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Nitric oxide inhibits hypothalamic luteinizing hormone-releasing hormone release by releasing γ-aminobutyric acid

(*in vitro* medial basal hypothalamic explants/sodium nitroprusside/hemoglobin/N^G-monomethyl-L-arginine/high K^+ medium)

A. SEILICOVICH*, B. H. DUVILANSKI*, D. PISERA*, S. THEAS*, M. GIMENO†, V. RETTORI†, AND S. M. MCCANN‡§

*Centro de Investigaciones en Reproducción, Facultad de Medicina, Piso 10., Universidad de Buenos Aires, 1121 Buenos Aires, Argentina; †Centro de Estudios Farmacologicos y Botanicos, Consejo Nacional de Investigaciones Cientificas y Tecnicas (CEFYBO-CONICET), Serrano 665, 1414 Buenos Aires, Argentina; and ‡Neuropeptide Division, Department of Physiology, The University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235-8873

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ABSTRACT **Nitric oxide synthase (NOS)-containing neurons, termed NOergic neurons, occur in various regions of the hypothalamus, including the median eminence–arcuate region, which plays an important role in controlling the release of luteinizing hormone-releasing hormone (LHRH). We examined the effect of NO on release of γ-aminobutyric acid (GABA) from medial basal hypothalamic (MBH) explants incubated *in vitro*. Sodium nitroprusside (NP) (300 μM), a spontaneous releaser of NO, doubled the release of GABA. This release was significantly reduced by incubation of the tissue with hemoglobin, a scavenger of NO, whereas hemoglobin alone had no effect on the basal release of GABA. Elevation of the potassium concentration (40 mM) in the medium increased GABA release 15-fold; this release was further augmented by NP. Hemoglobin blocked the increase in GABA release induced by NP but had no effect on potassium-induced release, suggesting that the latter is not related to NO. As in the case of hemoglobin, N^G-monomethyl-L-arginine (NMMA), a competitive inhibitor of NOS, had no effect on basal release of GABA, which indicates again that NO is not significant to basal GABA release. However, NMMA markedly inhibited the release of GABA induced by high potassium, which indicates that NO plays a role in potassium-induced release of GABA. In conditions in which the release of GABA was substantially augmented, there was a reduction in GABA tissue stores as well, suggesting that synthesis of GABA in these conditions did not keep up with release of the amine. Although NO released GABA, there was no effect of the released GABA on NO production, for incubation of MBH explants with GABA had no effect on NO release as measured by [^{14}C]citrulline production. To determine whether GABA had any effect on the release of LHRH from these MBH explants, GABA was incubated with the tissue and the effect on LHRH release was determined. GABA (10^{-5} or 10^{-6} M) induced a 70% decrease in the release of LHRH, indicating that in the male rat GABA inhibits the release of this hypothalamic peptide. This inhibition in LHRH release induced by GABA was blocked by NMMA (300 μM), which indicates that GABA converts the stimulatory effect of NO on LHRH release into an inhibitory one, presumably via GABA receptors, which activate chloride channels that hyperpolarize the cell. Previous results have indicated that norepinephrine stimulates release of NO from the NOergic neurons, which then stimulates the release of LHRH. The current results indicate that the NO released also induces release of GABA, which then inhibits further LHRH release. Thus, *in vivo* the norepinephrinergic-driven pulses of LHRH release may be terminated by GABA released from GABAergic neurons via NO.**

Constitutive nitric oxide synthase (NOS) occurs in hypothalamic neurons (1, 2). These neurons, which convert L-arginine to citrulline and NO, have been termed NOergic neurons (2, 3). NOergic neurons stimulate the release of a number of polypeptides from the hypothalamus including luteinizing hormone-releasing hormone (LHRH) (3), corticotropin-releasing hormone (4), somatostatin (5), growth hormone-releasing hormone (6), and prolactin-releasing factors (7).

Norepinephrine (3, 8) and glutamic acid (9, 10) induce LHRH release by stimulating NO release from NOergic neurons. γ-Aminobutyric acid (GABA) plays a dual role in LHRH control in female rats: it inhibits LHRH by action on LHRH neurons in the medial preoptic area and it has a stimulatory effect on LHRH in the arcuate nucleus–median eminence region (11). It also has dual effects on LHRH release in males (12). Therefore, we hypothesized that NO might also control the release of GABA from the medial basal hypothalamus (MBH) *in vitro*. Indeed, our results indicate that NO stimulates GABA release from the MBH of adult male rats.

Consequently, we evaluated the effect of GABA on the release of LHRH from similar MBH explants and discovered that this inhibitory neurotransmitter inhibited the release of LHRH and that this inhibition is mediated by NO since the inhibitory effect was prevented by hemoglobin, a scavenger of NO, or by N^G-monomethyl-L-arginine (NMMA), a competitive inhibitor of NOS (13). Taken together the results indicate that NO plays a crucial role not only in stimulating the release of GABA from the MBH but also in inhibiting the release of LHRH. The latter may be a mechanism for termination of the pulses of LHRH release, which are also driven by norepinephrine-induced stimulation of NO release. Thus, the results indicate that NO is involved not only in the stimulation of LHRH release by norepinephrine but in the inhibition of LHRH release induced by GABA as well.

MATERIALS AND METHODS

Male Wistar rats weighing 180–250 g were used. The animals were fed laboratory chow and water ad libitum and were maintained under controlled conditions of light (12-hr light/12-hr dark) and temperature (20–25°C).

The animals were killed by decapitation and the MBH was excised from the brain as described (3). Two MBHs were incubated in a Dubnoff shaker (60 cycles per min) at 37°C in an atmosphere of 95% O_2/5% CO_2 with 0.5 ml of Krebs–Ringer bicarbonate buffer (KRB) (118.46 mM NaCl/5 mM KCl/2.5 mM $CaCl_2$/1.18 mM NaH_2PO_4/1.18 mM $MgSO_4$/24.88 mM $NaHCO_3$, pH 7.4) containing 10 mM glucose, 25 mM Hepes, 1 mM ascorbic acid, and 0.1% bovine serum albumin. After a preliminary incubation of 15 min, the tissue was incubated for 30 min with fresh KRB. Then the medium was removed (basal efflux) and the fragments were incubated

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Abbreviations: NOS, nitric oxide synthase; LHRH, luteinizing hormone-releasing hormone; GABA, γ-aminobutyric acid; MBH, medial basal hypothalamus; NMMA, N^G-monomethyl-L-arginine; NP, sodium nitroprusside; NAME, nitroarginine methyl ester.

§To whom reprint requests should be addressed.

for a further 30 min in KRB containing 40 mM K^+ (balanced by reducing Na^+ concentration). At the end of the incubation period, the medium was aspirated (evoked efflux) and the tissue was quickly frozen on dry ice.

For the determination of GABA concentrations, the tissue was rapidly homogenized in distilled water and frozen on dry ice. The media and the tissue homogenates were heated for 10 min at 100°C and centrifuged at 10,000 × *g* for 10 min. The supernatants were stored at −70°C until the time of GABA determination by the [^{3}H]muscimol radioreceptor assay as described by Bernasconi *et al.* (14) (sensitivity range, 12.5–200 pmol). This radioreceptor method measures the concentration of endogenous GABA and other GABA receptor ligands metabolically related to GABA (15).

The effect of GABA on the production of NO from two MBH fragments incubated for 30 min in KRB was determined by a modification of the [^{14}C]citrulline method of Bredt and Snyder (16). This assay is based on the fact that NOS converts arginine into equimolor quantities of NO and citrulline.

To determine the effect of GABA on the release of LHRH, single MBHs were incubated as described above except that at the end of the 30-min preincubation time, the media were replaced by fresh medium containing various concentrations of GABA or NMMA. At the end of the experiment, the media were removed and boiled for 10 min in a water bath prior to storage at −20°C until assay for LHRH by RIA (3). Aliquots of the homogenates were used in determining protein concentration by the method of Lowry *et al.* (17).

Chemicals. The following were purchased from Sigma: sodium nitroprusside (NP), hemoglobin, NMMA, and GABA.

Statistics. The results were analyzed by one-way analysis of variance and the Student–Newman–Keuls multiple comparison test for unequal replicates. Differences with P values < 0.05 were considered significant.

RESULTS

Effect of NP on Release of GABA from MBH Explants. NP (300 μM), which spontaneously releases NO, evoked a doubling of the release of GABA from MBH explants (Fig. 1). Hemoglobin, a scavenger of NO, had no effect on the basal release of GABA but markedly and significantly reduced GABA release induced by NP.

Release of GABA was increased nearly 15-fold by incubation of the tissue in 40 mM K^+ medium. This K^+-induced release was nearly doubled by NP, so that the quantity of GABA released was increased by >30-fold (Fig. 2). Hemoglobin substantially inhibited the increase in GABA release induced by NP but had no significant effect on K^+-induced release alone.

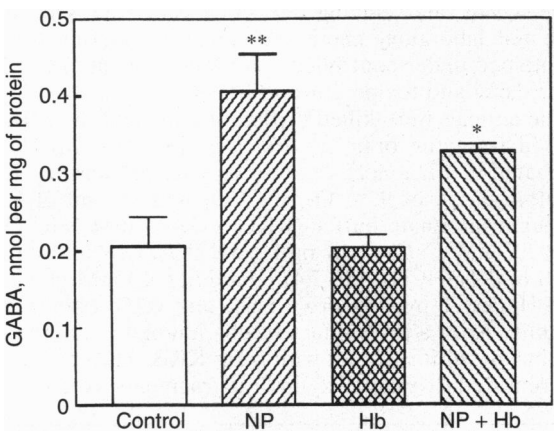

FIG. 1. Effect of NP (300 μM) and hemoglobin (20 μg/ml) on release of GABA from MBH explants. In this and subsequent figures, the height of the bar equals the mean; vertical lines equal SEM. *, $P < 0.05$ vs. control. **, $P < 0.01$ vs. control.

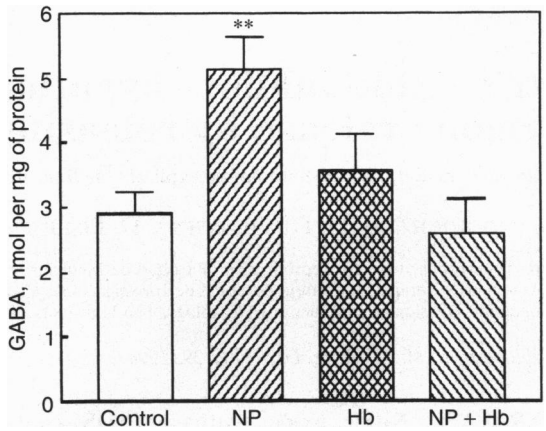

FIG. 2. Effect of elevated K^+ medium (40 mM) on NP (300 μM) and hemoglobin (20 μg/ml)-altered GABA release. **, $P < 0.01$ vs. control.

The tissue concentration of GABA in the MBH fragments incubated in high K^+ medium plus NP was significantly less than that in the tissue incubated in the presence of high K^+ medium alone (Fig. 3). The MBH content of GABA in tissue incubated with NP plus hemoglobin, which diminished the release of GABA, was significantly higher than in MBHs incubated with NP alone. The GABA content of MBH tissue incubated with hemoglobin plus high K^+ medium was similar to that of tissue incubated with high K^+ medium alone.

Effect of NMMA on GABA Release. NMMA had no significant effect on basal release of GABA from MBH tissue (Fig. 4). However, it markedly inhibited, but did not completely block, the release of GABA induced by incubation of the MBHs with high K^+ medium. This reduction in release of GABA was associated with a small increase in the tissue content of GABA (Fig. 4).

Effect of GABA on NO Release. NO release from MBH tissue was measured by the [^{14}C]citrulline method. This release was drastically curtailed by nitroarginine methyl ester (NAME; 300 μM), a competitive inhibitor of NOS (13) (Fig. 5). There was no effect of GABA (10^{-5} or 10^{-6} M) on the production of NO as estimated by conversion of arginine to citrulline (Fig. 6).

Effect of GABA on LHRH Release. GABA (10^{-5} or 10^{-6} M) had a highly significant inhibitory effect on release of LHRH. Both concentrations reduced LHRH release by ≈70% (Fig. 7).

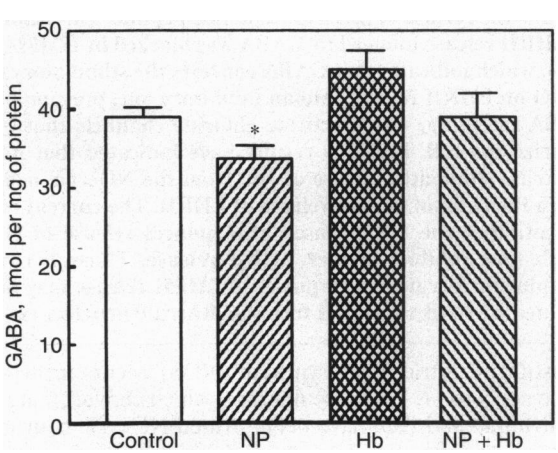

FIG. 3. Effect of NP and hemoglobin on GABA concentration in tissue at the end of the experiment illustrated in Fig. 1. *, $P < 0.05$ vs. control.

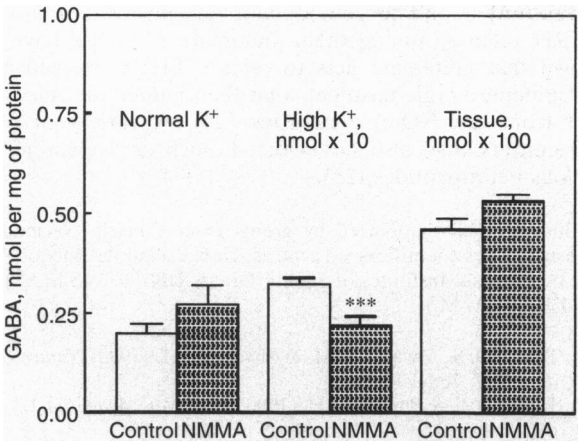

FIG. 4. Effect of NMMA (300 μM), an inhibitor of NOS, on GABA release from MBHs in normal and high K^+ media and on tissue concentration in the presence of high K^+. ***, $P < 0.005$ vs. control.

NMMA, which had no effect on basal LHRH release (data not shown), completely reversed the inhibitory effect of GABA on LHRH release (Fig. 7).

DISCUSSION

Because NP doubled the release of GABA from the MBH explants and the release was largely blocked by hemoglobin, a scavenger of NO, it is clear that NO stimulates the release of GABA from this tissue. It appears that under the conditions of incubation used here, effective concentrations of NO are not released spontaneously since hemoglobin, which would remove the NO produced, had no effect on GABA release. Furthermore, when NMMA in a concentration previously found to inhibit NOS (3) was incubated with the tissues, there was no inhibition of GABA release. That NO was being generated, even if not in effective concentrations, was evidenced by the formation of [^{14}C]citrulline and the partial blockade of its production by NAME.

As expected, elevation of extracellular K^+ concentration drastically stimulated the release of GABA from the tissue. This release was augmented by NO as revealed by the further dramatic increase in release induced by NP and since removal of NO released by NP by hemoglobin reduced GABA release. However, hemoglobin did not reduce high K^+-induced GABA release, perhaps because the concentration of hemoglobin used did not remove all of the NO produced. Evidence that NO plays a significant role in high K^+-induced release of GABA

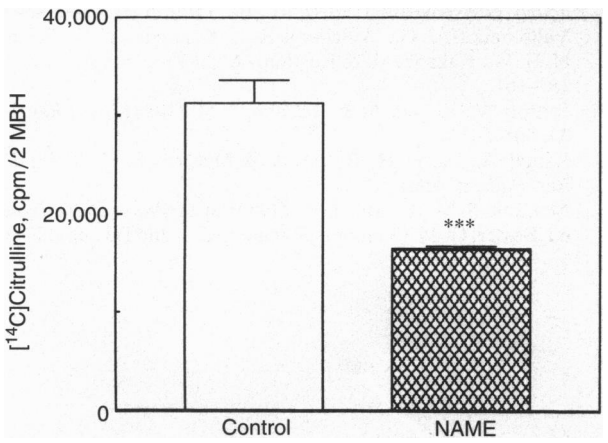

FIG. 5. Release of NO as measured by the [^{14}C]citrulline method and its inhibition by an inhibitor of NOS, NAME (300 μM). ***, $P < 0.005$ vs. control.

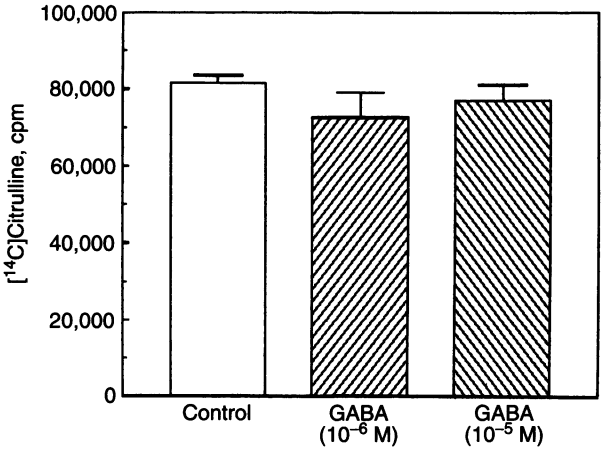

FIG. 6. Lack of effect of GABA on NO production measured by the [^{14}C]citrulline method at 10^{-5} and 10^{-6} M.

was the finding that inhibition of NOS by NMMA reduced high K^+-induced release of GABA.

High K^+-induced release of transmitters and polypeptides is thought to be due to opening of voltage-dependent Ca^{2+} channels as a result of K^+-induced depolarization of cell membranes (18). Presumably, NO acts to further increase intracellular Ca^{2+} in the presence or absence of increased extracellular K^+ to promote additional exocytosis of GABA. The accepted mechanism of action of NO in most tissues is via activation of soluble guanylate cyclase leading to generation of cGMP, which activates protein kinase G (1, 2, 13). Indeed, 8-monobutyryl cGMP (10^{-3}–10^{-2} M) significantly increased basal and high K^+-evoked GABA release and content, which supports the hypothesis that NO stimulates GABA release and synthesis via NO activation of soluble guanylate cyclase (unpublished data). The released cGMP in turn can cause an increase in intracellular Ca^{2+} (19). This mechanism appears to operate also in NO-induced release of the hypothalamic peptides LHRH (3, 8, 9) and somatostatin (5).

Since GABA inhibited the release of LHRH and its effect was blocked by the inhibitor of NOS, NMMA, it is apparent that NO is involved not only in the stimulation of LHRH release by norepinephrine shown earlier (3) but in the inhibition of LHRH release induced by GABA (Fig. 8) as well. In the intact animal, we visualize that norepinephrine acting via α_1 receptors stimulates NOergic neurons to release NO, which directly stimulates the LHRH terminals to release LHRH. After passage down the portal vessels, LHRH stimulates the

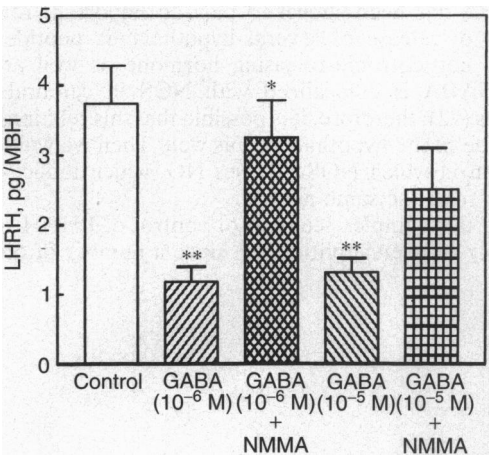

FIG. 7. Effect of GABA on release of LHRH from MBH explants and the ability of NMMA to prevent this inhibitory effect. **, $P < 0.01$ vs. control; *, $P < 0.05$ vs. control.

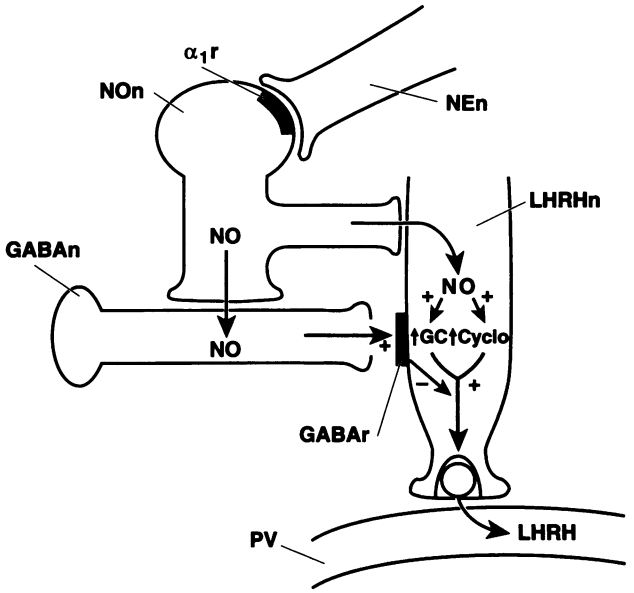

FIG. 8. Schematic diagram of the role of GABA in control of LHRH release. For description see *Discussion.* NEn, norepinephrine terminal; NOn, NOergic neuron; GABAn, GABAergic neuron; α_1r, α_1 norepinephrinergic receptor; GABAr, GABA receptor; LHRHn, LHRH terminal; PV, portal vessel; GC, guanylate cyclase; Cyclo, cyclooxygenase.

release of LH from the gonadotropes, but at the same time the NO released would also stimulate release of GABA, which would act directly on the LHRH terminal to inhibit the release of LHRH, thereby terminating the response to norepinephrine and thus the pulse of LHRH and LH. We postulate that the response of the LHRH terminal to the NO released is terminated only when the concentration of GABA increases to a threshold level after a pulse of LHRH has been released.

Consequently, when norepinephrinergic stimulation of NOergic neurons occurs, there are two effects. First, diffusion of NO to the LHRH terminal provokes release of LHRH. Second, stimulation of GABA release via NO occurs to terminate release of LHRH stimulated by NO. This inhibitory GABAergic mechanism accounts for the termination of the pulse. There may be other mechanisms as well, since it has been shown that LHRH has an autocrine action on cells that release it (20), thereby terminating LHRH release. After a delay, the next pulse would be induced by another barrage of norepinephrinergic firing, releasing additional norepinephrine to retrigger the NOergic neurons.

GABA has been shown to play an important role in the control of release of several hypothalamic peptides, which include corticotropin-releasing hormone as well as LHRH (21). GABA is colocalized with NOS in cerebral cortical neurons (22); therefore, it is possible that this relationship may hold true in the hypothalamus as well. Then we would have a situation in which NOS releases NO, which induces GABA release from the same neuron.

Even this complex scenario of control of LHRH release is probably an oversimplification, since a number of other neurotransmitters and neuromodulators are involved in pulsatile LHRH release, among them glutamate (23). We have just shown that glutamate acts to release LHRH by activating norepinephrinergic terminals that then induce the release of NO from the NOergic neurons (24). A variety of other transmitters may also be involved, such as dopamine and various neuropeptides (25).

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1. Bredt, D. S., Hwang, P. M. & Snyder, S. H. (1990) *Nature (London)* **347,** 768–770.
2. Bredt, D. S. & Snyder, S. H. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 682–685.
3. Rettori, V., Belova, N., Dees, W. L., Nyberg, C. L., Gimeno, M. & McCann, S. M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 10130–10134.
4. Karanth, S., Lyson, K. & McCann, S. M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 3383–3387.
5. Aguila, M. C. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 782–786.
6. Rettori, V., Belova, N., Yu, W. H., Gimeno, M. & McCann, S. M. (1994) *Neuroimmunomodulation* **1,** 195–200.
7. Rettori, V., Belova, N., Gimeno, M. & McCann, S. M. (1994) *Neuroimmunomodulation* **1,** 116–120.
8. Moretto, M., Lopez, J. F. & Negro-Vilar, A. (1993) *Endocrinology* **133,** 2399–2402.
9. Rettori, V., Kamat, A. & McCann, S. M. (1994) *Brain Res. Bull.* **33,** 501–503.
10. Mahachoklertwattana, P., Black, S. M., Kaplan, S. L., Bristow, J. D. & Grumbach, M. M. (1994) *Endocrinology* **135,** 1709–1712.
11. McCann, S. M. & Rettori, V. (1986) *Adv. Biochem. Psychopharmacol.* **42,** 173–179.
12. Masotto, C., Wisnieski, G. & Negro-Vilar, A. (1989) *Endocrinology* **125,** 548–553.
13. Knowles, R. G., Palacios, M., Palmer, M. J. & Moncada, S. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 5159–5162.
14. Bernasconi, R., Bittinger, H., Hied, J. & Martin, P. (1980) *J. Neurochem.* **34,** 614–618.
15. Mitchell, R., Grieve, G., Dow, R. & Fink, G. (1983) *Neuroendocrinology* **37,** 169–176.
16. Bredt, D. S. & Snyder, S. H. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 9030–9033.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–272.
18. Tan, K. N. & Tashjian, A. H. (1984) *J. Biol. Chem.* **259,** 418–426.
19. Xu, X., Star, R. A., Tortorici, G. & Muallem, S. (1994) *J. Biol. Chem.* **269,** 12645–12653.
20. Bedran de Castro, J. C., Khorram, O. & McCann, S. M. (1985) *Proc. Soc. Exp. Biol. Med.* **179,** 132–135.
21. Hoffman, G. E., Lee, W. S. & Wray, S. (1992) in *Neuroendocrinology*, ed. Nemeroff, C. B. (CRC, Boca Raton, FL), pp. 185–217.
22. Valtschanoff, J. G., Weinberg, R. J., Kharazia, V. N., Schmidt, H. H. W., Nakane, M. & Rustioni, A. (1993) *Neurosci. Lett.* **157,** 157–161.
23. Rettori, V., Kamat, A. & McCann, S. M. (1994) *Brain Res. Bull.* **33,** 501–503.
24. Kamat, A., Yu, W. H., Rettori, V. & McCann, S. M. (1995) *Brain Res. Bull.*, in press.
25. McCann, S. M. & Krulich, L. (1989) in *DeGroot Endocrinology*, ed. Besser, G. M. (Saunders, Philadelphia), 2nd Ed., pp. 117–130.