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

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Abstract

Neuronal nitric oxide synthase (nNOS) in the medial preoptic area (MPOA) has been implicated in various physiological functions, including male rat copulation. Based on their apparent sensitivity to gonadal steroid manipulation, we hypothesized that nNOS cells contain steroid receptors, and the testosterone-induced restoration of nNOS-immunoreactivity in castrates should accompany the restoration of copulation. In Experiment 1, we investigated co-localization of nNOS with the androgen receptor (AR) and the estrogen receptor alpha (ER\textsubscript{a}) using immunocytochemistry. We found regionally specific co-localizations of nNOS-AR and nNOS-ER\textsubscript{a}. In Experiment 2, we investigated the relationship between MPOA nNOS-immunoreactivity (ir) and copulatory measures in the testosterone-induced restoration paradigm in castrates. The restoration of various copulatory measures was accompanied by an increase in optical density of nNOS-ir, but not in the number of nNOS-ir cells. These data provide additional evidence supporting the role of MPOA nitric oxide in male rat copulation.

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1. Introduction

The medial preoptic area (MPOA) is an important structure for male copulatory behaviors in mammals and other species (reviewed in [10]). The importance of dopaminergic stimulation in the MPOA during male copulation is well documented (see [11]). One of the mediators of the dopamine (DA) release is nitric oxide (NO), a gaseous molecule produced from L-arginine by the enzyme nitric oxide synthase (NOS). NO regulates the release of various transmitters [17], including DA [31]. We have previously demonstrated that NO is involved in regulation of basal [13], as well as female-stimulated [14] DA levels in the MPOA. An NOS inhibitor in the MPOA interfered with copulation in both sexually naive and experienced animals [12,24]. Furthermore, we have demonstrated that NO mediated glutamate-stimulated DA release in the MPOA [4].

Our recent studies suggest that neuronal NOS (nNOS) might mediate at least some of the effects of gonadal steroids on male copulation. In the MPOA of long-term castrates, nNOS-immunoreactivity (ir) and extracellular levels of DA were reduced, while intracellular levels of DA were increased [5,7,21,22]. Therefore, gonadal steroids may maintain normal nNOS activity in the MPOA, and consequently normal DAergic stimulation. This effect of gonadal steroids is relatively slow. We have detected changes in the number of nNOS-ir cells at

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1 month after castration, but not at 2 weeks after castration [5].

If gonadal steroids directly modulate nNOS in the MPOA, nNOS cells should contain steroid receptors. The co-localization of nNOS and steroid receptors in the MPOA has been examined in hamsters [8] and mice [25], but not in rats. Furthermore, we have examined nNOS-ir only in a relatively small area around the medial preoptic nucleus (MPN) in the past [5,22]. Therefore, in Experiment 1, we examined co-localization of nNOS-ir and androgen receptor-ir (AR-ir) or estrogen receptor α-ir (ERα-ir) throughout the MPOA.

In our previous study using a T-restoration paradigm, we found that the T-induced restoration of copulatory behaviors was accompanied by the restoration of DA release in response to estrous females, as well as during copulation [20]. Given the regulation of nNOS by gonadal steroids, and facilitation of DA release by NO, we hypothesized that there is a change in nNOS during T-restoration of copulation. Thus, in Experiment 2, we examined the changes in nNOS-ir in the MPOA using the identical T-restoration paradigm.

2. Materials and methods

2.1. Animals

Adult male Long–Evans Blue–Spruce rats (250–300 g), purchased from Harlan (Indianapolis, IN), were individually housed in a temperature- and humidity-controlled colony room. Food and water were available ad libitum. The colony room was maintained on a 14:10 reverse light:dark cycle with lights off at 11:00 h.

All animals were screened for copulatory ability starting 1 week after arrival, using ovarioctomized and hormone-primed females [10 µg estradiol benzoate (Sigma-Aldrich, St. Louis, MO; sc, in olive oil vehicle), 48 h and 4 h, respectively, prior to testing]. Only the animals that achieved at least 3 ejaculations during a maximum of 30 min testing sessions were included in these studies. In Experiment 1, 8 gonadally intact animals were used for AR-ir/NOS-ir and for ERα-ir/NOS-ir stainings. In Experiment 2, 17 animals were used. All procedures were in accordance with the NIH Guidelines for the Use of Animals and approved by the local Institutional Animal Care and Use Committee (IACUC).

2.2. Perfusion and sectioning

The animals weighed 300–350 g at the time of perfusion. Animals were deeply anesthetized with sodium pentobarbital (50 mg/ml) and were perfused through the ascending aorta, with the descending aorta clamped, with 100–150 ml pre-wash [0.1 M sodium phosphate buffered saline (PBS), pH 7.4, with 0.9% NaCl] followed by 40–60 ml 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB).

After perfusion, brains were quickly removed, postfixed for 4 h in the same fixative, and cryoprotected in 20% sucrose in PBS for 24 h. Forty-micrometer coronal sections were cut on a cryostat and stored in PBS containing 0.02% sodium azide at 4 °C until further processing.

2.3. Primary antibodies

For nNOS staining, a rabbit polyclonal antibody against the C-terminal of human nNOS from DiaSorin (1:8,000, Stillwater, MN) was used, as in our previous studies [5,22]. For AR and ERα staining, rabbit polyclonal antibodies against the N-terminal of human AR (PG-21, 1 µg/ml) or against the C-terminal of rat ERα (C-1355, 1:15,000), both from Upstate Biotechnology (Lake Placid, NY), were used. PG-21 and C-1355 were chosen for their ability to recognize ligand-bound forms of the respective receptors in the tissue from gonadally intact animals [15,16,18]. Sections were incubated in anti-nNOS antibody for 36–48 h and in anti-AR or in anti-ERα antibody for 60–72 h, all at 4 °C.

2.4. Immunocytochemistry (ICC)

Briefly, sections were rinsed 3 × 5 min between steps in PBS with 0.3% Triton X-100. Sections were incubated in 0.3% H2O2 for 30 min then in 4% normal goat serum (Vector Laboratories, Burlingame, CA) in PBS for 30 min at room temperature (RT) prior to incubation with primary antibody. After the incubation in primary antibody, sections were incubated in biotinylated anti-rabbit secondary antibody raised in goat (1:200,Vector Laboratories, Burlingame, CA) for 2 h at RT followed by incubation in avidin–biotin complex solution (Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA), prepared according to the manufacturer’s instruction, for 1 h at RT. After a rinse in Tris buffered saline (TBS, pH 7.6) for 5 min, staining was visualized with 0.05% 3’,3’-diaminobenzidine (DAB) and 0.01% H2O2 in TBS to produce light brown staining. For AR and ERα staining, 0.04% NiCl was added to produce black staining. Following the DAB reaction, sections were quickly rinsed in 3 changes of TBS, mounted on gelatin-coated slides, air dried, dehydrated in ascending concentrations of alcohol, cleared with Hemo-De, and coverslipped with Permount. Due to the predominant distribution of nNOS throughout a cell, nNOS staining produced light brown cytoplasmic staining, while AR and ERα staining produced black nuclear staining. For double staining, AR or ERα staining was performed first followed by nNOS staining.

3. Experimental design and data analysis

3.1. Experiment 1

Sexually experienced, gonadally intact males were euthanized and brains processed for ICC for nNOS/AR.
(n = 4) or nNOS/ERα (n = 4). Every fourth section was stained for double labeling (nNOS and AR or ERα), nNOS alone, AR or ERα alone, or as control. An Olympus microscope, equipped with a CoolSnap digital camera and ImagePro Plus software (MediaCybernetics, Silver Spring, MD), was used to map the distribution of nNOS-ir cells, with and without AR-ir or ERα-ir, in the MPOA. Six 0.4 × 0.6 mm photomicrographs (3 per side) were taken for each brain section. There were 4 to 5 sections per animal that contained the MPOA. Neuronal NOS cells were manually tagged according to their single- (light brown cytoplasmic staining without black nuclear staining) or double-staining status (light brown cytoplasmic staining with black nuclear staining). The distributions of single- and double-stained cells were mapped on the best-matching sections from the rat brain atlas by Swanson [29]. In addition, photomicrographs containing each subnucleus (0.4 × 0.6 mm for MPN, 0.2 × 0.3 mm for other nuclei) were used as counting fields to quantify the number of nNOS-ir cells, as well as degrees of co-localization (Fig. 1a).

3.2. Experiment 2

The experimental design used for Experiment 2 was identical to one that we used previously [20]. Briefly, animals were castrated following initial screening. After 3 weeks, they were randomly assigned to one of four testosterone propionate (TP, 500 µg/day, sc, in olive oil vehicle, Sigma-Aldrich, St. Louis, MO) treatment conditions: no TP (0 day), 2-day TP treatment (2 days), 5-day TP treatment (5 days), and 10-day TP treatment (10 days). The TP injections were given 1 h before the end of light cycle. The day after the last injection, each animal was moved to a dark testing room with red lights and tested for copulatory behaviors for 30 min in his home cage. The behaviors were recorded by an experimenter blind to the treatment condition. The testing began 1 to 2 h from the start of the dark cycle. Following the copulation testing, the animals were left in the testing room for 1 h and perfused.

3.2.1. Behavioral analyses

The following behavioral measures were used: latency to the first mount (ML), latency to the first intromission (IL), latency from the first intromission to the first ejaculation (EL), mount frequency during the first ejaculatory series (MF1), intromission frequency during the first ejaculatory series (IF1), total mount frequency during the 30 min test (MFT), total intromission frequency during the 30 min test (IFT), total ejaculation frequency during the 30 min test (EF), and post-ejaculatory interval (PEI, the latency between the ejaculation and the first intromission of the next ejaculatory series). Since many animals did not exhibit mounts, intromissions, and/or ejaculations, the proportions of animals exhibiting each behavior were analyzed with 2-way Chi-square tests. Only the animals exhibiting the behavior were included in the subsequent analysis of respective latencies. In addition, the animals that failed to ejaculate were excluded from the analyses of MF1, IF1, and PEI. One-way between-subjects ANOVAs, followed by Student–Newman–Keuls multiple comparisons, were used for more detailed analysis of behaviors.

3.2.2. ICC data analysis

In Experiment 2, every fourth section was used for nNOS staining or control. Four 0.4 × 0.6 mm photomicrographs
from 2 representative brain sections containing the MPOA per animal (8 micrographs total per animal) were used for nNOS-ir cell counts and measurements of optical density (Fig. 1b). The photomicrographs contained the area where we previously found significant changes in nNOS-ir with hormonal manipulation [5,22]. Optical density was measured on 8-bit gray scale images taken under the constant lighting condition using ImagePro Plus and expressed in proportion to no light (1: image taken with shutter closed) and unstained part of the same section (0: part of striatum with no visible staining). Care was taken to avoid the optic chiasm (OC), the third ventricle (3V), and the anterior commissure (AC). Cell counting and optical density measurement were conducted by an experimenter blind to the treatment condition.

Based on the results of the Experiment 1, we initially analyzed the data based on a 2 × 2 (anterior–posterior × dorsal–ventral) ANOVA. However, the preliminary analyses of nNOS-ir cell count and optical density revealed no regional differences on these measures. Therefore, the data were pooled and the average nNOS-ir cell count and optical density per photomicrograph were used for statistical analysis. nNOS-ir cell count was analyzed with a 1-way between-subjects ANCOVA, with nNOS-ir cell count as a covariate, of nNOS-ir cell count on optical density, 1-way between-animal ANCOVA, with nNOS-ir cell count as a covariate, of nNOS-ir cell count on optical density, and 1-way between-animal ANCOVA, with nNOS-ir cell count as a covariate, of nNOS-ir cell count on optical density, and the pattern of co-localization from rostral to caudal levels, as well as from ventromedial to dorsolateral areas (Figs. 2a and b). The rostral part, especially near the OVLT, contained many nNOS-ir/ERα-ir cells, and a few nNOS-ir/ERα-ir cells were observed in the caudal MePN, ventral to the AC. In contrast to the nNOS-ir/ERα-ir double staining, only a small number of AR-ir cells or nNOS-ir/AR-ir cells were found in the MePN, and those were only in the rostral part.

4.1.3. Anteroventral periventricular nucleus (AVPV)

This nucleus showed significant regional differences in the pattern of co-localization from rostral to caudal levels, as well as from ventromedial to dorsolateral areas (Figs. 2a and b). Some nNOS-ir cells were observed in the AVPV, mostly in the dorsolateral part. Some nNOS-ir/ERα-ir cells were found in rostral to intermediate levels of the AVPV, while a relatively small number of them were found in the caudal part. Even though many AR-ir cells were seen in the rostral part of AVPV, only a small number of them were co-localized with nNOS-ir. On the other hand, the proportion of co-localized cells increased toward the caudal part of the AVPV, and the caudal part of the AVPV contained numerous nNOS-ir/AR-ir cells. Both nNOS-ir/ERα-ir and nNOS-ir/AR-ir cells were mostly found in the dorsolateral part of the AVPV, close to the MePN.

4.1.4. Anteroventral preoptic nucleus (AVP)

The AVP and its vicinity contained many nNOS-ir cells, many of them relatively larger (20 μm) than those in other parts of the MPOA (Figs. 2a and b). This group of cells seems to be continuous with the nNOS-ir cells located in the MePN and dorsolateral part of the AVPV. Some of them contained ERα-ir, while almost none of them contained AR-ir.

4.1.5. Anterodorsal preoptic nucleus (ADP)

The ADP and surrounding area contained a small number of nNOS-ir cells and very few ERα-ir and AR-ir cells (Figs. 2a and b). There was a cluster of medium-sized nNOS-ir/AR-ir cells between the ADP and the AC, while none of them co-localized with ERα-ir.
4.1.6. Medial preoptic nucleus (MPN)

Similarly to the AVPV, the MPN showed striking regional variation in co-localization of nNOS-ir with ERα-ir or AR-ir. The rostral part of the MPN contained some small, lightly stained nNOS-ir cells, while only a few of them contained either ERα-ir or AR-ir (Fig. 2b). In the intermediate part of the MPN (Fig. 2c), the number of nNOS-ir, ERα-ir, and AR-ir cells was more numerous than in the rostral part. Many nNOS-ir/ERα-ir cells were observed, especially in the ventral and the lateral parts of the MPN, with more double-stained cells located just lateral to the MPN. On the contrary, AR-ir cells were located more medially, with many of them co-localized with nNOS-ir. Toward the caudal part of the MPN (Fig. 2d), many nNOS-ir cells were observed, while only a small number of them were co-localized with ERα-ir. The cluster of nNOS-ir/ERα-ir cells extended dorsally from the ventrolateral part of the MPOA to the posterodorsal preoptic nucleus (PdPN). Co-localization with AR-ir was more prevalent at this level, with most such cells located in the lateral part of the MPN and just lateral to the MPN.

4.1.7. Posterodorsal preoptic nucleus (PdPN)

Some medium-sized nNOS-ir cells were located just ventral to the AC, in and around the PdPN and the parastral nucleus (PS). Only a few of them were co-localized with ERα-ir, while almost no co-localization was observed with AR-ir in this region (Fig. 2d).
4.2. Experiment 2

Behavioral measures are presented in Table 2. The proportions of animals showing intromission \( \chi^2(3) = 8.66, \) \( P = 0.034 \) and ejaculation \( \chi^2(3) = 10.43, \) \( P = 0.015 \) were significantly different between treatment conditions, while those of animals mounting \( \chi^2(3) = 7.37, \) \( P = 0.061 \) showed only a trend. Most of the animals in the 2-, 5-, and 10-day groups exhibited mounts, intromissions, and ejaculations. In contrast, only half of 0-day animals mounted, one of them intromitted, and none ejaculated.

One-way ANOVAs revealed significant effects of treatment conditions on ML \( F(3,11) = 14.66, \) \( P < 0.001 \), IL \( F(3, 9) = 4.95, \) \( P = 0.03 \), EF \( F(3, 13) = 5.75, \) \( P = 0.01 \), and IFT \( F(3,13) = 10.95, \) \( P < 0.001 \). Post-hoc tests revealed that ML was longer for 0-day than other groups; EF was lower in the 0-day group than in the 5- and 10-day groups; and IFT was larger in the 5- and 10-day groups compared to the 0- and 2-day groups.

There were no differences in nNOS-ir cell counts \( F(3,13) = 0.69, \) ns, Fig. 4a. On the other hand, 1-way ANCOVA revealed a significant effect of TP treatment on nNOS-ir optical density \( F(3, 12) = 11.63, \) \( P < 0.001 \), Fig. 4b. Student–Newman–Keuls multiple comparisons revealed greater optical density in the 10-day group than in the 0-day group.

5. Discussion

5.1. Co-localization of nNOS with AR or ERα

The current study demonstrates that AR and ERα are co-localized with nNOS in several subnuclei within the MPOA. In general, the distribution of AR-ir and ERα-ir was similar to the previously reported AR and ERα mRNA expression [26], with the highest concentration of AR and ERα cells in AVPV and MPN. A notable difference from the distribution of mRNA is the PdPN, where only small numbers of AR or ERα cells were found in this study. The distribution of nNOS cells was also consistent with previous reports [5,30]. Bands of medium-sized nNOS cells extended ventrolaterally from the MePN to the AVP, as well as dorsolaterally below and above the AC. Small sized nNOS cells were seen mostly between the two bands.

Most nNOS/AR co-localization was restricted to the dorsolateral part of the AVPV, as well as in the MPN. The nNOS/AR cells formed a diagonal band from the AVPV through the MPOA toward the BNST. In the previous study on Syrian hamsters, co-localization of nNOS and AR was observed in the MPN [8]. The current study suggests a similar pattern of nNOS/AR cell distribution in...
On the other hand, most nNOS/ERα co-localization was seen in the area dorsolateral to the AVPV and the ventral half of the MPN and its vicinity. Only a small number of nNOS/ERα cells were found in the dorsal half of the MPOA. This distribution seems to correspond to the area in which loss of nNOS-ir was detected in castrated Syrian hamsters [8].

5.2. T-restoration of nNOS-ir and copulation

The pattern of the restoration of copulatory behaviors in Experiment 2 was similar to that seen in our previous study [20]. The animals in the current study exhibited slightly more robust behaviors than those in our previous study. Nonetheless, the post-castration loss of behaviors observed in these two experiments was well within the wide range of individual differences reported elsewhere [3]. In the current study, most animals did not show intromission or ejaculation at 3 weeks following castration. Intromission and ejaculation were gradually restored over the course of TP treatment. The current study failed to show significant differences between 5- and 10-day treatments, most likely due to the ceiling effect, as the loss of behaviors following castration was less complete than in our previous study.

We found a significant increase in nNOS-ir in optical density per cell, but not in nNOS-ir cell count. This indicates that each nNOS-ir cell contained more detectable nOS proteins, although it is not clear if this reflects increased nOS expression or changes in exposure of epitope due to alteration of subcellular localization or association with other proteins. We did not detect significant changes in MPOA nNOS protein levels in Western blots following 1 month castration (unpublished observation). The lack of significant difference in number of nNOS-ir cells following 10 days of T-treatment is not surprising, given that we previously found significant differences after 3 weeks [20] or 1 month [5] of hormonal manipulations, but not after 2 weeks [5]. Thus, nNOS-ir optical density might be a more sensitive measure than nNOS-ir cell count. The increase in nNOS-ir density was gradual, reaching significance after 10 days of TP treatment; similar to the time course of restoration of several behavioral measures. This supports our hypothesis that

### Table 2: Behavioral measures

<table>
<thead>
<tr>
<th></th>
<th>0 day</th>
<th>2 days</th>
<th>5 days</th>
<th>10 days</th>
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<tbody>
<tr>
<td>Mount</td>
<td>2/4</td>
<td>4/4</td>
<td>5/5</td>
<td>4/4</td>
</tr>
<tr>
<td>ML (s ± SEM)</td>
<td>1279.5 ± 247.5</td>
<td>n = 2</td>
<td>407.8 ± 170.9*</td>
<td>n = 4</td>
</tr>
<tr>
<td>M1 (± SEM)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MFT (± SEM)</td>
<td>5.5 ± 4.9</td>
<td>n = 4</td>
<td>18.3 ± 7.3</td>
<td>n = 4</td>
</tr>
<tr>
<td>Intromissiona</td>
<td>1/4</td>
<td>3/4</td>
<td>–</td>
<td>3/4</td>
</tr>
<tr>
<td>IL (sec ± SEM)</td>
<td>1018 –</td>
<td>n = 1</td>
<td>580.3 ± 251.9</td>
<td>n = 4</td>
</tr>
<tr>
<td>IF1 (± SEM)</td>
<td>–</td>
<td>–</td>
<td>11.0 ± 1.7</td>
<td>n = 3</td>
</tr>
<tr>
<td>IFT (± SEM)</td>
<td>2.8 ± 2.8</td>
<td>n = 2</td>
<td>9.3 ± 3.3</td>
<td>n = 4</td>
</tr>
<tr>
<td>Ejaculationb</td>
<td>0/4</td>
<td>3/4</td>
<td>3/4</td>
<td>3/4</td>
</tr>
<tr>
<td>EL (sec ± SEM)</td>
<td>–</td>
<td>–</td>
<td>1116.3 ± 257.5</td>
<td>n = 3</td>
</tr>
<tr>
<td>EF (± SEM)</td>
<td>0.0 –</td>
<td>–</td>
<td>0.8 ± 0.3</td>
<td>n = 4</td>
</tr>
<tr>
<td>PEI (± SEM)</td>
<td>–</td>
<td>–</td>
<td>830.7 ± 166.4</td>
<td>n = 3</td>
</tr>
</tbody>
</table>

* Significantly different from 0-day group (P < 0.05).

b Significant difference in proportions of animals showing the behavior (P < 0.05).

c Significantly different from the 2-day group (P < 0.05).
restoration of nNOS, and thereby restoration of DAergic stimulation, as we previously demonstrated, accompanies the restoration of behaviors.

We expected a regional difference in nNOS-ir change in Experiment 2 for two reasons. First, we observed some differences in nNOS/AR and nNOS/ER co-localization in the Experiment 1. Second, we have shown that nNOS-ir in the MPOA is dependent on E2, but not on DHT [22]. However, we did not find any regional difference (anterior–posterior/dorsal–ventral) in this study. It is possible that such difference was too small in this study to detect. Longer T-treatment (as in [5]) might have revealed such differences.

One possible confound in the Experiment 2 is the behavior-induced changes in nNOS-ir, since the animals were perfused only 1 h after the end of behavioral testing. However, it seems unlikely that the differences in nNOS-ir were caused by the varying degrees of activity during the behavioral testing, as we have observed T-induced changes in nNOS-ir in the absence of behavioral testing [5]. In addition, we detected a behavior-induced increase in nNOS-ir in sexually naive, but not in sexually experienced animals [6]. Given that the animals in this study were sexually experienced, a behavior-induced change is probably not a significant factor.

5.3. NO as a mediator of the effects of gonadal steroids on MPOA DA and copulation

The current study provides additional evidence supporting the role of nNOS in male copulation, a gonadal steroid-sensitive behavior. As previously mentioned, there is an increase in extracellular DA in the MPOA of male rats in the presence of an estrous female and during copulation [9,23]. We have demonstrated that NO facilitates basal, as well as female-stimulated DA release in the MPOA [13,14]. Furthermore, administration of a NOS inhibitor impaired copulation in both naïve and sexually experienced animals [12,24]. Thus, NO is an important facilitator of MPOA DA release, which in turn facilitates male copulation. Our recent studies have implicated nNOS as a mediator of gonadal steroids’ influence on MPOA DA and copulation. First, castration reduced nNOS-ir [5,22] and impaired DA release in the MPOA [7,21]. This castration-induced alteration in nNOS-ir was dependent primarily on E2, while DHT seems to slightly attenuate the effects of castration on DA levels [22]. In the current study, we documented the presence of ERα and AR in the MPOA nNOS cells, providing evidence for a mechanism for direct effects of gonadal steroids on nNOS cells. Furthermore, we have shown that the restoration of nNOS-ir accompanies the restoration of copulatory behaviors, even with much shorter hormonal manipulations than previously employed in studies of nNOS. Therefore, gonadal steroids may modulate copulation, at least in part, through its effects on nNOS.

Currently, it is not clear how gonadal steroids modulate nNOS in the MPOA. The discrepancy in the results of several studies may provide some clues. Although we and others have seen decreases in nNOS-ir with castration and increases in the presence of T or E2 [5,8,22,25], Singh et al. [27] saw an increase in nNOS-ir with castration, which was attenuated by T or DHT-treatment. They also reported similar results for nNOS protein levels, nNOS mRNA expression, and NOS activity levels, although the NOS activity assay was not specific to nNOS. One consistent methodological difference between the study by Singh et al. and others is the nNOS antibody used. Singh et al. used a monoclonal antibody against N-terminal, while others used a polyclonal antibody against C-terminal of the nNOS protein. Given that the N-terminal of nNOS protein is the PDZ domain [1], increased exposure of this site may indicate dissociation from cellular membranes or other conformational changes. We suspect that the effects of gonadal steroids on nNOS may be rather indirect, possibly through associated proteins or changes in trafficking, given the lack of change in nNOS protein levels we observed with castration. The data on extracellular DA levels, an indirect measure of NO activity, also point to a loss of NO production after castration [7,20–22].

It should be noted that our results clearly indicate that nNOS may mediate some, but not all of the effects of gonadal steroids on MPOA DA and copulation. The MPOA extracellular DA levels corresponded better with copulatory performance than did nNOS-ir in both the T-restoration paradigm (Experiment 2 of this study and [20]) and the T-metabolites maintenance paradigm [21,22]. This probably reflects in part the effects of gonadal steroids on structures involved in olfactory and somatosensory inputs to the MPOA (e.g. MeA, BNST, spinal cord, and penis).

NO in the MPOA affects more than DA levels and copulation. The NO/cGMP pathway is also involved in regulation of GnRH release [19]. Thus, it is possible that NO coordinates both motor (via DA release) and endocrine (via GnRH release) responses (see [2] for example) in interactions with receptive females. Furthermore, NO has been shown to inhibit aromatase [28], the very enzyme that synthesizes E2. Given the presence of ERα in nNOS cells and E2-sensitivity of nNOS [22], this may represent a local negative feedback loop regulating E2 production in the MPOA.

In summary, we found that nNOS cells in the MPOA contain gonadal steroid receptors, indicating direct regulation of these cells by gonadal steroids. In addition, the T-induced restoration of nNOS-ir in castrates accompanied the restoration of copulation, even with much shorter hormonal manipulation than we previously employed in studies of nNOS-ir. The data from current and other studies suggest that NO in the MPOA plays an integrative role in motor and endocrine outputs associated with male sexual function.
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