of prothyrotropin-releasing hormone by the family of prohormone convertases. *J. Biol. Chem.* **272,** 19958–19968.
12. Danielson, P. B. and Dores, R. M. (1999). Molecular evolution of the opioid/orphanin gene family. *Gen. Comp. Endocrinol.* **113,** 169–186.
13. Jackson, I. M. (1998). The thyroid axis and depression. *Thyroid* **10,** 951–956.
14. Horita, A. (1998). An update on the CNS actions of TRH and its analogs. *Life Sci.* **62,** 1443–1448.
15. Carr, F. E., Fein, H. G., Fisher, C. U., Wessendorf, M. W., and Smallridge, R. C. (1992). A cryptic peptide (160-169) of thyrotropin-releasing hormone prohormone demonstrates biological activity in vivo and in vitro. *Endocrinology* **131,** 2653–2658.
16. Carr, F. E., Reid, A. H., and Wessendorf, M. W. (1993). A cryptic peptide from the preprothyrotropin-releasing hormone precursor stimulates thyrotropin gene expression. *Endocrinology* **133,** 809–814.
17. Yang, H. and Tache, Y. (1994). Prepro-TRH-(160-169) potentiates gastric acid secretion stimulated by TRH microinjected into the dorsal motor nucleus of the vagus. *Neurosci. Lett.* **174,** 43–46.
18. Ladram, A., Bulant, M., Montagne, J. J., and Nicolas, P. (1994). Distribution of TRH-potentiating peptide (Ps4) and its receptors in rat brain and peripheral tissues. *Biochem. Biophys. Res. Commun.* **200,** 958–965.
19. Ladram, A., Montagne, J. J., Nicolas, M. P., and Bulant, M. (1997). Specific binding sites for rat prepro-TRH-(160-169) on C6 glioma and BN1010 clonal neural cells. *FEBS Lett.* **403,** 287–293.
20. Harvey, S. and Cogburn, L. A. (1996). Cryptic peptides of prepro-TRH antagonize TRH-induced GH secretion in chickens at extrapituitary sites. *J. Endocrinol.* **151,** 359–364.
21. Redei, E., Hilderbrand, H., and Aird, F. (1995). Corticotropin release-inhibiting factor is preprothyrotropin-releasing hormone-(178-199). *Endocrinology* **136,** 3557–3563.
22. Roussel, J. P., Teresi, S., Vaudry, H., and Astier, H. (1994). A cryptic peptide of TRH prohormone inhibits TRH-induced GH release. *Comptes Rendus de L Academie des Sciences. Serie III, Sciences de la Vie* **317,** 270–276.
23. McGivern, R. F., Rittenhouse, P., Aird, F., Van de Kar, L. D., and Redei, E. (1997). Inhibition of stress-induced neuroendocrine and behavioral responses in the rat by prepro-thyrotropin-releasing hormone 178–199. *J. Neurosci.* **17,** 4886–4894.
24. Nillni, E. A., Aird, F., Seidah, N. G., Todd, R. B., and Koenig, J. I. (2001). PreporTRH178-199 and two novel peptides pFQ7 and pSE14) derived from its processing, which are produced in the paraventricular nucleus of the rat hypothalamus, are regulated during suckling. *Endocrinology* **142,** 896–206.
25. Mitsuma, T., Hirooka, Y., Kimura, M., and Nogimori, T. (1990). Failure to demonstrate the effect of various per-pro-TRH fragments on TSH and PRL release from rat pituitary in vitro. *Endocrinologia Experimentalis* **24,** 333–339.
26. Martinez de la Escalera, G. and Weiner, R. I. (1992). Dissociation of dopamine from its receptor as a signal in the pleiotropic hypothalamic regulation of prolactin secretion. *Endocrine Rev.* **13,** 241–255.
27. Ben-Jonathan, N. (1994). Regulation of Prolactin Secretion. In: *The pituitary gland.* Imura, H. (ed.). Raven Press, New York.
28. Villalobos, C., Nunez, L., Frawley, L. S., Garcia-Sancho, J., and Sanchez, A. (1997). Multi-responsiveness of single anterior pitutiary cells to hypothalamic-releasing hormones: a cellular basis for paradoxical secretion. *Proc. Natl. Acad. Sci. USA* **94,** 14132–14137.