

# Sexual Experience Increases Nitric Oxide Synthase in the Medial Preoptic Area of Male Rats

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Nitric oxide in the medial preoptic area (MPOA) is important for the expression and sensitization of male sexual behavior. In this article, the authors report that repeated sexual experience (mating for 2 hr on each of 3 days) increased levels of nitric oxide synthase (NOS) in the MPOA of male rats, regardless of whether they mated on the day they were given an overdose of sodium phenobarbital. This effect resulted from the previous experience and not acute mating, as NOS was not increased 2 hr after the first mating in previously naive males. Experience-induced increases in NOS in the MPOA may be one mechanism through which sexual experience facilitates sexual behavior in male rats.

*Keywords:* sexual behavior, learning, memory, glutamate, *N*-methyl-D-aspartate

Nitric oxide (NO) is important for male sexual function. Systemic inhibition of nitric oxide synthase (NOS) impaired copulation and decreased erections in rats (Hull et al., 1994). In the central nervous system one area where NO may facilitate sexual behavior is the medial preoptic area (MPOA), as evidenced by reports that increasing NO in the MPOA increased mounting rate (Y. Sato, Horita, Kurohata, Adachi, & Tsukamoto, 1998), whereas inhibiting NOS impaired mating (Lagoda, Muschamp, Vigdorichik, & Hull, 2004; Y. Sato et al., 1998). One way by which NO in the MPOA might facilitate behavior is by increasing extracellular dopamine (DA; Dominguez, Muschamp, Schmich, & Hull, 2004; Lorrain & Hull, 1993; Lorrain, Matuszewich, Howard, Du, & Hull, 1996), which in turn facilitates male sexual behavior (reviewed in Dominguez & Hull, 2005; Hull, Wood, & McKenna, 2006).

In addition to altering transmitter activity, NO also sculpts and maintains neural mechanisms resulting from experience, learning, and memory (reviewed in Susswein, Katzoff, Miller, & Hurwitz, 2004). Specifically, NO is important for long-term potentiation, synaptic plasticity, axonal outgrowth, and synaptogenesis at different levels of the central nervous system (reviewed in Schuman & Madison, 1994). We hypothesize that NO may also be important for sensitization of male sexual behavior. Compared with naive males, sexually experienced males display increased preference for receptive females rather than for males; require fewer mounts and intromissions to reach an ejaculation; have shorter latencies to

mount, intromit, and ejaculate; and require less time to resume copulation after ejaculating (reviewed in Hull & Dominguez, in press; Hull, Dominguez, & Muschamp, 2006). To examine whether NO in the MPOA is important for sensitization of male sexual behavior, Lagoda et al. (2004) blocked NOS activity in the MPOA of virgin male rats immediately before each of seven noncopulatory exposures to a receptive female. They found that the NOS antagonist blocked the exposure-induced enhancement of sexual behavior seen in vehicle-injected males, compared with males that were not exposed to females. Therefore, NO in the MPOA may play a role in the enhancement of sexual behavior resulting from exposure to a female. The present experiments examined whether sexual experience changes levels of NOS in the MPOA of male rats.

## Method

Two experiments were performed to assess the effects of sexual experience on NOS. Experiment 1 examined whether sexual experience changes NOS protein concentration in the MPOA, whereas Experiment 2 examined whether sexual experience changes the number of NOS-immunoreactive (ir) cells in the medial preoptic nucleus (MPN), a central nucleus in the MPOA. Levels of protein concentration or numbers of neuronal NOS (nNOS)-ir cells were measured in the following four groups of rats: naive no sex (NNS, no mating at any time before death), naive sex (NS, previously naive, mated for the first time 2 hr before death), experienced no sex (ENS, mated for 2 hr on three previous occasions but not on the day of death), and experienced sex (ES, mated for 2 hr on three previous occasions and also 2 hr before death). In Experiment 1, each group contained 6 male rats ( $N = 24$ ). In Experiment 2, NNS, ENS, and ES groups contained 5 rats, the NS group contained 6 ( $N = 21$ ; see Figure 1 for a diagram describing general experimental design).

## Subjects

Long-Evans/Blue Spruce adult male rats (Harlan, Indianapolis, IN) were housed individually in large plastic cages, in a climate-controlled room, on a 14:10 hr light–dark cycle, with lights off at 11:00 a.m. and on at 9:00 p.m. Food and water were freely available.

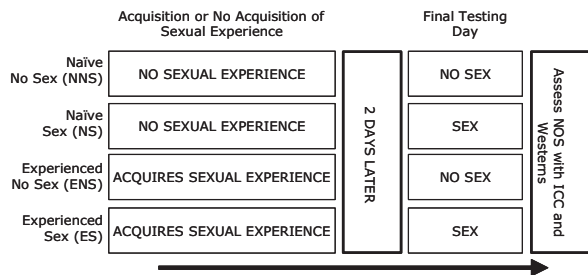
Conspecific females were ovariectomized under ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (4 mg/kg) anesthesia. They were

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**Figure 1.** Diagram describing the general experimental design. On experience days, rats acquired sexual experience (ENS, ES) or no experience (NNS, NS) on three separate occasions; on the final testing day rats copulated (NS, ES) or did not copulate (NNS, ENS) before they were killed. NOS = nitric oxide synthase; ICC = immunocytochemistry; Westerns = Western immunoblotting.

brought into behavioral estrus with 10  $\mu$ g estradiol benzoate 48 hr before and 500  $\mu$ g progesterone 4 hr before testing. Behavioral receptivity was confirmed by placing the female with a stud male shortly before the test began. All procedures were done in accordance with the National Institutes of Health Guidelines for the Use of Animals and were approved by the Institutional Animal Care and Use Committee of Florida State University.

All behavioral manipulations were performed in the male's home cage. Rats receiving sexual experience were taken from their colony to a test room where they were allowed to copulate with an estrous female, whereas sexually naive controls were taken to a similar but separate test room with no females. Sexual experience consisted of mating with a receptive female for 2 hr, on 3 successive days, for a total of 6 hr; on the last day of experience all rats in these groups reached at least three ejaculations. Two days separated the last experience day and the final test day. On the final test day, mated groups were allowed to copulate to one ejaculation; no-sex control groups were taken into the test room, but females were not introduced into their home cage. Use of these four groups allowed for isolation of any acute effects on NOS resulting from mating to one ejaculation from effects resulting from previous experience. All rats were killed by an overdose of sodium pentobarbital 2 hr after ejaculation or placement in the testing room.

### Western Immunoblotting

After the rats were killed, fresh brains were collected and tissue punches were obtained from the MPOA. Tissue punches were obtained from 1-mm thick sections at the level of the MPOA with an 18-gauge needle (1.2 mm inside diameter). Tissue was homogenized in ice cold buffer solution—50 mM Tris-hydroxymethyl (pH 8.0), 150 mM sodium chloride, 1% NP-40, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate (Sigma, St. Louis, MO) with protease inhibitors (Roche Complete Mini protease cocktail; Roche, Indianapolis, IN). The solution stayed on ice for 20 min and was then centrifuged at  $20,000 \times g$  for 20 min at 4  $^{\circ}$ C. Each sample included MPOA tissue pooled from two rats from the same treatment group; there were three samples for each group (three samples per group, two rats per sample, four groups;  $N = 24$ ). The supernatant was collected and stored at  $-80^{\circ}$ C until proteins were separated. Equal amounts of protein (10  $\mu$ g) were fractionated by 10% SDS-polyacrylamide gel electrophoresis and transferred overnight onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). Each step was preceded by rinses (three times for 15 min) in Tris-buffer saline with 0.05% Tween-20 (Fisher Scientific, Fairlawn, NJ). Membranes were blocked (Tris-buffer saline with 5% dry milk and 0.05% Tween-20) and incubated overnight in primary antisera against nNOS (1:2,000; Immunostar, Hudson, WI). The membranes were then incubated with horseradish

peroxidase-linked secondary antibody (goat anti-rabbit; Pierce Biotechnology, Rockford, IL) diluted 1:5,000 in blocking solution for 1 hr at room temperature. Next, signal was enhanced with an Amersham chemiluminescence kit (ECL; Amersham Biosciences, Piscataway, NJ). Images were acquired in dark by exposing membranes to Kodak BioMax film for 3 min. Film was scanned, and band densities were quantified with NIH-ImageJ (Version 1.33U; National Institutes of Health, Bethesda, MD). The PVDF was stripped of nNOS antibody and reprobbed with the actin antibody (1:2,000; Sigma, St. Louis, MO) as a loading control.

### Immunohistochemistry

For immunohistochemical staining in Experiment 2, we used 21 male rats, with 5 rats in the NNS, ENS, and ES groups and 6 rats in the NS group. They were run through the same procedure as were the rats in Experiment 1. After the last testing day, the rats were killed and their brains were perfused transcardially with saline, followed by 4% paraformaldehyde in 0.1 M PB. Brains were removed, postfixed for 1 hr in the same fixative at room temperature, and stored in 30% sucrose at 4  $^{\circ}$ C. Coronal sections were cut at 35  $\mu$ m and stored in cryoprotectant solution. Sections containing the MPN underwent immunohistochemical staining for nNOS. Washes in phosphate-buffered saline (PBS) (pH, 7.35), 4 times 5 min, preceded all incubations. Sections were incubated in 0.3%  $H_2O_2$  in PBS, and then blocked in PBS with 5% normal goat serum and 0.2% Triton X-100 (blocking solution). Sections were incubated overnight at room temperature in nNOS primary antibody (1:8,000; Immunostar, Hudson, WI) diluted in blocking solution. Next, anti-rabbit biotinylated secondary antibody (1:500 in blocking solution; Vector Labs, Burlingame, CA) preceded incubation with the avidin-biotin conjugate (Vectastain ABC Elite kit; Vector Laboratories, Burlingame, CA). Immunoreactivity was visualized with a diaminobenzidine-nickel chromogen solution (Sigma, St. Louis, MO) to yield a purple-black precipitate. Sections were dehydrated, mounted, and coverslipped with Permount. For negative controls, sections underwent the same immunostaining procedure, except the nNOS primary antibody was excluded. Light microscopy was used to quantify the number of nNOS-ir cells. The MPN (at AP level  $-0.51$ , according to Swanson, 2004) was examined bilaterally and labeled cells were counted in a  $300 \times 400 \mu$ m area by an experimenter blind to treatment conditions.

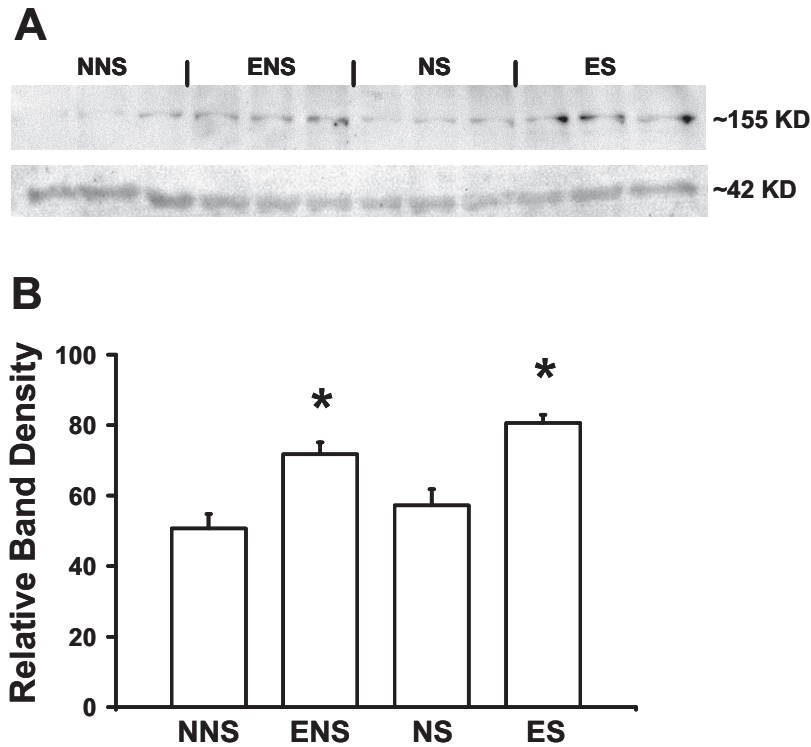
### Data Analysis

We conducted a two-way analysis of variance (ANOVA; Previous Experience  $\times$  Final Mating) to probe for differences in the number of ir cells or band densities between treatments. Newman-Keuls tests were used to probe for significant differences among individual means. Data analyses were performed with the SigmaStat data analysis program (Version 2.03; SPSS, Chicago, IL).

### Results

Previous sexual experience increased the concentration of nNOS protein in the MPOA of male rats. Specifically, comparison of band densities in Western blots with a two-way ANOVA in Experiment 1 revealed a significant effect of experience on levels of nNOS,  $F(1, 11) = 35.68$ ,  $p < .01$  (see Figure 2). Newman-Keuls tests revealed higher levels of nNOS in both experienced groups compared with previously naive groups ( $p < .05$ ). There were no significant differences as a result of mating on the day of death. Also, no significant changes were observed for levels of actin ( $p = .288$ ) as a loading control.

Results from Experiment 2 indicate that previous sexual experience increased the number of nNOS-ir cells in the MPN of male rats. Specifically, analysis with a two-way ANOVA revealed a



**Figure 2.** Protein concentration for neuronal nitric oxide synthase (nNOS) in the medial preoptic area (MPOA) of male rats. A: Western immunoblot of samples collected from the MPOA of male rats in the following groups: sexually naive rats that did (NS) or did not (NNS) mate on day of testing and sexually experienced rats that did (ES) or did not (ENS) mate on day of testing. Pictured on top are bands for nNOS (~155 kDa); below are bands for beta-actin (~42 kDa). B: Comparison of relative band densities from Western immunoblots. Values are expressed as  $M \pm SEM$ . \* $p < .05$ .

significant effect of experience on number of nNOS-ir cells,  $F(1, 17) = 30.52, p < .01$  (see Figure 3). Newman-Keuls tests revealed higher levels of nNOS in both experienced groups compared with previously naive groups ( $p < .05$ ). No significant differences were observed between rats that did or did not mate on the day of death, within the same experience condition. No immunoreactivity was observed in negative-control sections.

#### General Discussion

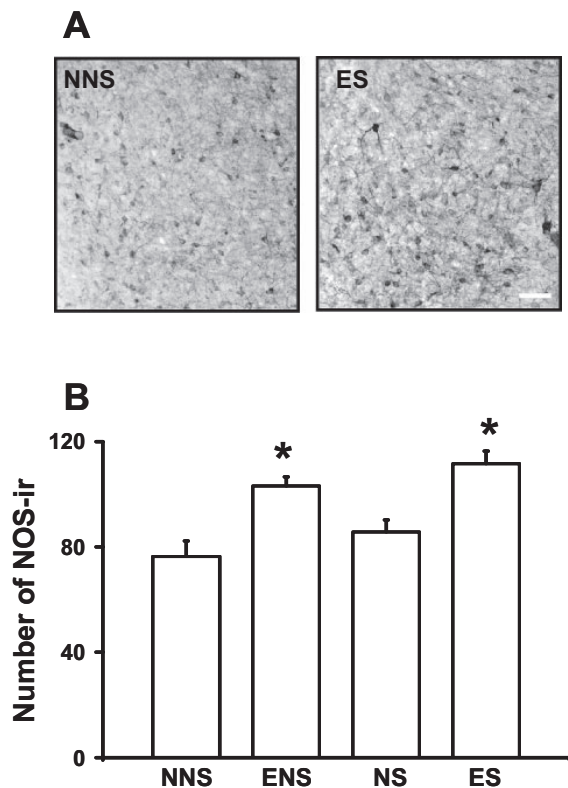
Sexual experience increased the number of nNOS-ir cells and nNOS protein concentration in the MPOA. Compared with the NNS control group, levels of nNOS in naive rats that copulated for the first time on the day of testing (NS group) were unchanged; however, levels were higher in rats that did not copulate on the day of testing but were already experienced (ENS group), suggesting that increased nNOS resulted from previous experience and not from acute sexual activity.

NO, unlike classical neurotransmitters, is not stored, does not undergo exocytosis or bind to membrane-associated receptors, and is not deactivated by reuptake or enzymatic mechanisms (reviewed in Dawson & Dawson, 1995). Instead, NO is membrane-permeable, is synthesized immediately after activation of NOS, and freely diffuses into nearby cells, where it can alter intracellular mechanisms. As NO cannot be regulated via traditional mecha-

nisms, the key to regulating NO is regulation of NOS (reviewed in Förstermann et al., 1995).

To date the three known isoforms of NOS are endothelial NOS, inducible NOS, and nNOS. Originally purified from neurons, expression of nNOS is considered to be constitutive. However, a growing body of literature suggests that expression of nNOS may be subject to regulation by numerous physiological conditions or stimuli (reviewed in Förstermann, Boissel, & Kleinert, 1998). A few of the factors that increase nNOS expression are calcium influx (Sasaki et al., 2000), electrical stimulation (Reiser, Kline, & Vaghy, 1997), feeding after food deprivation (Jahng et al., 2005), immobilization stress (Calza, Giardino, & Ceccatelli, 1993), neuronal activity (Tascedda, Molteni, Racagni, & Riva, 1996), protein kinase activity (Krainock & Murphy, 2001), sex hormones—estradiol (Ceccatelli, Grandison, Scott, Pfaff, & Kow, 1996; Putnam, Sato, Riolo, & Hull, 2005; Rosenfeld, Chen, Roy, & Liu, 2003) and testosterone (Hadeishi & Wood, 1996; Putnam et al., 2005; Reilly, Zamorano, Stopper, & Mills, 1997)—and now sexual experience.

nNOS is activated when intracellular  $Ca^{2+}$  interacts with calmodulin in the presence of oxygen and nicotinamide adenine dinucleotide phosphate diaphorase. Activation of nNOS results in oxidation of the guanidino nitrogen on L-arginine, which converts it to L-citrulline, and NO is produced. NO diffuses freely into



**Figure 3.** Number of neuronal nitric oxide synthase immunoreactive (nNOS-ir) cells in the medial preoptic area (MPOA) of male rats. **A:** Representative photomicrographs of sections stained for nNOS in the MPOA of sexually naive rats that did not mate on day of testing (NNS) versus sexually experienced rats that mated on day of testing (ES). **B:** Average number of nNOS-immunoreactive cells in the MPOA of male rats undergoing different experience and mating conditions. Values are expressed as  $M \pm SEM$ . ENS = sexually experienced rats that did not mate the last day of testing; NS = sexually naive rats that did mate the last day of testing. \* $p < .05$ .

nearby cells, where it can potentially alter genomic activity, protein synthesis, synaptogenesis, and transmitter release (reviewed in, e.g., Bogdan, 2001; Schuman & Madison, 1994). Indeed, NO has been reported to increase calcium-dependent and/or calcium-independent vesicular release of dopamine (DA; reviewed in Prast & Philippu, 2001; West, Galloway, & Grace, 2002) and to inhibit its reuptake (reviewed in Kiss & Vizi, 2001).

The MPOA is a central integrative site for the regulation of male sexual behavior. It receives indirect input from every sensory modality (Simerly & Swanson, 1986) and sends projections to structures critical for the initiation and patterning of copulation (Simerly & Swanson, 1988). Ablation of the MPOA inhibited male sexual behavior in all studied species; conversely, stimulation facilitated behavior (reviewed in Hull & Dominguez, 2003; Hull, Dominguez, & Muschamp, 2006; Hull, Wood, & McKenna, 2006). Stimulation of rats' MPOA also elicited erectile responses (Giuliano et al., 1997) and the urethrogenital reflex, a model for orgasm (Marson & McKenna, 1994). Finally, studies in which researchers have used electrophysiological recordings (Oomura, Aou, Koyama, Fujita, & Yoshimatsu, 1988; Shimura, Yamamoto,

& Shimokochi, 1994) or expression of immediate early genes (reviewed in Pfau & Heeb, 1997) indicate increased neural activity in the MPOA with sexual behavior. In fact, mating to one ejaculation elicited more neural activation (measured as Fos-ir) in the MPN of experienced males compared with naive males, even though the experienced males had fewer intromissions before ejaculating (Lumley & Hull, 1999). This suggests that sexual experience enhances mating-induced activation and responsiveness to sexual stimuli in the MPN.

NO in the MPOA is important for the expression and sensitization of male sexual behavior. Blocking NOS in the MPOA of rats impaired copulation, whereas administration of L-arginine, the precursor to NO, facilitated behavior (Y. Sato et al., 1998). In addition, an NO donor (sodium nitroprusside) reverse dialyzed into the MPOA of long-term castrates treated with dihydrotestosterone restored copulation to ejaculation in half of the rats; none of the controls copulated (S. Sato, Wersinger, & Hull, 2005).

Inhibition of NOS has somewhat different effects in sexually naive and in experienced males (Lagoda et al., 2004). Copulation was almost totally blocked by the NOS inhibitor nitro-L-arginine methyl ester (L-NAME) in sexually naive males; experienced males did copulate following L-NAME microinjections but were significantly impaired. In addition, inhibiting NOS in the MPOA of male rats before each of seven daily exposures to a female above their cage blocked the facilitative effects of those exposures, seen in vehicle-treated males compared with nonexposed males. However, experience-induced sensitization of the MPOA does not preclude a role for additional brain areas in the mediation of sexual learning.

It is not clear which upstream mechanisms are responsible for increased NOS resulting from sexual experience. One possibility is that testosterone mediates these effects. Indeed, studies indicate that sexually experienced male rats have a larger testosterone response when exposed to a receptive female and during copulation, compared with naive males (Bonilla-Jaime, Vazquez-Palacios, Arteaga-Silva, & Retana-Marquez, 2006; Kamel, Mock, Wright, & Frankel, 1975). Furthermore, numerous cells in the MPN of hamsters (Hadeishi & Wood, 1996) and rats (S. Sato, Braham, Putnam, & Hull, 2005) that contain NOS also contain androgen receptors. This, coupled with the fact that castration decreased and testosterone replacement increased NOS concentration in the MPN of hamsters (Hadeishi & Wood, 1996) and rats (Putnam et al., 2005), suggests that acute increases in testosterone may contribute to experience-induced increases in MPN NOS.

One way by which NO may facilitate sexual behavior is by increasing extracellular DA in the MPOA. DA in the MPOA is important for male sexual behavior (reviewed in Dominguez & Hull, 2005; Hull, Dominguez, & Muschamp, 2006; Hull, Wood, & McKenna, 2006). Reverse dialysis of L-arginine into the MPOA increased extracellular levels of DA; coadministration of N-monomethyl-L-arginine (a NOS antagonist) blocked the L-arginine-induced increase and lowered basal levels of DA when administered alone (Lorrain & Hull, 1993). Moreover, L-NAME, another NOS antagonist, blocked female-stimulated (Lorrain et al., 1996) and glutamate-evoked (Dominguez et al., 2004) release of DA in the MPOA, suggesting that NO in the MPOA mediates sex-relevant DA release.

Results presented here suggest that previous sexual experience increases levels of NOS in the MPOA of male rats. However, it is

not possible to rule out the effects of social novelty, per se, as males that copulated repeatedly were thereby exposed to a novel social stimulus. Further studies will be required to dissociate the specific factors that upregulate NOS.

In summary, the MPOA is critical for integrating sexually relevant sensory and endocrine stimulation, and it in turn sends information to regions important for coordinating copulation-specific motor output (reviewed in Hull, Dominguez, & Muschamp, 2006; Hull, Wood, & McKenna, 2006). NO facilitates sexual behavior, possibly by mediating release of neurotransmitters and by galvanizing genomic changes and protein synthesis or some combination thereof in the MPOA. In this article we report that sexual experience increased levels of NOS, the enzyme responsible for NO production. This increase might function to "prime" the system after sexual experience for appropriately responding to subsequent sexually relevant sensory or endocrine stimulation.

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