Testosterone Induction of Male-Typical Sexual Behavior Is Associated with Increased Preoptic NADPH Diaphorase and Citrulline Production in Female Whiptail Lizards

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ABSTRACT: **In rodents, male-typical copulatory behavior is generally dependent on gonadal sex steroids such as testosterone, and it is thought that the mechanism by which the hormone gates the behavior involves the gaseous neurotransmitter nitric oxide. According to one model, testosterone induces an up-regulation of nitric oxide synthase (NOS) in the preoptic area, increasing nitric oxide synthesis following exposure to a sexual stimulus. Nitric oxide in turn, possibly through its effect on catecholamine turnover, influences the way the stimulus is processed and enables the appropriate copulatory behavioral response. In whiptail lizards (genus *Cnemidophorus*), administration of male-typical levels of testosterone to females induces the display of male-like copulatory responses to receptive females, and we hypothesized that this radical change in behavioral phenotype would be accompanied by a large change in the expression of NOS in the preoptic area. As well as comparing NOS expression using NADPH diaphorase histochemistry between testosterone-treated females and controls, we examined citrulline immunoreactivity (a marker of recent nitric oxide production) in the two groups, following a sexual stimulus and following a nonsexual stimulus. Substantially more NADPH diaphorase-stained cells were observed in the testosterone-treated animals. Citrulline immunoreactivity was greater in testosterone-implanted animals than in blank-implanted animals, but only following exposure to a sexual stimulus. This is the first demonstration that not only is NOS up-regulated by testosterone, but NOS thus up-regulated is activated during male-typical copulatory behavior.** © 2006 Wiley Periodicals, Inc. J Neurobiol 66: 1156–1163, 2006

Keywords: **copulatory behavior; androgen; nitric oxide; preoptic area; *Cnemidophorus***

INTRODUCTION

In vertebrates with internal fertilization, males in breeding condition will respond to receptive females with a sequence of stereotyped courtship and copulatory behaviors culminating in sperm transfer. These male-typical behaviors are dependent on gonadal sex steroids, particularly testosterone, so that the levels of these behaviors displayed by animals following castration will diminish over a period of days and, in most individuals, disappear completely. In castrated, previously sexually active males, mating behavior can be reinstated by the administration of exogenous testosterone. This re-establishment also requires several days to complete, although the latency is dependent on the time elapsed since castration, sexual experience, and several other factors. The neuroanatomical substrates of male copulatory behavior have been extensively mapped in rodents, focusing on the pathways involved in the transmission of pheromonal and other olfactory information from the main and accessory olfactory bulbs via the olfactory component of

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the amygdala to the preoptic area, and thence to areas of the brainstem putatively controlling copulatory motor patterns. Because testosterone implants in the preoptic area can re-establish copulatory behavior in an animal systemically deprived of hormone, this area has been a focus of attention for researchers attempting to elucidate the cellular changes underlying the hormone-mediated gating of copulatory behavior.

According to one current model, the gating mechanism involves a testosterone-dependent increase in dopamine availability in the preoptic area. Hull and coworkers (e.g., Dominguez and Hull, 2005) have proposed that dopaminergic stimulation of preoptic neurons alters the processing of afferent information about the presence of a sexual stimulus. In turn, efferent signals from the preoptic area to the brainstem motor areas controlling copulatory behavior are altered in such a way as to augment the probability of a copulatory behavioral response. The same model posits that dopamine availability is influenced by nitric oxide (NO), and that an increase in the expression and action of the neuronal isoform of NO synthase (nNOS), the enzyme responsible for NO synthesis, is an important effect of testosterone's action in the preoptic area. Castration decreases NOS expression in the medial preoptic area in rats (Du and Hull, 1999) and Syrian hamsters (Hadeishi and Wood, 1996). Furthermore, pharmacological inhibition of this enzyme suppresses copulatory behavior in previously sexually active male rats (Benelli et al., 1995; Ratnasooriya et al., 2000), lending support to the idea that NO synthesis is an important component of the androgen-dependent gating of male sexual behavior.

Whiptail lizards of the genus *Cnemidophorus* offer some revealing insights into the androgen control of male-typical copulatory behavior (discussed by Crews, 2005). The genus includes normal gonochoristic species such as *C. inornatus*, and also several parthenogenic species. One of the parthenogenic species, *C. uniparens,* has been studied extensively because despite having all of the morphological and physiological characteristics of females, at certain times in the ovarian cycle, these animals exhibit towards receptive conspecifics a suite of copulatory behaviors identical to those shown by male whiptails. Male-like behavior can also be elicited from these animals in the laboratory by androgen treatment, but no androgens are detectable in unmanipulated parthenogens or females. Rather, the display of male-like behavior in the parthenogen is thought to be dependent on progesterone, which peaks around the time of ovulation when male-like behavior is observed. This unusual hormone-behavior relationship has stimulated the search for components of the neural system controlling male-typical copulatory behavior that might be targets of either androgens (in males or androgen-treated females) or progesterone (in parthenogens or progesterone-treated males). The NO system is a good candidate for such a target because it is androgen-regulated, involved in controlling male-typical copulatory behavior (as discussed above), and is known to be influenced by progesterone (Mani et al., 1994). A previous experiment had established that pharmacological inhibition of NO synthesis suppresses testosterone-induced malelike copulatory behavior in *C. uniparens* (Sanderson et al., 2005). The experiment described here was designed to test two predictions concerning the effect of testosterone treatment that induces male-like behavior in female whiptails: first that it would up-regulate NOS expression in the preoptic area and second that it would increase NOS activity in the same area following male-like copulatory behavior.

Two endpoints were compared between testosterone-treated females and controls: numbers of putatively NOS-containing cells were examined using NADPH diaphorase (NADPH-d) histochemistry, and NOS *activity* was assessed using citrulline immunohistochemistry. In the brain, NADPH-d is considered to be a reliable marker of nNOS, producing comparable distributions of stained cells to those marked by nNOS immunohistochemistry (Dawson et al., 1991), and has been used to mark nitrergic neurons in diverse vertebrates including the tilapia (Bordieri et al., 2003), the pigeon (Cuthbertson et al., 1999), and the gecko (Smeets et al., 1997), as well as rodents and primates. Citrulline is produced from arginine concomitantly with the synthesis of NO under the catalytic influence of NOS, and because other sources of the amino acid in the brain are not abundant, citrulline immunoreactivity is a reliable marker of NO production in the period immediately preceding sacrifice (Keilhoff et al., 2000; Martinelli et al., 2002). In order to distinguish a possible testosterone-induced increase in the activity of nitrergic cells *mediating copulatory behavior* from changes in activity in cells mediating other processes, citrulline immunoreactivity was examined in subsets of animals that had been exposed to sexual or nonsexual stimuli immediately before sacrifice.

MATERIALS AND METHODS

Animals and Hormone Treatments

Female *C. inornatus* were captured in the vicinity of Sanderson, Texas. They were held in environmentally controlled chambers over the winter of 2004 as described by Wade

and Crews (1991) and in May of 2005 were ovariectomized and implanted with 12 mm Silastic implants containing crystalline testosterone or an identical capsule containing no hormone. Such testosterone implants have previously been shown to produce systemic testosterone levels typical of breeding males in female whiptails (Lindzey and Crews, 1986) and to elicit male-typical copulatory behavior (Wade et al., 1993).

Behavioral Testing

Five weeks after testosterone implantation, each animal was given a single test, either with a female whiptail made receptive by intramuscular injection of 0.5 μg of estradiol dissolved in peanut oil, or else with a dummy comprising a plastic tube approximately the same size as a lizard. All animals with blank implants were given a test with a receptive female (BLANK+F group, $n = 8$), while animals with testosterone implants were divided into two groups, a T+DUMMY group ($n = 8$) tested with the dummy and a T+F group ($n = 9$) tested with receptive females. Animals' water bowls and planks were removed 10 min before the test to facilitate observation, and the test itself was allowed to proceed for 10 min after the introduction of the female. Latencies to the exhibition of mount and intromissive posture as described by Crews and Fitzgerald (1980) were recorded for those animals displaying these behaviors.

Perfusion and Tissue Processing

Immediately after the end of the 10 min testing period, the experimental animal was removed and injected with an overdose of sodium pentobarbital before perfusion through the ventricle with 10 mL of 0.9% saline and 10 mL ice-cold fixative compared of 1% glutaraldehyde, 0.2% sodium metabisulfite, and 3% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4. Brains were dissected from the skulls, postfixed in the same fixative at 4°C overnight, and then transferred to 30% sucrose in PBS for 2 to 3 days. After the brains sank they were blotted to remove excess sucrose, frozen in isopentane cooled with dry ice, and stored at −80°C. Brains were then mounted on a cryostat chuck in tissue-freezing medium (Triangle Biomedical Sciences, Durham, NC) and cut at 40 μm on an HM500 cryostat (MICROM, Waldorf, Germany). Sections were cut in four series into antifreeze containing 30% sucrose, 30% ethylene glycol, and 1% polyvinyl pyrrolidone in PBS and stored at −20°C. During sectioning, one brain was destroyed by a cryostat malfunction.

NADPH-d Staining

One series of sections from each brain was retrieved from antifreeze and rinsed in Tris-buffered saline (TBS), pH 7.4, before incubation at 37°C for 100 min in TBS containing approximately 0.5 m*M* NADPH (N-7505; Sigma), 0.4 m*M* Nitro Blue Tetrazolium (N-5514; Sigma), and 0.3% Triton-X. Sections were then washed in TBS at room temperature and stored in the same buffer at 4°C until mounting. Control sections were subjected to the same treatment except that the NADPH was omitted.

Citrulline Immunohistochemistry

Sections for citrulline immunohistochemistry were rinsed in PBS, incubated for 25 min in 75% methanol, 0.075% hydrogen peroxide, then in 0.5% sodium borohydride, 0.2% sodium metabisulfite for 1 h before blocking in PBS containing 0.3% Triton-X (PBST), 1% nonfat milk solids, and 10% normal goat serum. After 5 h blocking, during which period the primary antibody (rabbit anticitrulline, AB6464; Abcam U.K.) was preincubated with the same blocking solution, sections were transferred to primary antibody at a final concentration of 1:5000 and incubated for 12 h. Sections were then extensively washed in PBST, before incubation for 1 h in biotinylated goat antirabbit antibody (BA-1000; Vector Laboratories, Burlingame, CA) diluted 1:1000 in PBST containing 5% normal goat serum, and then 1 h in horseradish peroxidase-conjugated Avidin Biotin Complex (PK-6100; Vector) diluted to one-fifth of the manufacturer's recommended concentration with PBS, 0.1% Triton-X, and 0.05% bovine serum albumin. Staining was developed with the DAB substrate kit (SK-4100; Vector) following the manufacturer's directions. All steps were carried out at room temperature. Omission of primary antibody eliminated staining entirely. Of the first series to be processed, some slides were counterstained with toluidine blue to assist in locating regions of interest, and because the counterstaining interfered with optical density measurement, a second series was immunostained in the same way as the first, and all measurements were taken from this second series.

Cell Counting and Optical Density Analysis

Cells stained by NADPH-d histochemistry were counted using the optical fractionator routine included in the Stereo Investigator program (Microbrightfield, Williston, VT). The preoptic area, including the periventricular and medial preoptic areas, was delineated on the computer screen according to the atlas of Young et al. (1994) using the 10X objective of a Zeiss microscope, and cells included in counting frames spaced systematically by the computer within this area after a randomly selected start point were marked using the 100X oil immersion objective. Two sections were counted for each brain, a smaller number than is common for stereological studies (Mouton, 2002), because of the small size of the lizard preoptic area.

For citrulline immunostaining, which was expected to be distributed through the neuropil rather than confined to cell bodies, optical density was measured from micrographs taken with standardized illumination using a 10X objective on an Olympus microscope. Digital micrographs captured with a Microfire camera (Microbrightfield) and PictureFrame software, also supplied by Microbrightfield, were converted to eight-bit grayscale JPEG files and analyzed

using ImageJ software (Abramoff et al., 2004) on a Macintosh G4. ImageJ was used to delineate an area of interest corresponding to the preoptic area, to eliminate areas of the image corresponding to holes in the tissue or artifacts due to dirt, and to measure the optical density in comparison with a Kodak step tablet (Rasband, 1997–2005). Two sections through the preoptic area were sampled for each brain: one section just caudal to the rostral extremity of the third ventricle, and the section immediately caudal to that, which was generally the section in which the anterior commissure was first observed.

Slides were coded for quantitative analyses and the microscopist was blind to the experimental groups. After decoding the slides one coded identity was found not to correspond to an experimental subject and was not included in any further analysis. All statistical analysis was performed with the SAS program (SAS Institute, Cary, NC) on the University of Texas Windows Server.

RESULTS

Behavior

All testosterone-implanted individuals tested with a receptive female mounted the stimulus animal with latencies varying from 22 to 442 s (median = 114), and proceeded to assume the intromissive posture with latencies of 135 to 540 s (median = 245). No animal in either BLANK or DUMMY group mounted the stimulus animal.

NADPH-d

This technique produced vivid blue staining of cells and fibers in several areas of the brain, while control sections lacked the blue formazan staining characteristic of the technique. Distribution of stained cells and fibers was essentially similar to that reported by Smeets et al. (1997) for the gecko, with well-stained cells obvious in the striatum, the dorsal ventricular ridge, the amygdaloid complex, and the diagonal band of Broca, although stained cells were somewhat less numerous and fiber plexuses less dense than reported by Smeets et al. (1997). The target population of cells in the preoptic area was generally more lightly stained than other obvious populations of nitrergic cells, and was mostly arranged close to the third ventricle [Fig. 1(B)]. Total numbers of these periventricular NADPH-d-positive cells were estimated based on the density in the sampled sections and the estimated volume of the region of interest, and compared using the Wilcoxon test because of heterogeneity of variance and deviation from normality of the data. No difference was expected in this parameter between the T+DUMMY group and the T+F group, because they only differed in the nature of the test they were given immediately before sacrifice, and none was observed ($p = 0.740$). Accordingly, data from these two groups were pooled and compared with the BLANK+F group. Estimated cell numbers were significantly higher in the testosterone-treated groups than in the blank group ($p = 0.0002$; Fig. 1).

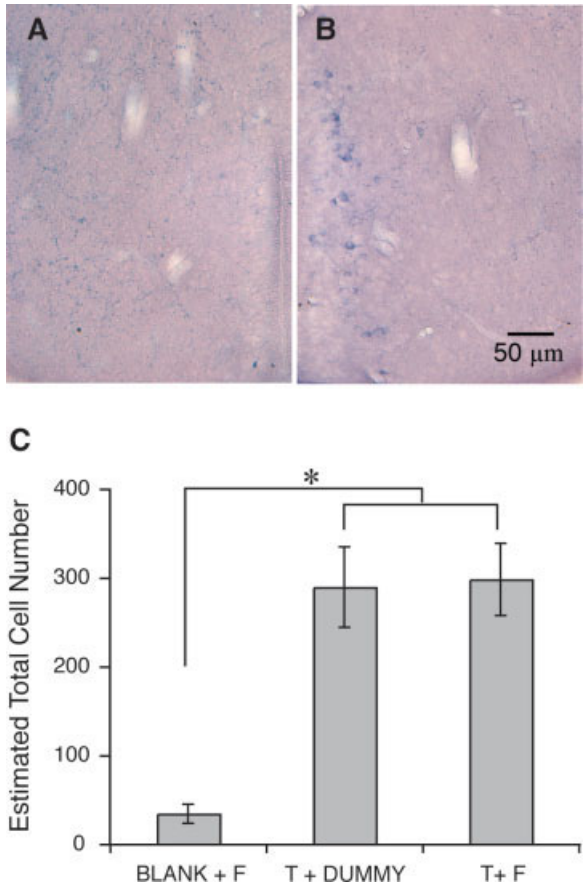

Figure 1 The effect of testosterone (T) treatment on NADPH diaphorase-positive cells in the periventricular preoptic area of ovariectomized female whiptail lizards (*Cnemidophorus inornatus*). Cells are rarely observed in ovariectomized animals given blank (BLANK) Silastic capsules (A), but can clearly be seen close to the third ventricle in an ovariectomized female given a Silastic capsule containing T (B). Figure 1(C) shows estimated total numbers of these periventricular NADPH-d cells in the three experimental groups. The asterisk signifies that the two T-treated groups are significantly higher than the BLANK-implanted group ($p = 0.0002$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Citrulline Immunostaining

In several areas of the brain, particularly the more rostral ventral forebrain, very clear staining of cells

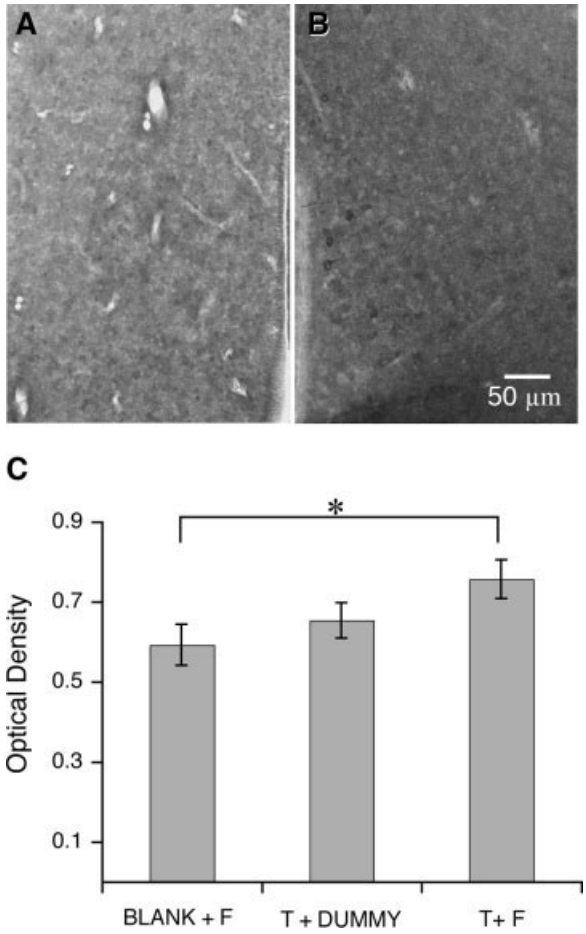

Figure 2 The effect of testosterone (T) treatment and copulatory behavior on citrulline immunoreactivity in the preoptic area of ovariectomized female whiptail lizards (*Cnemidophorus inornatus*). A similar region of the preoptic area as is depicted in Figure 1(A) and 1(B) is shown for an ovariectomized female given blank (BLANK) Silastic capsule (A), and for an ovariectomized female given a Silastic capsule containing T and exhibiting male-typical copulatory behavior toward an ovariectomized, estrogen-treated stimulus female immediately before sacrifice (B). Micrographs (A) and (B) were captured under similar lighting conditions and thenceforth processed identically. The neuropil is generally darker, suggesting greater citrulline immunoreactivity and putatively more nitric oxide synthesis in the animal having exhibited male-typical copulatory behavior (B), and a few darkly stained cells are visible close to the third ventricle. Optical densities measured over this area are compared between groups in (C). The asterisk signifies that the T+F group is significantly higher than the BLANK-implanted group ($p = 0.011$).

and fibers was observed with this technique. In the preoptic area that was the focus of this study, dark, diffuse staining was observed, with a few well-defined cells close to the ventricle in a subset of the brains [Fig. 2(B)]. The degree of staining was graded medio-laterally and rostro-caudally, with the darkest staining being apparent close to the ventricle and towards the rostral extremity of the preoptic area. Because optical density was almost universally greater in the more rostral of the two sections, optical densities were analyzed with a mixed model with section position (rostral or caudal) as a within-subject variable, using the MIXED procedure of the SAS program. Citrulline immunoreactivity as measured by optical density was substantially greater in the rostral preoptic area than in the caudal ($p = 0.0001$), but the interaction between rostro-caudal position and group was not significant. Accordingly, the main effect of group was examined with planned comparisons of the BLANK+F group with each of the testosterone-treated groups. This analysis suggested that citrulline immunoreactivity was significantly higher in the T+F group than in the BLANK+F group ($p = 0.011$), but that the difference between the BLANK+F group and the T+DUMMY group was not statistically significant ($p = 0.289$; Fig. 2).

DISCUSSION

If the NADPH-d staining is considered a reliable marker of nNOS expression, the present study demonstrates a robust effect of testosterone treatment up-regulating preoptic nNOS, consistent with results from the rat and hamster (Du and Hull, 1999; Hadeishi and Wood, 1996). This was not unexpected, because the NOS inhibitor L-NAME suppresses the expression of malelike copulatory behavior in androgen-treated *C. uniparens* (Sanderson et al., 2005), implicating the enzyme in the hormonal control of this behavior in this species. However, it has recently been reported that testosterone treatment has no effect on NOS expression in the preoptic area of male quail (Martini et al., 2005), and indeed that very little NOS immunoreactivity is observed in the medial preoptic area in this species. These observations prompted Martini et al. (2005) to speculate that the role of a NOS-dependent mechanism in controlling male-typical copulatory behavior might be limited to some subset of vertebrate taxa including rodents but excluding birds. The results of the present study demonstrate that whiptail lizards exhibit the pattern of testosterone up-regulation of preoptic NOS observed in rodents, and suggest that the set of taxa in which NOS has this role is a large one.

The pattern of putatively nitrergic, NADPH-d-positive cells and fibers revealed in this experiment was similar to that reported by Smeets et al. (1997) for the

gecko. For the purposes of the present study, the most important difference was that the technique stained noticeably fewer cells in the preoptic area in this study than were observed by Smeets et al. (1997). These authors reported observing several rows of NADPH-d-stained cells running parallel to the third ventricle, which is indeed what is observed with this technique in our hands using tissue that has been less extensively fixed. In this study NADPH-d-stained cells were distributed at the same distance from the ventricle as were the rows, but were less numerous, and generally the row structure was not very clear. As the intensity of fixation increases, the number of cells marked decreases (unpublished observations), and the simplest interpretation of the quantitative discrepancy between the results of Smeets et al. (1997) and our own is that NADPH-d activity was reduced by the intensive fixation in paraformaldehyde and glutaraldehyde used in the present study because of the requirements of the citrulline immunohistochemical technique (the citrulline antibody is raised against a citrulline-glutaraldehyde-protein conjugate and requires glutaraldehyde fixation of tissue to ensure antigenicity). It is thus possible that the study systematically underestimated the total number of NOS-containing cells.

The fact that citrulline immunoreactivity was greater in testosterone-implanted females than in blank-implanted females, but only following exposure to a sexual stimulus, strongly supports the hypothesis that an up-regulation of NO production is involved in the androgen-induced expression of male-typical copulatory behavior. The fact that citrulline immunoreactivity was intermediate in animals implanted with testosterone but not exposed to a sexual stimulus leaves open the possibility that NO production in this region of the brain following testosterone exposure may be up-regulated constitutively, in addition to the activity elicited by sexual behavior. A firm conclusion on this matter will require a study of sufficient statistical power to reveal definitely whether the testosterone-treated, sexually unexposed group resembles one of the other groups, or is genuinely distinct from either.

In female rats, mounting behavior and aggression are both activated by testosterone (van de Poll et al., 1986). The question therefore arises whether the mounting behavior displayed by testosterone-treated females exposed in this experiment to receptive females might be agonistic in nature, rather than copulatory. By extension, the putative NO production inferred from the increased citrulline immunoreactivity in the preoptic areas of the mounting animals might also be interpreted as aggression-related. This possibility was not considered in the design of this experiment, because the behavioral endpoints included the characteristic intromissive posture as well mounting. This behavior is not observed in agonistic contexts in this species (Crews et al., 1983; Lindzey and Crews, 1988), and because it was displayed toward the receptive stimulus female by every individual in the T+F group, it seemed reasonable to interpret the response as being copulatory in nature. However, agonistic behaviors share with male-typical copulatory behavior several key characteristics, including activation by androgens (van de Poll et al., 1986) and the involvement of NO and serotonin (Chiavegatto and Nelson, 2003), and discrimination between the two would require explicit comparison of the supposedly copulating group with a group exhibiting unambiguously aggressive behavior.

The distinction between NOS activity following copulatory behavior and constitutive activity is an important one. It is thought that most nNOS activity is dependent on calcium influx resulting from the activation of an NMDA receptor physically coupled to the enzyme by scaffolding proteins (reviewed by Kiss and Vizi, 2001). This suggests that specific glutamatergic afferents to an area might activate one set of NMDA receptors, and therefore one set of NOS moieties, while another set of afferents might affect a different population. Presumably, therefore, the cells in which NOS might be up-regulated by testosterone exposure, and subsequently stimulated with glutamatergic input during exposure to a sexual stimulus, are not the same set of cells that would produce NO if the same area of the brain were homogenized and assayed *in vitro*. In fact, there is good evidence that NOS in the preoptic area is activated during male-typical copulatory behavior by glutamatergic afferents from the medial amygdala (Dominguez et al., 2001). Although, to our knowledge, no previous studies have assessed the effect of castration or testosterone administration on NOS activity in the preoptic area following a copulatory stimulus, two have assayed *ex vivo* NOS activity in samples including preoptic tissue (Singh et al., 2000; Reynoso et al., 2002). In both cases, the authors reported that castration in fact decreased NOS activity or had no effect, but neither study was designed to examine the role of NO synthesis associated with sexual behavior. On the other hand, using microdialysis in awake, moving rats, Pu et al. (1996) found that NMDA infusion increased cGMP release (a marker of NO production) in intact but not in castrated males. This result agrees well with the results of this experiment, in which glutamatergic stimulation can be thought of as having been provided endogenously in response to the sexual stimulus. In summary, results of the present study

confirm and extend evidence from other species suggesting that an increase in NO synthesis capacity is a significant component of the androgen-dependent gating of male-typical copulatory behavior.

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