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Autocrine Regulation of Prolactin Secretion by Endothelins Throughout the Estrous Cycle*

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We have previously found that the ovarian steroid background determines the efficiency of the endothelin-mediated autocrine feedback regulation of prolactin (PRL) secretion. In this study, we investigated the role of endogenous endothelins in regulating PRL secretion during the estrous cycle. Adult female rats representing different stages of the 4-d cycle were sacrificed by decapitation, and the anterior pituitary cells were enzymatically dispersed using collagenase and hyaluronidase. PRL secretion of individual lactotrophs was measured in a PRL-specific reverse hemolytic plaque assay, and the influence of endogenous endothelins on PRL secretion was assessed by applying the selective ET_A receptor antagonist peptide, BQ123. Blocking the endothelin-mediated autocrine feedback resulted in an increase in PRL secretion when cells were obtained at proestrus, estrus, and diestrus-1, whereas PRL secretion was decreased at diestrus-2 by ET_A receptor blockade. These observations suggest that endogenous endothelins are predominantly inhibitory during proestrus, estrus, and diestrus-1, whereas at diestrus-2 their influence on PRL secretion is stimulatory. Whereas the bell-shaped concentration-response curves with BQ123 at proestrus and diestrus-1 may indicate a transition state in which endogenous endothelins can be both stimulatory and inhibitory, at estrus the influence of endogenous endothelins is unequivocally inhibitory in nature. We propose that intensification of the endogenous endothelin-mediated negative feedback at estrus may play a role in restraining PRL secretion following the estradiol-induced proestrous PRL surge.

Key Words: Prolactin; endothelin; estrous cycle; autocrine regulation.

Introduction

Besides the major pituitary hormones (luteinizing hormone, follicle-stimulating hormone, thyroid-stimulating hormone, growth hormone, prolactin [PRL], adrenocorticotropic hormone), the anterior lobe of the pituitary gland has an intrinsic capacity to produce a wide variety of peptides, cytokines, growth factors, and small molecules such as acetylcholine, GABA, adenosine triphosphate, and nitric oxide *(1–7)*. The expression of these well-known intercellular messengers indicates, intuitively, that an intense and elaborate cell-to-cell communication as well as autocrine regulation likely occurs within the anterior lobe of the pituitary gland. A plethora of studies indicates that this is indeed the case *(8–11)*. However, because of the complexity of the pituitary gland, discerning various components of these local control systems has been difficult. Although several test systems have been devised to study para-, juxta-, and/or autocrine actions among pituitary cells *(11)*, the reverse hemolytic plaque assay (RHPA) remains the most fruitful approach to demonstrate autocrine regulation *(12–18)*.

In our previous studies, by combining immunocytochemistry with RHPA, we were able to demonstrate unequivocally that endothelins (synthesized by lactotrophs) are capable of regulating PRL secretion in an autocrine fashion *(18)*. We have recently found that the autocrine regulation of PRL secretion by endothelins is strongly affected by the in vivo ovarian steroid background *(19)*. However, the physiologic context of the ovarian steroid–modulated autocrine feedback regulation of PRL secretion has not been established. In the present study, we investigated the role of endogenous endothelins in regulating PRL secretion during the estrous cycle. By applying RHPA to measure PRL secretion at the single-cell level, we provide evidence that the influence of endogenous endothelins on PRL secretion is largely determined by the stage of the estrous cycle.

Results

Effects of Endothelin-A Receptor Antagonism on PRL Secretion

The autocrine effects of endothelins on PRL secretion were assessed by blocking endogenous endothelins' action using a wide range of concentrations of the selective and endothelin-A (ET_A) receptor–specific endothelin antagonist, BQ123. In this experimental model, an increase in average plaque size and the rightward shift of the frequency distri-

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*This article is dedicated to the memory of L. Stephen Frawley.

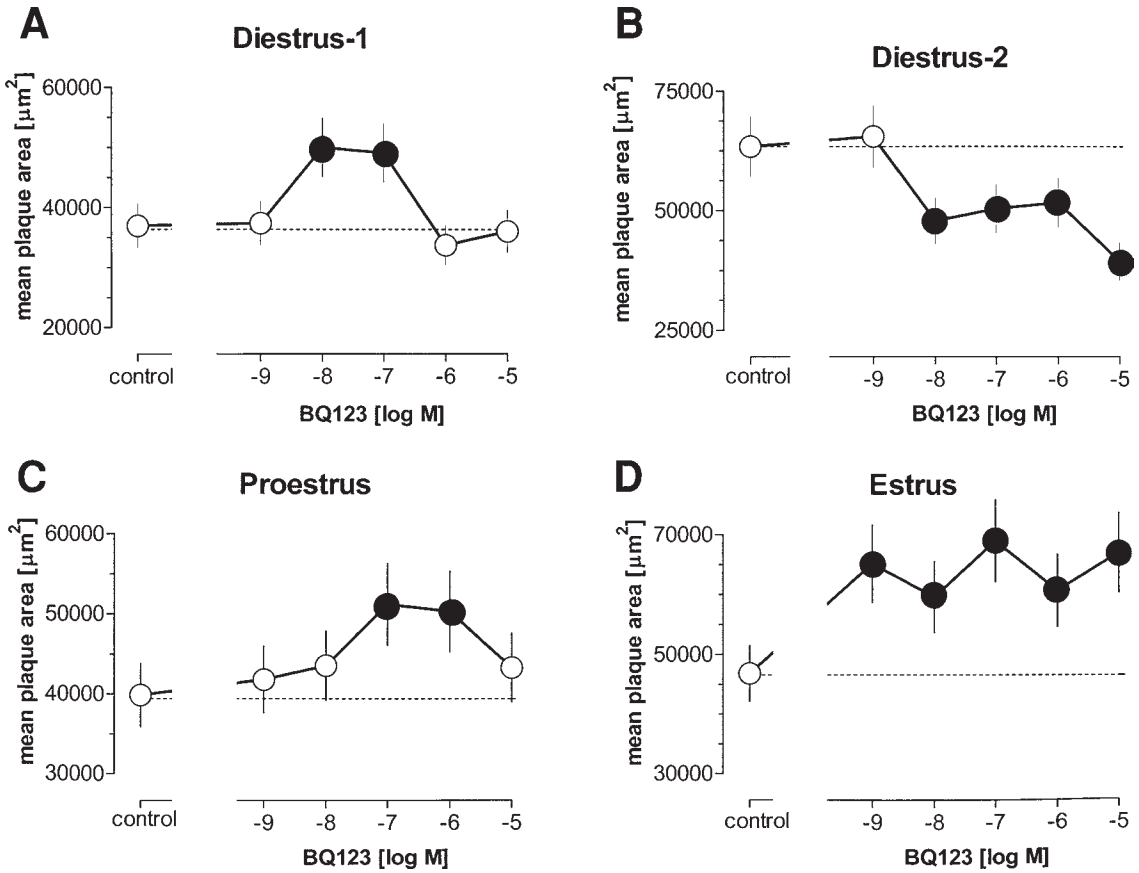

Fig. 1. Effects of ET_A receptor antagonism on PRL secretion throughout estrous cycle. Lactotrophs were obtained from female rats having a regular 4-d cycle on the days of (**A**) diestrus-1, (**B**) diestrus-2, (**C**) proestrus, and (**D**) estrus. PRL secretion of individual lactotrophs was measured by RHPA. Cells were challenged for 3 h in the absence (control) or presence of varying concentrations of BQ123. Each data point represents mean plaque area (SEM), expressed in square micrometers. Averages were calculated on a large number (>300) of individual plaques. Solid circles indicate significant ($p < 0.05$) difference from untreated control values.

bution curve of plaque sizes indicate increased PRL secretion caused by BQ123. Conversely, a decrease in average plaque size and a leftward shift of the frequency distribution curve of plaque sizes indicate decreased PRL secretion caused by ET_A antagonism.

Diestrus-1

ET_A receptor antagonism increased PRL secretion in a dose-dependent manner. The concentration response to BQ123 can be described best as a bell-shaped curve, reaching its maximum between 10^{-8} and 10^{-7} *M* (Fig. 1A). BQ123 predominantly affected the ascending phase of the frequency distribution curve causing a nonsymmetrical shift to the right (Fig. 2A).

Diestrus-2

When cells were obtained on the second day of diestrus, BQ123 caused a dose-related decrease in prolactin secretion (Fig. 1B). The minimal effective concentration was 10^{-8} *M*, while the largest inhibition was detected at 10^{-5} *M*. BQ123 treatment affected plaque size distribution throughout the entire spectrum and caused a symmetrical shift in the frequency distribution curve to the left (Fig. 2B).

Proestrus

The effect of ET_A receptor antagonism on PRL secretion was essentially similar to that of diestrus-1. BQ123 caused a concentration-dependent increase in mean plaque size, reaching maximum between a 10^{-7} and 10^{-6} *M* concentration (Fig. 1C). BQ123 affected predominantly the ascending phase of the frequency distribution curve (small plaque-forming cells), causing a nonsymmetrical shift to the right (Fig. 3C).

Estrus

Endothelin antagonism with the ET_A receptor antagonist BQ123 increased overall PRL secretion at estrus in all concentrations tested (from 10^{-9} to 10^{-5} *M*), indicating that endogenous endothelins are inhibitory at this stage (Fig. 1D). BQ123 treatment affected plaque size distribution throughout the entire spectrum and caused a symmetrical shift in the frequency distribution curve to the right (Fig. 2D).

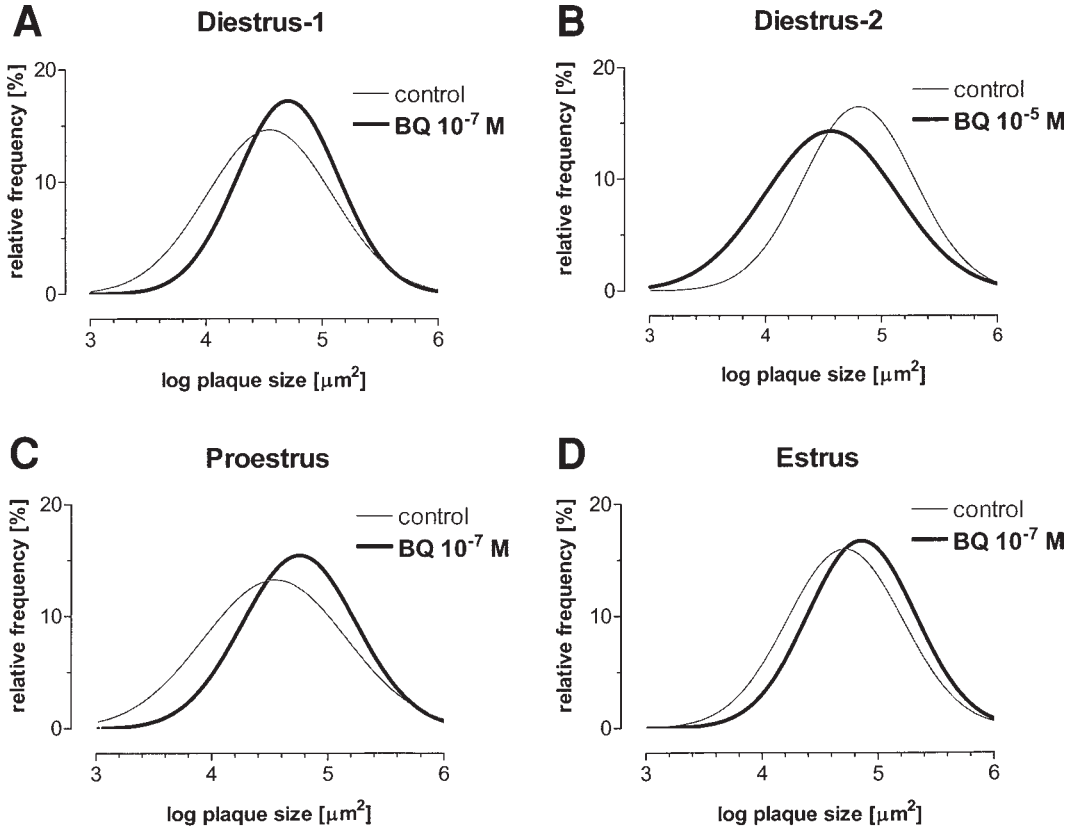

Fig. 2. Effects of ET_A receptor antagonism on the distribution of PRL plaque sizes. Logarithmically binned data (0.2 bin width) were fitted with a Gaussian function using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego CA). The mean plaque size in μm^2 was calculated by retransforming the logarithmic mean resulting from the nonlinear regression analysis (95% confidence intervals are in parentheses): (**A**) Diestrus-1: 35,318 (30,549–40,831), control; 50,816 (46,132–55,976), BQ123. (**B**) Diestrus-2: 64,121 (55,463–74,131), control; 36,813 (32,434–41,783), BQ123. (**C**) Proestrus: 36,643 (31,046–39,902), control; 56,885 (48,084–67,143), BQ123. (**D**) Estrus: 51,880 (44,463–60,674), control; 72,277 (64,714–80,723), BQ123.

Table 1
Average Number of Plaques in Cunningham Chambers[a]

	Control	10^{-9} *M* BQ123	10^{-8} *M* BQ123	10^{-7} *M* BQ123	10^{-6} *M* BQ123	10^{-5} *M* BQ123
Diestrus-2	122.5 (57.2–187.8)	112.0 (84.2–139.8)	101.0 (33.4–168.6)	106.8 (84.7–128.8)	89.8 (71.6–107.9)	73.7 (24.6–122.7)
Proestrus	179.0 (117.9–240.1)	239.3 (161.6–317.0)	244.9 (171.5–318.3)	233.0 (183.8–282.2)	219.8 (149.4–290.2)	260.1 (199.6–320.6)
Estrus	111.0 (62.5–159.5)	128.3 (58.1–198.4)	110.3 (84.2–136.4)	140.8 (130.6–150.9)	142.5 (37.11–247.9)	128.5 (96.9–160.1)
Diestrus-1	205.3 (82.2–328.5)	189.5 (95.2–283.8)	198.5 (105.6–291.4)	197.8 (109.5–286.2)	242.8 (118.0–367.7)	227.0 (122.1–331.9)

[a]Each average represents four to six chambers obtained from three independent cell dispersions (95 % of confidence limits in parentheses). Data were analyzed with one-way analysis of variance (ANOVA) for significant differences across the estrous stages or along the treatments. No significant differences were found ($p > 0.1$ in each case).

It is conceivable that the number of secreting lactotrophs changes across the cycle, or that treatment with varying concentrations of endothelin antagonist alters the ratio between secreting and nonsecreting lactotrophs. However, since there was no difference in the number of plaque-forming lactotrophs throughout the estrous cycle and endothelin antagonism did not affect lactotroph numbers (Table 1), our data do not support the notion of changes in the proportion of

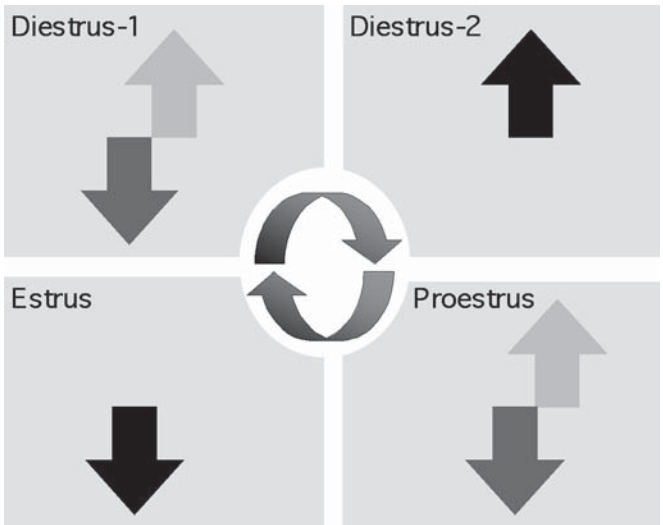

Fig. 3. Possible influence of endogenous endothelins on PRL secretion throughout the estrous cycle. According to this model, the endogenous endothelin tone is not steady but, rather, follows a characteristic pattern throughout the estrous cycle: stimulatory (diestrus-2)—biphasic (proestrus)—inhibitory (estrus)—biphasic (diestrus-1). These interpretations are based on the response to ET_A receptor antagonism (for details, see Results and Discussion). The upward solid arrow indicates stimulation (diestrus-2), while the downward solid arrow indicates inhibition (estrus). Semitransparent arrows indicate the bipotential character of endothelin's action on PRL secretion.

secreting lactotrophs. It cannot be excluded, however, that changes in secreting vs nonsecreting subpopulations did occur but were masked by the high variability in the incidence of plaque-forming lactotrophs.

Discussion

We assessed the autocrine effects of endothelins on PRL secretion throughout the estrous cycle by blocking endogenous endothelin's action with a selective endothelin ET_A receptor antagonist. Under this experimental paradigm, a decrease in PRL secretion by an endothelin antagonist suggests a stimulatory function of endogenous endothelins, whereas increased PRL secretion by endothelin receptor antagonism indicates inhibition by endogenous endothelins. When cells were obtained at diestrus-1, proestrus, and estrus, ET_A receptor antagonism elicited a dose-related increase in average plaque size and caused a rightward shift of the frequency distribution curve of plaque sizes, indicating increased PRL secretion. Conversely, on the second day of diestrus, a decrease in average plaque size and a leftward shift of the frequency distribution curve of plaque sizes was observed, indicating that ET_A antagonism decreases PRL secretion.

It is well established that the influence of endothelins on PRL secretion is predominantly inhibitory in nature *(20–23)*. It has also been suggested that endogenous endothelins may play an important role in the negative feedback regulation of PRL secretion in female rats *(20,23–25)*. The salient feature of our present finding is that in cycling female rats, the endothelin-mediated negative feedback regulation of the lactotrophs is not constant but, rather, follows a characteristic *transitional—stimulatory—transitional—inhibitory* pattern throughout the estrous cycle (Fig. 3).

During estrus or diestrus-2, the overall influence of the endogenous endothelins is unambiguous: it is either inhibitory (estrus) or stimulatory (diestrus-2). At diestrus-1 and proestrus, however, the endogenous endothelin-mediated regulation is in a transitional state, as reflected by the bell-shaped concentration-response curves with ET_A receptor antagonist. Although the latter type of concentration-relationship is not uncommon *(26)*, the general mechanism underlying bell- or U-shaped functions is not well understood *(27)*. The plausible and widely accepted interpretation of the bell-shaped concentration response is that a ligand can activate two receptors that mediate biologically opposite effects *(28)*. Lactotrophs, however, express ET_A receptors exclusively *(29–31)*. Therefore, it seems more likely that ET_A receptors could couple to two different signaling pathways with opposing biologic consequences.

In general, it appears that the effects of endothelins on lactotrophs are context specific since they are largely determined by the history of the cells *(32,33)*. Our preliminary observations indicate that the overall dopaminergic tone *(34)* and the ovarian steroid background *(19)* together play an important role in setting the responsiveness of lactotrophs to endogenous endothelins. Nevertheless, the most intriguing question—What is the mechanism by which the lactotroph alters the coupling efficiency of ET_A receptors toward the inhibitory and stimulatory pathways?—is still open.

It appears that the ovarian steroid milieu can have a profound effect on the self-regulatory mechanisms of lactotrophs. For instance, we previously reported that long-term 17β-estradiol replacement enhances the inhibitory nature of the autocrine regulation of PRL secretion by endothelins *(19)*. However, the mechanism(s) by which estradiol modulates the endothelin system in the pituitary is not yet known. It is still tempting to speculate that the observed changes in the endothelin-mediated autofeedback are being driven primarily by the rising and falling estradiol concentrations during the estrous cycle. Until there is further support from in vivo experiments, however, the relationship between the plasma ovarian steroid concentration and the activity of the endothelin system in the pituitary gland will remain conjectural.

Taken together, our present as well as previous observations reveal an endogenous endothelin-mediated feedback system in lactotrophs *(6,18,19)*. The precise physiologic function of the auto/paracrine regulation of lactotrophs is not yet clear, although several can be postulated. For instance, from a physiologic point of view, it is important to contain the duration and amplitude of the proestrous PRL surge, since

prolonged elevation of PRL secretion would disrupt the cycle by inducing pseudopregnancy *(10,35)*. Therefore, an estradiol-induced enhancement of the endothelin-mediated negative feedback would help to prevent a sustained elevation of PRL secretion following the proestrous PRL surge. In vivo experiments with cycling and ovariectomized estradiol-primed animals are currently under way to test this possibility.

Materials and Methods

Animals

Female Sprague-Dawley rats from Charles River (Raleigh, NC) were used. Animals were adapted to a daily 12-h light cycle (6:00 AM to 6:00 PM) and maintained under controlled temperature and humidity. Rat chow and water were provided ad libitum. The estrous cycle was monitored by daily vaginal smears, and only animals having three consecutive regular 4-d cycles were used. Animals representing each cycle stage were sacrificed between 9:00 and 10:00 AM.

Preparation of Pituitary Cells

Individual pituitary glands from three animals representing each cycle stage were collected after rapid decapitation. The anterior pituitary cells were dispersed using collagenase and hyaluronidase as described elsewhere *(36,37)*. Special care was taken to have an essentially monodispersed cell population. The viability of the dissociated cells was assessed with the trypan blue exclusion test. In spite of the repeated rigorous trituration, the percentage of viable cells was usually 85% or higher.

Hemolytic Plaque Assay

A PRL-specific RHPA was used to measure PRL secretion at the single-cell level, essentially as described previously *(16,38)*. Cunningham chambers were filled with 2% ovine red blood cells containing approx 50,000 pituitary cells/mL. To isolate autocrine regulation from potential paracrine influence, the concentration of pituitary cells should be kept low to minimize cell-to-cell interactions. However, the number of secreting cells should be high enough to allow frequency distribution analysis. We have previously found that in the case of cycling female donors, an initial density of 50,000 pituitary cells/mL will satisfy both criteria. To assess autocrine effects of endothelins, cells were incubated for 3 h in the presence or absence of the selective ET_A receptor antagonist BQ123 *(39)*.

Data Collection and Analysis

Data were collected from at least three separate dispersions for each cycle stage. Plaques were examined using a Leica microscope (Wetzlar, Germany) equipped with 2.5 and 5X phase-contrast objectives and a Spot camera (Diagnostic Instruments, Sterling Heights, MI). Numerical values of each plaque area (square micrometers) were obtained individually by Metamorph software (Universal Imaging, Downingtown, PA) and transported to a Microsoft Excel spreadsheet. For statistical analysis, one-way ANOVA followed by Dunnett multiple comparison test was applied by using the Prism 2.01 program from Graphpad (San Diego, CA), where $p < 0.05$ was considered the threshold of significance.

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References

1. Houben, H. and Denef, C. (1990). *Trends Endocr. Metab.* **1,** 398–403.
2. Renner, U., Pagotto, U., Arzt, E., and Stalla, G. K. (1996). *Eur. J. Endocrinol.* **135,** 515–532.
3. Carmeliet, P. and Denef, C. (1988). *Endocrinology* **123,** 1128–1139.
4. Houben, H., Tilemans, D., and Denef, C. (1990). *J. Endocrinol. Invest.* **13,** 855–863.
5. Carmeliet, P., Vankelecom, H., Van Damme, J., Billiau, A., and Denef, C. (1991). *Neuroendocrinology* **53,** 29–34.
6. Kanyicska, B., Lerant, A., and Freeman, M. E. (2001). *Endocrine* **14,** 263–268.
7. Cai, A., Bowers, R. C., Moore, J. P., and Hyde, J. F. (1998). *Endocrinology* **139,** 2452–2458.
8. Schwartz, J. and Cherny, R. (1992). *Endocr. Rev.* **13,** 453–475.
9. Ben-Jonathan, N. and Liu, J.-W. (1992). *Trends Endocr. Metab.* **3,** 254–258.
10. Freeman, M. E., Kanyicska, B., Lerant, A., and Nagy, G. (2000). *Physiol. Rev.* **80,** 1523–1631.
11. Schwartz, J. (2000). *Endocr. Rev.* **21,** 488–513.
12. Frawley, L. S. and Boockfor, F. R. (1986). *Nature* **321,** 793.
13. Nagy, G., Mulchahey, J. J., and Neill, J. D. (1988). *Endocrinology* **122,** 364–366.
14. Lerant, A. and Nagy, G. M. (1994). *Neuroprotocols* **5,** 198–208.
15. Frawley, L. S. and Neill, J. D. (1984). *Neuroendocrinology* **39,** 484–487.
16. Neill, J. D. and Frawley, L. S. (1983). *Endocrinology* **112,** 1135–1137.
17. Luque, E. H., Monoz de Toro, M., Smith, P. F., and Neill, J. D. (1986). *Endocrinology* **118,** 2120–2124.
18. Kanyicska, B., Lerant, A., and Freeman, M. E. (1998). *Endocrinology* **139,** 5164–5173.
19. Kanyicska, B., Sellix, M. T., and Freeman, M. E. (2001). *Endocrine* **16,** 133–137.
20. Kanyicska, B., Burris, T. P., and Freeman, M. E. (1991). *Biochem. Biophys. Res. Commun.* **174,** 338–343.
21. Samson, W. K., Skala, K. D., Alexander, B. D., and Huang, F.-L. S. (1990). *Biochem. Biophys. Res. Commun.* **169,** 737–743.
22. Samson, W. K., Skala, K. D., Alexander, B., and Huang, F.-L. S. (1991). *Endocrinology* **128,** 1465–1473.
23. Kanyicska, B., Burris, T. P., and Freeman, M. E. (1991). *Endocrinology* **129,** 2607–2613.
24. Stojilkovic, S. S. and Catt, K. J. (1992). *Trends Pharm. Sci.* **13,** 385–391.
25. Kanyicska, B., Burris, T. P., and Freeman, M. E. (1993). In: *Progress in endocrinology.* Mornex, R., Jaffiol, C., and Leclère, J. (eds.). Parthenon: New York.
26. Calabrese, E. J. and Baldwin, L. A. (2001). *Trends Pharmacol. Sci.* **22,** 285–291.

27. Pliska, V. (1994). *Trends Pharmacol. Sci.* **15,** 178–181.
28. Rovati, G. E. and Nicosia, S. (1994). *Trends Pharmacol. Sci.* **15,** 140–144.
29. Kanyicska, B. and Freeman, M. E. (1993). *Am. J. Physiol.* **265,** E601–E608.
30. Stojilkovic, S. S., Balla, T., Fukuda, S., et al. (1992). *Endocrinology* **130,** 465–474.
31. Samson, W. K. (1992). *Biochem. Biophys. Res. Commun.* **187,** 590–595.
32. Kanyicska, B., Freeman, M. E., and Dryer, S. E. (1997). *Endocrinology* **138,** 3141–3153.
33. Dymshitz, J., Laudon, M., and Ben-Jonathan, N. (1992). *Neuroendocrinology* **55,** 724–729.
34. Kanyicska, B., Livingstone, J. D., and Freeman, M. E. (1995). *Endocrinology* **136,** 990–994.
35. Freeman, M. E. (1994). In: *The physiology of reproduction.* Knobil, E. and Neill, J. D. (eds.). Raven: New York.
36. Vale, W., Grant, G., Amoss, M., Blackwell, R., and Guillemin, R. (1972). *Endocrinology* **91,** 562–572.
37. Gorospe, W. C. and Freeman, M. E. (1985). *Endocrinology* **116,** 1559–1564.
38. Smith, P. F., Luque, E. H., and Neill, J. D. (1989). In: *Neuroendocrine peptide methodology.* Conn, P. M. (ed.). Academic: San Diego.
39. Ihara, M., Noguchi, K., Saeki, T., et al. (1992). *Life Sci.* **50,** 247–255.