NITRIC OXIDE: Biology and Chemistry
Vol. 3, No. 5, pp. 366–374 (1999)
Article ID niox.1999.0251, available online at http://www.idealibrary.com on IDEAL®

Reproductive Function in Female Mice Lacking the Gene for Endothelial Nitric Oxide Synthase[1]

Deborah L. Drazen,*,[2] Sabra L. Klein,* Arthur L. Burnett,† Edward E. Wallach,‡ Julie K. Crone,† Paul L. Huang,§ and Randy J. Nelson*

**Behavioral Neuroendocrinology Group, Departments of Psychology and Neuroscience, †Department of Urology, and ‡Department of Obstetrics and Gynecology, The Johns Hopkins University, Baltimore, Maryland 21218; and §Cardiovascular Research Center, Massachusetts General Hospital, Boston, Massachusetts 02129*

Received April 26, 1999, and in revised form July 28, 1999

Nitric oxide (NO) acts as a neuronal messenger in both the central and peripheral nervous systems and has been implicated in reproductive physiology and behavior. Pharmacological inhibition of nitric oxide synthase (NOS) with the nonspecific NOS inhibitor, L-N^G-nitro-Arg-methyl ester (L-NAME), induced deficits in both the number of ovarian rupture sites and the number of oocytes recovered in the oviducts of mice. Female neuronal NOS knockout (nNOS−/−) mice have normal numbers of rupture sites, but reduced numbers of oocytes recovered following systemic injections of gonadotropins, suggesting that NO produced by nNOS accounts, in part, for deficits in ovulatory efficiency observed after L-NAME administration. Additionally, endothelial NOS knockout (eNOS−/−) mice have reduced numbers of ovulated oocytes after superovulation. Because endothelial NOS has been identified in ovarian follicles, and because of the noted reduced breeding efficiency of eNOS−/− mice, the present study sought to determine the role of NO from eNOS in mediating the number of rupture sites present after ovulation. Estrous cycle length and variability were consistently reduced in eNOS−/− females. The number of rupture sites was normal in eNOS−/− mice under natural conditions and after administration of exogenous GnRH. After exogenous gonadotropin administration, eNOS−/− females displayed a significant reduction in the number of ovarian rupture sites. Female eNOS−/− mice also produced fewer pups/litter compared to WT mice. These data suggest that NO from endothelial sources might play a role in mediating rodent ovulation and may be involved in regulation of the timing of the estrous cycle. © 1999 Academic Press

Key Words: **ovulation; fertility; estrous cycle; knockout; ovary.**

Among a wide range of physiological and behavioral processes, nitric oxide (NO)[3] has been implicated in reproductive function (reviewed in 1). For example, NO regulates male mating behavior (2, 3) and is involved in male sexual performance through its direct involvement in penile erectile function (4, 5). NO also appears to mediate female sexual behavior, specifically, lordosis (6). Additionally, NOS-containing neurons are localized in brain regions that are dense with GnRH neurons and fibers (7, 8). Recent evidence suggests that GnRH secretion may

[1] Support for this study was provided by USPHS Grants MH 57760 (R.J.N.), HL57818 (P.L.H.), and 1 K08 DK02568-01 (A.L.B.) and a Merck Clinician Scientist Award (A.L.B.).

[2] To whom correspondence should be addressed at Department of Psychology, Behavioral Neuroendocrinology Group, 225 Ames Hall, Johns Hopkins University, Baltimore, MD 21218-2686. Fax: (410) 516-6205. E-mail: drazen@jhu.edu.

[3] Abbreviations used: NO, nitric oxide; NOS, nitric oxide synthase; L-NAME, L-N^G-nitro-Arg-methyl ester; PMSG, pregnant mare serum gonadotropin; hCG, human chorionic gonadotropin; WT, wild type; LD, light–dark; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; PBS, phosphate-buffered saline; eNOS, endothelial NOS.

1089-8603/99 $30.00

be regulated through interactions among NO and other neural messengers (9–12).

NO is also involved directly in ovulatory processes. Treatment with the NOS inhibitor, L-N^G-nitro-Arg-methyl ester (L-NAME), reduces both the number of ovarian rupture sites and the number of oocytes recovered from the oviducts of female rats and rabbits (13, 14). Because L-NAME is nonspecific, inhibiting all three isoforms of NOS, the contribution of NO from neural or endothelial sources in ovulatory processes remains unspecified. Recently, our group has attempted to identify the role of individual NOS isoforms in ovulatory processes through the use of mice with targeted disruption of the genes encoding specific isoforms of NOS (15, 16). After administration of pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG), female mice lacking the gene for the neuronal form of NOS (nNOS−/−) have fewer oocytes recovered from the oviducts when compared to WT females, but nNOS−/− and WT females do not differ in the number of rupture sites on the ovaries after natural ovulation (17). These results suggest that NO produced by nNOS accounts, in part, for the deficits in ova release observed after L-NAME administration.

However, these results suggest that nNOS is not involved in mediating the number of ovarian rupture sites. Because nonspecific inhibition of NOS (but not specific inhibition of nNOS) reduces the number of rupture sites, we hypothesized that eNOS may mediate rupture of ovarian follicles. The ovarian vasculature undergoes many dramatic and rapid changes in vascular resistance and ovarian microcirculation induced by the ovulatory surge of gonadotropins (18–20). Endothelial NOS is responsible for producing NO in vascular endothelium and regulates vascular resistance, eliciting relaxation of the adjacent vascular smooth muscle (21–24). Immunofluorescent staining localizes eNOS specifically within the endothelium of the ovarian vasculature and within the theca interna that surrounds preovulatory follicles (25). During PMSG-induced follicle growth, eNOS concentration increases in the ovary by 2.5-fold relative to WT controls (26). Additionally, eNOS appears to be involved in mediating the number of oocytes recovered following superovulation (27). Furthermore, during the establishment of our eNOS−/− mouse breeding colony, low fertility was noted. Thus, the goal of the present study was to examine the role of eNOS in mediating ovarian rupture sites by examining ovarian function in mice lacking the gene for the endothelial form of NOS (eNOS−/−).

MATERIALS AND METHODS

Animals

Thirty-six adult (>60 days of age) female WT C57BL/6J mice and 36 adult endothelial nitric oxide synthase knockout (eNOS−/−) female mice of combined C57BL/6J and SV129 genetic backgrounds were obtained from the breeding colony at The Johns Hopkins School of Medicine. Endothelial NOS knockout mice were provided by Massachusetts General Hospital to establish a breeding colony at The Johns Hopkins School of Medicine (28). All animals were individually housed in polycarbonate cages (28 × 17 × 12 cm) in colony rooms with a 16:8-h light–dark (LD) cycle (lights on at 0600 h Eastern Standard Time). Temperature and relative humidity were held constant at 20 ± 2°C and 50 ± 5%, respectively. Food (Prolab 1000; Agway, Syracuse, NY) and tap water were provided *ad libitum* throughout the course of the experiment.

Procedure

Breeding Colony

Sexually mature (56–70 days) male and female eNOS−/− mice were combined in monogamous pair mating (29) and were typically paired together for the rest of their reproductive lives. Breeding cages were examined daily for the presence of pups. After the females whelped, the number of pups delivered by each was recorded. Pups were weaned at 25 days of age, segregated by sex, and the data recorded. Total colony fecundity was calculated as an average ± SE for all litters whelped in an 18-month period of time.

Experiment 1: Natural ovulation. After a 2-week acclimation period, daily vaginal cell samples were obtained from WT ($n = 10$) and eNOS−/− ($n = 12$) females between 1400 and 1500 h to determine the stage of estrus. Samples were transferred to microscope slides that were fixed, stained, and evaluated for estrous stage (estrus, diestrus I, diestrus II, and

proestrus). Only females with regular estrous cycles were used in this experiment. After six regular estrous cycles, to confirm vaginal proestrus, females were paired overnight with a WT male in a clear polycarbonate cage (28 × 17 × 12). Behavior was videotaped from 1900 to 0700 h. Females that mated, as determined by videotaped behavioral ejaculatory patterns and by the presence of sperm in the vaginal lavage, were killed 12 to 14 h after ovulation. The number of ovarian rupture sites was counted using a dissecting microscope (×25; Olympus SO, Tokyo, Japan).

Experiment 2: Exogenous gonadotropin-stimulated ovulation. Estrous cycles were monitored as described above. After six regular estrous cycles, WT (n = 9) and eNOS−/− (n = 12) female mice were administered an intraperitoneal (ip) injection of 5 IU PMSG (Sigma, St. Louis, MO) at 1500 h, followed by an ip injection of 5 IU hCG (Sigma) 48 h later to induce ovulation. Estrous stage at the time of injection was not taken into account (30). All animals were killed 20 h later, and the number of ovarian rupture sites was counted as described above.

Experiment 3: Ovulation after GnRH treatment. Estrous cycles were monitored as described above. After six regular estrous cycles, WT (n = 9) and eNOS−/− (n = 11) female mice received an injection of 25 ng of GnRH (Calbiochem, San Diego, CA) suspended in 0.1 ml of 0.9% sterile saline at 1400 h during diestrus. All animals were killed 19 h later, and the number of ovarian rupture sites was counted as described for Experiment 1.

Western Blot Analysis

The ovary and oviducts, uterine horns, uterocervical junction, and vagina were dissected and placed immediately in protease inhibitor buffer containing 1 mM EDTA, 1 mM EGTA, 1 mM Pefabloc, 5 μg/ml aprotinin, and 1 μg/ml leupeptin, at 4°C, and were homogenized. The homogenized samples were centrifuged at 10,000g for 4 min and the supernatant was collected. An aliquot was removed and assayed for total protein (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL). Protein (500 μg) was diluted with 1:1 with 2× Laemmli buffer and then subjected to 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), resolved by running at 15 mA, overnight. The proteins were transferred to Amersham Hybond-ECL nitrocellulose (Amersham, Arlington Heights, IL) on a Millipore Milliblot semidry transfer system (Milliporem Milford, MA) at 325 mA, for 1 h, optimizing for high-molecular weight proteins. Blots were probed with an antibody for neuronal nitric oxide synthase (diluted 1:5000), generated in a rabbit against a C-terminal synthetic peptide sequence corresponding to amino acids 1419–1433 of human nNOS coupled to keyhole limpet hemocyanin (Incstar, Stillwater, MN), developed by enhanced chemiluminescence (Amersham), and exposed on autoradiography film (Eastman Kodak, Rochester, NY). Rat cerebellum was used as a positive control. Analyses were performed by visual comparison of samples run simultaneously at identical exposures.

Immunohistochemistry

Mice were killed by cervical dislocation, and the reproductive tracts were removed *en bloc*. The tract was separated into three regions: ovary, oviducts, and uterine horns and embedded in O.C.T. compound (Sigma Chemical CO., St. Louis, MO). Specimens were cut into 6-μm-thick sections on a cryostat (−18°C) and mounted on slides coated with gelatin/chrome alum. Tissue-mounted slides were fixed in 4% paraformaldehyde for 15 min and then permeabilized in 0.4% Triton X-100 for 15 min. All slides were incubated in phosphate-buffered saline (PBS) with 1% (v/v) normal goat serum, for 30 min. Slides were washed with a 3% H_2O_2 in water to quench endogenous peroxides. Slides were incubated overnight in PBS containing a polyclonal antibody (diluted 1:250) generated in a rabbit against a synthetic peptide, corresponding to amino acids 559–613 of bovine endothelial nitric oxide synthase, coupled to keyhole limpet hemocyanin (Alexis Corporation, San Diego, CA). Staining was completed using a Vectastain Elite ABC Kit (Vectastain, Burlingame, CA) and diaminobenzidine as the chromogen (Dako Corporation, Carpinteria, CA). Primary antibody was omitted for negative controls.

Statistical Analyses

Average estrous cycle length was based on the first six regular estrous cycles for each animal. Es-

trous cycle variability was calculated based on the variance of the first six estrous cycle lengths for each individual. Two-tailed *t* tests were used to assess estrous cycle length and the number of rupture sites on the ovaries of WT and eNOS−/− female mice. Mann–Whitney *U* tests were used to assess estrous cycle variability and in cases where the assumptions of a normal distribution were violated. Treatment effects were considered statistically significant if $P < 0.05$.

RESULTS

Breeding Colony

The average litter size was smaller for eNOS−/− females (5.01 ± 0.22 pups) than what is generally reported for WT (6.3 ± 0.37 pups) (31) female mice. The weanling average in eNOS−/− mice was 3.3 ± 0.29. The percentage weaned was smaller in eNOS−/− (63.6%) compared to what is generally reported for WT female mice (88.5%) (32).

Experiment 1

Average estrous cycle length was shorter in eNOS−/− (3.88 ± 0.09 days) than in WT female mice (4.53 ± 0.21 days) ($P < 0.05$). Figure 1A presents the data for estrous cycle length from Experiments 1, 2, and 3 combined; the average estrous cycle length was shorter in eNOS−/− (4.15 ± 0.07 days) than that in WT female mice overall (5.05 ± 0.18 days) ($P < 0.05$). Estrous cycle variance was reduced in eNOS−/− (4.28 ± 0.39 standard errors of the mean (SE)) compared to WT female mice (7.22 ± 0.69 SE) ($P < 0.05$). Figure 1B presents the data for estrous cycle variance from Experiments 1, 2, and 3 combined; estrous cycle variance was reduced in eNOS−/− (4.6 ± 0.27 SE) compared to WT female mice overall (11.95 ± 1.69 SE) ($P < 0.05$). There was no significant difference in the number of ovarian rupture sites between eNOS−/− (7.6 ± 0.77) and WT females following natural ovulation (9.36 ± 0.94) (Fig. 2; $P > 0.05$).

Experiment 2

The average estrous cycle length was shorter in eNOS−/− (4.45 ± 0.13 days) than that in WT female mice (5.46 ± 0.39 days) ($P < 0.05$). (These data have been included in the combined cycle length; Fig. 1A.) Estrous cycle variance was reduced in eNOS−/− (5.51 ± 0.57 SE) compared to WT female mice (15.12 ± 4.0 SE) ($P < 0.05$). (These data have been included in the combined cycle variance; Fig. 1B.) The number of ovarian rupture sites was significantly reduced in eNOS−/− females (7.45 ± 0.75) compared to WT female mice following administration of exogenous gonadotropins (10.25 ± 0.73) (Fig. 3; $P < 0.05$ in all cases).

Experiment 3

The average estrous cycle length was shorter in eNOS−/− (4.12 ± 0.09 days) than that in WT female mice (5.22 ± 0.28 days) ($P < 0.05$). (These data have been included in the combined cycle length; Fig. 1A.) Estrous cycle variance was reduced in eNOS−/− (3.94 ± 0.26 SE) compared to WT female mice (14.04 ± 2.84 SE) ($P < 0.05$). (These data have been included in the combined cycle variance; Fig. 1B.) There was no significant difference in the number of ovarian rupture sites between eNOS−/− (8.67 ± 1.47) and WT females following GnRH administration (10.27 ± 1.04) (Fig. 4; $P > 0.05$).

Western Blot

Neuronal NOS protein was identified by Western blot analysis in the ovary/oviduct, uterine horn, uterocervical junction, and vagina of both eNOS−/− and WT mice. Neuronal NOS immunoreactivity was confirmed to a similar extent in respective reproductive tract organs in both WT and eNOS−/− mice (data not shown).

Immunohistochemistry

Ovary/Oviduct Staining

In the ovary, eNOS immunoreactivity was confirmed in WT but not eNOS−/− mice. Staining in the WT ovary (Fig. 5A) was detected in the thecal cell layers and in the ovarian stroma but not in the eNOS−/− ovary (Fig. 5C). Tissues incubated in the absence of primary antibody did not stain (Fig. 5B).

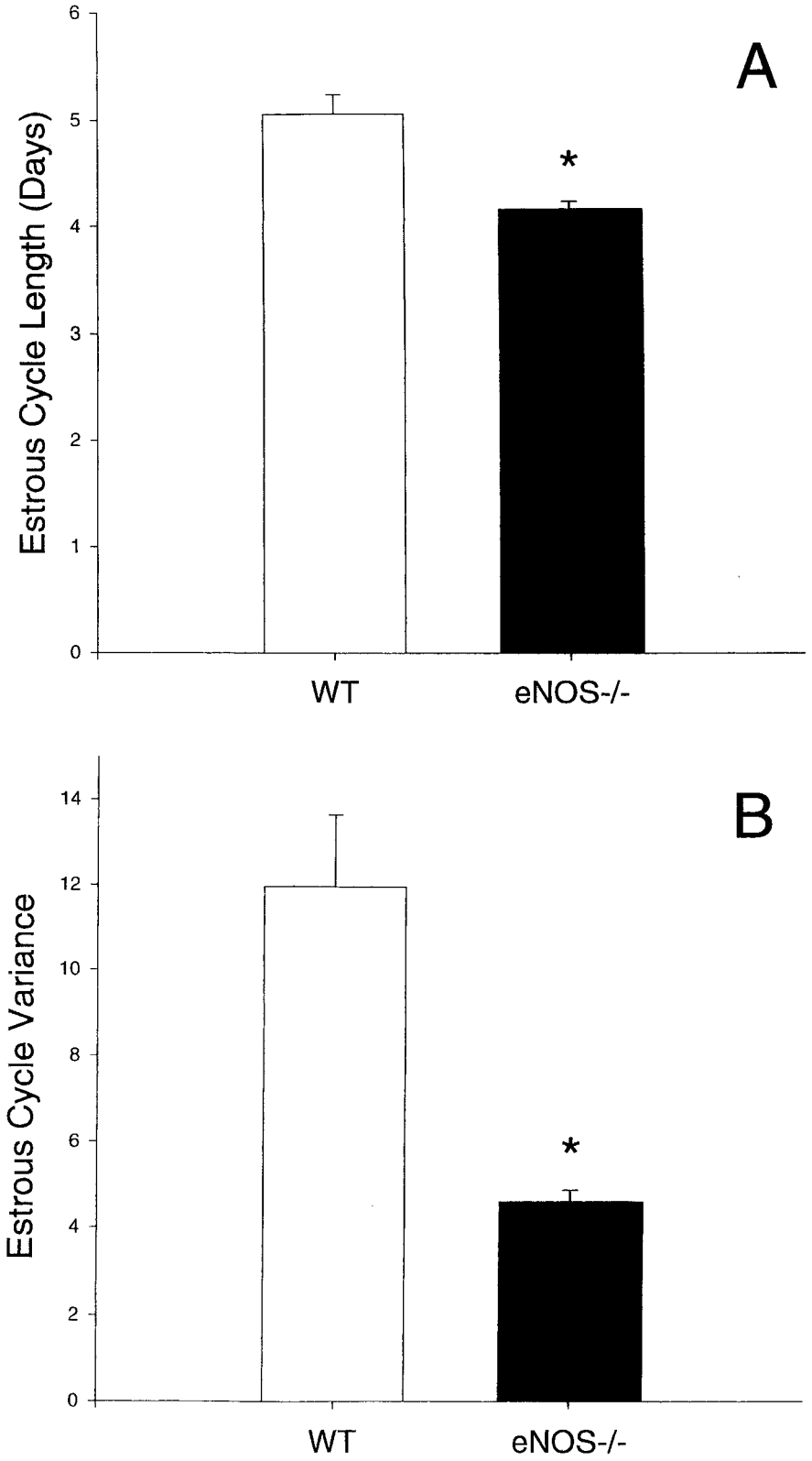

FIG. 1. (A) Mean (±standard errors of the mean (SE)) estrous cycle length of eNOS−/− females and WT females; data combined from Experiments 1, 2, and 3. (B) Mean (±SE) estrous cycle variance of eNOS−/− females and WT females; data combined from Experiments 1, 2, and 3. *Significantly different from WT controls ($P < 0.05$).

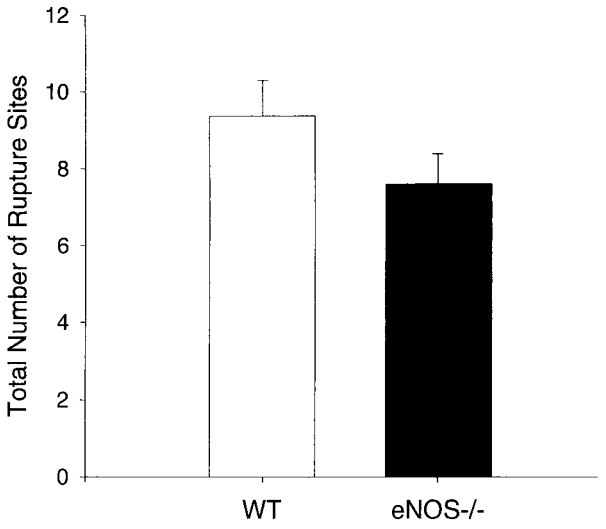

FIG. 2. Mean (±SE) number of rupture sites on the ovaries of eNOS−/− and WT females after natural ovulation.

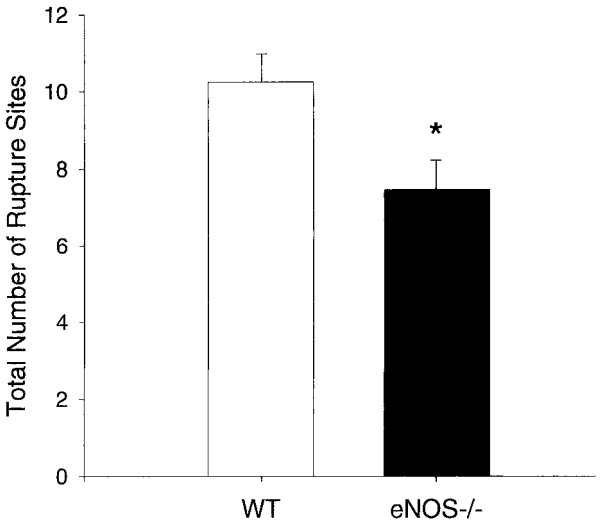

FIG. 3. Mean (±SE) number of rupture sites on the ovaries of eNOS−/− and WT females after exogenous gonadotropins. *Significantly different from WT controls ($P < 0.05$).

Uterine Horn Staining

In the uterine horns of WT mice, eNOS immunoreactivity was seen lining the luminal aspects of blood vessels distributed through the tissue (data not shown). Uterine horns from eNOS−/− mice incubated with primary antibody did not exhibit staining. Tissue incubated in the absence of primary antibody did not stain.

DISCUSSION

Female mice with targeted deletion of eNOS exhibited significantly shorter and less-variable estrous cycles than WT controls under all conditions. Ovulatory function, as measured by the number of ovarian rupture sites, under natural conditions, or following exogenous GnRH, was not impaired in eNOS−/− mice. However, when administered exogenous gonadotropins, eNOS−/− females displayed a significant reduction in the number of ovarian rupture sites. Additionally, eNOS−/− females had reduced numbers of pups/litter and a reduced weanling average when compared to WT females. Taken together, these results suggest that NO from endothelial sources plays a role in female rodent reproductive physiology.

Estrous cycle length and variability were consistently reduced in eNOS−/− females, suggesting that NO formed from eNOS is involved in the normal variability in the timing of estrous cycles. Presumably, stress and other intrinsic and extrinsic factors influence blood flow via NO formation. Although the circadian system plays a primary role in the timing of estrus in rodents (reviewed in 33–35), subtle changes in vascular tone (e.g., hypophyseal portal blood system and ovarian vasculature) can affect release and delivery of gonadotropins and steroid hormones that could affect the precise timing of estrus (36, 37). If NO formed from eNOS mediates

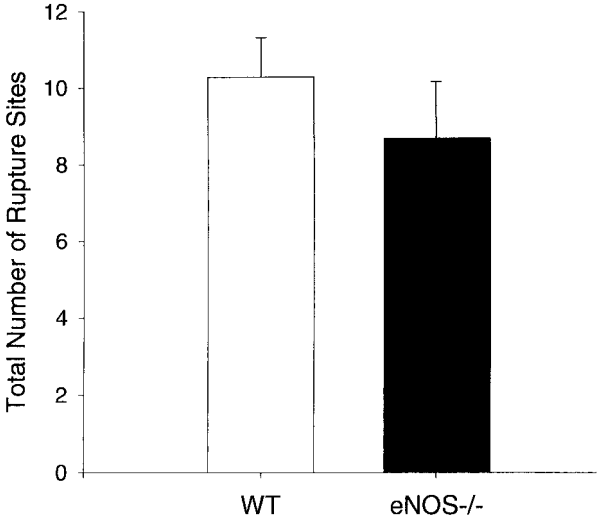

FIG. 4. Mean (±SE) number of rupture sites on the ovaries of eNOS−/− and WT females after GnRH treatment.

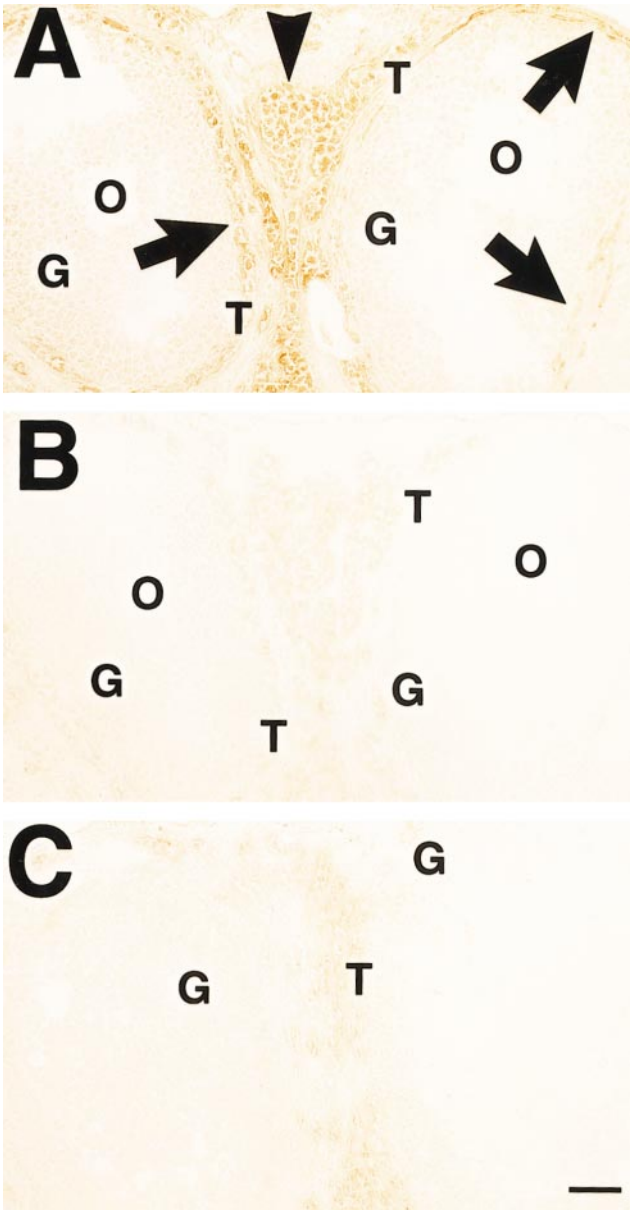

FIG. 5. eNOS immunostaining of the ovary from female WT and eNOS−/− mice. Serial sections of the ovary are shown from untreated animals. In the WT mouse (A), eNOS localizations are to the thecal cells (arrows) and the ovarian stroma (arrowheads). A negative control (B) incubated with normal goat serum shows no immunoreactivity. In the eNOS−/− mouse (C), tissue incubated with eNOS antiserum reveals only nonspecific background staining. G, granulosa cell layer; T, thecal cell layer; O, oocyte. Bar, 50 μm.

the effects of extrinsic and intrinsic factors that affect the timing of estrus, then estrous cycles of eNOS−/− mice may be more regular than those of WT mice because circadian regulation of these cycles is not obscured by other factors. Furthermore, NO could also affect circadian organization of estrus directly. Pharmacological inhibition of NOS with L-NAME prevented circadian phase shifts in response to glutamate and attenuated light-induced phase delays (38, 39). Additional studies are required to assess these hypotheses.

A significant reduction in the number of ovarian rupture sites was observed following artificial stimulation with PMSG and hCG. The study of ovarian function and, specifically, the morphology of the ovary is well controlled when using superovulation because it allows for the inspection of ovaries that contain follicles at synchronized stages of development (17, 40, 41). Although, in some cases, the ovary is distorted following superovulation (e.g., 17), this was not the case in the present study. Therefore, it is likely that the comparison of rupture sites between eNOS−/− and WT mice was most accurate following injection of PMSG and hCG. Because the number of rupture sites was normal after natural ovulation and following GnRH administration, it is possible that the results observed are a pharmacological effect associated with superovulation. However, this is unlikely given the recent findings that eNOS−/− females display reduced numbers of ovulated oocytes (27) and given that rupture sites and number of eggs ovulated are highly correlated (42, 43). It has recently been determined that eNOS−/− males exhibit pronounced abnormalities in ejaculatory function (44). It appears, therefore, that these problems in male erectile function may be responsible for the reduced breeding efficiency observed in the eNOS−/− breeding colony.

Wild-type mice treated with L-NAME show reduced numbers of both rupture sites and oocytes, whereas nNOS−/− mice only have reduced numbers of oocytes. The present study suggests that NO produced by eNOS plays a role in mediating the number of rupture sites. Because NO in endothelial cells acts as a vasodilator, it is possible that NO is necessary for normal ovarian blood flow, and therefore necessary for the regulation of ovarian rupture sites. Taken together, NO produced from eNOS appears to play a role in mediating ovulation.

Mice with targeted disruption of genes may use compensatory mechanisms that preserve the functions of the protein derived from the disrupted gene (5, 45–47). The possibility that nNOS was upregu-

lated to compensate for the lack of eNOS was examined; however, no upregulation of nNOS in reproductive tissue of the eNOS−/− females was observed. It is possible, though, that other compensatory mechanisms are responsible for the results of the current study. Because gene deletion occurs in an early embryonic stage, the missing gene is not expressed throughout development. The use of specific eNOS inhibitors or conditional gene knockouts will eliminate these potential confounds. However, studying the role of eNOS through the use of genetic knockouts allows for a precise ablation with few of the side effects associated with the use of pharmacological agents (reviewed in 45, 46). Data from knockout animals, such as those in the current study, when considered with converging evidence from other methods, such as pharmacological manipulations, can provide important insights into physiological mechanisms.

In sum, when combined with data from manipulations using L-NAME, it can be concluded that NO plays a role in mediating reproductive physiology in mice. NO from both eNOS and nNOS are involved in regulating ovulation; NO from endothelial sources also appears to affect the regulation of the timing of the estrous cycle and appears to play a role in normal fecundity.

ACKNOWLEDGMENTS

We thank Donna Bilu, Aliza Katz, and Sue Yang for technical assistance and Lance Kriegsfeld and Greg Demas for valuable comments on the manuscript. We also thank Ed Silverman for expert animal care.

REFERENCES

1. Nelson, R. J., Kriegsfeld, L. J., Dawson, V. L., and Dawson, T. M. (1997). Effects of nitric oxide on neuroendocrine function and behavior. *Front. Neuroendocrinol.* **18,** 463–491.
2. Hull, E. M., Lumley, M. A., Matuszewich, L., Dominguez, J., Moses, J., and Lorrain, D. S. (1994). The roles of nitric oxide in sexual function of male rats. *Neuropharmacology* **33,** 1499–1504.
3. Hadeishi, Y., and Wood, R. I. (1996). Nitric oxide synthase in mating behavior circuitry of male Syrian hamster brain. *J. Neurobiol.* **30,** 480–492.
4. Burnett, A. L., Lowenstein, C. J., Bredt, D. S., Chang, T. S., and Snyder, S. H. (1992). Nitric oxide: A physiologic mediator of penile erection. *Science* **257,** 401–403.
5. Burnett, A. L., Nelson, R. J., Calvin, D. C., Liu, J-X., Demas, G. E., Klein, S. L., Kriegsfeld, L. J., Dawson, V. L., Dawson, T. M., and Snyder, S. H. (1996). Nitric oxide-dependent penile erection in mice lacking neuronal nitric oxide synthase. *Mol. Med.* **2,** 288–296.
6. Mani, S. K., Allen, J. M., Rettori, V., McCann, S. M., O'Malley, B. W., and Clark, J. H. (1994). Nitric oxide mediates sexual behavior in female rats. *Proc. Natl. Acad. Sci. USA* **91,** 6468–6472.
7. Grossman, A. B., Rossansmith, W. G., Kabigting, E. B., Cadd, G., Clifton, D., and Steiner, R. A. (1994). The distribution of hypothalamic nitric oxide synthase mRNA in relation to gonadotropin-releasing hormone neurons. *J. Endocrinol.* **140,** R5–R8.
8. Herbison, A. E., Simonian, S. X., Norris, P. J., and Emson, P. C. (1996). Relationship of neuronal nitric oxide synthase immunoreactivity to GnRH neurons in the ovariectomized and intact female rat. *J. Neuroendocrinol.* **8,** 73–82.
9. Canteros, G., Rettori, V., Genaro, A., Suburo, A., Gimeno, M., and McCann, S. M. (1996). Nitric oxide synthase content of hypothalamic explants: Increased by norepinephrine and inactivated by NO and cGMP. *Proc. Natl. Acad. Sci. USA* **93,** 4246–4250.
10. McCann, S. M., and Rettori, V. (1996). The role of nitric oxide in reproduction. *Proc. Soc. Exp. Biol. Med.* **211,** 7–15.
11. Pu, S., Horvath, T. L., Diano, S., Naftolin, F., Kalra, P. S., and Kalra, S. P. (1997). Evidence showing that β-endorphin regulates cyclic guanosine 3′,5′-monophosphate (cGMP) efflux: Anatomical and functional support for an interaction between opiates and nitric oxide. *Endocrinology* **138,** 1537–1543.
12. Seilicovich, A., Duvilanski, B. H., Pisera, D., Theas, S., Gimeno, M., Rettori, V., and McCann, S. M. (1995). Nitric oxide inhibits hypothalamic luteinizing hormone-releasing hormone release by releasing γ-aminobutyric acid. *Proc. Natl. Acad. Sci. USA* **92,** 3421–3424.
13. Shukovski, L., and Tsafriri, A. (1994). The involvement of nitric oxide in the ovulatory process in the rat. *Endocrinology* **135,** 2287–2290.
14. Hesla, J. S., Preutthipan, S., Maguire, M. P., Chang, T. S. K., Wallach, E. E., and Dharmarajan, A. M. (1997). Nitric oxide modulates human chorionic gonadotropin-induced ovulation in the rabbit. *Fertil. Steril.* **67,** 548–552.
15. Huang, P. L., Dawson, T. M., Bredt, D., Snyder, S. H., and Fishman, M. C. (1993). Targeted disruption of the neuronal nitric oxide synthase gene. *Cell* **75,** 1273–1286.
16. Huang, P. L., Huang, Z., Mashimo, H., Bloch, K. D., Moskowitz, M. A., Bevan, J. A., and Fishman, M. C. (1995). Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* **377,** 239–242.
17. Klein, S. L., Carnovale, D., Burnett, A. L., Wallach, E. E., Zacur, H. A., Crone, J. K., Dawson, V. L., Nelson, R. J., and Dawson, T. M. (1998). Impaired ovulation in mice with targeted deletion of the neuronal isoform of nitric oxide synthase. *Mol. Med.* **4,** 658–664.

18. Hirokatsu, K., Yoshimura, Y., Wright, K. H., Santulli, R., and Wallach, E. E. (1985). Microvasculature of preovulatory follicles: Comparison of in situ and in vitro perfused rabbit ovaries following stimulation of ovulation. *Am. J. Obstet. Gynecol.* **152,** 889–895.

19. Janson, P. O. (1975). Effects of luteinizing hormone on blood in the follicular rabbit ovary as measured by radioactive microspheres. *Acta Endocrinol.* **79,** 122–123.

20. Murdoch, W. J., Nix, K. J., and Dunn, T. G. (1983). Dynamics of ovarian blood supply to periovulatory follicles of the ewe. *Biol. Reprod.* **28,** 1001–1006.

21. Snyder, S. H. (1992). Nitric oxide: First in a new class of neurotransmitters? *Science* **257,** 494–496.

22. Ignarro, L. J. (1989). Endothelium-derived nitric oxide: Actions and properties. *FASEB J.* **3,** 31–36.

23. Snyder, S. H. (1995). No endothelial NO. *Nature* **377,** 196–197.

24. Dawson, T. M., and Snyder, S. H. (1994). Gases as biological messengers: Nitric oxide and carbon monoxide in the brain. *J. Neurosci.* **14,** 5147–5159.

25. Powers, R. W., Chen, L., Russell, P. T., and Larsen, W. J. (1995). Gonadotropin-stimulated regulation of blood–follicle barrier is mediated by nitric oxide. *Am. J. Physiol.* **269,** E290–E298.

26. Jablonka-Shariff, A., and Olson, L. M. (1997). Hormonal regulation of nitric oxide synthases and their cell-specific expression during follicular development in the rat ovary. *Endocrinology* **138,** 460–468.

27. Jablonka-Shariff, A., and Olson, L. M. (1998). The role of nitric oxide in oocyte meiotic maturation and ovulation: Meiotic abnormalities of endothelial nitric oxide synthase knockout mouse oocytes. *Endocrinology* **139,** 2944–2954.

28. Nelson, R. J., Demas, G. E., Huang, P. L., Fishman, M. C., Dawson, V. L., Dawson, T. M., and Snyder, S. H. (1995). Behavioural abnormalities in male mice lacking neuronal nitric oxide synthase. *Nature* **378,** 383–386.

29. Bennett, J. P., and Vickery, B. H. (1970). Rats and mice. *In* Reproduction and Breeding Techniques for Laboratory Animals (Hafez, E. S. E., Ed.), pp. 310, Lea & Febiger, Philadelphia.

30. Redina, O. E., Amstislavsky, S. Y., and Maksimovsky, L. F. (1994). Induction of superovulation in DD mice at different stages of the oestrous cycle. *J. Reprod. Fertil.* **102,** 263–267.

31. Weir, J. A. (1960). Sex ratio factor in the house mouse that is transmitted by the male. *Genetics* **45,** 1539–1552.

32. Beamer, C. T. L., and Les, E. P. (1987). The laboratory mouse. *In* The UFAW Handbook on The Care and Management of Laboratory Animals (Poole, T. B., Ed.), 6th ed., pp. 275–308, Longman Scientific & Technology, Essex, England.

33. Turek, F. W., Van, and Cauter, E. (1994). Rhythms in reproduction. *In* The Physiology of Reproduction (Knobil, E., and Neill, J. D., Eds.), pp. 487–540, Raven Press, New York.

34. Carmichael, M. S., Nelson, R. J., and Zucker, I. (1981). Hamster activity and estrous cycles: Control by a single versus multipli circadian oscillator(s). *Proc. Natl. Acad. Sci. USA* **78,** 7830–7834.

35. Fitzgerald, K. M., and Zucker, I. (1976). Circadian organization of the estrous cycle of the golden hamster. *Proc. Natl. Acad. Sci. USA* **73,** 186–190.

36. Gallo, R. V. (1981). Pulsatile release during the ovulatory LH surge on proestrus in the rat. *Biol. Reprod.* **24,** 100–104.

37. Donham, R. S., and Stetson, M. H. (1991). The peripubertal golden hamster and the transition between daily and estrous cycle hormone rhythms. *Biol. Reprod.* **44,** 1108–1112.

38. Ding, J. M., Chen, D., Weber, T., Faiman, L. E., Rea, M. A., and Gillette, M. U. (1994). Resetting the biological clock: Mediation of nocturnal circadian shifts by glutamate and NO. *Science* **166,** 1713–1717.

39. Wantabe, A., Ono, M., Shibata, S., and Wantabe, S. (1995). Effect of a nitric oxide synthase inhibitor, *N*-nitro-L-arginine methylester, on light-induced phase delay of circadian rhythm of wheel-running activity in golden hamsters. *Neurosci. Lett.* **192,** 25–28.

40. Zarrow, M. X., Caldwell, A. L., Jr., Hafez, E. S. E., and Pincus, G. (1958). Superovulation in the immature rat as a possible assay for LH and hCG. *Endocrinology* **63,** 748–758.

41. Yoshinaga-Hirabayashi, T., Ishimura, K., Fujita, H., Kitawaki, J., and Osawa, Y. (1990). Immunocytochemical localization of aromatase in immature rat ovaries treated with PMSG and hCG, and in pregnant rat ovaries. *Histochemistry* **93,** 223–228.

42. Espey, L. I. (1978). Ovulation. *In* The Vertebrate Ovary: Comparative Biology and Evolution (Jones, R. E., Ed.), pp. 516–517, Plenum, NY.

43. Rowlands, I. W., and Weir, B. J. (1998). Mammals: Nonprimate eutherians. *In* Marshall's Physiology of Reproduction (Lamming, G. E., and Livingstone, C., Eds.), pp. 512–513.

44. Kriegsfeld, L. J., Demas, G. E., Burnett, A. L., and Nelson, R. J. Ejaculatory abnormalities in male mice lacking the gene for endothelial nitric oxide synthase, in press.

45. Nelson, R. J. (1997). The use of genetic "knockout" mice in behavioral endocrinology research. *Horm. Behav.* **31,** 188–196.

46. Huang, P. L., and Fishman, M. C. (1996). Genetic analysis of nitric oxide synthase isoforms: Targeted mutation in mice. *J. Mol. Med.* **74,** 415–421.

47. Irikurs, K., Huang, P. L., Ma, J., Lee, W. S., Dalkara, T., Fishman, M. C., Dawson, T. M., Snyder, S. H., and Moskowitz, M. A. (1995). Cerebrovascular alterations in mice lacking neuronal nitric oxide gene expression. *Proc. Natl. Acad. Sci. USA* **92,** 6823–6827.