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Estradiol and Progesterone Modulate the Nitric Oxide/Cyclic GMP Pathway in the Hypothalamus of Female Rats and in GT1-1 Cells

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Considerable evidence suggests that the nitric oxide (NO)/cGMP signaling pathway plays an important role in the expression of reproductive behavior and in gonadotropin-releasing hormone (GnRH) release from the hypothalamus The effects of the NO/cGMP pathway on GnRH release and gene expression have also been examined in GT1 cells. However, it is still controversial whether NO/cGMP signaling facilitates or inhibits GnRH release in these cells. The current study examined the effects of estradiol and progesterone on neuronal NO synthase (nNOS), soluble guanylyl cyclase (sGC), and NO-dependent cGMP production in the preoptic area (POA) and hypothalamus (HYP) as well as in GT1-1 cells. Ovariectomized female rats received vehicle, estradiol benzoate (48 h) and/or progesterone (3–4 h) before preparation of brain slices. GT1-1 cells were incubated with vehicle, estradiol (48 h), progesterone (3–4 h), or with both hormones. The combination of estradiol and progesterone increased the expression of nNOS protein in the POA and HYP. Hormones had little effect on the abundance of sGC. Estradiol and progesterone together greatly enhanced NO-stimulated sGC activity in HYP-POA slices. In GT1-1 cells, NO-stimulated sGC activity was significantly increased by estradiol and progesterone, alone or in combination, but sGC expression was not altered by hormones.

Key Words: Preoptic area; GnRH; reproductive behavior; neuronal nitric oxide synthase; soluble guanylyl cyclase.

Introduction

Nitric oxide (NO) is a unique biological messenger because of its fast diffusibility both within and between cells. It is synthesized by NO synthase (NOS) and activates soluble guanylyl cyclase (sGC) to convert GTP to cGMP *(1)*. Subsequently, targets of cGMP, such as cGMP-dependent protein kinase (PKG), cyclic nucleotide phosphodiesterases, and cyclic nucleotide-sensitive ion channels, are activated to continue the signal transduction. This NO/cGMP signaling pathway is crucial in many physiological functions, including immunological defense mechanisms, vasodilation, and neurotransmission *(2)*.

Another important function of NO/cGMP signaling is the neuroendocrine regulation of female reproductive function. Others and we have shown that the NO/cGMP pathway plays an important role in hormone-dependent regulation of lordosis (reproductive) behavior in female rodents *(3,4)*. The NO/cGMP system in the HYP is regulated by ovarian hormones, and may mediate the facilitatory effect of norepinephrine on lordosis behavior *(5)*. Estrogen priming increases neuronal NOS (nNOS) expression, mRNA levels, and activity in the preoptic area (POA) and hypothalamus (HYP) *(6–9)*, the two major brain sites involved in the hormonal regulation of lordosis behavior in female rodents *(10)*. Levels of nNOS mRNA and protein as well as enzyme activity are significantly elevated in the POA on the afternoon of proestrus *(11)*. About half of the NOS-immunoreactive cells in HYP also contain progesterone (P) receptor immunoreactivity in guinea pigs *(12)*, suggesting a role for P in regulating NO. HYP levels of cGMP rise significantly on the evening of proestrus, a time characterized by high levels of P and behavioral receptivity in gonadally intact rats *(13)*.

NO may also regulate the secretion of GnRH both in vivo and in vitro; blockade of NO production lowers GnRH release whereas an increase of NO in the HYP stimulates GnRH release *(14–20)*. Because GnRH neurons are widely scattered through the basal forebrain *(21)*, it can be difficult to study the regulation of GnRH secretion in vivo. A GnRH neuronal cell line, GT1, is thus developed by targeted tumorigenesis of GnRH neurons *(22,23)* to provide a homogeneous population of GnRH neurons. These cells are often used as a model to investigate the regulation of GnRH synthesis and secretion. However, the role of the NO/cGMP pathway in GnRH release from GT1 cells is unclear. Some studies indicate that agents which increase NO or cGMP enhance GnRH release in GT1 cells *(18,24)*, whereas these agents decrease stimulated GnRH release in other reports *(25)*.

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When GnRH mRNA is assessed in a subclone of GT1 cells, GT1-7, activation of the NO/cGMP pathway reduces GnRH mRNA levels *(26)*. In another subclone, GT1-1 cells, NO/cGMP increases GnRH mRNA *(27)*.

The purpose of the current study was to examine the expression of nNOS and sGC in POA and HYP as well as in GT1-1 cells in response to estradiol (E_2) and/or P. We also investigated hormonal effects on the activity of the NO/cGMP pathway by measuring NO-stimulated cGMP synthesis both in tissue slices and in GT1-1 cells. Our results indicate that E_2 and P together, but neither steroid alone, increases the expression of nNOS protein in both the POA and HYP. Ovarian hormones have little effect on the protein abundance of sGC, but the combination of E_2 and P greatly enhances NO-stimulated sGC activity in HYP-POA slices. In GT1-1 cells, NO-stimulated sGC activity is significantly increased by E_2 and P, alone or in combination, but sGC expression is not altered by the ovarian hormones. Therefore, our data suggest that the combination of E_2 and P enhances nNOS protein expression and NO-dependent sGC activity in the POA and HYP, and that GnRH neurons may be one cell type in which ovarian hormones regulate sGC activity.

Results

Expression of nNOS Is Increased in Both POA and HYP of E_2- and P-Treated Animals

Female rats were ovariectomized (OVX) and treated with vehicle (control) or with physiological doses of E_2 benzoate (EB, 48 h) and/or P (3.5–4 h) before preparation of POA and HYP slices. Tissue lysates were analyzed by immunoblotting for the expression levels of nNOS and sGC. Figure 1A shows that in the POA, hormone treatment significantly influences nNOS protein abundance ($p = 0.034$). Post-hoc tests indicate that expression of nNOS is significantly higher in rats injected with both EB and P compared to the control group ($p = 0.001$). EB or P alone does not alter the expression level of nNOS compared to the vehicle control. Figure 1B shows that hormone treatment also influences nNOS levels in the HYP ($p = 0.016$). Post-hoc tests indicate that nNOS expression is also significantly enhanced in HYP of rats primed with both EB and P compared to controls ($p = 0.013$).

Expression of sGC May Be Elevated in the POA by EB

Expression of the α1 subunit of sGC in the POA is affected by hormone treatment ($p = 0.026$). More specifically, 48 h of exposure to EB increases the α1 subunit expression relative to the control group ($p = 0.011$) (Fig. 2A). The effect of hormones on the expression of the β1 subunit does not reach significance ($p = 0.14$). Nevertheless, the expression pattern of the β1 subunit is very similar to that of the α1 subunit. On the contrary, hormones do not detectably affect the expression of either subunit of sGC in the HYP (Fig. 2B).

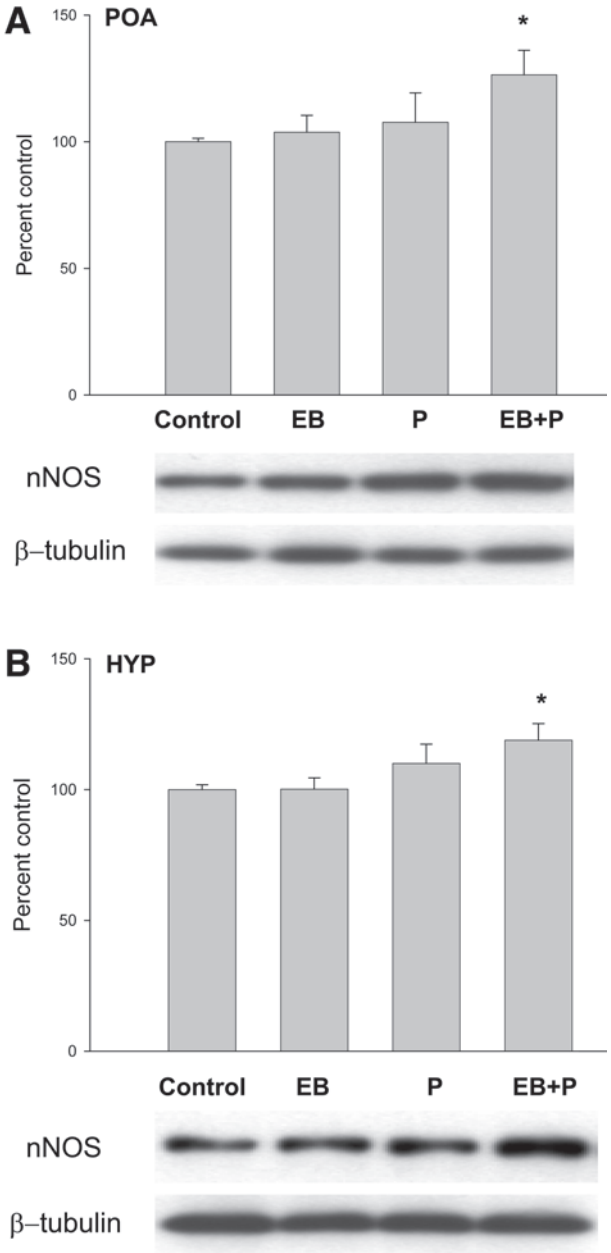

Fig. 1. Effects of hormone treatment on the expression of neuronal nitric oxide synthase (nNOS) in (**A**) the preoptic area (POA) and (**B**) the hypothalamus (HYP). Ovariectomized female rats were injected with estradiol benzoate (EB, 2 μg) or peanut oil (controls) 24 and 48 h before tissue preparation. Progesterone (P, 500 μg) or oil was injected 44 h after the first dose of EB. Rats were killed 3.5–4 h after P or oil, and the POA and HYP lysates were prepared as described in Materials and Methods. Quantification was done by calculating the ratio of the optical density (OD) of the nNOS band to the OD of the β-tubulin III band. This value was then expressed as a percentage relative to controls (no hormone treatment). Values presented are means ± SEM (n = 8–11); *$p < 0.05$ vs control. A representative immunoblot is shown for each brain area.

Stimulation of cGMP Accumulation Is Greatly Enhanced in Tissue Slices from EB- and P-Primed Rats

To measure NO-dependent sGC activity, slices from OVX, hormone-treated rats were incubated with the NO

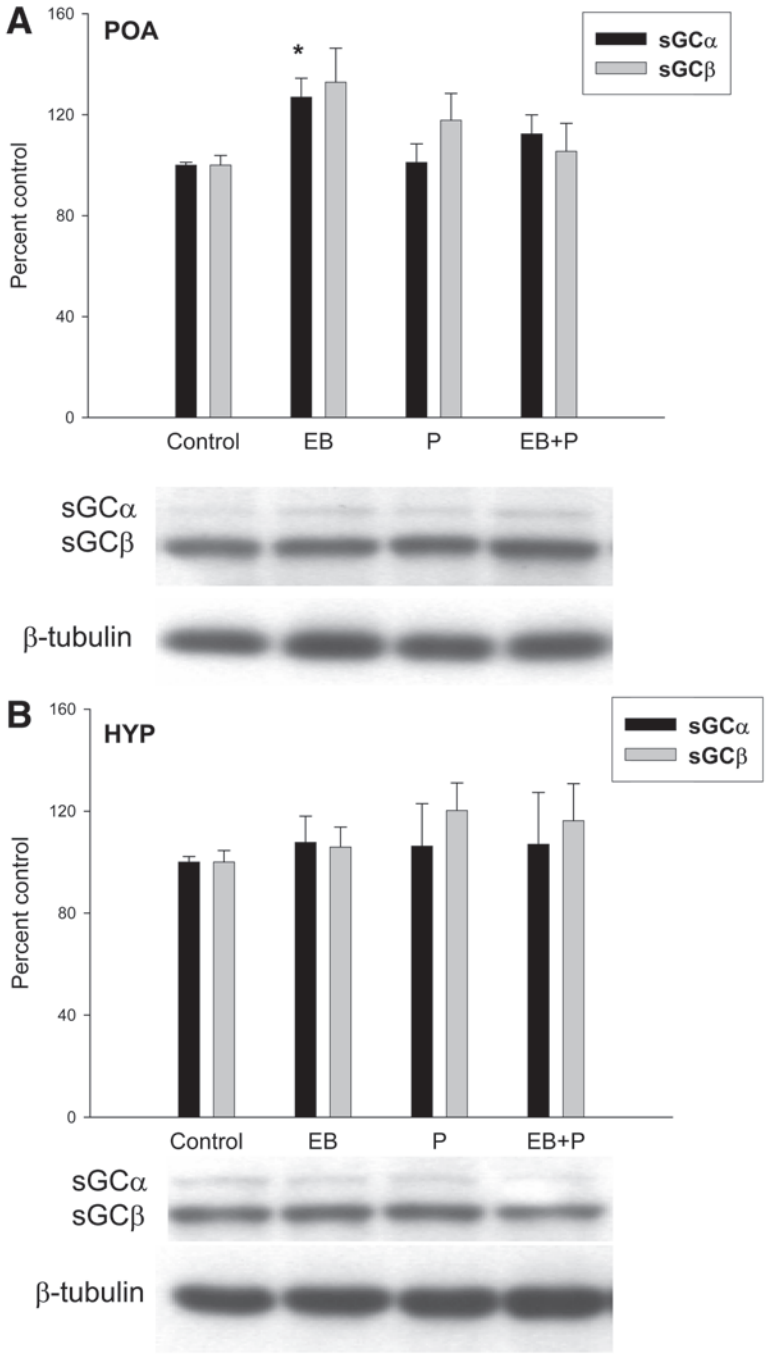

Fig. 2. Effects of hormone treatment on the expression of the two subunits of sGC in the POA (**A**) and HYP (**B**). Lysates of the POA and HYP were obtained from hormone-treated rats as described in Fig. 1. Abbreviations and quantification of protein are as in Fig. 1. Values presented are means ± SEM ($n = 8$); *$p < 0.05$ vs control. A representative immunoblot is shown for each brain region.

donor sodium nitroprusside (SNP) for 20 min, followed by quantitation of cellular cGMP (Fig. 3). A phosphodiesterase inhibitor was included in the incubation to prevent cGMP hydrolysis. Slices from control, EB-, or P- treated animals respond to 10 μ*M* SNP by increasing cGMP levels about 5- to 7-fold compared to the non-stimulated slices. However, slices from EB+P rats show a significantly higher response to SNP (15-fold over nonstimulated) than those from all other groups ($p < 0.05$). The basal cGMP levels are not affected by the different hormone treatments (data not shown).

Hormones Have No Effect on sGC Expression or Basal cGMP Levels in GT1-1 Cells

We next analyzed the effects of different hormone treatments on nNOS and sGC expression in GT1-1 cells incubated with vehicle, with E_2 alone for 48 h, with P alone for 3.5–4 h, or with both hormones. We are not able to detect

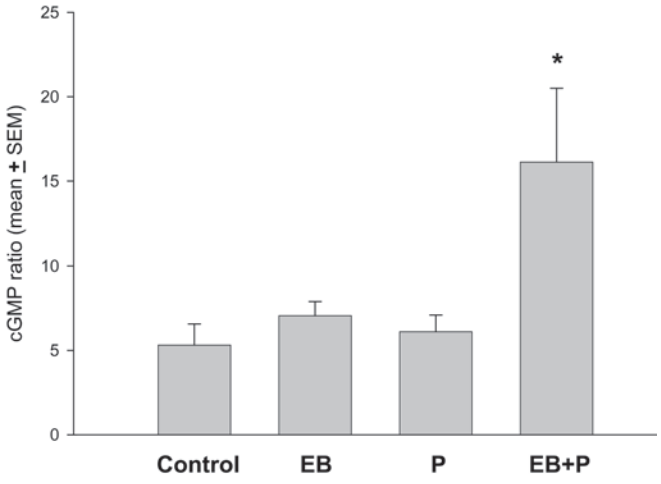

Fig. 3. Effects of hormone treatment on sodium nitroprusside (SNP)-stimulated cGMP production in HYP-POA slices. Hormone treatments and abbreviations are as described in Fig. 1. HYP-POA slices were incubated with SNP (10 μ*M*), a NO donor, to stimulate cGMP formation. Data are presented as the cGMP ratio, calculated by dividing the cGMP level in a SNP-treated slice by the cGMP level in an adjacent, vehicle-treated slice. Values presented are means ± SEM ($n = 4$); *$p < 0.05$ vs control.

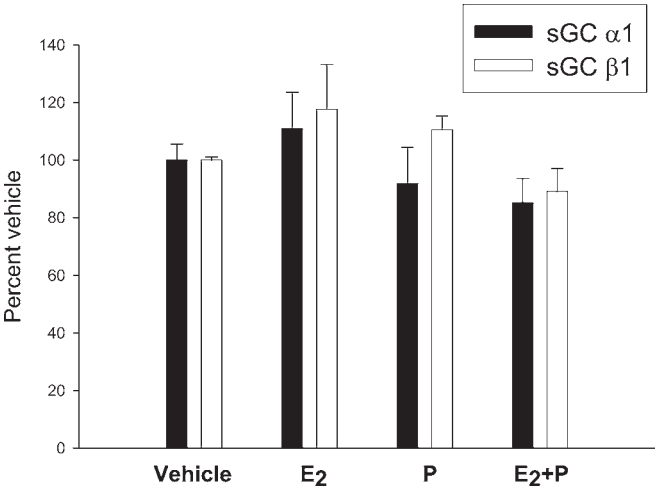

Fig. 4. Effects of hormone treatment on the expression of the two subunits of sGC in GT1-1 cells. GT1-1 cells were incubated with vehicle, with 1 n*M* E_2 alone for 48 h, with 20 n*M* P alone for 3.5–4 h, or with both hormones. Cell lysates were obtained from hormone-treated GT1-1 cells as described in Materials and Methods. Quantification was done as in Fig. 1. Values presented are means ± SEM ($n = 4$).

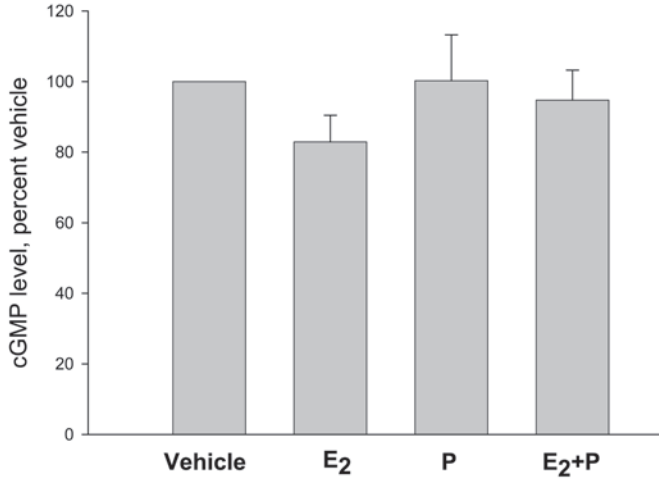

Fig. 5. Effects of hormone treatment on basal cGMP levels in GT1-1 cells. Cells were hormone-treated as in Fig. 4. About 4 h after P, cells were washed with phosphate-buffered saline (PBS). Culture medium was then collected, and cells were harvested for cGMP measurements. The cGMP data are expressed as a percentage of the cGMP level measured in cells without any hormone treatment harvested on the same day. Values presented are means ± SEM ($n = 4$-10).

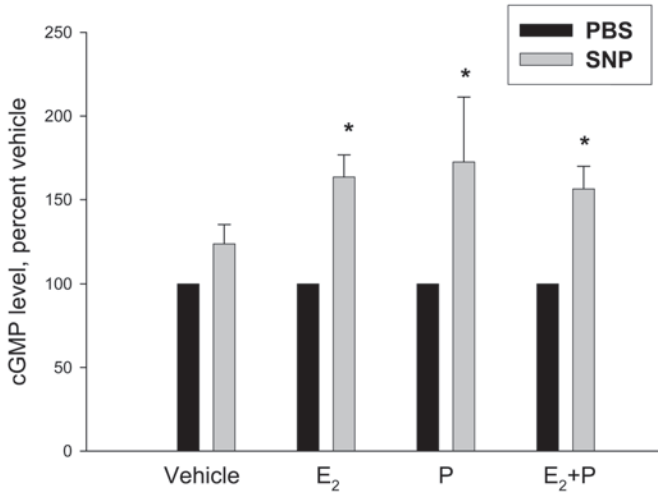

Fig. 6. Effects of hormone treatment on SNP-stimulated cGMP production in GT1-1 cells. Cells were hormone-treated and washed with PBS as described in Fig. 4. After washing with PBS, GT1-1 cells were stimulated with SNP (100 μ*M*) or PBS for 20 min. The cGMP data are expressed as a percentage of the cGMP level measured in cells without any hormone treatment harvested on the same day. Values presented are means ± SEM ($n = 4$–10). $p < 0.05$ vs PBS.

nNOS protein in GT1-1 cells. Both α1 and β1 subunits of sGC are detected in the GT1-1 cells, but the expression levels are not altered by any hormone treatment (Fig. 4). To investigate the hypothesis that hormone treatments modify the activity of sGC in GT1-1 cells, we first measured basal cGMP levels in GT1-1 cells after various hormone conditions. Figure 5 shows that basal cGMP levels in GT1-1 cells, which ranged from 5 to 10 pmole/dish, are not affected by any hormone treatment ($p = 0.27$).

Hormone Treatments Increase the cGMP Response to SNP in GT1-1 Cells

Next, we examined cGMP synthesis in response to SNP (100 μ*M*) stimulation. There is a significant effect of hormone treatment on the cGMP response to the NO donor (Fig. 6). Post-hoc tests demonstrate that E_2 alone, P alone, or both elevate the cGMP response to SNP stimulation in GT1-1 cells relative to phosphate-buffered saline (PBS)-treated control cells ($p = 0.004$, 0.013, and 0.017, respec-

tively). Contrary to the results in brain slices from OVX female rats with no hormone replacement (Fig. 3), vehicle-treated cells (no hormone group) do not respond to SNP with a significant increase of cGMP ($p = 0.13$).

Discussion

Our results suggest that the steroid hormones E_2 and P act at multiple levels to regulate the NO/cGMP signaling pathway in the POA and the HYP of female rats. A physiologically relevant combination of EB and P increases the expression of nNOS protein in both the POA and the HYP. However, with the exception of a small increase in the POA after EB treatment, these hormones do not detectably affect the expression levels of sGC. Rather, Fig. 3 indicates that EB and P together greatly enhance NO activation of sGC. The data further suggest that GnRH neurons may be one of the cell groups regulated by ovarian hormones in the HYP-POA. Even though the hormones do not change sGC protein levels in GT1-1 cells, the responsiveness of the cells to SNP stimulation is significantly increased by E_2 and P, alone or in combination. Therefore, the major mechanisms by which ovarian steroid hormones modulate the NO-cGMP pathway appear to be by increasing the protein expression of nNOS, and by altering the NO-sensitive catalytic activity of sGC without changing its protein abundance.

In vivo data point to a facilitatory role of hypothalamic NO and cGMP in female reproductive function. When the assayed end point is female reproductive behavior, results support a stimulatory role of the NO/cGMP pathway *(4,28–31)*, which is probably mediated by PKG *(28,29,32)*. In animals, activation of the NO/cGMP pathway also increases GnRH and luteinizing hormone (LH) release *(16,20,33)*. The current study, which demonstrates an increase of nNOS protein levels and sGC activity in the HYP and POA after EB and P treatments that would produce an LH surge and female reproductive behavior, are consistent with the hypothesis that the NO/cGMP pathway mediates a stimulatory effect on female reproductive functions.

Some of our results differ from earlier reports of hormonal regulation of nNOS. Estrogen alone was sufficient to increase nNOS expression in the POA and the HYP of female rats in several studies *(6–9)*. The dosages of EB used were much higher in those experiments (10–30 μg/d) than in the current study (2 μg/d). Although we did not measure plasma E_2 in the present study, the dosage of EB used produces 25–30 pg/mL E_2 in OVX female rats 48 h after the first EB injection (unpublished data). The methods used in the past and current studies are also different. The immunohistochemical approach in the previous studies demonstrates the detailed localization of nNOS expression. We used immunoblotting for better quantification but at the expense of anatomical resolution. Thus, we might miss changes in a small brain area because of tissue dissection for the cell lysate. Nevertheless, physiological doses of EB and P do produce detectable increases in nNOS expression in the whole POA and HYP during the expected time of the preovulatory LH surge and reproductive behavior.

The endogenous activators of the NO/cGMP in the central control of reproductive function are not well understood. This signaling pathway may mediate the effects of GABA *(19,34)*, dopamine *(35,36)*, norepinephrine *(5,37)*, opioid *(38,39)*, and NMDA *(15,40)* receptors on reproduction. In addition, there is strong evidence that NO produced by endothelial NOS in the median eminence regulates GnRH release into the portal system *(41)*. PKG, cyclic nucleotide sensitive ion channels, and phosphodiesterases are the known targets of cGMP. We have shown that blocking PKG reduces female reproductive behavior in OVX, hormone-treated rats *(28,29,32)*. Because many neurotransmitters engage the NO-cGMP pathway, and many downstream targets can propagate the cGMP signal, the ovarian hormones may act at multiple molecular levels within this pathway to optimize reproductive function.

The role of NO and cGMP in the modulation of GnRH release is still controversial, especially in GT1 cells. When GnRH mRNA is measured, NO or cGMP have inhibitory effects in GT1-7 cells *(26,42)* but stimulatory effects in GT1-1 cells *(27)*. When GnRH peptide is assessed, a stimulatory effect of NO-cGMP is shown in GT1-7 cells *(17,18)*, and both stimulatory and inhibitory effects are reported in GT1-1 cells *(24,25)*, depending on the experimental conditions. To further characterize the role of E_2, P, and the NO-cGMP pathway in GnRH neurons, we used GT1-1 cells. Our results suggest that there is little or no nNOS in these cells. This is consistent with previous in vivo findings that GnRH neurons do not express nNOS; rather, the nNOS-expressing cells surround the GnRH neurons *(43)*. However, nNOS mRNA and protein have been found in GT1-7 cells *(17)*. In addition, Mahachoklertwattana et al. *(24)* show that NOS inhibitors block NMDA-induced GnRH secretion in GT1-1 cells in the absence of hormone treatment, implying an active and complete NO/cGMP pathway in GT1-1 cells. Therefore, it is possible that the nNOS antibody we used is unable to detect a low level of protein expression in GT1-1 cells.

Hormone treatments have no effect on sGC expression in GT1-1 cells (Fig. 4), but the NO-dependent enzyme activity is modified by E_2 and P alone or in combination (Fig. 6). This differs from the results in HYP and POA, where sGC activity was greatly enhanced only in the slices from rats treated with both EB and P. This inconsistency may reflect the involvement of neighboring cells or afferent inputs in the tissue slices that are absent in GT1-1 cells. How the ovarian hormones modify sGC activity in GT1-1 cells is unknown. Estrogen and P receptors have been found in GT1-7 cells *(44–46)*. However, there are no reports of either estrogen or P receptors in GT1-1 cells.

In summary, we demonstrate in this study that E_2 and P differentially regulate the two key enzymes in the NO/cGMP

signaling pathway in the POA and the HYP of female rats, increasing levels of nNOS but not sGC. Moreover, E_2 and P act together to enhance NO-dependent cGMP synthesis in these brain regions. The results from GT1-1 cells suggest that GnRH neurons may be one of the target cells in which the NO-cGMP pathway, specifically NO-dependent activation of sGC, is modulated by ovarian steroid hormones.

Materials and Methods

Animals and Hormones Treatments

Female Sprague–Dawley rats weighing 150–175 g were purchased from Taconic Farms (Germantown, NY) and maintained under controlled temperature (22–24°C) and lighting (on 0600–2000) with food and water *ad libitum*. Animals were OVX under ketamine (80 mg/kg body weight) and xylazine (4 mg/kg body weight) anesthesia 1–2 d after arrival. Five or six days later, OVX animals received the first of the two daily injections of 2 μg EB in 0.1 mL of peanut oil vehicle or 0.1 mL of vehicle (controls) subcutaneously (sc). P (500 μg) in 0.1 mL of oil or oil vehicle was injected sc 42–44 h after the first EB or vehicle administration. Three to four hours after P or vehicle injection, animals were killed by decapitation. This time course of hormone treatment was chosen because OVX female rats reliably exhibit reproductive behavior with the combined administration of EB and P but not with either hormone alone *(28, 47)*. All procedures used in these experiments followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Albert Einstein College of Medicine.

Tissue Preparation for Immunoblotting

After hormone treatments, all animals were killed between 1130 and 1330 h, and the brain removed. Cuts were made along the hypothalamic sulci first. A horizontal cut was made at 2 mm depth with a dorsal–anterior angle from the most posterior end of the HYP. A final cut was made immediately posterior to the optic chiasm to separate the POA and the HYP. The POA and HYP were placed in ice cold lysis buffer (50 m*M* HEPES, 250 m*M* NaCl, 0.1% Triton X-100, 5 m*M* EDTA, 1 m*M* PMSF, 5 μg/mL aprotinin, 2.5 μg/mL leupeptin, 0.5 m*M* NaF, 100 m*M* Na_2VO_3 0.5 m*M* DTT, and 1 m*M* β-glycerophosphate), disrupted with an ultrasonic cell disruptor, and stored at –20°C until protein assay and Western immunoblot analysis.

Tissue Preparation for cGMP Determination

For assessment of cGMP levels in brain slices, OVX female rats were hormone-treated as above and killed between 1000 and 1100 h to control for the diurnal variation of cyclic nucleotides. A tissue block containing the entire HYP-POA was dissected, and eight slices of 350 μm were made with a McIlwain tissue chopper. Slices were incubated with 1 m*M* of the phosphodiesterase inhibitor 1,3-iso-butyl-1-methylxanthine (IBMX) to prevent cGMP hydrolysis. Because of the uneven distribution of cGMP levels along the anterior–posterior axis of POA-HYP *(5)*, every two consecutive slices were taken as a pair; one slice was treated with drug and the other was treated with vehicle. SNP (10 μ*M*) for 20 min was used as a NO donor to stimulate sGC. The slices were then lysed and homogenized in 0.1 *N* HCl. Cyclic nucleotides and protein were separated by centrifugation. The supernatant was collected and frozen for cGMP determination by enzyme immunoassay, and the precipitate was assayed by the Bradford method (BioRad, Hercules, CA) for protein content. The ratio of cGMP levels in the vehicle- and drug-treated slices in a pair was analyzed.

Cell Culture

GT1-1 cells were provided by Dr. J. Pollard (Albert Einstein College of Medicine), who originally obtained the cells from Dr. P. Mellon (University of California, San Diego). Cells were cultured on polystyrene culture plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. Three days later, cells were fed with steroid-depleted medium consisting of DMEM supplemented with 10% charcoal-stripped FBS and 1% penicillin–streptomycin for 3–4 d. Cells were then incubated with fresh stripped DMEM and 1 n*M* E_2 or vehicle (0.1% EtOH) for 43–44 h. P (20 n*M*, cyclodextrin-encapsulated, Sigma) or vehicle (PBS) was added to the cells for 3.5–4 h before harvesting. This hormone treatment mimics the in vivo hormone administration regimen for animals (see above). Cells were washed and collected in lysis buffer described above except with 0.5 m*M* PMSF, 2.5 μg/mL aprotinin, and without β-glycerophosphate for protein assay and Western immunoblot analysis.

For the determination of cGMP levels, GT1-1 cells treated with vehicle or hormones as described above were washed twice with PBS (20 min each wash) and preincubated for 30 min in fresh PBS containing 1 m*M* IBMX, a phosphodiesterase inhibitor. The PBS was then discarded and fresh PBS containing IBMX with or without drug (100 μ*M* SNP) was added to the cells for 20 min. The medium was then collected and placed on ice immediately. The cells were lysed, and cell extracts were collected by 20-min incubation in 0.1 *N* HCl, scraping, sonication, and centrifugation. All samples were stored at –20°C until enzyme immunoassay for cGMP was performed.

Immunoblotting

Proteins (25 or 50 μg for tissue or cell samples, respectively) were separated on an 8% SDS-PAGE gel and transferred to polyvinylidine difluoride membranes (PerkinElmer, Boston, MA). The membranes were first blocked with 5% non-fat milk for 30 min at room temperature and then incu-

bated with antiserum to rat sGC (Alexis, San Diego, CA or CalBiochem, La Jolla, CA) at 1:500 in 5% non-fat milk for 2 h at room temperature or overnight at 4°C, respectively. Membranes were washed and then incubated with anti-chicken secondary antiserum at 1:10,000 for 2 h at room temperature. The immunoreactive bands were visualized with enhanced chemiluminescence. The membranes were then stripped and reblotted with rat nNOS (BD Biosciences, San Jose, CA) at 1:2500 for 2 h at room temperature or overnight at 4°C, followed by anti-rabbit secondary antiserum at 1:5000 for 2 h at room temperature, and visualized with enhanced chemiluminescence. The membranes were last stripped and blotted with antiserum to β-tubulin type III (Sigma, St. Louis, MO) at 1:2000 and anti-mouse secondary antiserum at 1:5000 to correct for loading errors. All the bands on the film were quantified with Kodak 1D imaging software. Band densities were corrected by the tubulin values, and data were expressed relative to the vehicle-treated group.

cGMP Determinations

Duplicates of samples containing cGMP were assayed with enzyme immunoassay (Assay Design, Ann Arbor, MI). Assay procedures were based on the protocols provided by the manufacturer. This assay system provides a sensitive and specific assay for cGMP (detection limit, 0.08 pmole).

Statistical Analysis

All data were analyzed with Kruskal-Wallis test followed by Mann-Whitney U post-hoc tests with Bonferroni correction. Differences were considered significant if $p < 0.05$.

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