Endocrine, vol. 7, no. 2, 261–265, October 1997 0969-711X/97/7:261-265/$9.25

Serotonin Directly Stimulates Luteinizing Hormone-Releasing Hormone Release from GT1-1 Cells via 5-HT$_7$ Receptors

Micheline Héry, Anne Marie François-Bellan, Francis Héry, Paule Deprez, and Denis Becquet

Laboratoire de Neuroendocrinologie Expérimentale. INSERM U297,
Faculté de Médecine Nord, Bd Pierre Dramard, Institut Fédératif Jean Roche, 13916 Marseille Cedex 20, France

Luteinizing hormone-releasing hormone (LHRH release, which serves as the primary drive to the hypothalamic–pituitary gonadal axis, is controlled by many neuromediators. Serotonin has been implicated in this regulation. However, it is unclear whether the central effect of serotonin on LHRH secretion is exerted directly on LHRH neurosecretory neurons or indirectly via multisynaptic pathways.

The present studies were undertaken in order to examine whether LHRH secretion from immortalized LHRH cell lines is directly regulated by serotonin and, if so, to identify the receptor subtype involved. 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT), a 5-HT$_{1A/7}$ receptor agonist, stimulated LHRH release from GT1-1 cells. This effect was blocked by ritanserin, a 5-HT$_{2/7}$ receptor antagonist, but not by SDZ-216-525, a 5-HT$_{1A}$ antagonist. Basal LHRH release was not affected by the 5-HT$_2$ agonist DOI.

Reverse transcription and polymerase chain reaction technique (RT-PCR) was used in order to identify 5-HT$_{1A}$ and 5-HT$_7$ receptor mRNA in immortalized LHRH cell lines. GT1-1 cells express mRNA for the 5-HT$_7$, but not the 5-HT$_{1A}$ receptor subtypes. These results demonstrate a direct stimulatory effect of serotonin on LHRH release via 5-HT$_7$ receptor.

Key Words: GT1-1 cells; luteinizing hormone-releasing hormone; serotonin; 5-HT$_7$ receptor.

Introduction

The release of luteinizing hormone-releasing hormone (LHRH), which represents the final common pathway for regulation of luteinizing hormone (LH) secretion, is regulated by a variety of neuromediators through a complex network of afferent inputs *(1)*. It is well established that serotonin (5-HT) can influence LH secretion through changes in LHRH release *(2)*. Morphological studies demonstrated several potential sites of interaction between the 5-HT and LHRH systems, including the close overlap of 5-HT projection areas with the location of LHRH cells and terminals in the hypothalamus *(3)*, as well as synaptic contacts between 5-HT fibers and LHRH neurons in the medial preoptic area *(4)*. However, 5-HT impinges on other systems, such as the vasoactive intestinal peptide system in the suprachiasmatic nuclei, that may function as an intermediate in the regulation of LHRH secretion *(5,6)*. In addition to the uncertainty about the site of the central action of serotonin on LHRH release, the 5-HT receptor subtypes involved are also doubtful. We have recently reported a stimulatory effect of a 5-HT$_{1A}$ agonist, 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT), on LHRH-secretion from cultured fetal rat hypothalamic cells *(7)*; in the adult rat, however, Wright and Jennes *(8)* failed to detect any expression of 5-HT$_{1A}$ receptor mRNA in LHRH neurons. Since 8-OH-DPAT exhibits high affinity for both 5-HT$_{1A}$ and 5-HT$_7$ receptors *(9)*, the involvement of 5-HT$_7$ receptors in LHRH release stimulation described in our previous report cannot be excluded. A suitable model to investigate whether serotonin acts directly on LHRH-secreting neurons and to characterize the receptor subtypes involved in a such regulation seems to be GT1-1 LHRH neuronal cell lines, which were developed from a tumor induced by simian virus 40T antigen in a transgenic mouse by genetically targeted tumorigenesis *(10)*. These immortalized LHRH cell lines were shown to express neuronal, but not glial, cell markers, and to produce an appropriate precursor and process it into LHRH *(11)*. LHRH secretion from GT1-1 cells was shown to be pulsatile coupled to neuronal depolarization, and regulated by several neuromediators *(12–14)*. The present study was undertaken in order to examine whether LHRH secretion from immortalized LHRH cell lines is directly regulated by serotonin and, if so, to determine the receptors mediating this regulation.

Results

Effect of 8-OH-DPAT on LHRH Release

As shown in Fig. 1, treatment of GT1-1 cells with 8-OH-DPAT ($10^{-5}M$) for 30 min significantly increased

Received February 25, 1997; Revised April 4, 1997; Accepted April 4, 1997.
Author to whom all correspondence and reprint requests should be addressed: M. Héry, Laboratoire de Neuroendocrinologie Expérimentale, INSERM U297, Faculté de Médecine Nord, Bd Pierre Dramard, Institut Fédératif Jean Roche, 13916 Marseille Cedex 20, France. E-mail: herym@jean-roche.univ-mrs.fr

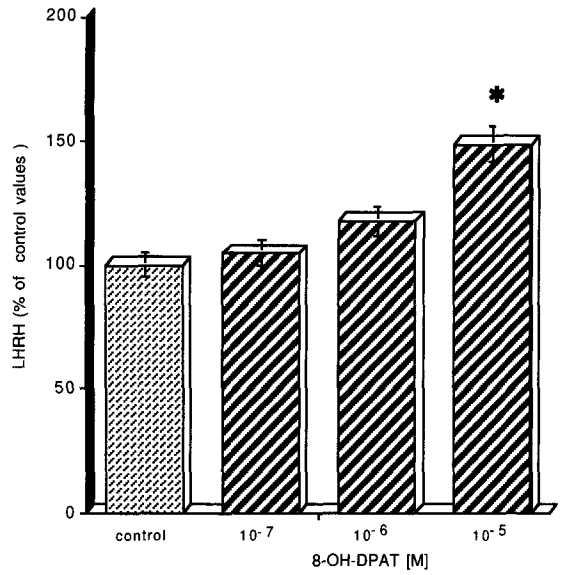

Fig. 1. Secretory response of GT1-1 cells to different concentrations of 8-OH-DPAT. 8-OH-DPAT was present in the medium for 30 min. LHRH levels are expressed as percent of control values. They are means ± SEM of data obtained in 18 wells for each group. $*p < 0.02$ when compared to control values.

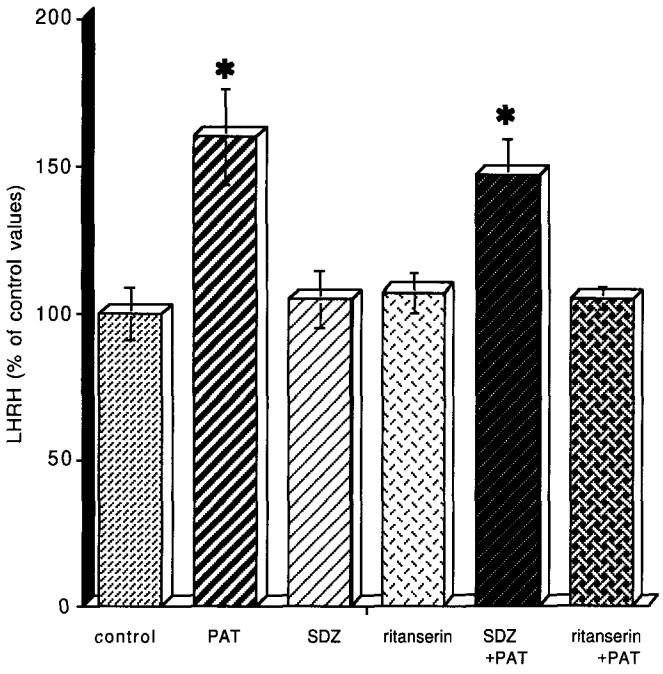

Fig. 2. Effect of SDZ 216-525 ($10^{-5}M$) or ritanserin ($10^{-5}M$) on LHRH release induced by 8-OH-DPAT ($10^{-5}M$, 30 min). SDZ 216-525 or ritanserin was present in the medium for 30 min before the addition of 8-OH-DPAT. Results are expressed as percent of control values. They are means ± SEM of data obtained in 12–18 wells for each group. $*p < 0.02$ when compared to control values.

LHRH release (50–60% over control values), whereas lower concentrations (10^{-7} and $10^{-6}M$) of the drug were ineffective. Taking into account the high affinity of 8-OH-DPAT for 5-HT_7 receptors, pharmacological investigations were then undertaken in order to determine whether 8-OH-DPAT increases LHRH release through 5-HT_{1A} and/or 5-HT_7 receptors.

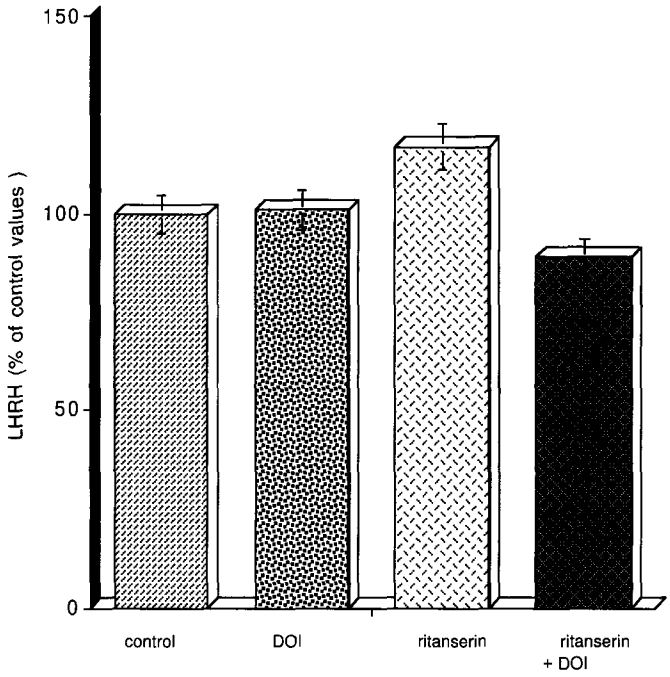

Fig. 3. Effect of DOI ($10^{-5}M$, 30 min) and ritanserin + DOI on. LHRH release. Ritanserin ($10^{-5}M$) was present in the medium 30 min before the addition of DOI ($10^{-5}M$). Results are expressed as percent of control values. They are means ± SEM of data obtained in 12 wells for each group.

Effects of SDZ 216-525 and Ritanserin on LHRH Release Induced by 8-OH-DPAT

When the 5-HT_{1A} antagonist SDZ 216-525 ($10^{-5}M$) was added to the incubating medium during the 60 min of incubation, it displayed no intrinsic effect on LHRH release and did not affect the LHRH response to $10^{-5}M$ 8-OH-DPAT applied during the last 30 min of incubation (Fig. 2).

Treatment of GT1-1 cells for 60 min with ritanserin ($10^{-5}M$) completely abolished the stimulatory effect of 8-OH-DPAT on LHRH release ($10^{-5}M$, 30 min). The 5-$HT_{2/7}$ antagonist treatment did not affect the basal level of LHRH secretion (Fig. 2). In order to eliminate a possible effect of ritanserin on LHRH secretion via the 5-HT_2 receptors, we tested the effect of 1-(2,5 dimethyl-4-iodophenyl)-2-aminopropane (DOI), a 5-HT_2 agonist. DOI ($10^{-5}M$, 30 min) did not affect the basal LHRH release (Fig. 3). When GT1-1 cells were pretreated with ritanserin ($10^{-5}M$, 60 min) before the addition of DOI in the incubating medium, LHRH release was similar to control values (Fig. 3).

5-HT_7 Receptor Subtype mRNA Present in GT1-1 Cells

The expression of 5-HT_{1A} and 5-HT_7 receptor mRNA was investigated using the method of RT-PCR. Results of agarose gel electrophoresis performed on amplified cDNA fragments of the 5-HT_{1A} and 5-HT_7 receptor subtypes are

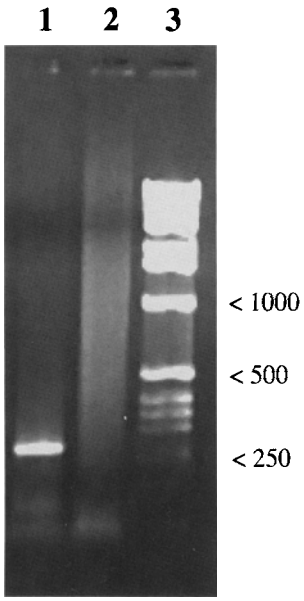

Fig. 4. One percent agarose gel stained with ethidium bromide showing PCR products amplified from cDNA (14 µL) derived from GT1-1 cells total RNA. Lane 1: the band located at approx 250 bp for the 5-HT_7 receptor corresponds to the expected length of the cDNA product produced by the oligonucleotide primer for this receptor. Lane 2: no band located at 635 bp where 5-HT_{1A} receptor was expected. Lane 3: DNA 1000-bp molecular size ladder.

shown in Fig. 4. One band is present for the 5-HT_7 receptor subtype, the location of which corresponds to the expected length of the cDNA products (253 bp) produced by the oligonucleotide primer for this receptor. In contrast, no band was observed for the 5-HT_{1A} receptor subtype whose expected size was 635 bp.

Discussion

In the present study, a direct stimulatory effect of 5-HT on LHRH secretion from the GT1-1 LHRH neuronal cell lines was characterized. We found that 8-OH-DPAT directly stimulates LHRH release and that this effects mediated by the activation of 5-HT_7 receptors. Recently, Nazian et al. *(15)*, analyzing the inhibition of different LHRH secretagogs by opioids, described a stimulatory effect of serotonin on LHRH secretion from GT1-7 cells. It is difficult to compare this results with ours, since these authors used the indolamine directly at a very high concentration ($10^{-3}M$). Nevertheless, it is noteworthy that high doses of 8-OH-DPAT ($10^{-5}M$) were necessary to induce an increase in LHRH release. Although this dose is in the range commonly reported for the effects of neurotransmitters or their agonists in acute in vitro studies *(16–18)*, we cannot explain the mismatch between the affinities of serotonin or 8-OH-DPAT for both 5-HT_7 and 5-HT_{1A} receptors (K_d = 1 nM) and the concentration of agonist required to induce LHRH release. However, the possible involvement of nonserotoninergic mechanism in the effect of 8-OH-DPAT, particularly the possible involvement of an adrenergic mechanism, was ruled out in our previous work describing a stimulatory effect of 8-OH-DPAT on LHRH release from cultured fetal rat hypothalamic cells *(7)*.

Binding profiles of the cloned 5-HT_7 receptor show that SDZ 216-525 and ritanserin, 5-HT_{1A} and 5-$HT_{2/7}$ receptors antagonists, respectively, are good tools to distinguish between the 5-HT_7 and the 5-HT_{1A} receptor subtypes *(19)*. Therefore, we examined whether SDZ 216-525 or ritanserin could antagonize the stimulation of LHRH release by 8-OH-DPAT. Pretreatment of GT1-1 cells with ritanserin abolished the stimulation of LHRH release by 8-OH-DPAT, whereas SDZ 216-525 had no effect. Since basal LHRH release was unaffected by DOI, a 5-HT_2 receptor agonist, our pharmacological results suggest that activation of the 5-HT_7 receptor is responsible for stimulation of LHRH release by 8-OH-DPAT.

RT-PCR analysis showed that GT1-1 cells express the 5-HT_7, but not the 5-HT_{1A} receptor mRNA. These results are consistent with our pharmacological data and reinforce the idea of a functional coupling of 5-HT_7 receptors to LHRH secretion in GT1-1 cells.

Serotonin was shown to be an important neuroregulator of LH secretion, probably through changes in LHRH release *(2)*. A stimulatory effect of 5-HT on LHRH secretion was previously reported by Vitale et al. *(20)*. The addition of 5-HT induced the secretion of LHRH from median eminence tissue obtained from proestrus rats, and this effect was abolished by methiothepin. Data obtained by Meyer et al. *(17)* confirmed the stimulatory effect of 5-HT on LHRH secretion, and suggested the involvement of 5-HT_{1A} and or 5-HT_7 receptors in this effect. Using 8-OH-DPAT, these authors reported an in vitro increase in LHRH secretion from the medio-basal hypothalamus from ovariectomized estradiol-treated rats. These results are consistent with previous findings by Johnson and Kevin *(21)*, who demonstrated that the stimulatory effect of 5-HT on LH release in ovariectomized estradiol-treated rats involved at least partly the activation of 5-HT receptors activated by 8-OH-DPAT.

Finally, we reported previously a stimulatory effect of 8-OH-DPAT on LHRH release from fetal hypothalamic cells *(7)*. Taking all these results into account, we recently proposed that the decrease in hypothalamic LHRH content we observed "in vivo" 30 min following 8-OH-DPAT administration was associated with an increased release of the neurohormone and that the increase in phasic LH release *(22)* resulted at least partially from an action of 8-OH-DPAT in the hypothalamus. Our results with GT1-1 cells are consistent with these findings. The use of such immortalized LHRH cells lines in the present study allows us to suggest that the serotoninergic system could act directly on LHRH neurons and that 5-HT_7 receptor, rather than 5-HT_{1A} receptor subtypes, are involved in the stimu-

latory effect of 8-OH-DPAT on LHRH or LH secretions reported both in vivo and in vitro *(8,17,22)*. These results ascribe a physiological function to the 5-HT fibers synapsing on LHRH immunoreactive neurons in the medial preoptic area *(4)* and to the abundant 5-HT_7 receptor expression in the hypothalamus *(9)*. To date, the similarities between hypothalamic and GT1-1 cell physiologies have been well documented. Although this cell line appears to be a good tool to improve our knowledge concerning the neuroendocrine regulation of gonadotrophin secretion, further studies comparing data obtained in animal tissues to those obtained in cell lines are necessary.

Materials and Methods

Cell Culture

Immortalized LHRH neurons of the GT1-1 subtype were cultured at 37°C in a water-saturated atmosphere of 95% O_2 and 5% CO_2. The culture medium was a Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 4.5 mg glucose/mL, 0.6 mg L-glutamine/mL, 100 U penicillin/mL, 100 µg streptomicin/mL, and 10% fetal bovine serum (FBS; Gibco). GT1-1 cells were seeded directly on 35-mm plastic Petri dishes and cultured until they reached 50–70% confluence. The medium was then replaced by a defined medium (Opti-MEM-Gibco) without serum for 2 d.

Release Experiments

On the day of the experiment, the medium was withdrawn, and cells were incubated for 1 h with 1 mL of fresh medium. The medium collected at the end of the first hour of incubation period was used to determine LHRH basal release in each dish. Cells were then incubated for another hour with 1 mL of fresh medium containing serotoninergic drugs. All drugs tested were made up as 50-fold concentrates in medium and added in 20-µL aliquots to 1 mL medium. The two antagonists tested (SDZ 216-525, a 5-HT_{1A} antagonist, and ritanserin, a 5-$H_{2/7}$ antagonist) were present in the medium all along the second hour of incubation. The two agonists tested, 8-OH-DPAT ($5HT_{1A/7}$ agonist and DOI 5-HT_2 agonist), were added 30 min following the beginning of the second hour of incubation and for 30 min. Media were snap-frozen and stored at –20°C until radioimmunoassay (RIA) analysis for LHRH levels.

LHRH Assay

LHRH was measured using a radioimmunoassay procedure *(23)*. LHRH antiserum no. B 73 was kindly provided by V. D. Ramirez. Intra- and interassay variabilities were 6.4 and 7.8% respectively.

RNA Preparation and First-Strand cDNA Synthesis

Total RNA was extracted from cells by a single-step method using the procedure of Chomczynski and Sacchi *(24)*. Briefly, cells were rinsed with PBS and lysed in guanidium solution (4 *M* guanidium isothiocyanate, 0.1 *M* β-mercaptoethanol, and 25 m*M* sodium citrate, pH 7.0). Lysates were extracted with phenol-chloroform, and RNA was precipitated with isopropanol. In order to eliminate any residual genomic DNA, RNA samples were treated with RNase-free DNase I, followed by inactivation of the DNase by heating and phenol extraction.

This RNA preparation was then divided into two equal parts, one of which was used to synthesize cDNA with the oligo $(dT)_{15}$ primer, and the other one to synthesize cDNA with the $(dN)_6$ random primer. Using RNAs as the templates, first-stand cDNAs were synthesized with Moloney Murine Leukemia Virus Reverse Transcriptase at 37°C for 4 h. After incubation, the reaction mixtures using either oligo (dT) or random primers were combined and used as PCR template.

PCR Amplification

Oligonucleotide primers used for PCR amplification of 5-HT_{1A} and 5-HT_7 receptor subtypes were those described by Pierce et al. *(25)*. Approximately 600 ng of cDNA were used as the PCR template. The cDNA together with 1 µ*M* of each primer, 0.4 m*M* of four deoxynucleoside triphosphates, and 2 U of *Taq* DNA polymerase were used in each PCR reaction in a total volume of 50 µL containing 10 m*M* Tris-HCl (pH 9.0 at 25°C), 1.5 m*M* $MgCl_2$, and 50 m*M* KCl.

The PCR temperature cycle on a Biometra Thermoblock was as follows: first denaturing at 94°C for 3 min, then denaturing at 91°C for 1 min, annealing at 60°C for 1 min, and extension at 74°C for 1 min, total 35 cycles. Finally, a 15-min extension at 74°C was conducted. In order to determine the ability of the synthesized oligonucleotide primers to amplify cDNA, the primers were tested in PCR amplification of cDNA from rat brain mRNA. The primers tested positive in rat brain mRNA control

Aliquots of the PCR products (14 µL) were analyzed on 1% agarose gel in TBE buffer (89 m*M* Tris, 89 m*M* boric acid, and 2.5 m*M* EDTA, pH 8.0) containing 0.05 µg/mL ethidium bromide. Gel electrophoresis results were visualized under uv light and photographed. Since internal standards were not included during reverse transcription of amplification, amplified products were only qualitatively compared. The positively 270 bp detected PCR band had been previously sequenced and shown to be identical to the published 5-HT_7 receptor sequence *(25)*. In our experiment, the band was subcloned into plasmid vector pCR2.1 using the TA Cloning kit (Invitrogen), and its restriction map was checked and found to correspond to the one previously published *(26)*.

Statistical Analysis

Data from three to four release experiments were pooled and expressed in percent of control values. Differences between the experimental groups were analyzed using one-way analysis of variance (ANOVA) followed by Scheffe test.

Drugs

8-OH-DPAT, DOI, and ritanserin were obtained from RBI (Natick, MA). SDZ 216-525 was generously obtained from Sandoz.

Acknowledgments

The authors wish to thank P. Boulenguez for advice during the preparation of the manuscript and M. Seguin for the typing.

References

1. Weiner, R. I., Findell, P. R., and Kordon, C. (1988). In: *The Physiology of Reproduction*. Knobil, E. and Neill, J. D. (eds.). Raven: New York, pp. 1235–1281.
2. Vitale, M. L. and Ciocchio, S. R. (1993). *Endocr. Rev.* **14,** 480–493.
3. Jennes, L., Beckman, W. C., Stumpf, W. E., and Grzanna, R. (1982). *Exp. Brain Res.* **46,** 331–338.
4. Kiss, J. and Halasz, B. (1985). *Neuroscience* **14,** 69–78.
5. Bosler, O. and Beaudet, A. (1985). *J. Neurocytol.* **18,** 105–113.
6. Van der Beek, E. M., Wiegan, V. M., Van der Donk, H. A., Van der Hurk, R., and Buijs, R. M. (1993). *J. Neuroendocrinol.* **5,** 137–144.
7. Héry, M., Becquet, D., François-Bellan, A. M., Deprez, P., Fache, M. P., and Héry, F. (1995). *Neuroendocrinology* **61,** 11–18.
8. Wright, D. E. and Jennes, L. (1993). *Neurosci. Lett.* **163,** 1–4.
9. Ruat, M., Traiffort, E., Leurs, R., Tardivel-Lacombe, J., Diaz, J., Arrang, J. M., and Schwartz, J. C. (1993). *Proc. Natl. Acad. Sci. USA* **90,** 8547–8551.
10. Mellon, P. L., Windle, J. J., Goldsmith, P. C., Padula, C. A., Roberts, J. L., and Weiner, R. I. (1990). *Neuron* **5,** 1–10.
11. Wetsel, W. C., Mellon, P. L., Weiner, R. I., and Negro Villar, A. (1991). *Endocrinology* **129,** 1584–1586.
12. Martinez de la Escalera, G., Choi, A. L. H., and Weiner, R. I. (1992). *Endocrinology* **131,** 1397–1402.
13. Martinez de la Escalera, G., Choi, A. L. H., and Weiner, R. I. (1992). *Proc. Natl. Acad. Sci. USA* **89,** 8,9, 1852–1855.
14. Martinez de la Escalera, G., Gall, F., Choi, A. L. H., and Weiner, R. I. (1992). *Endocrinology* **131,** 2965–2971
15. Nazian, S. J., Landon, C. S., Muffly, K. E., and Cameron, D. F. (1994). *Mol. Cell Neurosci.* **5,** 642–648.
16. Condon, T. P., Ronnekleiv, O. K., and Kelly, M. S. (1989). *Neuroendocrinology* **50,** 51–58.
17. Meyer, D. C., Mc Ree, C., and Jacobs, M. (1992). *Brain Res. Bull.* **28,** 853–860.
18. Petiti, N. and Etgen, A. M. (1992). *Neuroendocrinology* **55,** 1–8.
19. Kawahara, F., Saito, H., and Katsuki, H. (1994). *J. Physiol.* **478,** 67–73.
20. Vitale, M. L., Parisi, M. N., Chiocchio, S. R., and Tramezzani, J. H. (1986). *J. Endocrinol.* **3,** 309–315.
21. Johnson, J. H. and Kevin, S. (1987). *Anat. Rec.* **218,** 67,68.
22. Héry, M., François-Bellan, A. M., and Héry, F. (1995). *Endocrine* **3,** 751–754.
23. Nett, T. M., Akbar, A. M., Niswender, G. D., Heldum, M. T., and White, W. F. (1973). *J. Clin. Endocr. Metab.* **36,** 880–885.
24. Chomczynski, P. and Sacchi, N. (1987). *Ann. Biochem.* **162,** 156–159.
25. Pierce, P. A., Xie, G. X., Levine, J. D., and Peroutka, S. J. (1996). *Neuroscience* **70,** 553–559.
26. Shen, Y., Monsma, F. J., Metcalf, M. A., Jose, P. A., Hamblin, M. W., and Sibley, D. R. (1993). *J. Biol. Chem.* **268,** 18,200–18,204.