Roles of Estrogen Receptor α and Androgen Receptor in the Regulation of Neuronal Nitric Oxide Synthase

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ABSTRACT
In brain and peripheral tissues, steroid hormones regulate nitric oxide synthase (nNOS). We asked whether estrogen receptor-α (ERα) and/or androgen receptor (AR) regulated nNOS immunoreactivity in mouse brain. First, we quantified cells singly labeled for nNOS immunoreactivity or labeled dually with ERα-immunoreactive (-ir) or AR-ir cells in the nucleus accumbens (Acb), preoptic area (POA), bed nucleus of the stria terminalis (BNST), posterior dorsal and posterior ventral regions of the medial amygdala (MePD and MePV, respectively), and paraventricular nucleus (PVN). The POA and MePD contained the greatest number of double-labeled cells. More nNOS-ir cells were colabeled with ERα immunoreactivity compared with AR immunoreactivity. Next, by using a double mutant mouse in which males lacked functional ERα, AR, or both, we investigated the roles of these steroid receptors in nNOS-ir cell numbers and immunoreactive area staining under testosterone (T) and estradiol (E2) conditions. Our data show that functional ERα is correlated with more nNOS-ir cells under T conditions and more immunoreactive area staining in the POA under both T and E2 conditions. However, ERα decreases nNOS-ir cell number in the BNST under E2 treatment. In summary, the data suggest that AR has organizational actions on nNOS-ir cell numbers in the MePV, that interactions between ERα and AR genes occur in PVN, and that sex differences in nNOS-ir area staining are limited to the POA. Thus, we show that ERα and AR interact to regulate nNOS in male and female brain in a site-specific manner. J. Comp. Neurol. 453:336–344, 2002.

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Indexing terms: estrogen receptor β; sexual behavior; dopamine; knockout mouse; testicular feminization mutant

Neuronal nitric oxide synthase (nNOS), by means of the production of nitric oxide (NO), is important for many reproductive functions. Release of gonadotropin-releasing hormone (GnRH) is triggered by NO (McCann et al., 1999). Reproductive behavior is also modulated by nNOS. Competitive inhibitors of nNOS attenuate lordosis in female rats (Mani et al., 1994), reduce mount rates (Sato et al., 1998), and prevent ejaculations (Benelli et al., 1995) in male rats. In addition, disruption of the nNOS gene in knockout mice results in inappropriate sexual behavior in males (Nelson et al., 1995). Conversely, treatment of rats with L-arginine, a natural nNOS substrate, facilitates sexual behavior in males (Benelli et al., 1995; Sato et al., 1998), whereas treatment with a NO donor facilitates sexual behavior in females (Mani et al., 1994). Neuronal NOS is also involved in behaviors such as circadian activity, locomotor behavior, and aggression (Nelson et al., 1995; Demas et al., 1997; Kriegsfeld et al., 1997, 1999; Gammie and Nelson, 1999; Gammie et al., 2000).

Neuronal NOS is regulated by circulating steroid hormones (Hull et al., 1997). Testosterone (T) acts either through the androgen receptor (AR) or after aromatization to estradiol by means of estrogen receptors (ERα and ERβ). Castration in rats leads to an increase in nNOS activity and mRNA levels in the combined hypothalamus and preoptic (POA) areas. In addition, the number of immunoreactive cells in the hypothalamus but not in POA

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increased after castration, whereas T and dihydrotestosterone (DHT) treatment reversed these effects (Singh et al., 2000). In general, these data suggest that AR is responsible for the down-regulation of nNOS. Yet, in hamsters (Hadeishi and Wood, 1996) and rats (Du and Hull, 1999), castrated males have fewer nNOS-positive neurons in the POA compared with castrates treated with T. Estradiol increases nictinamide-adenosine-dinucleotide phosphate-diaphorase (NADPHd) staining, which is an indicator of NOS activity, in the POA of female guinea pigs (Warembourg et al., 1999), increases nNOS mRNA in the ventrolateral subdivision of the ventromedial nucleus in female rats (Ceccatelli et al., 1996; Rachman et al., 1998), and up-regulates nNOS protein expression in neutrophils of both sexes (Garcia-Duran et al., 1999). Although these data are not in complete agreement, they have been generated in different species and sexes and by using different experimental paradigms. Yet, all support the hypothesis that steroid hormones play a strong role in the regulation of nNOS.

Outside the brain, steroids regulate epithelial and inducible NOS (eNOS and iNOS, respectively). In cardiac (Nuedling et al., 1999) and skeletal tissue (Rubanyi et al., 1997), estrogen is responsible for the regulation of eNOS, and more specifically, ERα up-regulates eNOS in vascular tissue (Rubanyi et al., 1997). In addition, in myocytes (Nuedling et al., 1999) and in inflammatory responses (Cuzzocrea et al., 2001; Vegato et al., 2001), estrogen has been shown to regulate iNOS. We hypothesize that estrogen acts in the same manner, i.e., by means of ERα, in brain to regulate nNOS expression. This premise is well established in females where ovarioectomy results in decreased numbers of nNOS-immunoreactive (-ir) cells, whereas estradiol (E2) treatment increased nNOS-ir cells (Rachman et al., 1998; Ishihara et al., 2001). In males, a paradigm of castration and reimplantation of T (Du and Hull, 1999; Singh et al., 2000) has been used. Where these data establish a role for T, they cannot be used to distinguish between actions of T on AR vs. ERα (or ERβ).

To elucidate the role of steroid receptors on nNOS expression, we used a different experimental approach. We generated unique double knockout mice in which ERα, AR, or both, genes were dysfunctional. In the first experiment, we described the distribution of ERα and AR colocalized in nNOS-ir cells in brains of normal C57BL/6J male mice. In the second and third experiments, we quantified nNOS immunoreactivity in brains of littermates that were either wild-type (WT) males or females or males with the ERα gene disruption (ERαKO), an AR point mutation (The), or double mutants lacking both ERα and AR function (double knockout mice; DKO).

**MATERIALS AND METHODS**

**Animal care**

Mice were housed singly at weaning (18–20 days of age) in a temperature- (23 ± 1°C) and light-controlled (12:12; L:D) facility. At all times, animals received Purina mouse chow (#5001) and water ad libitum. All animal maintenance and procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

**Production of mice**

Females carrying the Tfm mutation were originally purchased from Jackson Laboratory in 1998 (C57BL/6J-\(A_{w}^{+/-}\).Ta +/+ Ar\(^{Tfm}\)). The Tfm mutation is a result of a spontaneous point mutation in the AR gene, the deletion of C at the 1112 position. Tfm mice by definition are males with the mutated AR gene on the X chromosome. Androgen receptor mutants have been described in rats, mice, and humans (Olsen, 1992). In all cases, if the mutation is complete and the AR gene is not functional, XY individuals appear externally as females with small abdominal testes (Olsen, 1992). We produced Tfm males by mating heterozygous females, which carry one functional copy of the AR gene located on an X chromosome, with normal C57BL/6J males.

For Experiments 2 and 3, Tfm carrier females were mated to males that were heterozygous for the ERα (+/-) gene. ERαKO (Lubahn et al., 1993) heterozygotic breeding pairs were originally provided in 1996 by Dr. Dennis Lubahn (University of Missouri). The mice had been backcrossed into the C57BL/6J strain for at least 10 generations. From that cross, we obtained offspring female that were both Tfm carriers and heterozygotic for the ERα gene disruption. These females were mated with heterozygotic ERαKO males. The cross produced offspring of the following genotypes: WT males (ERα +/-; AR+/+); and females (ERα +/-; AR+/+), ERαKO males (ERα-/-; AR+/-) and females, Tfm males (ERα +/-; AR-), Tfm/ERαKO (referred to as DKO) males (ERα-/-; AR-), ERα heterozygous males and females, Tfm carrier females, Tfm carrier/ERα heterozygous females, Tfm carrier/ERαKO females, and Tfm/ERα heterozygous males.

**Mouse genotyping**

For the Tfm screening, tail DNA was isolated, amplified, and then digested with a cutting enzyme. A set of primers were designed to amplify a section of the AR gene that included the mutation point and also contained three cutting sites for the restriction enzyme Mwo1, one of the cutting sites coincided with the site of point mutation. The primers used were: forward primer 5'-GAA CAT CTG AGT CCA GGG GA-3'; reverse primer 5'-GTT CTC CAG CGN NNGC. . .3'; 3'. . .CGN NNNNGC. . .5', cutting sites between bases 956-957, 957-965, and 1110-1111. On digestion of the amplified AR DNA with Mwo1, the resulting products were 215-bp band + 198-bp band in males (who have only one X chromosome; thus AR-). In females, the products were 215-bp band, 198-bp band, and 155-bp band (because they have one functional copy of the ERα gene). In Experiment 1, five adult, gonad-intact WT C57BL/6J males from the ERα breeding cross were used. Males were 75–90 days of age at the time of sacrifice. In Experiments 2 and 3, males were littermates produced in the cross previously described (Table 1). In Experiment 2, 7 WT males and WT females, and 6 Tfm, ERαKO, and DKO
males were used (total n = 32). In Experiment 3, 7 WT males, Tfm, ERKO, DKO males, and 6 WT females were used (total n = 34). Between 60 and 90 days of age, animals were gonadectomized by means of a midline incision under general anesthesia (100 mg/kg ketamine and 10 mg/kg xylazine injected i.p.). At the time of gonadectomy, animals received implants made from Dow Corning Silastic Laboratory Tubing (1.02 mm ID × 2.16 mm OD) packed with 5 mm of T (1:1 T:cholesterol; Experiment 2) or 5 mm of 17β-estradiol (E2; 1:1 E2:cholesterol; Experiment 3). These implants were inserted in the midsagittal region s.c. Animals were killed 4–6 weeks later.

Tissue preparation

Mice in Experiment 1 were deeply anesthetized with halothane and immediately decapitated. Brains were removed and immersed in 5% acrolein fixative in Tris-buffered saline (TBS; pH 7.4) for 10 minutes. After blocking, brains were placed in acrolein and gently agitated for a total of 4 hours, with one change to fresh acrolein after 2 hours, before cryoprotection in 30% sucrose. Mice in Experiments 2 and 3 were deeply anesthetized with a lethal dose (0.5 mg) of sodium pentobarbital. Animals were perfused with heparinized-saline solution (approximately 35 ml) followed by 2% acrolein (180–200 ml). After perfusions, brains were removed and post-fixed in 2% acrolein (20 ml) for 1 hour, then placed in 30% sucrose overnight. Cryoprotected brains were frozen in 2-methylbutane and stored at −70°C until sectioning. Tissue was cut in a coronal plane at 30 μm on a frozen cryostat. Sections were collected into a series of three vials for Experiments 1 and 3 and into a series of alternating sections in Experiment 2. Tissue was stored at −20°C in antifreeze until processing.

Immunocytochemistry

For Experiment 1, two of the three wells of tissue were used for immunocytochemistry with antiserum against either ERα or AR followed by incubation with nNOS antiserum 48 hours later. Sections were rinsed in TBS and treated with sodium borohydride (1% NaBH4 in cold TBS). Tissue was then incubated for 48 hours at 4°C in primary antibody, ERα (C1355, generously provided by Dr. M. Shupnik, 1:5,000), or AR (Affinity Bioreagents; catalog no. PA1-111A; 1:800), respectively, in TBS carrier solution containing 0.25% λ-carrageenan and 0.1% sodium azide, 0.5% bovine serum albumin (BSA), and 1.5% Triton X. After 48 hours, brain sections were rinsed in TBS and incubated in biotinylated goat anti-rabbit immunoglobulin G (1:500, Vector Labs, Burlingame, CA) for 45 minutes. Carrier solution for secondary antiserum was the same as described above, except no azide was used. Tissue was rinsed and incubated in avidin-biotin-peroxidase complex (ABC 1:1,000, Vector Elite Kit, Vector Labs) for 1 hour.

ABC carrier was the same as the secondary carrier with the exception of BSA. Immunoreactivity was visualized for ERα and AR by using nickel-intensified (3% of 0.05 M Ninhydrin (0.15% DAB) with 0.001% hydrogen peroxide as the chromogen. Next, tissue processed for ERα immunoreactivity and AR immunoreactivity was incubated in nNOS antiserum (1:1,000) after extensive rinsing. The secondary antibody concentration was 1:250, and staining was visualized by using 0.04% DAB (without nickel) with hydrogen peroxide. In Experiments 2 and 3, one well of brain tissue was processed for nNOS immunoreactivity. We used the primary antiserum at a concentration of 1:10,000 (DiaSorin; catalog no. 24287), all other steps were as described for Experiment 1. Within each experiment, all tissue was developed in DAB for the identical length of time. All antiserum had been validated previously for use in rodent (ERα, Moffatt et al., 1999; AR, Prins et al., 1992; nNOS, Du and Hull, 1999).

Image analysis and counting

Metamorph Image Analysis software was used to quantify immunoreactivity (Universal Imaging West Chester, PA). For all the experiments, we selected the regions for quantification based on the Franklin and Paxinos (1997) mouse brain atlas. In Experiment 1, best matched, unilateral sections containing the nucleus accumbens (Acb; Plate 25; analyses restricted to the ventral lateral portion of the Acb), preoptic area (POA; Plate 30; area included all portions of the medial preoptic nucleus and the entire periventricular nucleus of the hypothalamus), bed nucleus of the stria terminalis (BNST; Plate 32; area included all portions of the BNST except the medial ventral BNST), paraventricular nucleus (PVN; Plate 36), and postero dorsal section of the medial amygdala (MePD; Plate 45) were captured at low magnification (Franklin and Paxinos, 1997; Fig. 1). The unit of area for the quantification was defined by the boundaries of the superimposed area (312,352 μm²), as illustrated in Figure 1. Double- and single-labeled nNOS-IR cells were included in the counts only if a proximal fiber was visibly extending from the cell. We counted double-labeled cells in which the nickel-stained nucleus was completely surrounded by brown cytoplasmic staining at a higher magnification within the same region specified above. These cells were selected only if the cell body and nucleus were in the same focal plane. In Experiments 2 and 3, best-matched, unilateral sections in the POA, BNST, posteroventral portion of the medial amygdala (MePV), and PVN were captured at low magnification (Fig. 1). Manual counts were conducted for cell body staining, and automated measurements were taken for the area of immunoreactive staining in all regions except PVN, where prominent individual cells were present but few fibers were noted. Immunoreactive area staining includes both stained cells and fibers. The immunoreactive area was expressed as micrometers squared (μm²).

Statistics

In Experiment 1, data were analyzed by using one-way analysis of variance (ANOVA) to detect differences between and within brain regions with ERα/nNOS immunoreactivity and AR/nNOS immunoreactivity. Fisher’s least significant difference (LSD) post hoc comparisons were used for pair comparisons. In Experiments 2 and 3, data were analyzed by one- and two-way ANOVAs followed by Fisher’s LSD post
ERα and AR regulation of nNOS-IR

**RESULTS**

**Experiment 1**

Colocalization of either ERα immunoreactivity or AR immunoreactivity and nNOS immunoreactivity was greatest in the POA and MePD. In gonad-intact C57BL/6J males, the regions containing the greatest numbers of ERα-ir and nNOS-ir double-labeled cells were the POA and MePD. There was a significant difference in the number of double-labeled cells between brain areas (F(4,23) = 92.17; P < 0.000001; Table 2). The POA contained more ERα-ir/nNOS-ir double-labeled cells than all other regions (P < 0.05). In addition, MePD contained a significantly greater number of double-labeled cells compared with Acb, BNST, and PVN (P < 0.05) but significantly fewer than POA (P < 0.05).

The pattern of regional coexpression of AR immunoreactivity and nNOS immunoreactivity was similar to that seen for ERα immunoreactivity and nNOS immunoreactivity in these same animals. The number of AR-ir/nNOS-ir double-labeled cells varied by brain area (F(3,19) = 21.28; P < 0.000008; Table 2). The POA and MePD had significantly greater numbers of double AR-ir/nNOS-ir cells compared with BNST and PVN (P < 0.05).

In the two areas with the largest concentration of nNOS immunoreactivity, the POA and MePD, there were more ERα-ir cells, compared with AR-ir cells, double labeled with nNOS immunoreactivity (F(7,39) = 86.89; P < 0.000001). Because nNOS-ir cell numbers were the same in the POA, MePD, BNST, and PVN in sections labeled for ERα immunoreactivity vs. AR immunoreactivity, differences in numbers of double-labeled cells between brains were not a result of differences in numbers of single-labeled nNOS-ir cells. Both the POA and MePD (P < 0.05) had significantly greater numbers of ERα-ir/nNOS-ir-labeled cells than AR-ir/nNOS-ir-labeled cells (Fig. 2). There were no differences between the number of double-labeled ERα-ir/nNOS-ir cells vs. AR-ir/nNOS-ir cells in BNST or PVN.

**Experiment 2**

Functional ERα gene was associated with more nNOS-ir cells and increased immunoreactive area staining in the POA under T conditions. A one-way ANOVA revealed a group effect on nNOS-ir cell numbers in the POA (F(4,31) = 6.26; P < 0.001; Table 3). This effect was due to Tfm male brains having greater numbers of nNOS-ir cells compared with all other genotypes (P < 0.05). Next, a two-way ANOVA for the presence or absence

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**Note:** Table 2 is not included in this text due to the size of the image. However, the context suggests that it contains data related to the distribution of double-labeled cells and nNOS immunoreactivity across different brain regions, as indicated by the presence of religious numbers and statistical significance levels. The text summarizes these findings, emphasizing the patterns and differences observed in the distribution of ERα and AR immunoreactivity and nNOS immunoreactivity across various brain regions.
of ERα and AR was conducted with data from male mice only. This analysis revealed a significant effect of the ERα gene (F(1,24) = 8.97; P < 0.01; Fig. 3A) on nNOS-ir cell counts in the POA and a significant interaction between ERα and AR genes (F(1,24) = 6.26; P < 0.05). Males possessing functional copies of the ERα gene had significantly more nNOS-ir cell numbers in the POA. As noted in the one-way ANOVA, brains of male mice with functional ERα and the AR point mutation (Tfm) had more nNOS-ir cells than males of any other genotype (P < 0.05). There was no significant effect of the AR gene (F(1,24) = 2.38) alone. Finally, no significant group effect and no significant effect of the ERα and/or AR genes on nNOS-ir cell counts were noted in any other brain areas.

A similar analysis was conducted by using a one-way ANOVA on nNOS-ir area staining in the POA. A significant interaction between the presence of functional ERα and mutant AR, resulting in greater nNOS-immunoreactive cell numbers in Tfm brains compared with all other male groups, P < 0.05. b, Brains from males with functional ERα have significantly more nNOS-ir area staining compared with males with the ERα gene disruption, P < 0.01.

Fig. 2. Photomicrographs of male mouse preoptic area (A,B); medial amygdala posterior dorsal (C,D) and paraventricular nucleus (E,F). A,C,E represent brain areas double labeled for nitric oxide synthase (nNOS; light brown) and estrogen receptor α (black) immunoreactivity. B,D,F represent brain areas double labeled for nNOS (light brown) and androgen receptor (black) immunoreactivity. Single-labeled nNOS-immunoreactive (-ir) cells are marked by black arrowheads. Double-labeled steroid-receptor/nNOS-ir cells are marked by white arrowheads. These plates of double-labeled cells are a sub-area of the total area counted and were minimally processed by increasing (10%) the contrast between black and brown staining in Adobe Photoshop 4.0. This minimal processing was done to make the captured images appear like the images viewed in the microscope. Scale bar = 50 μm in B (applies to A–F).

Fig. 3. Mean (± SEM) nitric oxide synthase–immunoreactive (nNOS-ir) cell number in A and nNOS-ir area staining (μm²) in B. Data are from the preoptic area (POA) of castrated, testosterone-treated wild-type (ERα+/AR+), androgen receptor (AR) point mutation (ERα+/AR-), estrogen receptor α (ERα) gene disruption (ERα-AR-), and double knockout (ERα-/AR-) males. a, Significant interaction between the presence of functional ERα and mutant AR, resulting in greater nNOS-immunoreactive cell numbers in Tfm brains compared with all other male groups, P < 0.05. b, Brains from males with functional ERα have significantly more nNOS-ir area staining compared with males with the ERα gene disruption, P < 0.01.

TABLE 3. Mean (±SEM) Number of nNOS-ir Cells in Testosterone Treated Mouse Brains

<table>
<thead>
<tr>
<th>Brain Area</th>
<th>WT male mean ± SEM</th>
<th>WT female mean ± SEM</th>
<th>Tfm mean ± SEM</th>
<th>ERαKO mean ± SEM</th>
<th>DKO mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medial preoptic area</td>
<td>38.4 ± 6.5</td>
<td>24.6 ± 4.5</td>
<td>70.7 ± 12.5₂</td>
<td>34.5 ± 5.5</td>
<td>26.8 ± 5.7</td>
</tr>
<tr>
<td>Bed nucleus of the stria terminalis</td>
<td>20.2 ± 5.2</td>
<td>19.3 ± 3.3</td>
<td>18.5 ± 2.1</td>
<td>18.8 ± 3.5</td>
<td>18.8 ± 3.3</td>
</tr>
<tr>
<td>Medial amygdala posterior ventral</td>
<td>20.1 ± 5.2</td>
<td>20.1 ± 5.0</td>
<td>32.8 ± 10.5</td>
<td>36.0 ± 7.7</td>
<td>46.7 ± 8.2</td>
</tr>
<tr>
<td>Paraventricular nucleus</td>
<td>67.1 ± 3.3</td>
<td>70.3 ± 6.9</td>
<td>72.2 ± 9.3</td>
<td>71.6 ± 7.0</td>
<td>76.8 ± 4.6</td>
</tr>
</tbody>
</table>

₁nNOS, nitric oxide synthase-ir, immunoreactive; WT, wild-type; Tfm, testicular feminization mutation; ERαKO, estrogen receptor α knockout; DKO, double knockout.

₂Significantly different from all groups within the same brain area (P < 0.05).
significant effect of group was noted (F(4,31) = 20.85; P < 0.00001). Brains of WT and Tfm (mean ± SEM = 32,362 ± 2,792 and 35,431 ± 3,321, respectively) males had significantly more immunoreactive area staining compared with all other genotypes (P < 0.05). In addition, WT female brains (mean ± SEM = 25,890 ± 813) had significantly less nNOS-ir area staining compared with WT and Tfm males, but more area staining when compared with ERαKO and DKO males (mean ± SEM = 20,264 ± 1,331, and 17,569 ± 336, respectively; P < 0.05). Next, a two-way ANOVA conducted on male data only revealed a significant effect of the status of the ERα gene (F(1,24) = 67.21; P < 0.0000001; Figs 3B, 4). There was no significant effect on nNOS-ir area staining in the POA under E2 conditions. A one-way ANOVA revealed a group effect on nNOS-ir area staining in the POA (F(4,31) = 4.19; P < 0.01; the staining pattern is the same as T-treated male brains as shown in Fig. 4). As noted in Experiment 2, WT and Tfm males (mean ± SEM = 20,537 ± 1,981 and 23,054 ± 3,331, respectively) had more nNOS-ir area staining compared with ERαKO and DKO males (mean ± SEM = 14,318 ± 744 and 14,081 ± 519, respectively; P < 0.05). WT females (mean ± SEM = 16,267 ± 1,955) were again intermediate in immunoreactive area staining, exhibiting less area staining than WT and Tfm males and more area staining than ERαKO and DKO males. The only significant difference was found between females and Tfm males (P < 0.05). A two-way ANOVA restricted to data from males showed that functional ERα resulted in greater nNOS-ir area staining in the POA (F(1,26) = 14.43; P < 0.001). There was no effect of the AR gene (F(1,24) = 0.33) and no interaction between ERα and AR genes (F(1,26) = 0.47). No effect of the presence or absence of steroid receptor genes was noted in any other area.

**Experiment 3**

*Presence of ERα increased nNOS-ir area staining in the POA under E2 conditions.*

The presence or absence of functional ERα gene affected neuronal NOS in mice that received E2. A one-way ANOVA revealed a group effect on nNOS-ir area staining in the POA (F(4,31) = 2.49) in the POA. Neuronal NOS-ir area staining was not significantly different based on group and was not affected by ERα and/or AR gene interactions in any other area.

**DISCUSSION**

These results show that steroid receptors regulate NOS immunoreactivity in both cell bodies and fibers. Moreover, this regulation is site-specific and receptor-specific. Estrogen receptor-α and AR both play important roles in the regulation of NOS expression.

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**Table 4. Mean (±SEM) Number of nNOS-ir Cells in Estradiol-Treated Mouse Brains**

<table>
<thead>
<tr>
<th></th>
<th>WT male</th>
<th>WT female</th>
<th>Tfm</th>
<th>ERαKO</th>
<th>DKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medial preoptic area</td>
<td>186.8 ± 16.2</td>
<td>218.8 ± 21.6</td>
<td>172.9 ± 11.9</td>
<td>162.9 ± 15.2</td>
<td>186.1 ± 17.8</td>
</tr>
<tr>
<td>Bed nucleus of the stria terminalis</td>
<td>46.4 ± 6.0</td>
<td>46.1 ± 3.9</td>
<td>36.3 ± 0.7</td>
<td>62.1 ± 10.2</td>
<td>72.0 ± 11.2</td>
</tr>
<tr>
<td>Medial amygdala posterior ventral</td>
<td>163.0 ± 12.0</td>
<td>162.3 ± 16.3</td>
<td>127.9 ± 13.4</td>
<td>195.0 ± 27.5</td>
<td>137.0 ± 13.8</td>
</tr>
<tr>
<td>Paraventricular nucleus</td>
<td>162.9 ± 9.9</td>
<td>140.3 ± 20.8</td>
<td>123.4 ± 10.5</td>
<td>145.1 ± 15.0</td>
<td>169.9 ± 11.2</td>
</tr>
</tbody>
</table>

1nNOS, nitric oxide synthase; ir, immunoreactive; WT, wild-type; Tfm, testicular feminization mutation, ERαKO, estrogen receptor α knockout; DKO, double knockout.
2Significantly different from WT males (P < 0.05).
3Significantly different from WT males and females and Tfm males (P < 0.05).
roles in nNOS regulation, and interesting interactions between ERα and AR were revealed by the use of double mutant mice.

**Role of ERα in nNOS regulation**

The most striking effects of steroids on nNOS immunoreactivity were found in the POA. Regardless of hormone treatment, nNOS-ir area staining in POA was greater in animals possessing a functional ERα gene compared with ERαKO and DKO males. As shown in Figure 4, the majority of immunoreactive staining (including fibers and cell bodies) is present in the medial portion of the POA in WT and Tfm males, whereas the more lateral portion of the POA appears to have fewer fibers and less nNOS-ir cells. This pattern existed regardless of hormone treatment. This pattern of staining is not present in ERαKO and DKO males, which appear to have an even distribution of nNOS-ir cells but few fibers throughout both the lateral and medial portions of the POA. Thus, ERα could play a role in regulating both the amount of nNOS present as well as regulate the distribution of nNOS.

There are two possible explanations for this finding. One is that ERα acts within the POA to up-regulate production of nNOS and, thus, increases amounts present in fibers. An alternative explanation is that mice with functional ERα are receiving more nNOS-containing projections from other areas, a pattern that could have been established in adulthood or during neonatal development. It has been shown that, in neonatal tissue, testosterone can attract projections from the BNST to the POA (Ibanez et al., 2001), and this mechanism could be working by means of ERα. The finding that ERα regulates nNOS expression compliments our colocalization data that ERα-ir and nNOS-ir cells are present together in 90% of the nNOS-ir cells in the POA. Therefore, it is most likely that, in the POA, ERα is directly affecting nNOS immunoreactivity.

In addition, in the POA, our results reveal an interaction between AR and ERα. Neuronal NOS-ir cell numbers are greatest in mice that had a functional ERα gene but also possess the AR point mutation. In fact, in WT males, functional AR was sufficient to decrease nNOS-ir cells when ERα was present. This finding suggests that, when both AR and ERα are activated by administration of T, AR may down-regulate nNOS-ir cells in the POA. Moreover, in males treated with E2, we did not detect this effect of AR.

Unlike the POA, nNOS-ir in the BNST was negatively impacted by functional ERα protein under E2 conditions. We noted fewer nNOS-ir cells when ERα was functional. Yet, only 17% of nNOS-ir cells in this nucleus also contain ERα immunoreactivity. Thus, ERα may indirectly regulate nNOS expression in this area. The BNST receives projections from various brain areas, one of which is the medial amygdala (Wood and Swann, 2000). In the MePD, 53% of the nNOS-ir neurons are also colocalized with ERα immunoreactivity. Thus, ERα-containing neurons could be influencing nNOS-ir cells in the BNST by means of projections from the MePD.

**Role of AR in nNOS regulation**

Androgen receptor also has a critical role in regulating nNOS-ir cell number in different brain areas. After E2 treatment, mice with a functional AR had more nNOS-ir cells in the MePV. It is important to note that it is not likely that AR is being activated in brain by E2 treatment; thus, AR may exert its effects before castration. Organizational effects of AR have been described previously in the central nervous system (Goldstein and Sengelaub, 1992; Watson et al., 1998). Androgen receptor also interacted with ERα gene in PVN. In this region, having a functional ERα but lacking AR resulted in fewer nNOS-ir cells. Both ERα and AR immunoreactivity were colocal-
ERα and AR regulation of nNOS-IR

ized in the same low (10%) frequency within nNOS-IR cells in this area. These findings support the idea of site-specific steroid receptor regulation of nNOS expressing cells. In male rats, 50% of the nNOS-IR cells also contain AR-ir in POA (Sato and Hull, 2000). In male hamsters, AR-ir and nNOS-IR cells are colocalized in a subset of neurons in the POA and MeP (a larger area that includes both MePV and MePD; Hadeishi and Wood, 1996). Both studies suggest that AR may play a role in regulating nNOS. In male rats, nNOS activity and mRNA increase after castration and decrease after DHT treatment in the hypothalamus (Singh et al., 2000). In addition nNOS-IR cell numbers in the hypothalamus, but not in the POA, increased 2 weeks after castration. In contrast, when brains were examined 2 months, as opposed to 2 weeks, after castration, numbers of nNOS-IR cells in the POA were decreased compared with gonad-intact animals (Hadeishi and Wood, 1996; Du and Hull, 1999). These data suggest that T is required to increase nNOS-IR cells. However, in these studies, no distinction was made as to whether T was increasing nNOS-IR cell numbers by means of AR or ERα. Our data suggest that T is acting by means of ERα to up-regulate and by means of AR to down-regulate nNOS-IR cell numbers in POA.

Sex differences in nNOS expression

The greater amount of nNOS-IR area staining in the POA of WT and Tf males compared with WT females suggests an organizational effect for ERα, because all animals had the same concentration of T in plasma at the time of sacrifice. That WT females had more nNOS-IR area staining compared with ERαKO and DKO males suggests that activation effects of ERα also influence the expression of nNOS-IR staining. Taken together, the data suggest that ERα is required both during neonatal development and in adulthood to affect nNOS-IR expression. One caveat of using knockout mice is that organizational effects cannot be distinguished from activational effects. However, with our paradigm, we are able to make reliable predic-

tions about the effects of nNOS on specific social behaviors.

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LITERATURE CITED


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