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Preoptic neuronal nitric oxide synthase induction by testosterone is consistent with a role in gating male copulatory behavior

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Abstract

Copulatory behaviors are generally dependent on testicular androgens in male vertebrates, being eliminated by castration and re-instated by testosterone administration. It is postulated that a critical factor in this hormonal gating is up-regulation of neuronal nitric oxide synthase (nNOS) in the preoptic area, and consequent enhanced nitric oxide synthesis in response to stimuli associated with a receptive female. Previous studies have suggested that nNOS protein is more abundant in behaviorally relevant preoptic regions of testosterone-exposed animals than in hormone-deprived controls. This study sought to elucidate the molecular events underlying this apparent up-regulation by examining preoptic nNOS mRNA abundance at several time points following testosterone administration in a castration and replacement paradigm. Castrated male whiptails (*Cnemidophorus inornatus*) were implanted with testosterone, and at four time points over the subsequent 18 days their sexual behavior was tested. A rostral periventricular area previously implicated in hormonal gating of male-typical copulatory behavior was then excised by laser microdissection, and nNOS transcript abundance was assessed by quantitative PCR. As neither this technique nor nNOS mRNA measurements have previously been performed in this area of the brain, expression was concommitantly assayed on adjacent sections by *in situ* hybridization or NADPH diaphorase histochemistry. Results are consistent with transcriptional up-regulation of nNOS by testosterone and a central role for the enzyme in mediating hormonal gating of copulatory behavior.

Introduction

Male-typical copulatory behaviors such as mounting and intromission are activated by testicular androgens, being eliminated by castration, and reinstated by exogenous testosterone (Hull *et al*., 2002). The preoptic area (POA) appears to be critical in the hormonal gating of the behavior, as testosterone implants in this locus will reinstate behavior in castrated animals as with systemic hormone (Wood, 1998). The mechanism may involve local aromatization of testosterone, and some effects of testosterone can be mimicked by estradiol (Hull *et al*., 2002).

According to one model (Hull & Dominguez, 2006), the probability of a copulatory response to a receptive female is influenced by dopamine in the medial preoptic area (MPOA). Extracellular dopamine levels are increased by nitric oxide, whose levels are determined by neuronal nitric oxide synthase (nNOS). Calcium influx sufficient to activate the enzyme occurs following activation of NMDA receptors by glutamate from amygdalar inputs conveying information about sexual stimuli. The gating effect of testosterone is achieved via regulation of the abundance of nNOS.

Comparisons between castrated controls and hormone-exposed, sexually active animals have generally confirmed that nNOS protein in the POA is more abundant in the latter (Du & Hull, 1999; Scordalakes *et al*., 2002; Putnam *et al*., 2005; Sanderson *et al*., 2006). However, the only study to examine the effect of androgen on preoptic *nNOS* mRNA found the opposite effect (Singh *et al*., 2000), although that study's utility in elucidating the role of nNOS in hormonal gating has been questioned (Putnam *et al*., 2005).

The present study examined the time course of up-regulation of nNOS in comparison with behavioral reinstatement following testosterone administration. First, we studied whether the expected increase in protein abundance was paralleled by mRNA abundance and whether the up-regulation was transcriptional or translational in nature. Secondly, if mRNA up-regulation was observed, would its temporal pattern reveal information about the mechanisms involved: abundance of nNOS might slowly increase with testosterone exposure, consistent with direct transcriptional induction; there might be no effect initially, and then an increase in abundance at the same time as behavioral reinstatement was observed, consistent with indirect induction, perhaps by steroid-induced transcription factors; and nNOS abundance might reach its ceiling significantly earlier than behavioral reinstatement, which would be inconsistent with the hypothesis that nNOS abundance is the limiting factor in controlling behavior. Thirdly, delineation of an early time point at which testosterone has an effect at the molecular level is a vital step in enabling future studies of the molecular mechanisms of androgen gating.

Subjects were whiptail lizards (*Cnemidophorus inornatus*), in which hormonal gating of sexual behavior has been extensively studied (Crews, 2005), and in which nitric oxide has been implicated

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in the control of androgen-dependent male-typical copulatory behavior (Sanderson *et al*., 2005). Because transcriptional response to testosterone was expected to differ by region, analysis was restricted to a rostral periventricular preoptic (PvPOA) population of nitrergic cells (approximately the same location as the rat anteroventral periventricular nucleus or AVPv), previously implicated in androgen gating of copulatory behavior in the whiptail lizard (Sanderson *et al*., 2006).

Materials and methods

Animals, hormone treatment and experimental design

All behavioral experiments and tissue collection were completed within the breeding season of the species. Adult *Cnemidophorus inornatus* were captured near Sanderson, Texas, transported to the University of Texas Austin campus, and individually housed with *ad libitum* access to water and food as described by Lindzey & Crews (1986). After 2 weeks to acclimatize to the laboratory, males were castrated as described by Wade & Crews (1991). Seven weeks later they were given 10-min tests with receptive females on three consecutive days to verify the absence of courtship behavior, and then implanted subcutaneously under hypothermic anesthesia with silicone tubing (length 12 mm, ID 1.46 mm, OD 1.97 mm; Helix Medical, Carpinteria, CA, USA) packed with crystalline testosterone for 10 mm of its length and sealed with silicone adhesive for 1 mm at each end, or with empty implants. Such implants have been shown to result in physiologically relevant levels of testosterone in male *C. inornatus* and to induce male-typical copulatory behavior (Lindzey & Crews, 1986). Controls were implanted with empty capsules. One, 3, 9 or 18 days later, animals were tested with receptive females for reinstatement of copulatory behavior and then immediately killed by decapitation. The 1-day time-point was chosen as the earliest at which testosterone levels have been reported to reach their ceiling after Silastic implantation (Ketterson *et al*., 1991). Eighteen days was chosen as the last time-point on the grounds that most individuals in this species express copulatory behavior after this length of testosterone exposure, and the 3- and 9-day time-points were chosen to enable the time-course analysis described in the Introduction. Sexual experience was thus equivalent among all groups, other than the test immediately before the animal was killed. Brains were then sectioned as described below in four series to enable independent assays of *nNOS* mRNA and protein expression in adjacent sections from the same animals. Group sizes are thus the same for all endpoints, except where a data point was lost. For all time points, the control group was smaller than the hormone-treated group, in order to maximize the statistical power available for elucidating the time-course of hormone action, while minimizing the number of animals used. It was assumed that no biologically important difference would be seen in nNOS expression among control groups that differ only in the length of time for which they have been exposed to no exogenous hormone. All procedures were approved by the University of Texas IACUC in accordance with NIH guidelines.

Sectioning and microdissection

Brains were rapidly dissected from skulls, frozen in tissue freezing medium on dry ice and stored at −80 °C. Brains were sectioned at 20 μm in four series, of which three were thaw-mounted onto Superfrost plus slides (Fisher) held at −20 °C in the cryostat during the sectioning operation and then transferred to −80 °C for storage, and one was similarly mounted onto membrane-covered slides for immediate laser microdissection (P.A.L.M. Microlaser Technologies AG, Germany), without storage. Before use, these membrane-coated slides were prepared according to the supplier's instructions, including 2 h of baking at 180 °C to inactivate RNAses, 30 min UV exposure under a PCR hood, poly-L-lysine treatment and air drying. Slides for *in situ* hybridization or NADPHd were stored at −80 °C for several weeks before use.

Behavioral observations

Animals were tested in their home tanks with stimulus females rendered receptive by injection of 0.5 μg of estradiol on two sequential nights before the test. Tests followed a standard paradigm of 10 min of interaction with the female during which latencies to approach and mount behaviors were recorded if they occurred (Lindzey & Crews, 1986).

Cloning of whiptail nNOS

A 900-bp fragment of the nNOS gene was cloned from whole-brain lizard cDNA by a degenerate PCR strategy using primers based on available human, teleost, and *Xenopus* sequences. A radioactive subclone of this fragment prepared by PCR in the presence of 32-P labeled dCTP (Sambrook & Russell, 2001) was used to probe the cDNA library prepared from pooled cDNA from whole brains of *Cnemidophorus* lizards of both sexes by a commercial company (Lambda ZAP II, Stratagene, La Jolla, CA, USA). Plaque-lifts were performed according to the library manufacturer's instructions, and probing was achieved using the method described by Sambrook & Russell (2001), yielding a 2155-bp fragment. This fragment was sequenced on both strands beginning with primers matching the M13 sites present in the cloning vector, and then using internal primers designed from the resulting sequence. An additional 892 bases corresponding to the 5′ end of the coding region and 5′ untranslated region was cloned by 5′ RACE (Clontech, Palo Alto, CA, USA; cat. no. 634914) according to the manufacturer's instructions. The additional fragment was sequenced on both strands using M13 primers, and the entire sequence submitted to GenBank (accession number DQ141603).

Laser microdissection and RNA isolation

Sections on membrane-covered slides were taken directly from the cryostat, fixed for 5 min in 70% ethanol at −20 °C, dipped in 20% ethanol, stained with 0.5% toluidine blue for 2 min, rapidly dehydrated in an ascending ethanol series and air-dried in a laminar flow hood. Sections were then visualized on a microdissection microscope (P.A.L.M. Microlaser Technologies AG), and an area corresponding to the rostral periventricular preoptic area excised and captured with the laser (see Fig. 1C, and Supplementary material, Fig. S1). This target area was generally found on two or three sequential sections in each brain, the most rostral being approximately at the rostral extremity of the third ventricle, and the most caudal being slightly rostral to the anterior commissure, where the dorso-ventral height of the ventricle exceeds 500 μm. Once capture was complete, 30 μL of TRIZOL (Invitrogen, Carlsbad, CA, USA) was added to the caps in which the fragments were captured, incubated for 5 min, and then spun down into the tube, after which total RNA was isolated according to the manufacturer's instructions.

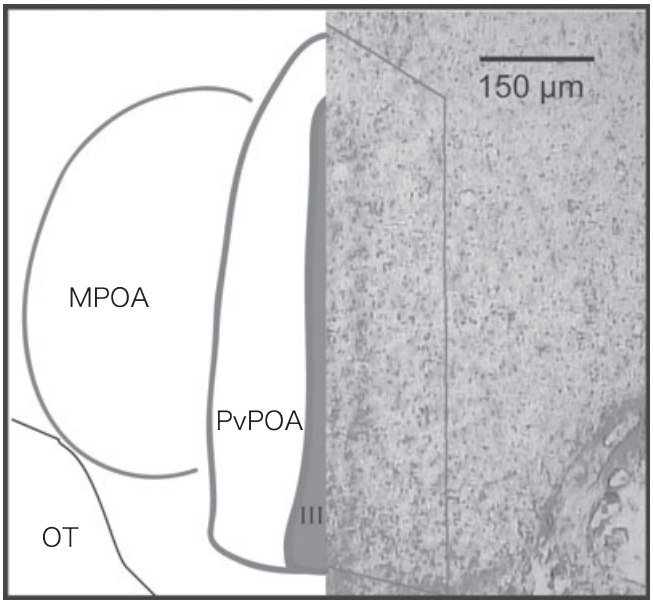

FIG. 1. Regions of interest in the whiptail brain at the level of the preoptic area. Schematic view of medial preoptic area (MPOA), periventricular preoptic area (PvPOA) is depicted on left. Toluidine-blue-stained section prior to laser capture microdissection is shown on right. The third ventricle (III) is faintly delineated by darker staining cells, and the optic tract (OT) can be made out in the bottom right corner. The area outlined in red including the periventricular preoptic area (PvPOA) is the fragment excised by the laser, and RNA from tissue contained within the outline was extracted and reverse transcribed for measurements of nNOS and 18S transcript levels (see Fig. 2B). Numbers of cells marked by *in situ* hybridization or NAPDH diaphorase histochemistry counted within a similar region are shown in Figs 3 and 4, respectively.

Reverse transcription and quantitative PCR

Each RNA sample was treated with DNAse I (Ambion, Austin, TX, USA) according to the manufacturer's instructions, with the addition of 20 units of RNAse inhibitor (RNAse OUT, Invitrogen) to degrade genomic DNA contamination. The resulting RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen) in a 40-μL reaction using a mixture of oligo dT primers and primers specific for *nNOS* and 18S RNA. These gene-specific reverse transcription primers were designed to be 100–200 bp 5′ to the primer used in quantitative PCR on the non-coding strand. Positive controls used 1 ng of whole-brain RNA in place of RNA derived from laser microdissected samples, and in negative controls the reverse transcriptase was omitted. Samples were incubated at 50 °C for 60 min, the reaction was terminated by incubation at 70 °C for 12 min, and excess primers and salts were removed by dilution and re-concentration in Microcon YM30 columns (Millipore, Bedford, MA, USA).

For each laser microdissected sample, 18S RNA abundance and *nNOS* abundance were each measured in triplicate in a 384-well plate in an ABI PRISM 7900HT real-time PCR cycler (ABI SDS 2.2.1 software), using SYBR Green I dye (Invitrogen) to reveal the amount of product present after each cycle of amplification. Amplification primers for quantitative PCR were designed to span fragments of approximately 115 bases of the target cDNA. Primers for 18S were designed from the *Cnemidophorus* 18S rRNA (GenBank AY217941, spanning bases 1173–1287), and for *nNOS* from the cloned sequence described above (spanning bases 1924–2036). An arbitrary threshold SYBR signal was established within the range corresponding to geometric amplification for each gene, and the cycle threshold (number of cycles of amplification required for the SYBR signal to exceed this threshold) recorded for each sample (i.e. each well of the plate). Standard curves (amount of template plotted against cycle threshold) were constructed using known dilutions of cDNA, and amplification efficiency, i.e. $10^{(-1/\text{slope of the standard curve})}$, calculated for the two primer pairs. For each individual, median values from the 18S and *nNOS* triplicates were used to calculate the relative transcript abundance of *nNOS* using the modified delta CT method that allows for correction of differential gene PCR efficiencies, following Shoemaker *et al*. (2007). This method uses the formula:

$$\text{Normalized expression} = (E_{\text{ref}}{}^{\wedge}Ct_{\text{ref}})/(E_{\text{target}}{}^{\wedge}Ct_{\text{target}}),$$

where E = gene-specific PCR efficiency, Ct = cycle threshold from each independent sample, target = gene of interest (i.e. nNOS), and ref = constitutively expressed reference gene (i.e. 18S). For two individuals, the reference gene signal was less than the smallest value of the standard curve, and these individuals were excluded from further analysis. After amplification, qPCR products were sequenced on both strands to confirm the identity of the target. Sequences of primers used for reverse transcription and for amplification during qPCR are given in supplementary Appendix S1.

Northern hybridization

A 641-bp fragment of the *nNOS* sequence described above (bases 1638–2278) was subcloned into pCR II-TOPO, and a digoxygenin-labeled probe prepared by *in vitro* transcription using Ambion's Megascript kit. Its specificity was checked by Northern hybridization. Whole brains were rapidly dissected from two intact male *C. inornatus* after decapitation under hypothermic anesthesia and stored in RNAlater (Ambion) at 4 °C overnight, and then frozen at −80 °C. Subsequently, brains were homogenized in TRIZOL and RNA extracted according to the manufacturer's instructions. Five micrograms of total RNA was electrophoresed through a formaldehyde agarose gel along with 2 μg of Millenium Marker (Ambion) for size information, and then electroblotted onto positively charged nylon membrane. After cross-linking with UV light, the size standard lane was cut off and visualized with methylene blue, while the membrane including the brain RNA lane was prehybridized with Ultrahyb (Ambion) and then probed with approximately 10 ng/mL of *nNOS* probe. After hybridizing at 68 °C overnight and washing, the probe was visualized with the CDPstar chemiluminescent system (Roche Applied Science, Indianapolis, IN, USA).

In situ *hybridization*

Slides were taken from −80 °C, fixed in ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS), treated with 0.25% acetic anhydride in 0.1 M triethanolamine, dehydrated in an ascending ethanol series and air-dried. Slides were incubated overnight at 62 °C overlaid with 100 μL of hybridization buffer containing 1.5×10^6 c.p.m./min of a 35-S labeled riboprobe prepared by reverse transcription using the same 641-bp template as above. For control slides, antisense probe was replaced with 2×10^6 c.p.m./min of sense probe. Post hybridization, the slides were washed in descending concentrations of buffer, dehydrated in an ascending ethanol series and air-dried overnight. After imaging in a phosphorimager to confirm signal, slides were dipped in Kodak NTB emulsion, dried at 55 °C, and then maintained in darkness at 4 °C. After 4 weeks, emulsion was developed according to Kodak's recommendations, and sections were

counterstained with toluidine blue before dehydration, clearing in xylene and coverslipping with Permount.

NADPH diaphorase histochemistry

Slides were taken from −80 °C, air-dried briefly, and fixed for 20 min in ice-cold 0.1 M phosphate buffer, pH 7.4 (PB) containing 4% paraformaldehyde and 1% glutaraldehyde. After rinsing well in PB, slides were incubated for 2 h at 37 °C in PB containing 0.3% triton-X, 0.1 mg/mL nitro blue tetrazolium, and 0.25 mg/mL β-NADPH. They were then rinsed, dehydrated, cleared with xylene and coverslipped with Permount.

Cell counting and statistical analysis

Cells labeled by NADPH diaphorase staining or by *in situ* hybridization were counted with similar procedures using the Fractionator routine of the Stereo Investigator software package (Microbrightfield, Williston, VT, USA). A region of interest (shown in Fig. 1A) was defined under low power, and then, under higher magnification, positive cells were counted that fell within 75-μm-square counting frames. Counting frames are placed systematically by the computer every 80 μm (i.e. with 5 μm between each counting frame) within the region, after a randomly chosen start-site. Cells expressing *nNOS* mRNA were clearly marked by dense clusters of silver grains after *in situ* hybridization, and were counted using a 40× objective. Cells stained by NADPH diaphorase histochemistry were less distinct, and were counted using a 60× immersion objective. Slides were coded, and processed by a blinded observer.

Differences between the proportions of individuals displaying male-typical copulatory behavior in testosterone-treated and control groups were subjected to Fisher's exact test. As only a minority of animals expressed any copulatory behavior, particularly at the earlier time points, no attempt was made to analyse the precise latencies to various behavioral endpoints from animals that did display them. Data from all three quantitative assays were analysed using two-way analysis of variance (ANOVA) with time between implantation and death (TIME), and implant type (testosterone or blank: HORMONE) as independent variables. Where justified by the omnibus ANOVA, contrasts were examined with Dunnett's test. These *posthoc* analyses reflected the rationale, described above in the experimental design, that control groups were equivalent across time points, and control groups were therefore pooled. To test the validity of this assumption, the simple effect of TIME was tested across the blank-implanted groups and was confirmed to be non-significant.

Results

Behavioural reinstatement and nNOS induction have similar time-courses

None of the blank-implanted individuals displayed any male-typical copulatory behavior except one individual on day 3 (mount latency 40 s). Among the testosterone-implanted individuals, the number displaying mounting behavior increased with testosterone exposure (Fig. 2A). Only for day 18 was the difference in behavior significantly different between testosterone-treated and control groups (Fisher's exact test, two-sided probability, $P < 0.048$), although the absence of a significant difference at the day 9 time point is quite conceivably due to the small size of the control group. Copulatory responses of those animals that did mount were typical for this species (median mount

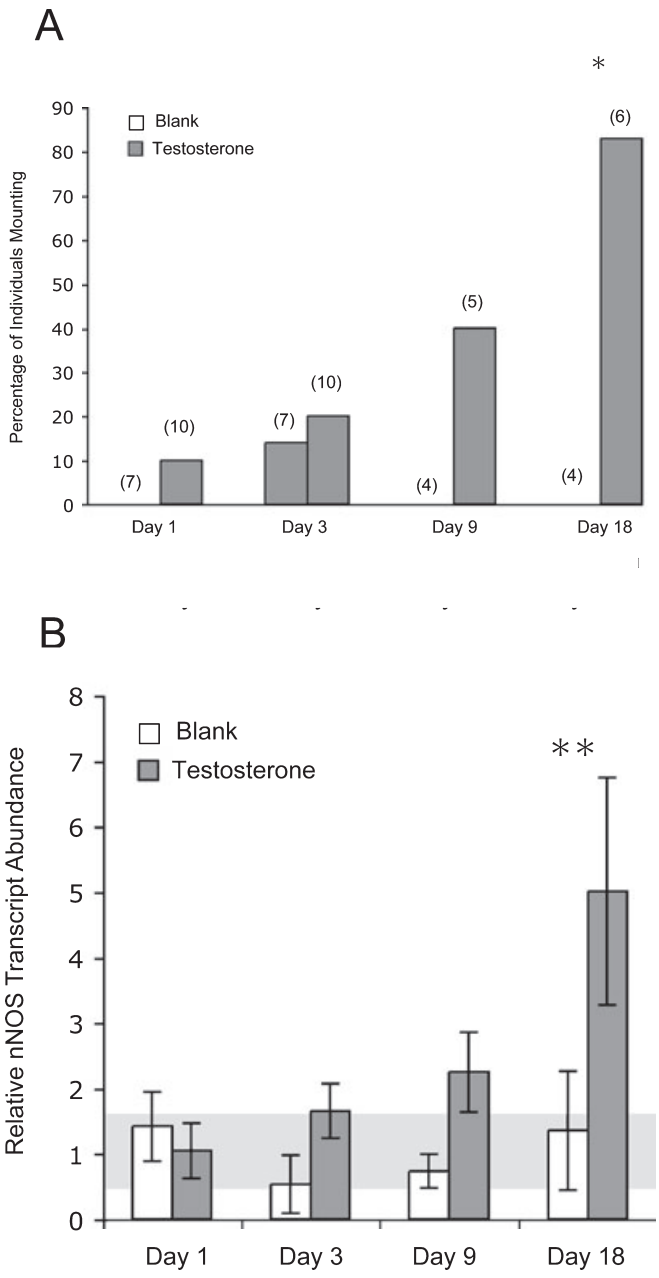

FIG. 2. Relationship between reinstatement of behavior and nNOS expression in castrated male whiptails after castration and hormone replacement. (A) Percentage of individuals exhibiting male-typical copulatory behavior after various lengths of time following implantation of testosterone-filled (grey bars) or blank (empty bars) Silastic capsules. Numbers in parentheses above bars are group sizes. Asterisk indicates that the proportion of individuals displaying behavior is greater for the testosterone-implanted than control group at the same time point ($P < 0.05$). (B) Relative *nNOS* transcript abundance in laser microdissected fragments of periventricular preoptic tissue of the same animals as in A. Raw measures of nNOS transcript abundance are normalized relative to 18S ribosomal RNA abundance and calibrated to the mean value of the blank-implanted controls to yield fold-change over baseline (so that the average individual among all controls would have a value of 1). Error bars show SE, and the 95% confidence interval around the global mean of the blank-treated animals is shown as a light grey band. Asterisks indicate significant difference between testosterone-implanted animals at that time-point and the pooled control group (**$P < 0.0001$).

latency, 244 s; range, 2–282 s) Mount latencies for the 11 individuals that mounted were not obviously different between time points, although numbers were insufficient, particularly at the early time

points, to test this hypothesis rigorously, and both the longest and the shortest latencies were displayed by day 18 in testosterone-treated individuals.

Measurements of *nNOS* mRNA abundance in laser microdissected fragments of PvPOA tissue are illustrated in Fig. 2B. The apparent trend was a steady increase in *nNOS* levels over time in testosterone-implanted animals, and no increase among blank-implanted animals. Two-way analysis of variance revealed significant main effects of HORMONE, with testosterone animals higher than controls ($F_{1,43} = 7.19$, $P = 0.01$), while the effects of TIME and the interaction between HORMONE and TIME were marginal ($F_{3,43} = 3.76$, $P = 0.05$; and $F_{3,43} = 2.26$, $P = 0.09$, respectively). Each of the testosterone-implanted groups was compared with the pooled blank-implanted group using Dunnett's test for multiple comparisons, revealing that only at day 18 was *nNOS* expression significantly higher than in controls ($P < 0.0001$).

Up-regulation of nNOS expression is observed in the periventricular cell population after 3 days of testosterone exposure

The probe derived from the whiptail *nNOS* clone recognized a single band at around 12 kb, a similar size to the dominant human *nNOS* transcript found in brain and other tissues (Park *et al*., 2000). Supplementary Fig. S2 shows the binding pattern in the whole brain RNA lane, together with the size information derived from the Millenium Marker. After *in situ* hybridization using this probe, the distribution of *nNOS*-expressing cells covered in silver grains matched well the previously reported distribution of NADPH-diaphorase-positive cells in androgen-exposed female whiptails, and other lizards (Sanderson *et al*., 2006, and references therein). Dense clusters of grains were visible even under low magnification in the striatum, the dorsal ventricular ridge and the amygdaloid complex in all subjects regardless of treatment, while the septum and the cortex were almost devoid of signal. In the PvPOA, *nNOS* expression was dramatically induced by three or more days of testosterone exposure (Fig. 3). Expression of *nNOS* in this cell population was indistinguishable from blank-implanted animals after 1 day of testosterone exposure (Fig. 3A), but after 18 days was dramatically up-regulated (Fig. 3B). Cell counts are shown in Fig. 3C. Subjecting these counts to two-way ANOVA revealed a main effect of HORMONE ($F_{1,42} = 20.04$, $P < 0.0001$). Neither the effect of TIME nor the interaction was significant.

NADPH diaphorase histochemistry yielded a distribution of stained cells similar to that identified by *in situ* hybridization, and to that previously described for NADPH diaphorase using perfusion-fixed tissue (Sanderson *et al*., 2006). In testosterone-exposed animals, lightly stained cell bodies were visible under high magnification in the periventricular preoptic area, mostly from 20 to 100 μm from the ventricle wall (Fig. 4B). In animals deprived of testosterone or exposed for only 1 day, these cells are scarcely detectable (Fig. 4A). Numbers of these cells are displayed by group in Fig. 4C. The pattern of up-regulation is similar to that revealed by *in situ* hybridization: expression increases with time in testosterone-exposed animals, but

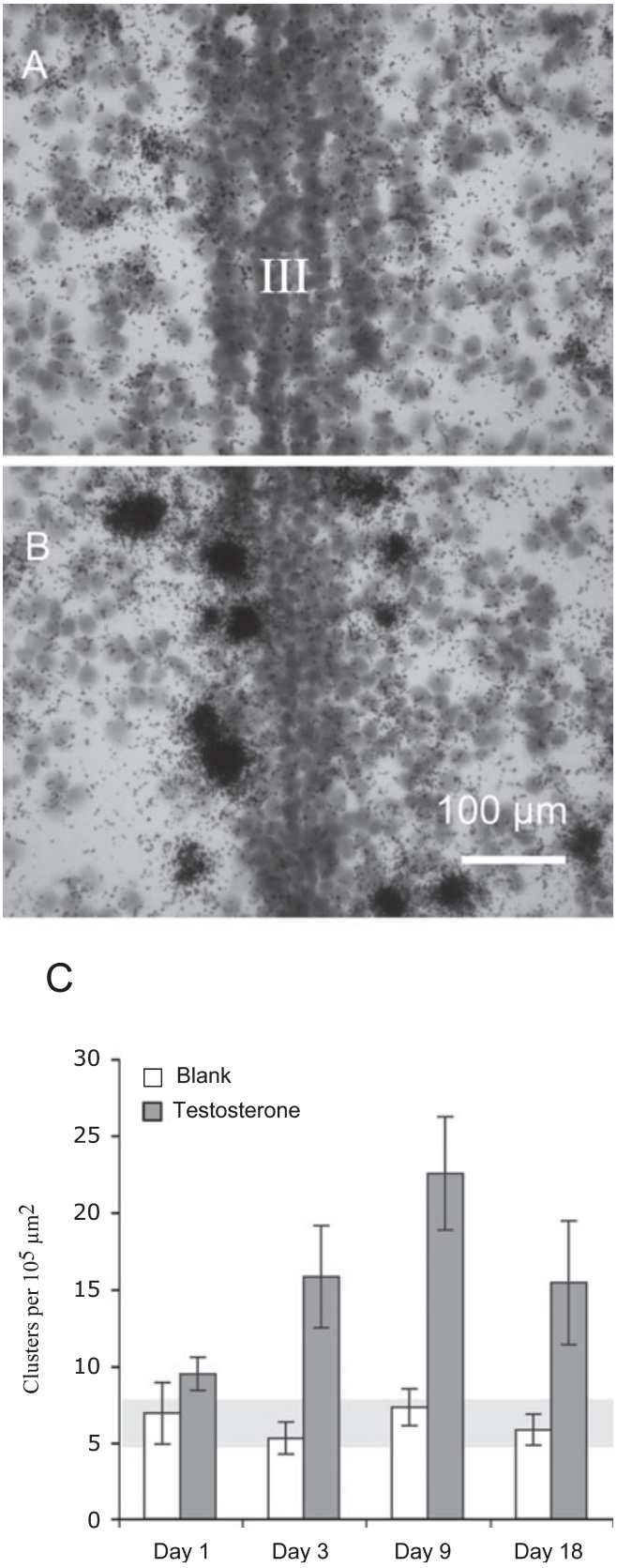

FIG. 3. Expression of *nNOS* in the periventricular preoptic area (PvPOA), as revealed by *in situ* hybridization. (A) Coronal section through the rostral preoptic area of an animal exposed to testosterone for one day, showing area adjacent to the third ventricle (III). very little signal is observed other than scattered silver grains. (B) Similar micrograph from an animal exposed to testosterone for 18 days. A population of cells close to the third ventricle shows dramatically increased *nNOS* expression. (C) Histogram showing numbers of *nNOS*-expressing cells inferred from silver grain clusters in the PvPOA. For comparison, the 95% confidence interval around the global mean of the blank-treated animals is shown as a light grey band.

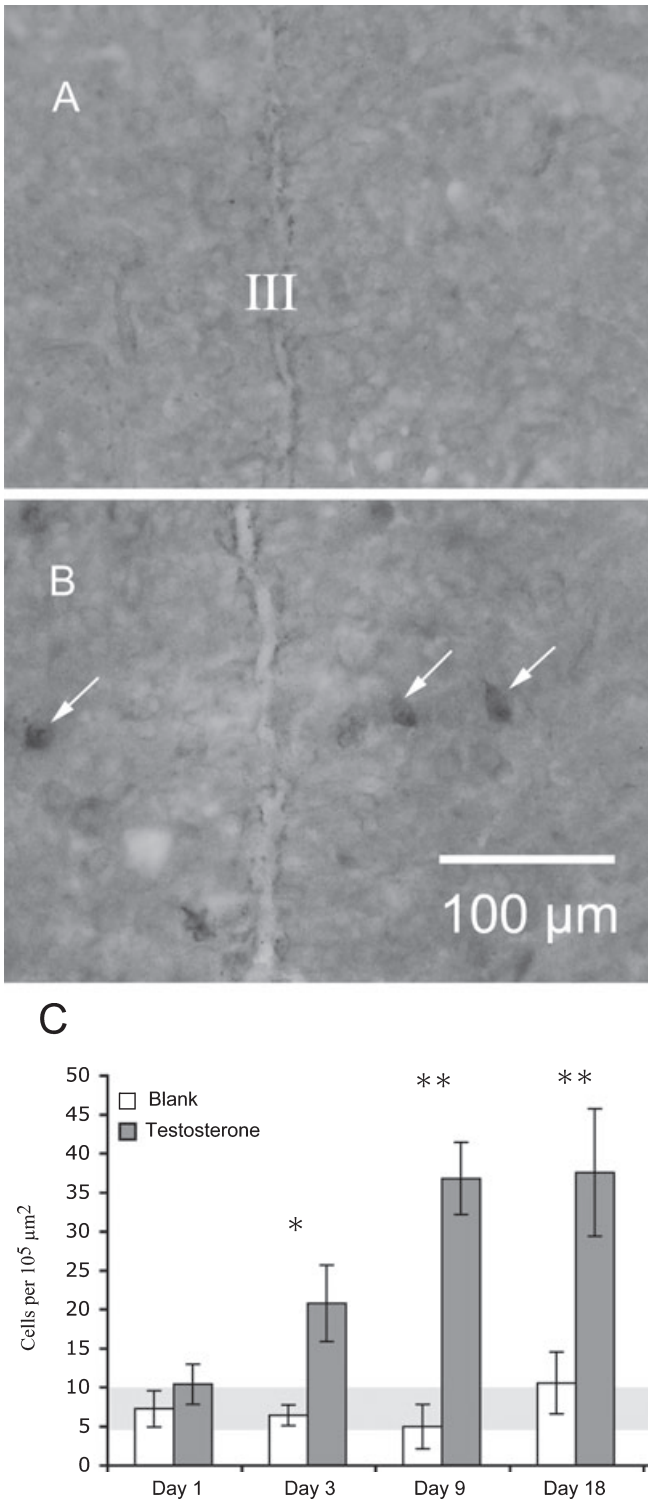

FIG. 4. Expression of nitric oxide synthase as inferred from NADPH diaphorase histochemistry (NADPHd). (A) Photomicrograph of area adjacent to third ventricle of a 1-day testosterone-exposed animal showing few NADPHd-positive cells. (B) Similar micrograph from an animal after 18 days of testosterone exposure, showing NADPHd-positive cells (white arrows). (C) Histogram of numbers of NADPHd-positive cells in the periventricular preoptic areas of animals implanted with testosterone-filled (grey bars) or blank (empty bars) Silastic capsules for various lengths of time before death. Asterisks indicate significant difference between testosterone-implanted animals at that time-point and the pooled control group (*$P < 0.01$, **$P < 0.0001$). For comparison, the 95% confidence interval around the global mean of the blank treated animals is shown as a light grey band.

not in controls. Two-way ANOVA revealed significant effects of HORMONE ($F_{1,42} = 30.51$, $P < 0.0001$) and TIME ($F_{3,42} = 4.24$, $P = 0.01$), and a significant interaction between the two ($F_{3,42} = 3.73$, $P = 0.02$). Each of the testosterone-implanted groups was compared with the pooled blank-implanted group using Dunnett's test for multiple comparisons, revealing that from day 3 onwards, more NADPHd-positive cells were observed in testosterone-treated groups (day 3, $P = 0.006$; day 9 and day 18, $P < 0.0001$).

Discussion

The results presented suggest unequivocally that nNOS in the whiptail preoptic area is up-regulated by testosterone, at the mRNA as well as protein level, with a time-course consistent with a role in gating male-typical copulatory behavior.

Comparison of the time-courses of the various endpoints is also informative. Both endpoints that relied on counting numbers of cells were increased over controls within 3 days of testosterone exposure, and by 9 days had reached their maximum values. By contrast, levels of total nNOS mRNA, as measured by quantitative PCR, continued to increase robustly until the 18-day time point, when the highest level of copulatory behavior was also observed. A simple interpretation of these results is that a defined population of cells responds to testosterone stimulation by expressing nNOS. After 9 days of exposure, the majority of these cells are expressing at a high enough level to become visible to the staining techniques used, and therefore countable. Thereafter, although the number of labeled, countable cells does not change, expression in each cell as revealed by qPCR continues to increase. As the proportion of animals expressing male-typical copulatory behavior also continues to increase until 18 days, it is plausible that the higher level nNOS expression is behaviorally relevant. However, with reference to the possible patterns of up-regulation considered in the Introduction, the increased expression that was observed after 3 days by the two cell-labeling techniques is consistent with direct transcriptional control of nNOS expression by ligand-bound steroid hormone receptor.

This interpretation is consistent with the results of Sato *et al*. (2005), who found that 10 days of testosterone exposure had no effect on the numbers of nNOS-immunoreactive cells in the MPOA of castrated male rats, but did increase the optical density per cell. A similar effect was reported by Scordalakes *et al*. (2002), who examined the expression of nNOS in gonadectomized mice with various steroid receptor-knockout genotypes, exposed to testosterone for several weeks. Wild-type mice and estrogen receptor-knockouts had similar numbers of nNOS-immunoreactive cells in the MPOA, but the area of immunoreactive tissue was greater in the wild-types, suggesting that testosterone (acting after aromatization, via the estrogen receptor) increases nNOS expression in each cell, but does not change the number of expressing cells.

The results of the present study are the opposite of those reported by Singh *et al*. (2000), with respect to the effect of androgens on *nNOS* expression. This previous study, the only one to date to examine the effect of androgens on preoptic nNOS mRNA, found that castration increased nNOS expression as measured by enzymatic activity, nNOS immunoreactivity or a semiquantitative PCR blotting measure of mRNA abundance, and that this effect was attenuated by dihydrotestosterone administration. There are several important differences between that study and the present one, in addition to the obvious difference in species. The various technical differences are probably not the reason for the discrepancy, as each study supports its conclusion on the basis of several independent techniques. Perhaps the

most important difference for future consideration is the anatomical location from which tissue was taken in the two studies: inasmuch as rostro-caudal positions can be compared between the rat and lizard preoptic areas, the tissue sample analysed by Singh *et al*. (2000) was more caudal than the area examined in the present study, which is entirely rostral to the anterior commissure. The target area of the present study was chosen on the basis of a previous study suggesting that the expression of male-typical copulatory behavior in testosterone-exposed female whiptails was associated with nitric oxide synthesis in this location (Sanderson *et al*., 2006).

Although evidence concerning the androgenic regulation of *nNOS* transcription in the POA is limited, the phenomenon has been examined in other tissues. Several studies have established that androgens increase *nNOS* mRNA in the penis (e.g. Park *et al*., 1999). Sato *et al*. (2004) reported that in mice with genetic ablation of the androgen receptor (AR), the expression of *nNOS* mRNA was reduced in a tissue sample described as 'hypothalamus'. By contrast, Scordalakes *et al*. (2002) found no difference in preoptic nNOS immunoreactivity between wild-type mice and mice with a loss of function mutation in the AR, a difference that is presumably due to the different anatomical locations from which the samples were derived.

The relationship between anatomical location and hormonal control of nNOS expression is a complex one. The area of the brain labeled 'preoptic' appears to include various populations of cells in which nNOS expression is suppressed by androgen (Singh *et al*., 2000), increased by androgen (Scordalakes *et al*., 2002, and references therein; present study), suppressed by ovarian hormones associated with the luteinizing hormone surge (Ishihara *et al*., 2002) and increased by ovarian hormones associated with the luteinizing hormone surge (Lamar *et al*., 1999). The possibility that response to hormones might differ by location was examined explicitly by Ishihara *et al*. (2002), who reported that the effect of ovarian hormones on *nNOS* expression was detectable in sections 60 or 120 μm caudal to the organum vasculosum of the lamina terminalis (OVLT), but not at the level of the OVLT itself, or in sections 180 μm caudal to the OVLT. These observations are perhaps not surprising in view of the numerous neural and behavioral processes in which preoptic nitric oxide synthesis has been implicated. These include the control of gonadotropin secretion (McCann *et al*., 2003), sleep (Stenberg, 2007) and maternal behaviors including pup retrieval (Service & Woodside, 2007) and aggression (Gammie & Nelson, 2000), in addition to the control of sexual behavior already discussed.

The possible existence of multiple, independently regulated, nitrergic systems within the POA would imply that traditionally dissected tissue samples, such as those used by Singh *et al*. (2000) and Lamar *et al*. (1999), may include nNOS-expressing cells from more than one population, potentially with disparate responses to steroids. In the whiptail, the population of nitrergic cells putatively involved in hormonal gating of male copulatory behavior is limited to a small periventricular area with a rostro-caudal extent of only a few hundred microns (Sanderson *et al*., 2006). Methods based on cell counting, such as *in situ* hybridization or immunohistochemistry, enable the precise localization of cells being assayed and may be more appropriate for the anatomically heterogeneous hypothalamus/preoptic area. By contrast, quantitative differences that are hard to measure precisely with these techniques may also be important. For example, the difference between the two-fold up-regulation of *nNOS* revealed by qPCR after 9 days of testosterone exposure in this study, and the five fold up-regulation seen after 18 days of exposure appears to be important behaviorally, as the latter was associated with a significantly higher proportion of individuals copulating. In this and similar situations requiring the combination of anatomical precision and reliable quantitative measurements over a wide range of expression levels, the combination of laser microdissection and quantitative PCR promises to be a valuable tool.

In summary, we conclude that in this species, nNOS expression is up-regulated by testosterone exposure, with a time-course consistent with direct transcriptional up-regulation by ligand-bound steroid hormone receptor. The effect of testosterone on nNOS is observed by some measures after as little as 3 days of testosterone exposure, making this a useful time point at which to look for the earliest effects of hormone at the molecular level in future studies.

Supplementary material

The following supplementary material may be found on http://www.blackwell-synergy.com

Fig. S1. A coronal section through the whiptail brain at level of the rostral preoptic area, as seen through the laser microdissection microscope.

Fig. S2. Northern hybridization using the whiptail nNOS probe that was used in *in situ* hybridization to probe a membrane on which eletrophoretically separated whole whiptail brain RNA has been electroblotted.

Appendix S1. Primers for Reverse Transcription and Quantitative PCR for nNOS and 18S rRNA.

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Abbreviations

MPOA, medial preoptic area; nNOS, neuronal nitric oxide synthase; POA, preoptic area; PvPOA, periventricular preoptic area.

References

Crews, D. (2005) Evolution of neuroendocrine mechanisms that regulate sexual behavior. *Trends Endocrinol. Metab.*, **16**, 354–361.

Du, J. & Hull, E.M. (1999) Effects of testosterone on neuronal nitric oxide synthase and tyrosine hydroxylase. *Brain Res.*, **836**, 90–98.

Gammie, S.C. & Nelson, R.J. (2000) Maternal and mating-induced aggression is associated with elevated citrulline immunoreactivity in the paraventricular nucleus in prairie voles. *J. Comp. Neurol.*, **418**, 182–192.

Hull, E.M. & Dominguez, J.M. (2006) Getting his act together, roles of glutamate, nitric oxide, and dopamine in the medial preoptic area. *Brain Res.*, **1126**, 66–75.

Hull, E.M., Meisel, R.L. & Sachs, B.D. (2002) Male sexual behavior. In: Pfaff, D.W., Arnold, A.P., Etgen, A.M., Fahrbach, S.E. & Rubin, R.T. (Eds), *Hormones, Brain and Behavior*, Vol. 1. Academic Press, San Diego, pp. 3–137.

Ishihara, T., Orikasa, C., Araki, T. & Sakuma, Y. (2002) Sex difference in the expression and regulation of nitric oxide synthase gene in the rat preoptic area. *Neurosci. Res.*, **43**, 147–154.

Ketterson, E.D., Nolan, V. Jr, Wolf, L., Ziegenfus, C., Dufty, A.M. Jr, Ball, G.F. & Johnsen, T.S. (1991) Testosterone and avian life histories: the effect of experimentally elevated testosterone on corticosterone and body mass in dark-eyed juncos. *Horm. Behav.*, **25**, 489–503.

Lamar, C.A., Bhat, G.K., Mahesh, V.B. & Brann, D.W. (1999) Evidence that neuronal nitric oxide synthase but not heme oxygenase increases in the hypothalamus on proestrus afternoon. *Neuroendocrinology*, **70**, 360–367.

Lindzey, J. & Crews, D. (1986) Hormonal control of courtship and copulatory behavior in male *Cnemidophorus inornatus*, a direct sexual ancestor of a unisexual, parthenogenetic lizard. *Gen. Comp. Endocrinol.*, **64**, 411–418.

McCann, S.M., Haens, G., Mastronardi, C., Walczewska, A., Karanth, S., Rettori, V. & Yu, W.H. (2003) The role of nitric oxide (NO) in control of LHRH release that mediates gonadotropin release and sexual behavior. *Curr. Pharm. Des.*, **9**, 381–390.

Park, K.H., Kim, S.W., Kim, K.D. & Paick, J.S. (1999) Effects of androgens on the expression of nitric oxide synthase mRNAs in rat corpus cavernosum. *BJU Int.*, **83**, 327–333.

Park, C.S., Krishna, G., Ahn, M.S., Kang, J.H., Chung, W.G., Kim, D.J., Hwang, H.K., Lee, J.N., Paik, S.G. & Cha, Y.N. (2000) Differential and constitutive expression of neuronal, inducible, and endothelial nitric oxide synthase mRNAs and proteins in pathologically normal human tissues. *Nitric Oxide*, **4**, 459–471.

Putnam, S.K., Sato, S., Riolo, J.V. & Hull, E.M. (2005) Effects of testosterone metabolites on copulation, medial preoptic dopamine, and NOS-immunoreactivity in castrated male rats. *Horm. Behav.*, **47**, 513–522.

Sambrook, J. & Russell, D.W. (2001) *Molecular Cloning: a Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanderson, N.S., Le, B.D. & Crews, D. (2006) Testosterone induction of male-typical sexual behavior is associated with increased preoptic NADPH diaphorase and citrulline production in female whiptail lizards. *J. Neurobiol.*, **66**, 1156–1163.

Sanderson, N.S., Weissler, E. & Crews, D. (2005) The nitric oxide synthase inhibitor L-NAME suppresses androgen-induced male-like pseudocopulatory behavior in whiptail lizards. *Brain Res.*, **1052**, 236–239.

Sato, S., Braham, C.S., Putnam, S.K. & Hull, E.M. (2005) Neuronal nitric oxide synthase and gonadal steroid interaction in the MPOA of male rats: co-localization and testosterone-induced restoration of copulation and nNOS-immunoreactivity. *Brain Res.*, **1043**, 205–213.

Sato, T., Matsumoto, T., Kawano, H., Watanabe, T., Uematsu, Y., Sekine, K., Fukuda, T., Aihara, K., Krust, A., Yamada, T., Nakamichi, Y., Yamamoto, Y., Nakamura, T., Yoshimura, K., Yoshizawa, T., Metzger, D., Chambon, P. & Kato, S. (2004) Brain masculinization requires androgen receptor function. *Proc. Natl Acad. Sci. USA*, **101**, 1673–1678.

Scordalakes, E.M., Shetty, S.J., Rissman. E.F. (2002) Roles of estrogen receptor alpha and androgen receptor in the regulation of neuronal nitric oxide synthase. *J. Comp. Neurol.*, **453**, 336–344.

Service, G. & Woodside, B. (2007) Inhibition of nitric oxide synthase within the medial preoptic area impairs pup retrieval in lactating rats. *Behav. Neurosci.*, **121**, 140–147.

Shoemaker, C.M., Queen, J. & Crews, D. (2007) Response of candidate sex-determining genes to changes in temperature reveals their involvement in the molecular network underlying temperature-dependent sex determination. *Mol. Endocrinol.*, **21**, 2750–2763.

Singh, R., Pervin, S., Shryne, J., Gorski, R. & Chaudhuri, G. (2000) Castration increases and androgens decrease nitric oxide synthase activity in the brain, physiologic implications. *Proc. Natl Acad. Sci. USA*, **97**, 3672–3677.

Stenberg, D. (2007) Neuroanatomy and neurochemistry of sleep. *Cell Mol. Life Sci.*, **64**, 1187–1204.

Wade, J. & Crews. D. (1991) The effects of intracranial implantation of estrogen on receptivity in sexually and asexually reproducing female whiptail lizards, *Cnemidophorus inornatus* and *Cnemidophorus uniparens*. *Horm. Behav.*, **25**, 342–253.

Wood, R.I. (1998) Integration of chemosensory and hormonal input in the male Syrian hamster brain. *Ann. NY Acad. Sci.*, **855**, 362–372.