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Serotonin (5-HT) Stimulates Thyrotropin-Releasing Hormone (TRH) Gene Transcription in Rat Embryonic Cardiomyocytes

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Thyrotropin-releasing hormone (TRH) and its mRNA have been identified in the rat heart, and TRH can enhance cardiomyocyte contractility in vivo. At present, little is known about cardiac TRH gene transcriptional regulation in the heart. Hormones and neurotransmitters, including thyroid hormone (T_3), glucocorticoids, testosterone, and 5-HT initiate effects not only in the cardiovascular system, but also in the regulation of hypothalamic TRH. To clarify the potential roles of these modulators upon the cardiac TRH gene transcription, rat TRH promoter activity was assessed in rat embryonic myocyte cells (H_9C_2) by transient transfection assays. TRH promoter activity was stimulated significantly by dexamethasone ($10^{-4}M$) and testosterone ($10^{-5}M$), and was inhibited by T_3 ($10^{-7}M$). Interestingly, the neurotransmitter 5-HT stimulated TRH promoter activity in H_9C_2 cells, but not in HTB-11 cells. To further clarify this selective role of 5-HT on TRH promoter transcriptional activity in cardiac cells, 5-HT receptor antagonists and agonists were tested. A selective 5-HT_2 receptor antagonist blocked 5-HT stimulation, whereas 5-HT agonist analogs caused augmentative effects when combined with 5-HT. Neither 5-HT nor any antagonists or agonists influenced H_9C_2 cell growth or morphology. These data suggest that 5-HT is an important transcriptional regulator of the cardiac TRH gene.

Key Words: Serotonin; TRH gene transcription; rat cardiomyocyte H_9C_2.

Introduction

Thryotropin-releasing hormone (TRH), the tripeptide pyroGlu-His-ProNH_2, is synthesized predominantly in the central nervous system (CNS), particularly in the paraventricular nuclei (PVN) of the hypothalamus *(1–3)*. TRH is the principal regulator of thyrotropin hormone (TSH) synthesis and may also contribute to the control of prolactin secretion *(4–6)*. In addition to the CNS, TRH gene transcription has also been detected in several other extra-CNS sites *(7,8)*, including the rat heart *(9)*.

The regulation of hypothalamic TRH synthesis and secretion by T_3 has been investigated. Thyroid hormones (T_3/T_4) have been found to negatively regulate both hypothalamic TRH gene transcription and secretion *(3,10)*. TRH is also positively regulated by several other hormones and neurotransmitters in the CNS, including glucocorticoids (DEX), testosterone (TEST), and 5-HT *(11–13)*. Although cardiac TRH and TRH mRNA have been quantitated in all heart chambers *(9,14)*, little is known about cardiac TRH gene transcriptional regulation. Knowledge about TRH gene control and TRH physiological function in the heart is of considerable interest, in view of the prior demonstration that TRH mRNA expression in the rat heart atria is greater than that in ventricle, and by the finding that both are induced by DEX and TEST in vivo *(9,15)*.

Rat cardiomyocyte H_9C_2 cells, derived from embryonic heart tissue, have been employed as an in vitro model for elucidating cardiomyocyte function *(16)*. These cells share characteristics of both cardiac and striated muscle cells and have been utilized successfully for exploring cardiac gene transcriptional regulation *(17–21)*. For the present study, it was hypothesized that T_3, DEX, TEST, and 5-HT have regulatory roles in the cardiac TRH gene transcription, in addition to their roles in hypothalamic TRH gene regulation *(22)*. It is reported here that the transfected TRH gene promoter activity is inhibited by T_3, and stimulated by DEX, TEST, and 5-HT, selectively via 5-HT_2 receptor subtypes.

Results

When a rat TRH-luciferase (LUC) promoter construct was transiently transfected into H_9C_2 cells, DEX ($10^{-4}M$), or TEST ($10^{-5}M$) stimulated TRH-LUC promoter activity significantly ($p < 0.01$), demonstrated by a 67% increase above control for DEX treatment, and a 42% increase above control for TEST treatment. T_3 ($10^{-7}M$) caused significant promoter inhibition of 35% below control ($p < 0.05$, Fig. 1).

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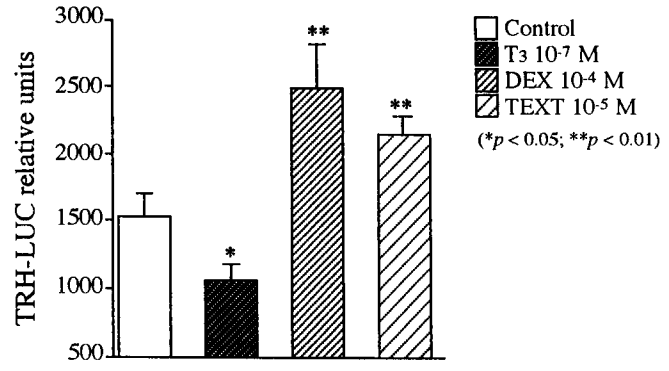

Fig. 1. Response of the rTRH-LUC promoter chimera to T_3, DEX, and TEST in H_9C_2 cells. T_3 resulted in significant inhibition, whereas, DEX and TEST exerted a significant stimulatory effect on TRH promoter activity, compared to nontreated H_9C_2 cells.

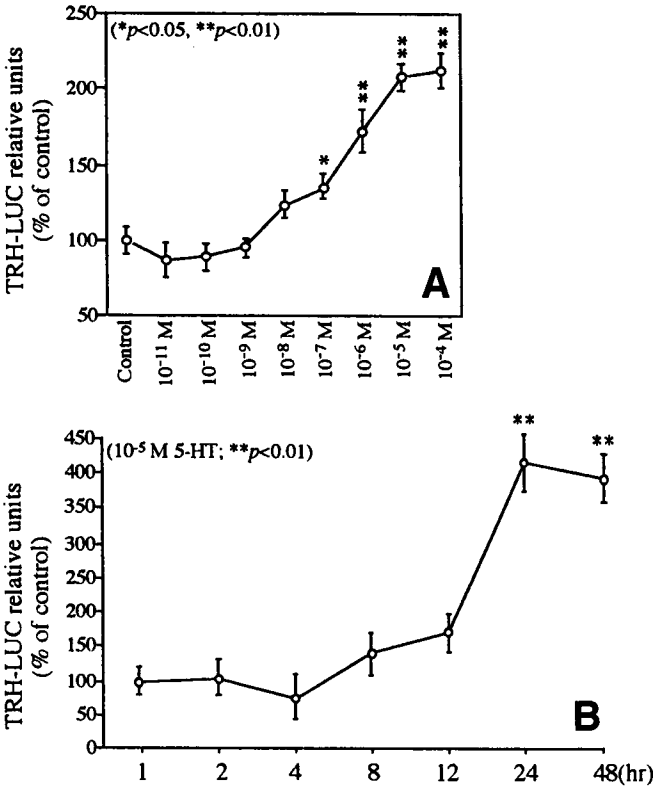

Fig. 2. TRH-LUC reporter activities in response to graded 5-HT treatment. **(A)** Significant stimulation occurred between 10^{-7} to $10^{-4}M$ 5-HT, with half-maximal stimulation at $5 \times 10^{-7}M$. **(B)** The time course of TRH-LUC stimulation by 5-HT ($10^{-5}M$) showed a latency of 8 h and a maximal response at 24 h, sustained through 48 h.

TRH promoter activity was stimulated by 5-HT in a dose-dependent manner (Fig. 2). A peak response to $10^{-5}M$ 5-HT was apparent after 24 or 48 h exposure (Fig. 2A). After 24 h of incubation, 5-HT exhibited a graded stimulation of the TRH promoter activity, between concentrations of 10^{-8}–$10^{-5}M$, with the half-maximal stimulation occurring at 5-HT $10^{-7}M$ (Fig. 2B). In contrast, TRH promoter activity was not affected by 5-HT in HTB-11 cells under identical conditions (Fig. 3).

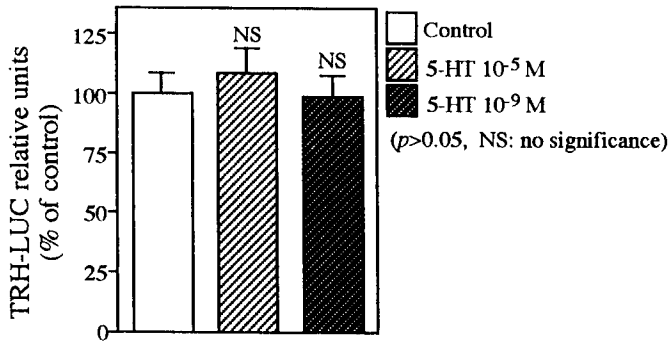

Fig. 3. TRH-LUC reporter activity in response to 5-HT treatments in HTB-11 cells. Neither 10^{-9} nor $10^{-5}M$ 5-HT concentrations caused any significant stimulation compared to control (left bar).

Table 1
Selective Antagonists and Agonists for Serotonin Receptor Subtypes

5-HT Receptors	Chemical compounds	Receptor specificity
Antagonists		
C-112	Cyproheptadine HCl	5-HT_2/5-HT_{1c}
S-006	Ketanserin tartrate	5-HT_2/5-HT_{1c}
M-149	Methiothepin mesylate	5-HT_1
P-110	Propranolol HCl (S-)	5-HT_{1A}
T-113	3-Tropanyl-indole-3-carboxylate methiodide	5-HT_3
T-104	3-Tropanyl-indole-3-carboxylate HCl	5-HT_3
Agonists		
D-101	DOI HCl (±)	5-HT_2/5-HT_{1c}
M-110	α-Methylserotonin maleate	5-HT_2 > 5-HT_1
C-144	1-(m-Chlorophenyl)-biguanide HCl	5-HT_3
H-133	5-HTQ iodide	5-HT_3

Selective antagonists and agonists for 5-HT receptors, used previously for investigating the 5-HT receptors in vivo and in vitro. Their chemical names and receptor specificities were shown.

To examine further which 5-HT subtype receptors are involved in these 5-HT stimulatory effects, transfected cells were incubated with six specific 5-HT receptor antagonists (Table 1), in the presence or absence of 5-HT ($10^{-5}M$). Four of these antagonists (C-112, S-006, M-149, and P-110) blocked the stimulatory effect of 5-HT on TRH promoter activity significantly compared to 5-HT ($10^{-5}M$) administration alone (Fig. 4). C-112 ($10^{-11}M$) and S-006 ($10^{-9}M$), 5-HT_2 receptor antagonists, caused significant serotonin antagonism (Fig. 4A,B). M-149 and P-110, 5-HT_1 and 5-HT_{1A} receptor antagonists, required a higher concentration ($10^{-9}M$) for a significant blocking effect (Fig. 4C,D). In contrast, the two 5-HT_3 receptor antagonists, T-113 and T-104, failed to inhibit 5-HT stimulation at either 10^{-11} or $10^{-9}M$ (Fig. 4E,F). In the absence of 5-HT, none of these receptor's antagonists had any significant effect on the rat

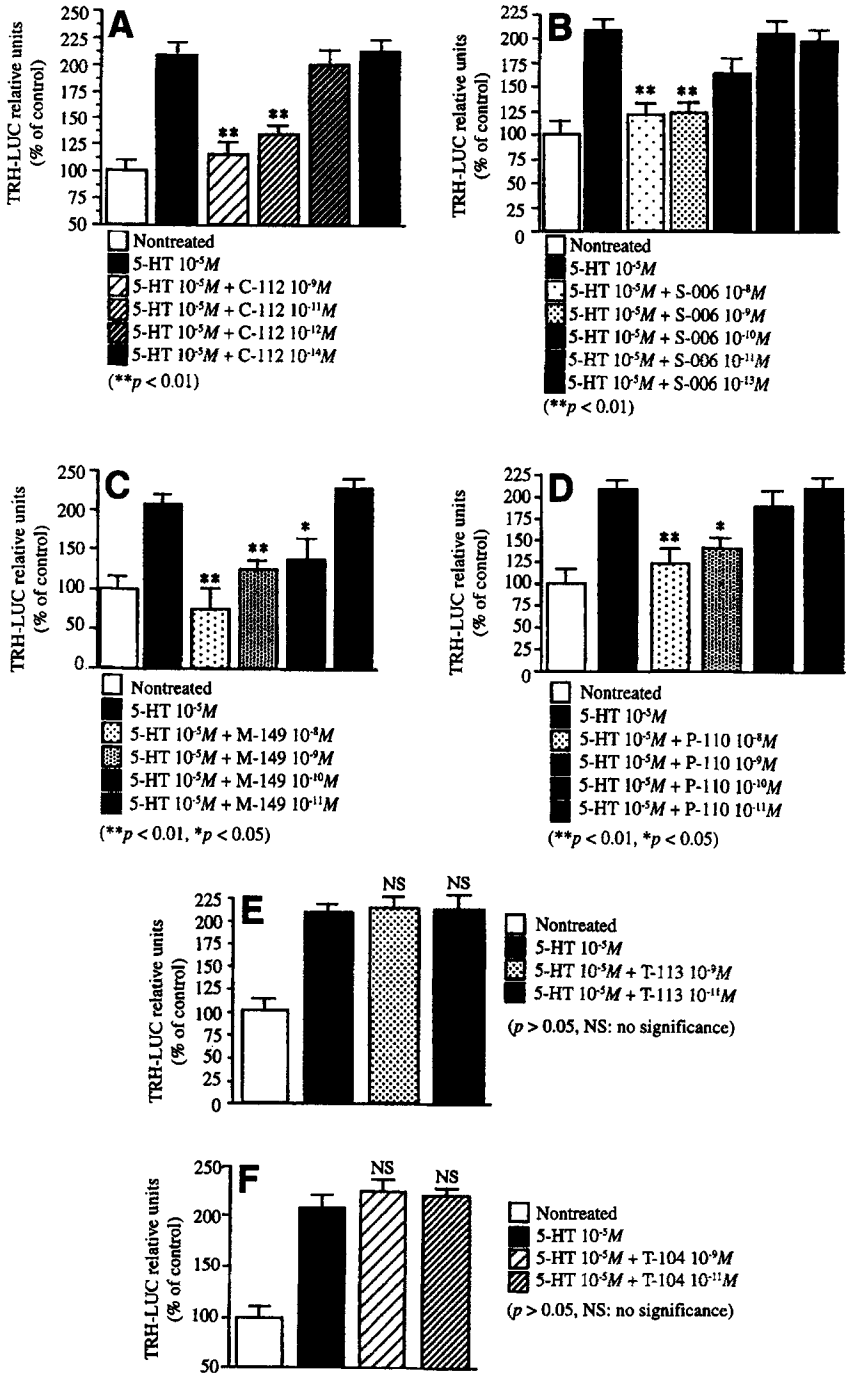

Fig. 4. Effects of 5-HT antagonists on 5-HT-induced TRH-LUC promoter activity in H_9C_2 cells. Significant inhibition of 5-HT-induced stimulation by the 5-HT_1 receptor antagonists C-112 **(A)**. Significant inhibition by 5-HT_2 receptor antagonists S-006 **(B)**, M-149 **(C)**, and P-110 **(D)** under identical conditions. 5-HT_3 receptor antagonists **(E)** T-113 and **(F)** T-104 failed to inhibit 5-HT stimulation of TRH-LUC promoter activity.

TRH-LUC promoter activity under identical cell culture conditions.

Each of four 5-HT receptor agonists: C-144, M-110, D-101, and H-133 (Table 1), potentiated 5-HT-induced stimulation of TRH promoter activity at a concentration of $10^{-7}M$ in the presence of $10^{-5}M$ 5-HT. No stimulatory effect was observed when these receptors agonists were administrated without 5-HT (Fig. 5).

Transfected H_9C_2 cells, after exposure for 24 h to T_3, DEX, TEST, or 5-HT or its receptor analogs, appeared to be unaffected morphologically by any treatments. Hence, the observed effects on TRH gene promoter transcriptional

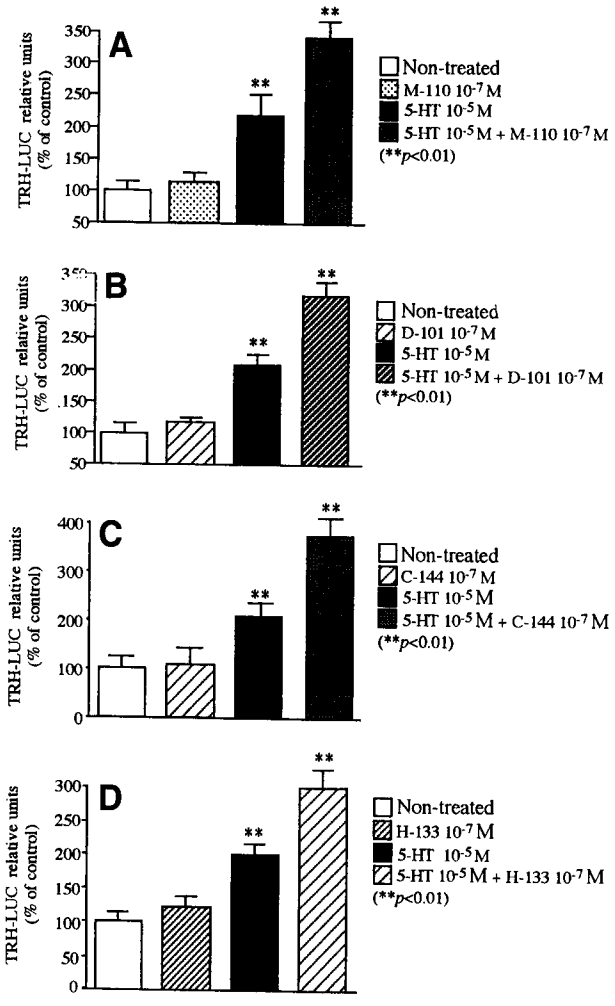

Fig. 5. Effects of four 5-HT agonists on the TRH-LUC promoter activity in H_9C_2 cells. **(A)** M-110, **(B)** D-101, **(C)** C-144, and **(D)** H-133. In the presence of 5-HT $10^{-7}M$, responses to agonists were augmented. Agonists exerted no significant effects at $10^{-7}M$ in the absence of 5-HT.

activity were unlikely to be a result of either toxicity or death of transfected cells.

Discussion

The principal finding of the present study is that serotonin, 5-HT, acts as a potent stimulator of TRH gene promoter transcription in rat cardiomyocyte H_9C_2 cells. Although H_9C_2 cells are derived from rat embryonic heart tissue and have been characterized by properties of both striated and cardiac muscle cells *(17,20)*, their validity as a model for studying the TRH gene regulation in heart is supported strongly by the fact that both DEX and TEST are able to activate TRH gene promoter transcriptional activity in this cell line in vitro, as reported previously in the normal rat heart in vivo *(9)*. Moreover, the H_9C_2 cells are one of the major models for studying other cardiac related genes in the heart *(16,17,19,20)*.

However, important differences exist in the regulation of the TRH gene in normal heart cells compared to hypothalamus *(3,6)*. Particularly notable is that T_3 is able to inhibit TRH gene expression in hypothalamic model cells, HTB-11, whereas, excessive dose of T_3 administration in vivo did not show inhibitory effect on the TRH gene expression of the heart tissue *(9)*. Therefore, the ability of T_3 to suppress TRH gene promoter activity in H_9C_2 cells herein is intriguing and is compatible with the concept that cardiac TRH gene expression occurs primarily in cells other than cardiomyocytes, as suggested by our recent in situ hybridization studies *(23)*. The expression TRH mRNA has been localized predominantly to atrial endocardium and vascular adventitial tissue, which provides a structural basis for the dissociation of these T_3 regulatory influences in vitro and in vivo. On the other hand, the cardiac roles of T_3 in vivo, not only upon the TRH gene, but also upon other cardiac T_3-target genes including myosin-heavy chain (MHC) and Na+/K+ ATPase *(24,25)*, is a complex process involving several different components *(26–28)*, including the quantities of TRs isoforms (TRα1, 2 and β1, 2) in both cardiomyocytes and nonmyocyte cells of the heart and certain tissue-specific transcriptional factors *(29)*. Thus, the absence of T_3 downregulation may reflect events occurring in nonmyocyte structures that can not be explored in vitro with H_9C_2 cells. Because of this discrepancy, transfection studies with TR-T_3 complexes in cultured atrial endocardial and vascular adventitial cells are under development.

It has been demonstrated herein for the first time that 5-HT stimulates TRH gene promoter transcription in cardiac H_9C_2 cells, but not in neuroblastoma HTB-11 cells. Moreover, the TRH gene promoter response to 5-HT is both dose- and time-dependent. Of interest, 5-HT stimulation of TRH promoter activity appears to be mediated principally through 5-HT_2 receptors, and to a lesser degree 5-HT_1 receptors, and not through the 5-HT_3 receptor subtype. The current findings are consistent with previous studies, indicating that 5-HT in the heart acts to enhance cardiac contractility predominantly through 5-HT_2 receptors *(30,31)*. Serotonin via its specific receptors (5-HT_{1-4} receptor subtypes) acts as an important modulator of TRH secretion in the CNS, and, 5-HT can also modulate the release of other hormones *(32–36)*. Therefore, the stimulation of cardiac TRH promoter activity by serotonin in cardiomyoctye cells raises the possibility that TRH may mediate, in part, stimulation of cardiac contractility by serotonin.

The enhancement of TRH gene transcription activity by DEX and TEST in vitro suggests that TRH may participate, respectively, in cardiac responses to stress stimuli and cardiac hypertrophy caused by anabolic steroids. Although molecular mechanisms for DEX and TEST effects on the TRH gene regulation, are not known, cardiac cells possess glucocorticoid receptors (GRs) and estrogen receptors (ERs) *(37–39)*. Previously, it has been established that glucocorticoids can modulate some cardiovascular functions *(40,41)*. Similarly, anabolic–androgenic steroids can

induce cardiac hyperthyophy, their abuse can result in cardiotoxicity in man *(42)*. The regulation of the cardiac TRH gene by hormones and neurotransmitters opens a new arena for investigation concerning the role of TRH both in normal cardiac physiology and in heart disease, including cardiac hypertrophy and congestive heart failure.

Materials and Methods

Rat H_9C_2 cells (ATCC, Rockville, MD) were cultured in D-MEM media with antibiotics (penicillin/streptomycin/fungizone, GIBCO-BRL), 10% fetal bovine serum (FBS) (charcoal-treated), and in an atmosphere of 5% CO_2, at 37°C. HTB-11 cells, generated from human neuroblastoma tissue (ATCC), were cultured in D-MEM media with antibiotics and 10% FBS as described previously *(43)*. HTB-11 cells have been characterized as having a typical neuron-like appearance *(44)*.

Transient Transfection Assays

Transient transfections were performed in triplicate as described previously with cultured cells in 100 mm culture dishes *(19,44)*. Five mcgs of rTRH-LUC DNA chimeric construct, containing the rat prepro-TRH gene sequence extending from –554 to +84 bp cloned upstream of the luciferase coding region *(45)*, was transiently transfected by calcium phosphate precipitation. Efficiencies of transfection were monitored by cotransfection of a pRSV promoter construct (1 μg) containing the β-galactosidase gene (Promega Co.). The LUC reporter activity was determined by luciferase generated light units, and normalized by β-galactosidase activity.

Transfected H_9C_2 and HTB-11 Cells Treated with T_3, DEX, or TEST

Hormones were dissolved at a concentration of $2 \times 10^{-2}M$ and stored at –20°C no longer than 1 wk. Transfected cells were exposed to various hormones for 24 h, including T_3 $10^{-7}M$, DEX $10^{-4}M$ or TEST $10^{-5}M$. Cells were harvested with lysing buffer (2.5 m*M* glycylglycine, 0.15 m*M* $MgSO_4$, 0.4 m*M* EGTA, 0.05% Triton-X, and 1 m*M* DTT) after a washing by 1X PBS buffer.

5-HT Treatments of Transfected H_9C_2 and HTB-11 Cells

5-HT was utilized in graded concentrations from 10^{-4} to $10^{-11}M$, and treated cells were harvested after 24 h. A time course for assessing 5-HT effects also was performed in a separate set (triplicate) of transfected H_9C_2 cells with $10^{-5}M$ 5-HT administration for durations of 1, 2, 4, 8, 12, 24, and 48 h.

Application of 5-HT Receptor Antagonists and Agonists

Selective 5-HT receptor subtype antagonists and agonists, listed in the Table 1, were purchased from Research Biochemicals International (Natick, MA). Antagonists or agonists were coincubated in the presence or absence of 5-HT ($10^{-5}M$) for 24 h before cells were harvested.

Statistical Analyses

Relative LUC light units corresponding to the TRH-LUC reporter activity were expressed as mean ± S.E.% of controls. Statistical significance was assessed by two way analysis of variance between the nontreated controls and the various treated groups, followed by Duncan's multiple range test *(46)*. A p value <0.05 was considered significant.

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References

1. Lee, S. L., Stewart, K., and Goodman, R. H. (1986). *J. Biol. Chem.* **231,** 159–165.
2. Segerson, T. P., Kauer, J., Wolfe, H. C., Mobtaker, H., Wu, P., Jackson, I. M. D., and Lechan, R. M. (1987). *Science* **238,** 78–80.
3. Dyess, E. M., Segerson, T. P., and Lechan, R. M. (1988). *Endocrinology* **123,** 2291–2297.
4. Rivier, C. and Vale, W. (1974). *Endocrinology* **95,** 978–983.
5. Visser, T. J. (1985). *Front Horm. Res.* **14,** 100–115.
6. de Greef, W. J., Rondeel, J. M., van der Vaart, P. D. M., van der Schoot, P., Lamberts, S. W., and Visser, T. J. (1989). *Endocrinology* **125,** 612–617.
7. Aratan-Spire, S., Wolf, B., and Czernichow, P. (1984). *Acta. Endocrinol.* **106,** 102–108.
8. Tavianini, M. A., Gkonos, P., Lampe, T. H., and Roos, B. (1989). *Mol. Endocrinol.* **3,** 605–610.
9. Satoh, T., Lathrop, M., Feng, P., Li, Q. L., Feldman, A. M., Aversano, T., and Wilber, J. F. (1994). In *Heart and Thyroid,* Braverman, L. E., Eber, O., and Langsteger, W. (eds.). pp. 24–32.
10. Lechan, R. M., Wu, P., Jackson, I. M. D., Wolf, H., Cooperman, S., Mandel, G., and Goodman, R. H. (1986). *Science* **231,** 159–161.
11. Arimura, A., Gordin, A., and Schally, A. V. (1976). *Fed. Proc.* **35,** 782A.
12. Bauer, K., Gräf, K. J., Faivre-Bauman, A., Beier, S., Tixier-Vidal, A., and Kleinkauf, H. (1978). *Nature* **274,** 174,175.
13. Anderson, M. S., Bowers, C. Y., Kastin, A. J., Schalch, D. S., Schally, A. V., Snyder, P. J., Utiger, R. D., Wilber, J. F., and Wise, A. J. (1971). *N. Engl. J. Med.* **285,** 1279–1283.
14. Carnell, N., Feng, P., Kim, U., and Wilber, J. F. (1992). *Neuropeptides* **22,** 209–212.
15. Wilber, J. F., Feng, P., Li, Q. L., and Shi, Z. X. (1996). *Trends Endocrinol. Metabol.* **7,** 93–100.
16. Heads, R. J., Latchman, D. S., and Yellon, D. M. (1984). *J. Mol. Cell Cardiol.* **26,** 695–699.
17. Brady, A. J. (1991). *Physiol. Rev.* **71,** 413–428.
18. Greenwald, J. E., Needleman, P., Siegel, N., Tetens, E., Biel, B., and Ritter, D. (1992). *Biochem. Biophy. Res. Commun.* **188,** 644–654.
19. Mestril, R., Chi, S.-H., Sayen, M. R., O'Reilly, K., and Dillmann, W. H. (1993). *J. Clin. Invest.* **93,** 759–767.
20. Mestril, R., Chi, S.-H., Sayen, M. R., and Dillmann, W. H. (1994). *J. Biochem.* **298,** 561–569.
21. Kurosaki, M., Calzi, M., Scanziani, E., Garattini, E., and Terao, M. (1995). *Biochemical J.* **306,** 225–234.
22. Burgus, R., Dunn, T. F., DeSiderio, D., Ward, D. W., Vale, W., and Guillemin, R. (1970). *Nature* **226,** 321–325.

23. Shi, Z. X., Xu, W., Mergner, W. J., Li, Q. L., Cole, K. H., and Wilber, J. F. (1997). *Pathbiology,* in press.
24. Rohrer, D. and Dillmann, W. H. (1988). *J. Biol. Chem.* **263,** 6941–6944.
25. Ojamaa, K. and Klein, I. (1993). *Endocrinology* **132,** 1002–1006.
26. Parvin, J. D., Timmers, H. T. M., and Sharp, P. A. (1992). *Cell* **68,** 1135–1144.
27. Marks, M. S., Hallenbeck, P. L., Nagata, T., Segars, J. H., Appella, E., Nikodem, V. M., and Ozato, K. (1992). *EMBO. J.* **111,** 1419–1435.
28. Baniahmad, A., Ha, I., Reinberg, D., Tsai, S., Tsai, M. J., and O'Malley, B. W. (1993). *Proc. Natl. Acad. Sci. USA* **90,** 8832–8836.
29. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., Evans, R. (1995). *Cell* **83,** 835–839.
30. Ono, H., Ono, S. S., and Fukuda, H. (1991). *Eur. J. Pharmacol.* **203,** 283–285.
31. Choi, D. S., Colas, J. F., Kellermann, O., Loric, S., Launay, J. M., Rosay, P., and Maroteaux, L. (1994). *Cell. Mol. Biol.* **40,** 403–411.
32. Imura, H., Nakai, I., and Yoshimi, T. (1973). *J. Clin. Endocrinol. Metabol.* **39,** 1–5.
33. Assadian, H., Ishikawa, Y., Shimatsu, A., Tanoh, T., and Imura, H. (1991). *J. Auto. Nervous Sys.* **35,** 193–198.
34. Saphier, D., Welch, J. E., Farrar, G. E., Nguyen, N. Q., Aguado, F., Thaller, T. R., and Knight, D. S. (1994). *Psychoneuroendocrinology* **19,** 779–797.
35. Arvidsson, U., Cullheim, S., Ulfhake, B., Luppi, P. H., Kitahama, K., Jouvet, M., and Hokfelt, T. (1994). *J. Chem. Neuroanat.* **7,** 3–12.
36. Mota, A., Bento, A., Penalva, A., Pombo, M., and Dieguez, C. (1995). *J. Clin. Endocrin. Metabol.* **80,** 1973–1977.
37. Gardner, D. G., Gertz, B. J., Deschepper, C. F., and Kim, D. Y. (1988). *J. Clin. Invest.* **82,** 1275–1281.
38. Lin, A. L. and Shain, S. A. (1985). *Arteriosclerosis* **5,** 668–677.
39. Orimo, A., Inoue, S., Ikegami, A., Hosoi, T., Akishita, M., Ouchi, Y., Muramatsu, M., and Orimo, H. (1993). *Biochem. Biophy. Res. Commun.* **195,** 730–736.
40. Chrousos, G. P. and Gold, P. W. (1992). *JAMA* **267,** 1244–1252.
41. Bamberger, C. M., Schulte, H. M., and Chrousos, G. P. (1996). *Endocr. Rev.* **17,** 245–261.
42. Melchert, R. B., Herron, T. J., and Welder, A. A. (1992). *Med. Sci. Sports Exer.* **24,** 206–212.
43. Li, Q. L., Feng, P., Koch, C., Shi, Z. X., and Wilber, J. F. (1996). *Thyroid* **6,** 233–236.
44. Feng, P., Kim, J., Yamada, M., Carnell, E., and Wilber, J. F. (1991). In *Proceedings of the 10th International Thyroid Congress*. Gordon, A., Gross, J., and Hennemann, G. (eds.). Progress in thyroid research. A. A. Balkema Publishers. Brookfiled, pp. 83–87.
45. Wood, W. M., Ocran, K. W., Gordon, D. F., and Ridgway, E. C. (1991). *Mol. Endocrinol.* **5,** 1049–1061.
46. Duncan, D. B. (1955). *Biometrics* **11,** 1–42.