Physiology & Behavior, Vol. 67, No. 4, pp. 561–566, 1999

0031-9384/99/$–see front matter

PII S0031-9384(99)00100-6

Ejaculatory Abnormalities in Mice Lacking the Gene for Endothelial Nitric Oxide Synthase (eNOS−/−)

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Received 18 February 1999; Accepted 7 May 1999

KRIEGSFELD, L. J., G. E. DEMAS, P. L. HUANG, A. L. BURNETT AND R. J. NELSON. *Ejaculatory abnormalities in mice lacking the gene for endothelial nitric oxide synthase (eNOS−/−).* PHYSIOL BEHAV **67**(4) 561–566, 1999.—Nitric oxide (NO) has been established as a neurotransmitter in both the central and peripheral nervous systems. Three isoforms of its synthetic enzyme, NO synthase (NOS), have been identified: 1) in the endothelial lining of blood vessels (eNOS), 2) an inducible form found in macrophages (iNOS), and 3) in neurons (nNOS). Previous studies using pharmacological agents that block all three isoforms of NOS have revealed that NO mediates several aspects of reproductive physiology and behavior, including anomalies in male sexual behavior and erectile function. To determine the specific contribution of the endothelial isoform of NOS in male reproductive behavior, we studied mice missing the gene for only eNOS (eNOS−/−). Wild-type (WT) and eNOS−/− animals were placed with an estrous WT female and observed for 45 min. Both WT and eNOS−/− mice displayed equivalent motivation to mount the stimulus female. However, eNOS−/− mice exhibited striking anomalies in ejaculatory function. A higher percentage of eNOS−/− than WT mice ejaculated during the testing period ($p < 0.001$). This increased propensity to ejaculate was apparently due to reduced stimulation required to elicit ejaculation; eNOS−/− mice required significantly fewer mounts ($p < 0.003$) and intromissions ($p < 0.001$) to ejaculate compared to WT mice. Taken together, these results suggest that NO synthesized by eNOS may be involved in ejaculatory physiology, but not sexual motivation. © 1999 Elsevier Science Inc.

Mating Erection Rodent Reproduction Copulatory behavior Gene knockout

NITRIC OXIDE (NO) is an endogenous gas that acts as a neurotransmitter in both the central and peripheral nervous systems (9,31,35). Because NO is extremely labile, most studies to date have investigated the actions of NO by manipulating its synthetic enzyme, nitric oxide synthase (NOS). Currently, three isoforms of NOS have been identified: 1) in the endothelial tissue of blood vessels (eNOS), 2) an inducible form in macrophages (iNOS), and 3) in neural tissue (nNOS) (23, 24).

Previous studies have blocked the formation of NO by eliminating arginine or by administering a potent NOS inhibitor such as L-N^G-nitro-Arg-methyl ester (L-NAME). Studies with L-NAME have revealed a role for NO in hippocampal long-term potentiation (28) and entrainment (i.e., synchronization) of circadian rhythms (1,13,25,37). Recently, NO has been implicated in male reproductive behavior and physiology. Administration of L-NAME or other nonselective NOS inhibitors produces a number of reproductive anomalies including deficits in the ability of male rats to copulate (3,20,32). Apparently, the deficits in sexual behavior observed in rats given L-NAME are not due to decreased sexual motivation or motor abnormalities, but are due to deficits in penile erection (20). Likewise, male rats injected with L-NAME exhibit increased numbers of ex copula seminal emissions, and decreased latency to first seminal emission (20). Administration of general NOS inhibitors leads to the abolition of electrophysiologically induced penile erections (6).

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In addition to acting peripherally to modulate penile erection and ejaculation, NO may act centrally on the neural substrate regulating male reproductive behavior. NO has been localized to several brain regions responsible for regulating mating behavior in male Syrian hamsters (*Mesocricetus auratus*), including the medial amygdaloid nucleus (Me), the bed nucleus of the stria terminalis (BNST), and the medial preoptic area (MPOA) in hamsters (16). Likewise, a specific subpopulation of NOS-positive neurons in the medial preoptic nucleus (MPN) is reduced after castration (16). Additionally, NO appears to function as a major regulator of GnRH synthesis and release [reviewed in (27)].

Not only is NOS present in neuronal populations involved in male motivated reproductive behavior, but there is increasing evidence that central NO can directly influence male copulatory behavior. For example, dopamine (DA) is released in the MPOA of male rodents during copulation (18). Elevated DA during copulation is prevented when L-NAME is applied to the MPOA via microdialysis, but not by its inactive isomer D-NAME (22). Likewise, local infusions or microinjections of DA antagonists into the MPOA impair rat copulatory behavior, while local DA agonists infusions enhance male sexual behavior (17,19,36). In addition, local delivery of the NO precursor, L-arginine by reverse dialysis also leads to increased DA release (21), and infusions of L-arginine into the MPOA of rats facilitate male sexual behavior (32). Taken together, these data suggest that NO may act both centrally, on the neural circuitry modulating male copulatory behavior, as well as peripherally to modulate penile erection and ejaculation. However, treatment with L-NAME and other general NOS inhibitors has many nonspecific side effects; for example, L-NAME affects all three isoforms of NOS and affects systemic blood flow throughout the brain.

The present study used mutant mice with targeted disruption of the gene encoding the endothelial isoform of NOS to determine the specific role of NO synthesized by eNOS in male reproductive behavior. During the establishment of our eNOS−/− breeding colony, low fertility was noted. This low fertility was not attributable to deficits in reproductive behavior or ovulatory physiology of female eNOS−/− mice (14). Therefore, the goal of the present study was to examine whether or not reproductive deficits in male eNOS−/− mice may be responsible for reduced fertility among eNOS−/− animals. If eNOS modulates male reproductive behavior, penile erection, and ejaculation, then eNOS−/− males should exhibit pronounced abnormalities in their ability to achieve penile intromissions and ejaculate. If eNOS acts in conjunction with nNOS to mediate erectile function and ejaculation, then eNOS−/− males may only exhibit minor impairments in their ability to intromit and ejaculate during copulation. Finally, it is possible that an isoform of NOS other than eNOS serves to modulate male reproductive behavior and physiology, and no impairment will be observed in male eNOS−/− mice. These possibilities were examined in the present study.

MATERIALS AND METHODS

Animals

Twenty-one mutant mice with targeted disruption of the eNOS gene (eNOS−/−) and 21 wild-type (WT) animals (C57B6J), 4–8 months of age at the time of testing, were housed individually in polycarbonate cages (28 × 17 × 12 cm), and were maintained in LD 14:10 photoperiods (lights on 0700 h EST) at 20 ± 2°C with relative humidity of 50 ± 5%. Food and tap water were available ad lib for the duration of the study.

Mating Tests

Males were placed in a clear aquarium (38.5 × 26.5 × 30.7 cm) for a 15-min acclimation period. After this acclimation period, an estrous WT female was introduced into the mating arena. The following information was recorded during the 45-min test: latency to begin mounting behavior, latency to first intromission, latency to first ejaculation (calculated from first intromssion), number of intromissions to ejaculation, number of mounts until ejaculation, and the number of ejaculations during the test period. Males were observed during a single 45-min test with an estrous WT female.

A mount was operationally defined as the male assuming the copulatory position, but failing to achieve intromission. Intromission was defined as the males' penis entering the vagina in association with thrusting behavior. Intromissions were behaviorally defined as the male mounting the female, associated with slow, rhythmic thrusting behavior. In mice, mounts may be distinguished from intromission because the thrusting behavior associated with mounting (without intromission) is qualitatively distinct from thrusting behavior associated with intromissions. Ejaculation was behaviorally defined by the culmination of vigorous thrusting behavior and the male's arching his spine and lifting his forepaws off the female prior to withdrawal. Ejaculation was verified by the presence of a sperm plug followed by a refractory period of ⩾5 min before the next mounting (29).

One day prior to the mating test, stimulus females received an s.c. injection of estradiol-17β (0.05 mg suspended in 0.05 cc of sesame seed oil). Six hours prior to the mating tests, the females were injected s.c. with 0.1 mg progesterone (suspended in 0.05 cc sesame seed oil) to induce behavioral estrus. Mating tests began 6 h after the injection of progesterone at onset of the dark period. Behavioral observations were made by two individuals who were unaware of the genotype of the males being tested.

Statistics

All parametric pairwise comparisons were analyzed with a series of independent two-tailed *t*-tests. Nonparametric comparisons (i.e., percentage data) were accomplished with a chi-square test. Treatment effects were considered statistically significant if $p < 0.05$.

RESULTS

Both WT and eNOS−/− mice exhibited equivalent levels of motivation to mount the estrous stimulus female ($p > 0.05$). An equivalent percentage of WT and eNOS−/− males mounted the female and did not differ in their latency to mount the stimulus female ($p > 0.05$ in each case; Figs. 1 and 2). Likewise, a similar percentage of WT and eNOS−/− mice were motivated to and capable of achieving intromission with the stimulus female; and equal proportion of WT and eNOS−/− mice achieved intromission with the estrous female and exhibited an equivalent latency to first intromission ($p > 0.05$ in each case; Figs. 1 and 2).

However, a greater proportion of eNOS−/− mice ejaculated during the testing period compared to WT mice ($p < 0.05$) (Fig. 1). This increased likelihood of ejaculating during the testing period in eNOS−/− mice compared to WT males was apparently due to reduced stimulation required for ejacu-

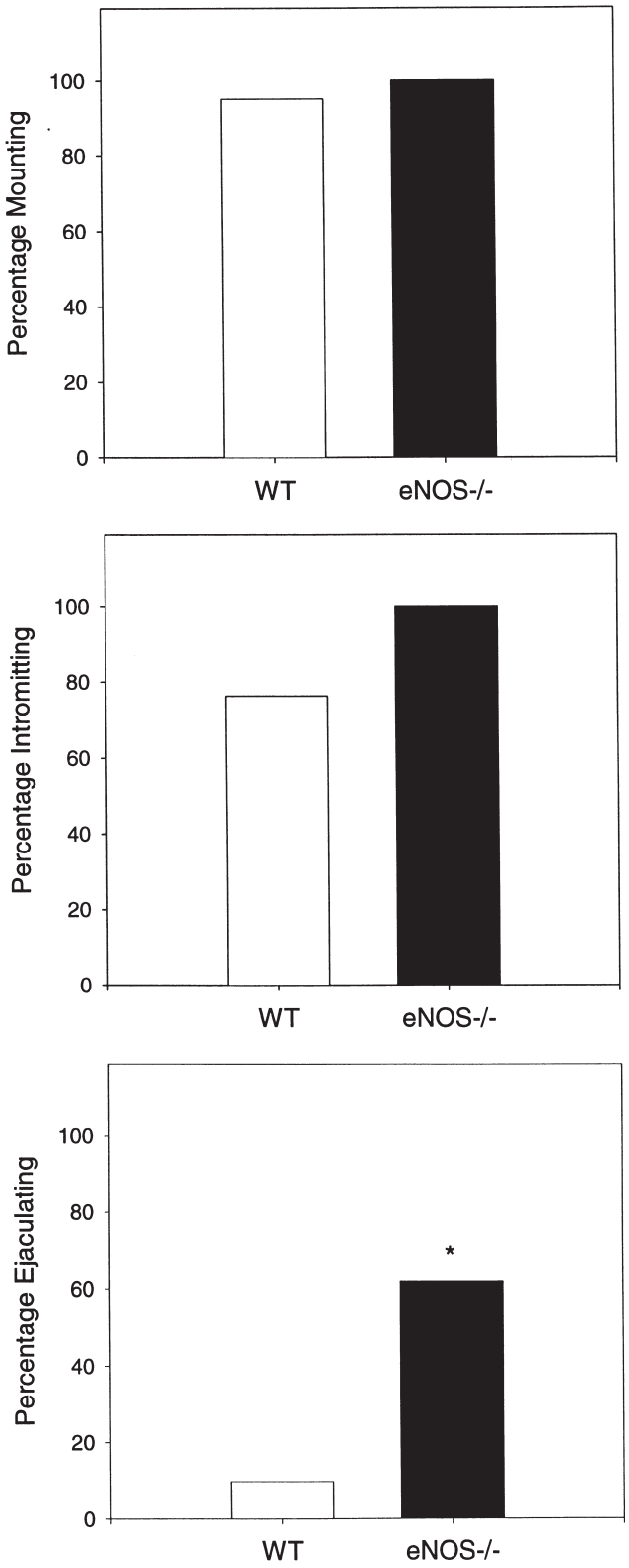

FIG. 1. Percentage of eNOS−/− and WT mice mounting (top panel), intromitting (middle panel), and ejaculating (bottom panel) during a 45-min test with an estrous WT female. *Significantly greater than WT mice ($p < 0.05$).

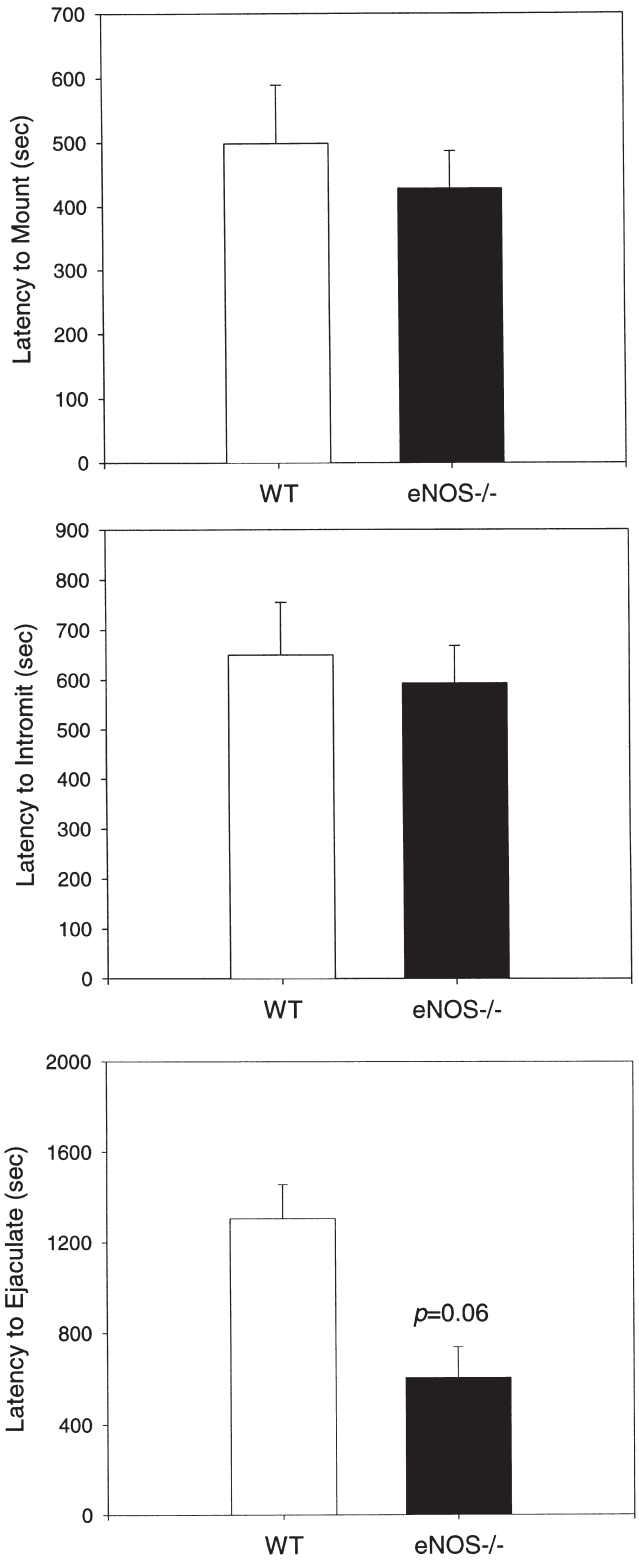

FIG. 2. Latencies (in seconds) to mount (top panel) intromit, (middle panel), and ejaculate (bottom panel) in eNOS−/− and WT mice during a 45-min test with an estrous WT female.

lation to occur. eNOS−/− mice exhibited a dramatic reduction in the number of mounts and intromissions required for ejaculation relative to WT animals ($p < 0.05$ in each case; Fig. 3). This reduction in the number of mounts and intromissions required for ejaculation was accompanied by a marginally significant decrease in the latency to ejaculate in eNOS−/− mice compared to WT animals ($p < 0.06$; Fig. 2).

DISCUSSION

The results of the present study suggest that NO synthesized by eNOS is involved in ejaculation in male mice. Male mice missing the gene for eNOS exhibit pronounced abnormalities in ejaculatory function. This abnormality is associated with a decrease in the amount of stimulation required to elicit ejaculation in eNOS−/− males compared to WT mice. eNOS−/− mice ejaculate after a shorter latency and require fewer mounts and intromissions to ejaculate compared to WT mice. Taken together, these data suggest that, in nongenetically altered mice, NO from eNOS may inhibit ejaculatory function by decreasing sympathetic nervous system activity to prevent premature ejaculation. Support for this contention comes from studies in which sympathetic nonadrenergic contractions of rabbit and human corpus cavernosum induced by electrical stimulation are inhibited by the presence of nitric oxide (8). A general inhibitor of nitric oxide synthase allows contraction of corpus cavernosum tissue during electrical stimulation, while this response is delayed in control tissues (8).

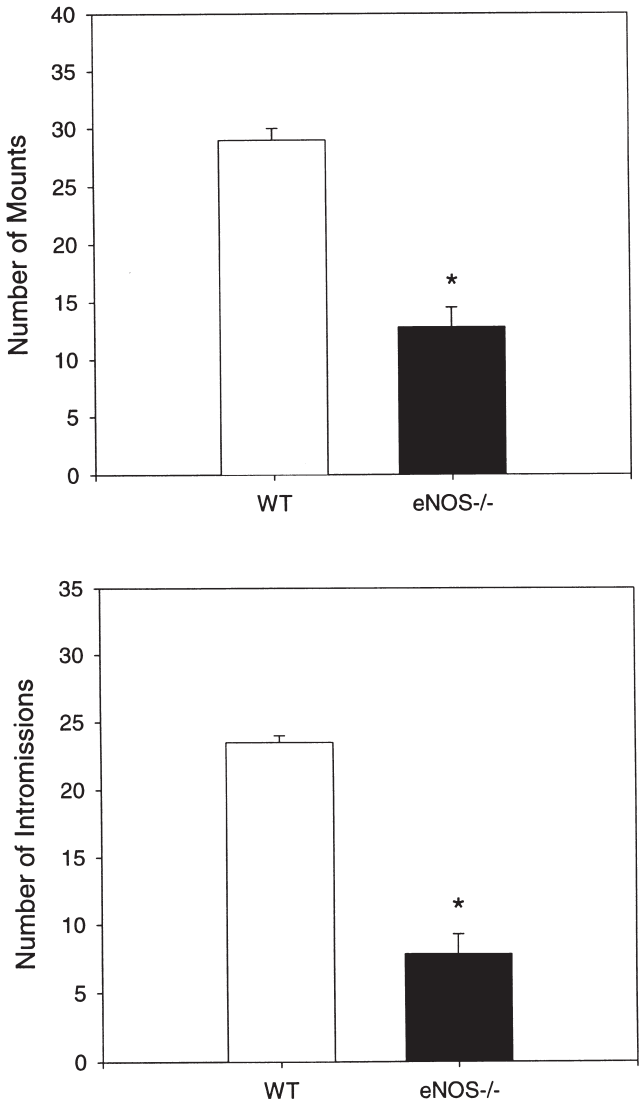

FIG. 3. Total number of mounts (top panel) and intromissions (bottom panel) until ejaculation in eNOS−/− and WT mice during a 45-min test with an estrous female. *Significantly less than WT mice ($p < 0.05$).

In addition to affecting ejaculation through sympathetic nervous system activity, eNOS may be localized to structures and smooth muscle responsible for the ejaculatory reflex. The ejaculatory reflex requires coordinated contractions of the bulbospongiosus and ischiocavernosus somatic muscles as well as smooth musculature of the vas deferens, ejaculatory ducts, proximal urethra, and bladder neck (2). Because smooth muscle relaxation requires the formation of cyclic GMP (cGMP), and NO relaxes smooth muscle of blood vessels by stimulating the formation of cGMP [reviewed in (33)], eNOS is an excellent candidate enzyme for regulating NO production to modulate ejaculation. eNOS has been localized to penile musculature and vasculature in rodents and humans (4,15,30), including the endothelium of penile vasculature and sinusoidal endothelium within the corpora cavernosa (7). Likewise, an enzyme (i.e., heme oxygenase-2; HO2) responsible for the formation of another endogenous gas, carbon monoxide (CO), has been localized to genitourinary tissue of mice and has been linked to ejaculatory function (5). HO2−/− mice exhibit diminished bulbospongiosus muscle activity, a muscle that mediates ejaculation, after electrical stimulation, along with diminished ejaculatory behavior (5). The fact that CO also acts by increasing cGMP levels (9,38) suggests that eNOS may affect ejaculation through a similar mechanism.

The ejaculatory abnormalities observed in the present study are unlikely to be related to anomalous sensorimotor ability or anxiety differences between eNOS−/− and WT mice. eNOS−/− mice have no deficits in olfactory ability, balance, or coordination (11). In fact, eNOS−/− mice display increased forelimb strength and an increased ability to turn in a narrow, "blind" alley compared to WT mice (11). Finally, alterations in ejaculatory function are unlikely to be related to anxiety alternations in eNOS−/− mice; there are no differences between eNOS−/− and WT mice in performance on an elevated-plus maze task (11).

Because nonspecific NOS inhibitors block penile erection (6), it was initially surprising that eNOS−/− mice were capable of achieving vaginal intromissions and ejaculating. Neuronal NOS is localized to autonomic neurons and vascular endothelium of the genitourinary tract (7). However, nNOS−/− mice display normal erectile function presumably because of a compensatory increase in eNOS (7). Considered together, these results suggest several possibilities: 1) eNOS is responsible for regulating penile erection; 2) nNOS and eNOS normally work in conjunction to regulate penile erection, and either isoform of the enzyme is sufficient to modulate erectile function; or 3) nNOS normally modulates penile erection, but eNOS is capable of supporting erectile capability when nNOS is not present. Future studies using selective pharmacological agents that target specific isoforms of the enzyme responsible for NO formation are necessary to distinguish among these possibilities.

The results from the present study also suggest that reduced fertility among eNOS−/− mice may be the result of anomalous sexual behavior seen in eNOS−/− males. To sup-

port corpora lutea function and maintain pregnancy, female mice require a threshold of vaginal stimulation prior to ejaculation (12). Likewise, duration of mating is positively correlated with fertility in house mice (10). Thus, the present finding suggests that reduced fertility among eNOS−/− mice may be due to insufficient vaginal stimulation of eNOS−/− females due to rapid ejaculation by eNOS−/− males.

eNOS−/− mice did not display any abnormalities in sexual motivation or performance, excluding ejaculatory abnormalities. The neural circuitry regulating motivated reproductive behavior is more likely to be influenced by NO synthesized by nNOS. As previously mentioned, NO has been localized to neurons in several brain regions responsible for regulating mating behavior in rodents (16). Likewise, abnormal sexual behavior is apparent in male nNOS−/− mice; nNOS−/− males persistently mount nonestrous female mice to a vastly greater extent than WT mice (26). eNOS is found primarily in the endothelial lining of blood vessels in the central nervous system and periphery, and is unlikely to be responsive for alterations in motivated reproductive behavior. However, eNOS may be an important regulator of ejaculatory function and may provide novel insights into the mechanisms responsible for premature ejaculation. Ejaculatory problems are a common clinical problem in adult males (34). If eNOS regulates ejaculation, then drugs that can be administered peripherally to increase eNOS selectively may be effective agents in treating these dysfunctions with minimal central nervous system side effects.

ACKNOWLEDGEMENTS

The authors thank Debbie Drazen for technical assistance and valuable comments during the preparation of this manuscript. Expert animal care was provided by Edward Silverman. This work was supported by USPHS Grants MH57535 and MH57760 (R.J.N.), DK02565-01 (A.L.B.), and USPHS Grant NS33335 (P.L.H.).

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