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Physiology

Nitric oxide inhibits the release of norepinephrine and dopamine from the medial basal hypothalamus of the rat

(nitroprusside/N^G-monomethyl-L-arginine/elevated-[K^+] medium/tissue catecholamine concentration/hemoglobin)

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ABSTRACT **Previous research indicates that norepinephrine and dopamine stimulate release of luteinizing hormone (LH)-releasing hormone (LHRH), which then reaches the adenohypophysis via the hypophyseal portal vessels to release LH. Norepinephrine exerts its effect via α_1-adrenergic receptors, which stimulate the release of nitric oxide (NO) from nitricoxidergic (NOergic) neurons in the medial basal hypothalamus (MBH). The NO activates guanylate cyclase and cyclooxygenase, thereby inducing release of LHRH into the hypophyseal portal vessels. We tested the hypothesis that these two catecholamines modulate NO release by local feedback. MBH explants were incubated in the presence of sodium nitroprusside (NP), a releaser of NO, and the effect on release of catecholamines was determined. NP inhibited release of norepinephrine. Basal release was increased by incubation of the tissue with the NO scavenger hemoglobin (20 μg/ml). Hemoglobin also blocked the inhibitory effect of NP. In the presence of high-potassium (40 mM) medium to depolarize cell membranes, norepinephrine release was increased by a factor of 3, and this was significantly inhibited by NP. Hemoglobin again produced a further increase in norepinephrine release and also blocked the action of NP. When constitutive NO synthase was inhibited by the competitive inhibitor N^G-monomethyl-L-arginine (NMMA) at 300 μM, basal release of norepinephrine was increased, as was potassium-evoked release, and this was associated in the latter instance with a decrease in tissue concentration, presumably because synthesis did not keep up with the increased release in the presence of NMMA. The results were very similar with dopamine, except that reduction of potassium-evoked dopamine release by NP was not significant. However, the increase following incubation with hemoglobin was significant, and hemoglobin, when incubated with NP, caused a significant elevation in dopamine release above that with NP alone. In this case, NP increased tissue concentration of dopamine along with inhibiting release, suggesting that synthesis continued, thereby raising the tissue concentration in the face of diminished release. When the tissue was incubated with NP plus hemoglobin, which caused an increase in release above that obtained with NP alone, the tissue concentration decreased significantly compared with that in the absence of hemoglobin, indicating that, with increased release, release exceeded synthesis, causing a fall in tissue concentration. When NO synthase was blocked by NMMA, the release of dopamine, under either basal or potassium-evoked conditions, was increased. Again, in the latter instance the tissue concentration declined significantly, presumably because synthesis did not match release. Therefore, the results were very similar with both catecholamines and indicate that NO acts to suppress release of both amines. Since both catecholamines activate the release of LHRH, the inhibition of their release by NO serves as an ultra-short-loop negative feedback by which NO inhibits the release of the catecholamines, thereby reducing the activation of the NOergic neurons and decreasing the release of LHRH. This may be an important means for terminating the pulses of release of LHRH, which generate the pulsatile release of LH that stimulates gonadal function in both male and female mammals.**

Norepinephrine, and to a lesser extent dopamine, play a controlling role in release of luteinizing hormone (LH)-releasing hormone (LHRH) from the hypothalamus (1, 2). LHRH is secreted into the hypophyseal portal vessels and reaches the gonadotropes of the anterior pituitary gland, thereby mediating the release of LH, which in turn activates steroid secretion from the gonads and induces ovulation (3). The release of LHRH is pulsatile and is thought to be driven by norepinephrine, which acts on hypothalamic nitricoxidergic (NOergic) neurons (4–7). The NO diffuses to the LHRH terminals in the arcuate-median eminence region to release LHRH by activating guanylate cyclase and cyclooxygenase (6, 8). Dopamine can also stimulate LHRH release, but the physiologic significance of this is less clear (1, 2).

We hypothesized that NO released from the NOergic neurons in the arcuate-median eminence region alters the release of norepinephrine and/or dopamine from the basal hypothalamus. Therefore, we measured the effect of NO on release of dopamine and norepinephrine from medial basal hypothalamic explants incubated *in vitro*. Indeed, incubation of this tissue with sodium nitroprusside (NP), which spontaneously releases NO (9, 10), inhibited release of both catecholamines, and their release was increased by blocking NO synthase (NOS) with a competitive inhibitor of constitutive NOS present in the NOergic neurons, N^G-monomethyl-L-arginine (NMMA) (9, 10). We believe that this NO-induced inhibition of release of norepinephrine and dopamine constitutes an ultra-short-loop negative feedback mechanism to inhibit the pulsatile release of these catecholamines and thereby terminate the pulsatile release of LHRH.

MATERIALS AND METHODS

Male Wistar rats weighing 180–250 g were used. The animals were fed laboratory chow and water ad lib and were main-

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Abbreviations: LH, luteinizing hormone (lutropin); LHRH, LH-releasing hormone (luliberin); NOergic, nitricoxidergic; NOS, NO synthase; NP, sodium nitroprusside; NMMA, N^G-monomethyl-L-arginine; MBH, medial basal hypothalamus; GABA, γ-aminobutyric acid.

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tained under controlled conditions of light (12 hr light and 12 hr darkness) and temperature (20–25°C). The animals were killed by decapitation and the medial basal hypothalamus (MBH) was excised from the brain as previously described (6).

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 (KRB) buffer (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, 10 μM tyrosine, and 0.1% bovine serum albumin. After a preliminary incubation of 15 min the tissue was incubated for 30 min in fresh KRB buffer containing the compounds studied. Then the medium was removed (basal efflux) and the tissues were incubated for a further 30 min in KRB containing 30 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.

NMMA, a competitive inhibitor of NOS (9, 10), and hemoglobin, a scavenger of NO (9, 10), were purchased from Sigma.

The media were collected in 0.1 M perchloric acid (final concentration), and both the media and the tissue were stored at −70°C. The tissue was homogenized in 0.1 M perchloric acid immediately before assay. Norepinephrine and dopamine concentrations were determined by high-pressure liquid chromatography with electrochemical detection according to Reinhard and Roth (11), using dihydroxybenzilamine hydrobromide as internal standard. Aliquots of the homogenates were used in determining protein concentration by the method of Lowry *et al.* (12).

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

RESULTS

Effect of NP on Norepinephrine Release and Tissue Concentration. NP releases NO, and therefore it was used to see the effect of increased NO concentrations on the release of norepinephrine from the MBH. NP (300 μM) decreased norepinephrine release by over 50% ($P < 0.01$ vs. control). Hemoglobin (20 μg/ml), a scavenger of NO, increased norepinephrine release by nearly a factor of 2 ($P < 0.01$) and, in the presence of NP, elevated the concentration above that in the presence of NP alone ($P < 0.01$) (Fig. 1). In the case of the addition of NP, there was a slight but significant ($P < 0.05$) reduction in norepinephrine concentration in the tissue in the presence of NP (Fig. 2), but the other treatments were without effect.

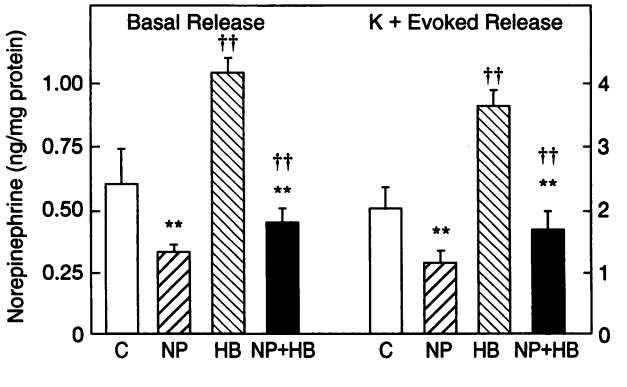

FIG. 1. Effect of NP (300 μM) on norepinephrine release from MBH explants (mean ± SEM). In this and subsequent figures there were six individually incubated MBH explants per group. C, control; HB, hemoglobin (20 μg/ml); K^+-evoked release, release in the presence of 40 mM potassium. **, $P < 0.01$ versus respective control without NP; ††, $P < 0.01$ versus respective control without HB.

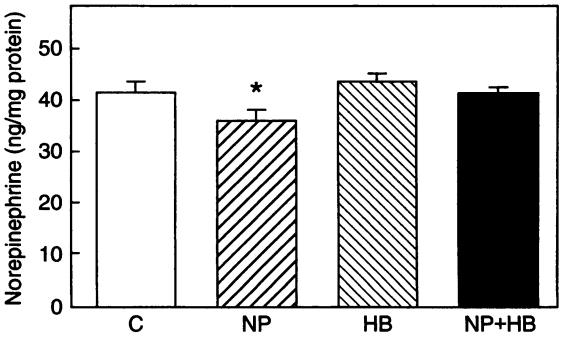

FIG. 2. Effect of NP on the tissue concentration of norepinephrine. C, control; NP, 300 μM NP; HB, hemoglobin at 20 μg/ml. *, $P < 0.05$ versus control.

In the presence of a depolarizing potassium concentration (40 mM) in the medium, norepinephrine release was increased by a factor of 3, and as before, this release was decreased by approximately 40% ($P < 0.01$) by NP. The release was highly significantly increased by hemoglobin and the decrease of release in the presence of NP was reversed ($P < 0.01$) (Fig. 1).

Effect of NMMA on Norepinephrine Release and Tissue Concentration. This competitive inhibitor of NOS (300 μM) caused a significant ($P < 0.05$) increase in basal release of norepinephrine and a highly significant increase in the release of norepinephrine evoked by increased extracellular potassium concentration in the medium (Fig. 3). This increased release induced by NMMA was associated in the case of the potassium-induced release with a reduction in the tissue concentration of norepinephrine compared with that measured with high potassium alone (Fig. 3).

Effect of NP on Dopamine Release. NP (300 μM) decreased dopamine release slightly but not significantly; however, it was significantly elevated by hemoglobin (20 μg/ml), the scavenger of NO (Fig. 4). The addition of NP in the presence of hemoglobin had no effect on dopamine release, but the release was significantly greater than that obtained in the presence of NP alone (Fig. 4).

The tissue concentration of dopamine was significantly increased by NP, but not by hemoglobin, and was reduced below that in the presence of NP alone by hemoglobin (Fig. 5).

Effect of NMMA on Dopamine Release and Tissue Concentration. When NMMA was incubated with the tissue to block NOS, basal release of dopamine was increased by a factor of 3 ($P < 0.001$) (Fig. 6). As in the case of other transmitters, including norepinephrine, the release was increased dramatically by increasing the potassium concentration in the medium, and this was further markedly augmented to 170% of the

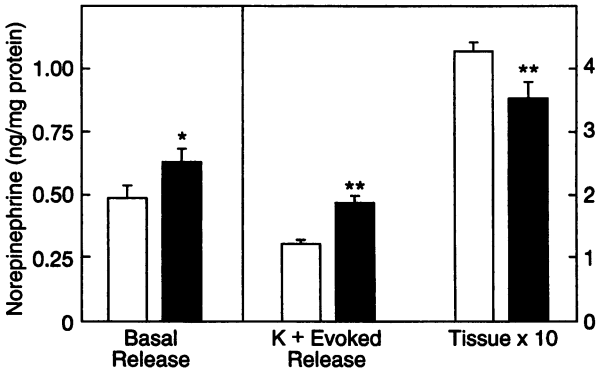

FIG. 3. Effect of NMMA (300 μM) (black bars) on norepinephrine release and tissue concentration (note that for tissue the scale on the right ordinate goes to 4.0 ng/mg of protein). *, $P < 0.05$; **, $P < 0.01$ versus control (white bars).

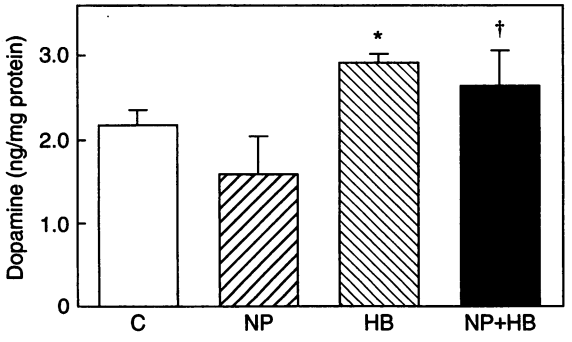

FIG. 4. Effect of NP on the release of dopamine from MBH explants. C, control; NP, 300 μM NP; HB, hemoglobin at 20 μg/ml. *, $P < 0.05$ versus C; †, $P < 0.05$ versus NP.

K^+-evoked release in the presence of NMMA ($P < 0.01$). This increased release of dopamine in the presence of NMMA was accompanied by decreased tissue content (Fig. 6).

DISCUSSION

The results clearly show that NP, which spontaneously releases NO, can inhibit the basal and potassium-stimulated release of norepinephrine; the inhibitory effect in both cases was abolished by hemoglobin, a scavenger of NO. The release of norepinephrine under basal and potassium-stimulated conditions was enhanced by NMMA, a competitive inhibitor of NOS. Increased release was associated with decreased tissue concentration in the presence of the inhibitor NMMA, suggesting that when release was stimulated, the content in the tissue was depleted, since the increased release was not accompanied by sufficient *de novo* synthesis to maintain tissue content. The small decrease in tissue content following incubation with NP is unexplained.

The effects on dopamine were similar. However, the decrease in dopamine release induced by NP was not statistically significant. Hemoglobin evoked a significant increase and prevented the decline in dopamine release caused by NP. Furthermore, marked increases in both basal and potassium-evoked release of dopamine were observed when NOS was inhibited by NMMA. In the case of tissue concentration, this was reduced when release was augmented by NMMA in the presence of high potassium. The changes in dopamine concentration in the tissue of NP-incubated explants were consistent in that NP, which inhibited release, resulted in an increase in tissue content; however, hemoglobin, which increased release, had no effect on tissue content but reduced the increase in tissue content induced by NP. This is consistent with a lesser inhibition of release in this situation.

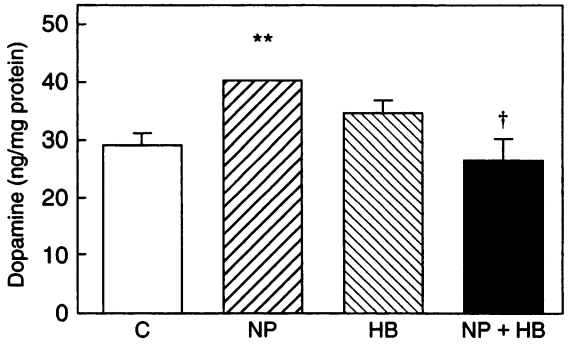

FIG. 5. Effect of NP (300 μM) on tissue concentration of dopamine. C, control; NP, 300 μM NP; HB, hemoglobin at 20 μg/ml. **, $P < 0.01$ versus control; †, $P < 0.05$ versus NP.

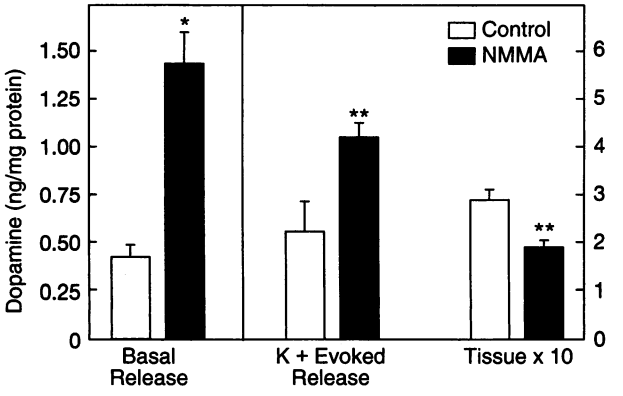

FIG. 6. Effect of NMMA on dopamine release and tissue concentration. *, $P < 0.001$ versus control; **, $P < 0.01$ versus high-potassium or tissue control.

NO was able not only to inhibit basal release of the catecholamines but also to block high-potassium-induced release. Elevated potassium concentrations in the media increase release of virtually all transmitters in the central nervous system. Since there are few data on the effect of specific transmitters on release of catecholamines from the hypothalamus, we chose high-potassium medium as a convenient stimulator of release of the catecholamines. The mechanism of the release is thought to be K^+-induced depolarization of the cell membrane, which opens voltage-gated calcium channels, leading to increased intracellullar calcium ion concentrations, which induce exocytosis of catecholamine-containing synaptic vesicles (13).

Thus, release of NO from the NOergic neurons in the medial basal hypothalamus *in vitro* has a tonic inhibitory action to decrease the release of norepinephrine and dopamine from the explants and to block the high-potassium stimulation of the release of catecholamines. Presumably, the NO produced by the NOergic neurons diffuses to the terminals of the catecholaminergic neurons, where it acts on soluble guanylate cyclase within the terminals to activate the enzyme and cause the production of cyclic guanosine monophosphate (cGMP) (9, 10). This cGMP may cause a decrease in intracellular free calcium in the cell, which may block the depolarization of the catecholaminergic terminals associated with release of the catecholamines from storage vesicles (9, 10). The mechanism of the inhibition needs further study.

Previous evidence indicates that noradrenergic stimulation of LHRH release is mediated by α_1-adrenergic receptor stimulation of the NOergic neurons by increasing intracellular free calcium, which combines with calmodulin to activate NOS, leading to the release of NO (Fig. 7) (6). Indeed, we have demonstrated that NO production measured by the citrulline method is increased by norepinephrine by α_1 receptor stimulation, since it can be blocked by the α_1 receptor blocker prazosine (6). The released NO diffuses to the LHRH terminals in the arcuate-median eminence region, enters the terminals, and activates guanylate cyclase. This causes the release of cGMP, which we speculate increases intracellular free calcium and activates phospholipase A_2 (PLA_2). Although cGMP often lowers intracellular Ca^{2+}, which is the basis for its relaxation of vascular smooth muscle (10), Muallem's group demonstrated by incubating pancreatic acinar cells that low concentrations lowered Ca^{2+} but high concentrations increased it (15). Recent evidence indicates that cGMP can increase Ca^{2+} by means of cyclic ADP-ribose, which is the ligand for the ryanodine–Ca^{2+} channel (16).

The activated PLA_2 enzyme converts membrane phospholipids into arachidonate, and at the same time the NO acting on the heme group of cyclooxygenase activates it, resulting in

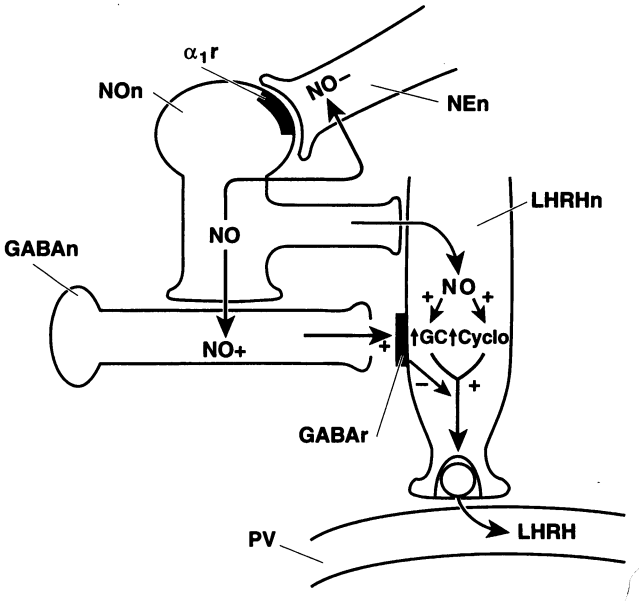

FIG. 7. Diagram illustrating the role of NO in stimulating LHRH release by activating guanylate cyclase and cyclooxygenase, and its ability to stimulate the release of γ-aminobutyric acid (GABA), which then, via GABA receptors, blocks the activation of the LHRH neuron via NO. It also illustrates the further action of NO to feed back and inhibit the release of norepinephrine. These two mechanisms terminate the pulsatile release of LHRH and thereby the pulsatile release of LH. PV, portal vessel; GABAr, GABA receptor; GC, guanylate cyclase; Cyclo, cyclooxygenase; GABAn, GABA neuron; NEn, norepinephrine neuron; α_1r, α-adrenergic type 1 receptor; NOn, NOergic neuron; +, stimulatory action; −, inhibitory action. The figure is modified from ref. 14.

conversion of arachidonate to prostaglandin E_2 (PGE_2). PGE_2 activates exocytosis of LHRH secretory granules, presumably by activation of adenylate cyclase and generation of cAMP, which in turn activates protein kinase A. We believe this is the means for initiation of pulsatile discharge of LHRH (1–3).

The data presented here indicate that as the NOergic neurons are activated by α_1-adrenergic receptors, the NO produced diffuses to the noradrenergic terminals and inhibits them as described here, thus providing an ultra-short-loop negative feedback to terminate the release of norepinephrine and consequently the release of LHRH. In addition, we have recently demonstrated that the NOergic neurons, by release of NO, stimulate the release of GABA, which then acts on the LHRH neuron to terminate its response to NO, thereby acting as a feedforward inhibition to terminate the LHRH pulse (14). Glutamic acid also activates LHRH via NOergic neurons, but it does not act directly; instead it activates the noradrenergic terminals, which in turn activate the NOergic neurons (17).

The role of dopamine in pulsatile LHRH release is less clear. There are tuberoinfundibular dopaminergic neurons in the hypothalamus which have been shown to stimulate the release of LHRH from male rat hypothalami *in vitro* (18); however, other studies indicate that at certain concentrations and in different hormonal states such as in the castrate rat, both dopamine and norepinephrine can inhibit LHRH release (19, 20). Whether dopamine is released prior to each LH pulse to stimulate NO release and LHRH release has yet to be determined, but this may contribute to release of NO and augment pulsatile LHRH release, since dopamine receptor blockers can block LH release (21). It is quite apparent that a number of transmitters and neuromodulators are released simultaneously to bring about the pulse of LHRH (1). Therefore, the schema presented here is an oversimplification of the *in vivo* events.

In summary, we have evidence not only that the catecholamines induce the pulsatile LHRH release but also that the NO released from the NOergic neurons, which stimulates LHRH release and initiates the pulse, also acts back on the norepinephrine and dopaminergic neurons to terminate the pulse. This is coupled with the negative feedforward of GABA released by NO to bring about the termination of the pulse.

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1. McCann, S. M. & Krulich, L. (1989) in *De Groot: Endocrinology*, ed. Besser, G. M. (Saunders, Philadelphia), 2nd Ed., pp. 117–130.
2. Negro-Vilar, A., Advis, J. P., Ojeda, S. R. & McCann, S. M. (1982) *Endocrinology* **111,** 932–938.
3. McCann, S. M. (1991) in *Brain Endocrinology*, ed. Motta M. (Raven, New York), 2nd Ed., pp. 1–30.
4. Rettori, V., Belova, N., Dees, W. L., Nyberg, C. L., Gimeno, M. & McCann, S. M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 10130–10134.
5. Rettori, V., Belova, N., Kamat, A., Lyson, K. & McCann, S. M. (1994) *Neuroimmunomodulation* **1,** 86–91.
6. Canteros, G., Rettori, V., Franchi, A., Genaro, A., Cebral, E., Saletti, A., Gimeno, M. & McCann, S. M. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 3416–3420.
7. Moretto, M., Lopez, F. J. & Negro-Vilar, A. (1993) *Endocrinology* **133,** 2399–2402.
8. Rettori, V., Gimeno, M., Lyson, K. & McCann, S. M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11543–11546.
9. Dawson, T. M. & Snyder, S. H. (1994) *J. Neurosci.* **14,** 5147–5159.
10. Bredt, D. S. & Snyder, S. H. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 9030–9033.
11. Reinhard, J. F. & Roth, R. H. (1982) *J. Pharm. Exp. Ther.* **221,** 541–546.
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–272.
13. Darnell, J., Lodish, H. & Baltimore, D. (1990) *Molecular Cell Biology*, Scientific American Books (Freeman, New York), 2nd Ed., pp. 790–794.
14. Selicovich, A., Duvilanski, B. H., Pisera, D., Theas, S., Gimeno, M., Rettori, V. & McCann, S. M. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 3421–3424.
15. Xu, X., Star, R. A., Tortorici, G. & Muallem, S. (1994) *J. Biol. Chem.* **269,** 12645–12649.
16. Berrige, M. J. (1993) *Nature (London)* **365,** 388–389.
17. Kamat, A., Yu, W. H., Rettori, V. & McCann, S. M. (1995) *Brain Res. Bull.* **37,** 233–235.
18. Ojeda, S. R., Negro-Vilar, A. & McCann, S. M. (1979) *Endocrinology* **104,** 617–624.
19. Vijayan, E. & McCann, S. M. (1978) *Neuroendocrinology* **25,** 221–235.
20. McCann, S. M. (1982) *Annu. Rev. Pharmacol. Toxicol.* **22,** 491–515.
21. Ojeda, S. R., Harms, P. G. & McCann, S. M. (1974) *Endocrinology* **94,** 1650–1657.