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Effects of testosterone on neuronal nitric oxide synthase and tyrosine hydroxylase

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Abstract

Male rat copulatory ability decreases dramatically following castration. This may be due in part to the impairment of medial preoptic area (MPOA) dopamine (DA) release. Previous studies showed that extracellular DA levels in the MPOA of castrates were lower than in intact males, both during basal conditions and in the presence of a receptive female. However, tissue levels of DA in the MPOA were higher in castrates than in intact males, suggesting that DA synthesis may be normal or increased in castrates, but that release may be compromised. The current study found that neither long term (2 months) nor short term (2 weeks) castration had any effect on the number of neurons in the DA A_{14} area that were immunoreactive (ir) for tyrosine hydroxylase (TH), the rate limiting enzyme for DA synthesis. Therefore, castration may not affect DA synthesis in the MPOA. Tissue levels of neurotransmitter reflect release, as well as synthesis. We previously reported that nitric oxide (NO) may increase DA release in the MPOA. The present study tested whether castration affected the number of NO producing cells in the MPOA. Long term, but not short term, castration significantly decreased the number of NADPH-d (nicotinamide adenine dinucleotide phosphate diaphorase) positive neurons and brain nitric oxide synthase immunoreactive (bNOS-ir) neurons in the medial preoptic nucleus (MPN). This suggests that in gonadally intact animals testosterone may activate NOS, which increases the production of NO. Long or short term castration had no effect on the numbers of bNOS-ir neurons in the paraventricular nucleus (PVN) or medial amygdala. However, short term castration decreased bNOS-ir neurons in the bed nucleus of stria terminalis (BNST). Thus, one means by which testosterone promotes male sexual behavior may be by increasing production of NO in the MPOA, which increases local DA release.

Keywords: Male rat; Castration; Dopamine; Tyrosine hydroxylase; Medial preoptic area; Nitric oxide synthase; NADPH-d; Paraventricular nucleus; Bed nucleus of stria terminalis; Amygdala

1. Introduction

Testosterone is a major factor driving male sexual behavior. Following castration, male rats gradually lose their copulatory ability, which can be restored by testosterone replacement. Male rat sexual behavior is also affected by dopamine (DA) released in the medial preoptic area (MPOA). The DA agonist apomorphine, administered systemically [6,33] or microinjected into the MPOA [19], facilitated the rate and efficiency of copulation, while microinjection of the DA antagonist *cis*-flupenthix-Ol into the MPOA impaired copulation, penile reflexes and sexual motivation [34,44]. Moreover, a microdialysis study revealed an increase in extracellular DA in the MPOA when a sexually receptive female was presented behind a barrier, and a further increase during the copulatory period [20]. Thus, DA is released in the MPOA during a sexual encounter and facilitates all aspects of male sexual behavior.

Our previous studies have shown that testosterone may affect DA function in the MPOA. Extracellular MPOA DA levels were increased in the presence of an estrous female in those 1-week castrates that copulated, but not in the 1- or 2-week castrates that did not copulate [20]. Testosterone replacement restored the MPOA DA response to the estrous female, as well as copulatory behavior. Basal extracellular DA levels were also affected by castration. An in vivo quantitative microdialysis (no net flux) study showed that castrates, compared to gonadally intact rats, had lower extracellular DA levels during basal conditions [9]. Thus, both basal and female-stimulated levels of extracellular DA are lower in castrates. This may result from impaired

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DA release, rather than decreased synthesis in the MPOA, because castrates had more DA in MPOA tissue punches, almost all of which is intracellular, as well as greater amphetamine-stimulated MPOA DA release [9]. Thus, castrates had less extracellular DA in the MPOA, but more stored in tissue.

The increased tissue DA levels in castrates may result from decreased release, increased synthesis, or both. Experiment 1 investigated the effects of castration and hormonal replacement on DA synthesis in the DA A_{14} area, including the MPOA, by examining tyrosine hydroxylase (TH) immunoreactivity in this area of male rats. TH is the first and rate-limiting enzyme in the catecholamine biosynthetic pathway. TH immunoreactive (TH-ir) cell bodies were found in the MPOA and appeared to be dopaminergic [39].

Testosterone may regulate DA release in the MPOA by increasing the production of nitric oxide (NO). Numerous in vivo and in vitro studies have shown that NO may act as a messenger molecule in the central nervous system (reviewed in [38]). The NO precursor (L-arginine) increased extracellular levels of DA in the MPOA, and this increase was blocked by an nitric oxide synthase (NOS) inhibitor [23]. In addition, when a NOS inhibitor was retrodialysed into the MPOA through the microdialysis probe, it reduced the amount of DA released during copulation [24]. Furthermore, hormones affect NOS. Castration in male hamsters markedly decreased the number of neurons in the MPOA that were stained for NADPH-d (nicotinamide adenine dinucleotide phosphate diaphorase, a marker for NOS) or for brain NOS immunoreactivity (bNOS-ir) [16]. Similarly, ovariectomy in female rats resulted in a dramatic loss of NOS in the medial preoptic nucleus (MPN) [32].

Experiment 2 investigated the effects of castration and hormone replacement on NADPH-d positive neurons (Experiment 2A) and NOS immunoreactive (NOS-ir) neurons (Experiment 2B) in the MPN, the posterior dorsal portion of the medial amygdala, the principal nucleus of the bed nucleus of stria terminalis (BNST) and the paraventricular hypothalamic nucleus (PVN) of male rats. The MPN is the largest nucleus in the MPOA and contains the sexually dimorphic nucleus of the preoptic area [15]. The medial amygdala is an important processing area for olfactory stimulation from the receptive female, which is then relayed to the MPOA (reviewed in Ref. [27]). It contributes to the regulation of male sexual behavior, especially ejaculation [13,17]. The BNST is an important relay station for some of the sensory inputs from the amygdala to the MPOA (reviewed in Ref. [27]). Lesions of this area impaired copulatory behavior of male rats [7]. The role of the PVN in the regulation of penile erection and yawning has been studied extensively [28,29]. The PVN also contains steroid hormone receptors [5] as well as DA neurons (reviewed in Ref. [30]). Experiment 3 examined the relationship between the NOS-containing neurons and TH-containing neurons in the MPOA and PVN.

2. Materials and methods

2.1. Subjects

The subjects were sexually mature male Long–Evans rats (about 250 g). They were housed in individual, clear plastic cages in a temperature and humidity controlled environment on a 1410 h light–dark cycle (lights off at 1100 h). Food and water were available ad libitum. They were weighed daily to check their health.

2.2. Experimental overview

Experiment 1 examined the effects of 2-week and 2-month castration and hormone replacement on the number of TH-ir neurons in the DA A_{14} area. Thirty-six sexually inexperienced male rats were used in this experiment, with 18 subjects each in Experiments 1A and 1B. In Experiment 1A, 12 of the 18 were castrated, and the other six were sham castrated. Among the 12 castrates, six of them were given daily testosterone propionate (TP) (500 μg/animal) injections, while the other castrates and six sham castrates were given oil (0.1 ml/animal) daily injections for 2 weeks. Among the 18 subjects of Experiment 1B, six castrates had TP replacement for 2 months, and the remaining six castrates and six sham castrates had oil replacement for 2 months. At the end of hormone treatments, rats were sacrificed, perfused and fixed. Brains were removed and sliced into 40 μm sections. Four sets of brain slices were collected; two were used in these experiments. One set was used for TH staining in Experiment 1. The primary antibody used in Experiment 1 was raised in mouse against TH (Incstar, 1:2000). The second antibody was raised in goat against mouse (Vector Laboratory, 30 ml/10 ml). The other set of brain slices was used in Experiments 2B and 2C, which examined the effects of castration and hormone replacement on brain NOS-ir neurons.

Experiment 2A examined the effects of 1-month castration and hormone replacement on NADPH diaphorase activity. NADPH is a molecule in the respiratory metabolism cycle; NADPH diaphorase (NADPH-d) is an enzyme that transfers an electron from NADPH to nitroblue tetrazolium chloride to form a blue reaction product, which is used as a histochemical marker. NADPH-d has been shown to be the bNOS in fixed tissue and is used widely as a marker for NOS in the brain [10,26,41]. Because a significant reduction in NADPH-d positive neurons was found in 1-month castrates in Experiment 2A, Experiments 2B and 2C examined the effects of a shorter (2-week, Experiment 2B) and a longer (2-month, Experiment 2C) interval post-castration. However, because NADPH-d histochemistry appeared to be less sensitive and more variable than immunocytochemistry, Experiments 2B and 2C examined the effects of castration and hormone replacement on brain nitric oxide synthase immunoreactiv-

ity (bNOS-ir). The primary antibody used in Experiments 2B and 2C was raised in rabbit against bNOS (Incstar, 1:8000), and the second antibody was raised in goat against rabbit (Vector Laboratory, 30 ml/10 ml).

Experiment 3 used double labeling to determine the relationship between TH- and NOS-containing cells. Subjects were seven gonadally intact, sexually inexperienced male rats, with five in Experiment 3A (TH-ir and NADPH-d) and two in Experiment 3B (TH-ir and bNOS-ir). Techniques were similar to those in the previous experiments and are described below.

2.3. Castration

Animals were anesthetized with ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (4 mg/kg). A longitudinal midscrotal incision was made, and the testes and surrounding fatty tissue were tied off. A cut was made distal to the ligature to remove the testes and surrounding tissue. In the sham castrates, the incision was made, but the testes were not taken out. The skin was sutured and spread with Bacitracin ointment. Immediately after the surgery, castrated rats were given subcutaneous injections of the antibiotic Gentamicin and either oil (0.1 ml/animal) or TP (500 mg/animal). The sham castrated rats were given Gentamicin and oil (0.1 ml/animal). The incision sites were checked daily for 7 days after the surgery.

2.4. Perfusion and tissue preparation

On the day of perfusion, subjects were deeply anesthetized with sodium pentobarbital (50 mg/1.00 ml/animal) and perfused through the ascending aorta with about 300 ml saline, followed by about 200 ml of freshly made 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB). The brains were removed and postfixed for 3 h in the perfusion fixative and cryoprotected for 48 h in 20% sucrose in 0.1 M PB at 4°C. Coronal brain sections (40 μm) were cut on a cryostat at −14°C; four sets of brain slices were collected in 0.1 M PB containing 0.01% sodium azide and stored at 4°C. Two sets of the brain slices were used in these experiments, with one set used in Experiment 1 for TH staining and the other set used in Experiment 2 for bNOS staining. Separate animals were used in Experiment 3.

2.5. Immunocytochemistry

Collected slices were placed into a basket, which was placed into a six-well plate and washed 3 times for 5 min each time (3×5 min) in 0.1 M PB with 0.3% Triton X-100 (PBT). Slices then were incubated in 0.03% hydrogen peroxide (H_2O_2) in 0.1 M PBT for 30 min and washed 3×5 min, followed by incubation in the blocking serum (goat serum) for 30 min. Blocking serum was used to occupy nonspecific binding sites for the primary antibody, thereby reducing the background signal. Goat serum (150 ml) was added to 10 ml 0.1 M PBT. Slices were then incubated in primary antibody for 48 h at 4°C. After the incubation in the primary antibody, slices were washed 3×5 min in PBT. Slices then were incubated for 2 h at room temperature with diluted biotinylated second antibody solution (30 ml into 10 ml 0.1 M PBT), followed by 3×5 min washes in PBT. Slices were incubated for 1 h with Vectastain ABC reagent and washed again (3×5 min). Slices were incubated for 3 min in 3′,3-diaminobenzidine tetrahydrochloride (DAB) solution (50 mg DAB/100 ml 50 mM Tris (pH 7.5) + 0.01% H_2O_2 + 50 ml 8% NiCl). Sections were briefly rinsed in distilled water and wet mounted on subbed slides. After the slices were dried, they were dehydrated, cleaned with Hemo-D and coverslipped with Permount. A set of brain slices from one of the animals was used as a control for background signal and specificity of the staining. It was processed as mentioned above, except that it was incubated in PBT instead of primary antibody.

2.6. NADPH-d histochemistry

In Experiment 2A, slices were thaw mounted onto slides. Mounted slices were incubated at 37°C in PB containing 0.3% Triton X-100, 0.5 mg/ml nitroblue tetrazolium chloride and 1.0 mg/ml b-NADPH to produce a dark-blue reaction product over NADPH-d-containing neurons. The reaction was stopped after 50–60 min by rinsing the sections in water 3×5 min, at room temperature. After rinsing, sections were dehydrated in alcohol, cleared in Hemo-D and coverslipped with Permount.

2.7. Cell counting

The distribution of TH-ir and bNOS-ir positive cell bodies was illustrated using the Neuro-lucida program in Dr. Susan Udin's Lab. The Image-Pro program was used to snap the designated brain areas and to count the number of neurons.

In Experiment 1, since TH-ir positive neurons were scattered through the whole MPOA, five comparable MPOA sections across the animals were chosen, and TH-ir positive neurons were counted in these five sections. The total number of TH-ir positive neurons in these five sections was taken as an index for the number of neurons in the MPOA (the anterior portion of the DA A_{14} area). For the periventricular area (the posterior portion of the DA A_{14} area), three comparable sections (posterior to the MPOA but anterior to the DA A_{12} area) were chosen, and the number of TH-ir positive neurons counted in these three sections was used as the estimate of the total number of TH-ir positive neurons in the periventricular portion of DA A_{14} area.

In Experiment 2A, the area identified as the MPN was selected as representative of the MPOA, and NADPH-d-containing-neurons were counted there. The MPN does not have a clear boundary from the surrounding MPOA.

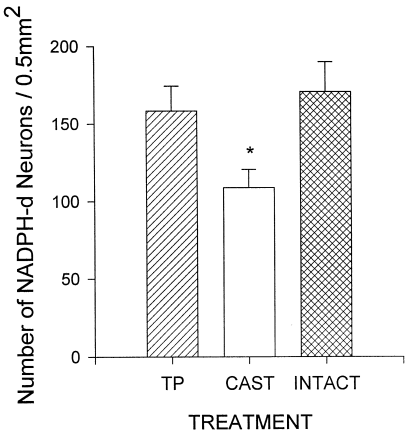

Fig. 1. Numbers of NADPH-d neurons in the MPN of male rats, castrated 1 month previously and treated with either TP (500 μg/day, s.c.) or oil vehicle (CAST), compared with gonadally intact males. Castration significantly reduced the number of NADPH-d neurons in the MPN, which was restored by TP. (NADPH-d is a marker for NOS.) * $p < 0.001$.

Therefore, cell bodies in a fixed size of frame were counted across the animals. For counting bNOS-ir neurons in Experiments 2B and 2C, matched sections of the MPOA, PVN, amygdala or BNST across the animals were chosen. These four brain areas all contain NADPH-d positive neurons [43]. For the MPOA, bNOS-ir neurons in the fixed frame of MPN were counted. bNOS-ir positive cell bodies were quantified in the posterior dorsal portion of the medial amygdala, since this area has been shown to be rich in androgen receptors [45]. NOS-ir positive neurons in the principal nucleus of the BNST were counted, since this area contains the most NOS-ir positive neurons of the BNST. NOS-ir positive cell bodies in the PVN were also counted in this study. For each brain region examined, a consistent box (MPN) or box according to the boundary of the brain region (PVN, BNST and amygdala) was placed around the designated area to be quantified. The number of bNOS-ir cells per designated area (number of cells/0.5 mm^2) was determined. Photomicrographs of the areas of interest were taken, using an Olympus camera.

2.8. Double labeling

TH-ir and NADPH-d: The free-floating sections were collected and incubated in the NADPH solution for 30 to 60 min as described. Slices then were rinsed for three times in the 0.1 M PB and incubated in the blocking serum (goat serum), primary antibody (anti-TH, raised in mouse, Incstar), second antibody (anti-mouse, raised in goat), ABC solution and DAB solution (as described above) consecutively, with three rinses of PBT between incubations. Slices were then mounted, dehydrated and coverslipped.

TH-ir and bNOS-ir: Brain slices were first stained for bNOS-ir neurons using glucose oxidase (GOD) instead of hydrogen peroxide without the presence of nickel chloride. The end reaction products were golden staining. Then they were stained for TH-ir positive neurons with hydrogen peroxide and nickel chloride enhancement, but no GOD. The end reaction products appeared as dark brown staining. The protocol for staining NOS-ir or TH-ir positive neurons with GOD solution was basically the same as the protocol using hydrogen peroxide as described above.

2.9. Data analysis

For Experiments 1A and 1B, one-way ANOVAs were conducted separately for the MPOA and periventricular area to determine if there was a statistically significant difference among the numbers of TH-ir positive neurons in 2-week (1A) or 2-month (1B) castrates with testosterone replacement, 2-week (1A) or 2-month (1B) castrates with oil replacement and gonadally intact rats. For Experiment 2, one-way ANOVAs were performed to determine if there

Table 1
Number of bNOS neurons in the MPN, BNST, PVN and amygdala
Males castrated 2 months previously had fewer bNOS-ir neurons in the MPN than did intact or TP treated males. Males castrated 2 weeks previously had fewer bNOS-ir neurons in the BNST than did intact males. Castration did not affect the numbers of bNOS-ir neurons in the PVN or the amygdala.

	Condition	Number of bNOS neurons per 0.5 mm^2 (mean ± S.E.M.) after 2 weeks castration	Number of bNOS neurons per 0.5 mm^2 (mean ± S.E.M.) after 2 months castration
MPN	Intact	382.3 ± 19.3	664.8 ± 42.6
	Castrated	360.4 ± 26.8	478.0 ± 26.8*
	TP	341.2 ± 13.6	569.33 ± 45.9
BNST	Intact	495.2 ± 20.9	620.3 ± 57.7
	Castrated	375.1 ± 13.1*	577.7 ± 44.0
	TP	394.0 ± 39.5	667.2 ± 56.9
PVN	Intact	508.7 ± 34.5	573.2 ± 10.9
	Castrated	546.3 ± 15.6	572.8 ± 17.3
	TP	541.2 ± 34.7	548.8 ± 22.1
Amygdala	Intact	759.5 ± 38.5	993.0 ± 72.7
	Castrated	815.2 ± 35.1	1034.9 ± 23.7
	TP	735.1 ± 34.1	945.1 ± 25.7

* $p < 0.05$.

was a statistically significant difference in the number of NADPH-d neurons in the MPN (Experiment 2A) and in the number of bNOS-ir positive neurons of the MPN, BNST, PVN and amygdala (Experiments 2B and 2C) among the sham castrates, castrates with testosterone replacement, and castrates without testosterone replacement. Because the tissues for the different experiments were processed at different times, and because staining was variable across experiments, statistical comparisons were not made across experiments (i.e., for 2-week vs. 2-month post-castration intervals). Newman–Keuls post-hoc tests were used to probe significant main effects.

3. Results

3.1. Experiment 1: effects of castration on TH-ir neurons

Neither 2-week nor 2-month castration or hormone replacement had any effect on numbers of TH-ir cell

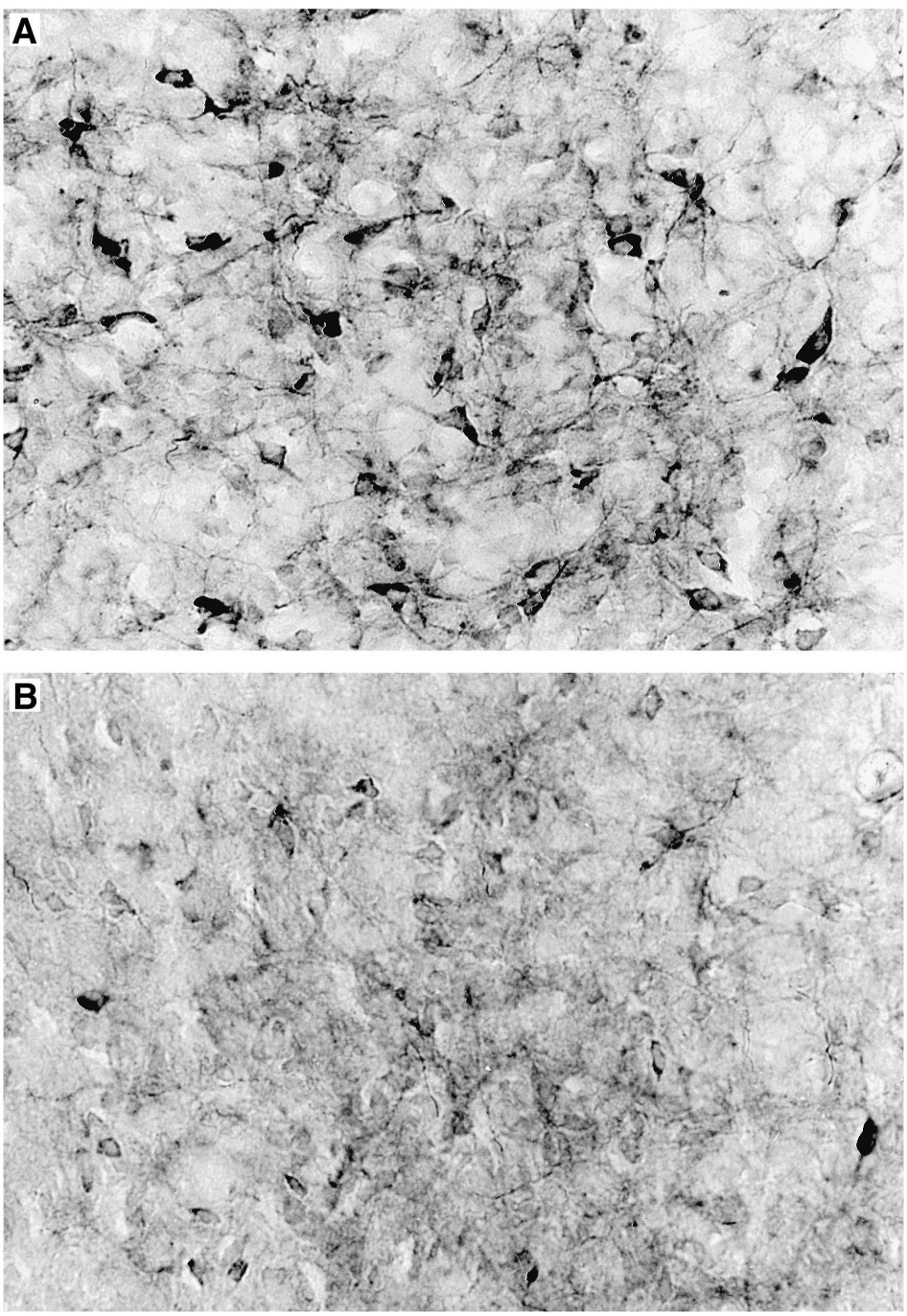

Fig. 2. BNOS-ir neurons in the MPN of male rats, castrated 2 months previously and treated with either (A) TP (500 μg/day, s.c.) or (B) oil vehicle, compared with (C) gonadally intact males. Castration decreased the number of BNOS-ir neurons in the MPN, which was restored by TP.

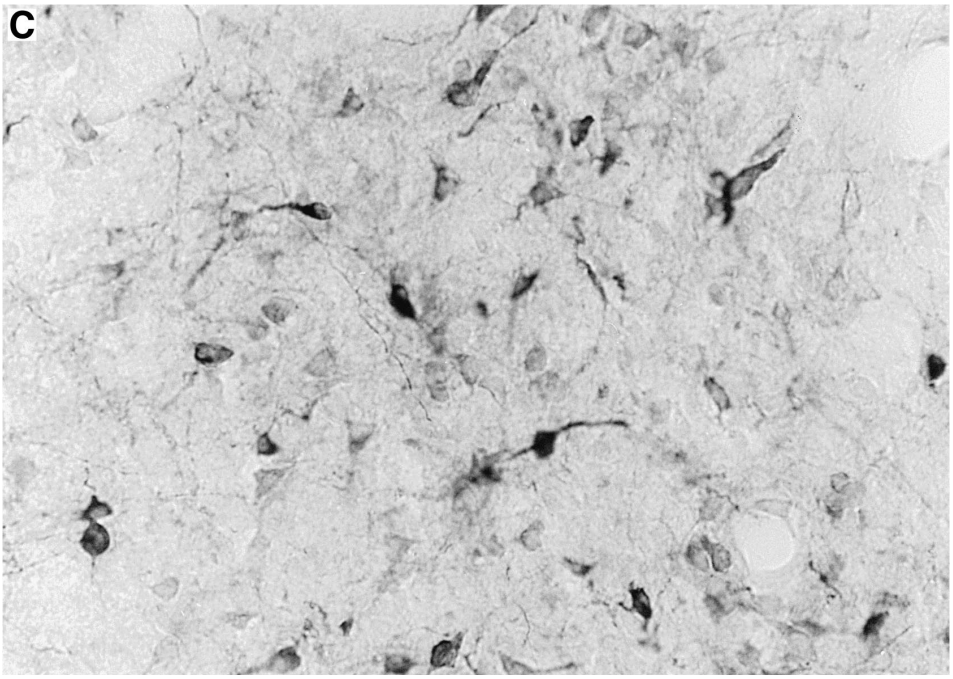

Fig. 2 (continued).

bodies in the DA A_{14} MPOA or periventricular area. The distribution of neurons stained for TH-ir in the MPOA of male rats was similar to that described by Simerly et al. [39]. The TH-ir positive neurons were found in the parastrial nucleus, anteroventral preoptic nucleus, and along the third ventricle, the periventricular zone. A few TH-ir stained cells were seen occasionally inside the domain of MPN, but none was found inside the MPNc. TH-ir fibers were seen throughout the whole MPOA. Even though the MPN contained only occasional TH-ir cell bodies, it did have numerous TH-ir fibers.

Cell bodies of the posterior portion of A_{14} DA neurons were located in the periventricular area, which surrounds the entire rostrocaudal extent of the third ventricle. The characteristics of TH-ir cell bodies in the periventricular area were different from those seen in the MPOA. TH-ir cell bodies in the periventricular area had longer neuronal processes, compared to those in the MPOA. Also, TH-ir cell bodies and fibers were more densely stained in this area, compared to the periventricular zone of the MPOA.

3.2. Experiment 2: effects of castration on NOS neurons

In Experiment 2A, NADPH-d neurons were found in the MPN. A one-way ANOVA, followed by post-hoc tests, revealed that 1-month castrates had significantly fewer NADPH-d neurons in the MPN, relative to both gonadally intact males and castrates with testosterone replacement ($F_{2/19} = 14.0,\ p < 0.001$) (Fig. 1).

In Experiment 2B, 2-week castration did not affect the numbers of bNOS-ir neurons in the MPN, PVN or medial amygdala, but significantly reduced it in the principle nucleus of the BNST, compared to intact males ($F_{2/15} = 6.32,\ p < 0.05$) (Table 1). In Experiment 2C, 2-month castration significantly reduced the number of bNOS-ir neurons in the MPN ($F_{2/17} = 5.64,\ p < 0.05$), compared to intact and testosterone-replaced males, but had no effect on numbers of bNOS-ir neurons in the BNST, PVN or the medial amygdala (Table 1). Representative photos of the MPN of a 2-month testosterone-treated castrate, a 2-month oil-treated castrate, and an intact male are shown in Fig. 2.

NOS-ir positive neurons were seen throughout the whole MPOA, the posterior portion of the BNST, PVN and amygdala. Neuronal processes stained for bNOS were found in the same brain regions that contained bNOS-ir positive neurons. Neurons with bNOS-ir expression were often bipolar, although monopolar and multipolar neurons were also observed.

3.3. Experiment 3: relationship of NOS neurons and DA neurons

In Experiment 3A, no clear colocalization of NADPH-d and TH-ir was found within the same neurons in the MPOA or the PVN. However, those two kinds of neurons and their processes were frequently found side by side. The BNST and medial amygdala contained NADPH-d positive neurons and TH-ir positive fibers, but no TH-ir cell bodies.

In Experiment 3B, TH-ir positive neurons and bNOS-ir positive neurons were found in the MPOA and the PVN. In some cases, those two types of neurons and their processes were found adjacent to each other; however, it was not possible to resolve any contacts between them at

the magnification used (20×). No TH-ir positive cell bodies were found in the BNST or the amygdala, only bNOS-ir positive cell bodies and fibers and TH-ir positive fibers.

4. Discussion

The present experiments show that the numbers of TH-ir positive neurons in the MPOA or in the periventricular area were not affected by either long term (2-month) or short term (2-week) castration. However, long term castration significantly decreased the number of bNOS-ir positive neurons in the MPN, while testosterone replacement restored it. This effect was not seen in the BNST, PVN or amygdala. On the other hand, short term castration had no effect on the number of bNOS-ir positive neurons examined in the MPN, PVN and the amygdala, but it decreased the number of bNOS-ir positive neurons in the BNST. Male rats show a variable decline in the ability to copulate after castration. These animals were not tested for copulation before sacrifice; therefore, it is impossible to know whether the 2-week castrates would have copulated.

The current study is consistent with the previous suggestion [9] that the increased stored DA in the MPOA of castrates is probably due primarily to decreased release, rather than increased synthesis, since long or short term castration did not cause any changes in the number of TH-ir positive neurons in the MPOA and the periventricular area. This may indicate that castration does not affect the synthesis of DA in this brain area. Even though the intensity of the TH-ir staining was not quantitatively determined in this study, there was no obvious difference in intensity among the castrates with 2 weeks or 2 months replacement of testosterone, castrates with oil replacement and gonadally intact animals. These findings are consistent with other studies that have reported increases or decreases in TH mRNA or protein in adrenergic or noradrenergic neurons, but not in dopaminergic neurons, as a result of chronic stress or drug regimens (reviewed in Ref. [14]). Thus, dopaminergic neurons appear to be less responsive than adrenergic or noradrenergic neurons to various factors that alter the production of TH. On the other hand, dopaminergic neurons may regulate TH activity through phosphorylation (reviewed in Ref. [14]), which would not affect TH-ir. We have very recently found that TH-ir is colocalized with androgen receptors (AR-ir) in only 0.5 to 3% of neurons in the A_{13} and A_{14} DA cell groups [37]. Those data, together with the lack of effect of castration in the present experiment, suggest that testosterone has little or no influence on DA synthesis in the MPOA.

DA activity in two other areas, besides the MPOA, has been correlated with male rat sexual behavior. Extracellular DA in the nucleus accumbens (NAcc, ventral striatum) increased in the presence of a receptive female and increased further during copulation [35]. Extracellular DA in the dorsal striatum increased only during copulation and may reflect motor activity [35]. Castration has produced mixed effects on measures of DA activity in these areas. Both basal and amphetamine-stimulated extracellular DA levels were increased in the ventral striatum (NAcc) of castrates [18]. On the other hand, measures of DA turnover in tissue punches [1] or amphetamine-stimulated DA levels in microdialysate [2] from the striatum were reported to be unaffected by castration. However, an increase in striatal DA release following castration was also reported [8]. Differential response of DA activity in the different brain areas to the effect of castration may be due to the functional differences among brain areas and/or the time period since castration in the different studies. There have been no studies of TH-ir in those areas following castration.

Tissue levels of DA are affected by release, as well as by synthesis. One factor that may regulate DA release in the MPOA is NO [23,24]. The present study examined the effect of castration and testosterone replacement on NOS activity in the MPOA, BNST, PVN and amygdala of male rats. In the MPN the numbers of NADPH-d containing neurons of 1-month castrates and of bNOS-ir positive neurons in 2-month castrates were significantly reduced, compared to gonadally intact animals. Testosterone replacement restored the numbers of neurons to those of intact males. Previous studies have shown similar effects of gonadectomy on preoptic NOS levels in male hamsters [16] and female rats [32]. Furthermore, bNOS and androgen receptors were reported to be colocalized in cells of the medial amygdala and MPN of male hamsters [16]. Similarly in rats, the MPOA contained not only bNOS-ir neurons, as described in this study, but also androgen receptors [5]. This provides anatomical evidence suggesting that NOS is regulated by steroid hormones in the brain.

Hormonal regulation of NO production was also demonstrated in studies using cGMP levels as a measure of NO activity. When NO is produced, it binds to and activates soluble guanylyl cyclase, which increases cGMP levels in target cells [11,22,25,40]. The extracellular cGMP levels analyzed through microdialysis in discrete brain sites have been used as a reliable measure of basal and stimulated NO activities [4,25,42]. Basal cGMP levels in the MPOA were significantly higher in gonadally intact male rats than in 2-week castrates [36]. This provides additional evidence that NO production in the MPOA is augmented by steroids that may exert their modulatory influence directly on the NOS-producing cells.

The current study also showed that 2-week and 2-month castration and testosterone replacement had no effect on the numbers of bNOS-ir positive neurons in the PVN and amygdala. However, 2-week, but not 2-month castration significantly decreased the number of bNOS-ir positive neurons in the BNST. Thus, the NO activity in the PVN and amygdala may not be regulated by steroid hormones. It is not clear whether NO regulates DA release in the

BNST. No study has examined the relationship between the DA release in the BNST and male rat copulatory behavior, even though DA terminals were found there [12]. NO may activate mechanisms other than DA release, under the influence of testosterone, to regulate either sexual behavior or other kinds of behavior. In addition, the lack of hormonal effects on BNST NOS in 2-month castrates may have been due to some type of recovery.

NO has been suggested to play an important role in regulating male rat sexual behavior. Peripheral administration of L-arginine, the precursor for NOS, significantly shortened mount and intromission latencies and post-ejaculatory intervals, whereas L-NAME, a NOS inhibitor, reduced the percentages of mounting and ejaculating subjects, as well as the efficiency of copulation [3,21]. The effects of those systemically administered drugs on male rat sexual behavior were probably mainly due to its action on penile erection, since i.c.v. administration of neither L-NAME nor L-arginine significantly modified any index of copulatory behavior in sexually experienced male rats [3]. However, in sexually naive animals central administration of L-NAME worsened, while central administration of L-arginine did not improve sexual performance. Furthermore, in the same study there was a striking difference in amount of NOS mRNA expression in the magnocellular component of the PVN between the sexually potent and sexually impotent male rats. Finally, microinjection of L-NMMA, another NOS inhibitor, into the MPOA of male rats increased the number of seminal emissions in an ex copula test (probably by increasing sympathetic nervous system activity) and decreased the number of sexually naive animals that copulated [31]. Thus, a role of central NO in regulating male rat sexual behavior was suggested by these data.

One-way for NO to regulate male rat sexual behavior is probably by eliciting DA release in the MPOA. Numerous in vivo and in vitro studies have shown that NO may act as a messenger molecule in the central nervous system (reviewed in Ref. [38]). When L-arginine, the NO precursor, was given through the microdialysis probe into the MPOA, it significantly increased extracellular DA levels; this increase was blocked by a NOS inhibitor, which also decreased basal DA release when administered alone [23]. Furthermore, administration of a NOS inhibitor into the MPOA via reverse dialysis blocked the copulation-induced increase in DA release observed with the inactive isomer [24]. In addition, the present study found that TH-ir and NOS containing neurons and fibers were located near each other in the MPOA and the PVN, although synaptic contacts could not be observed with this level of magnification.

In summary, long-term castration of male rats significantly decreased the amount of brain NOS-ir in the MPOA. However, DA synthesis in MPOA, estimated by the number of TH-ir neurons, appears to be unaffected by castration. Thus, both the decreased extracellular DA levels and the increased stored DA in the MPOA of castrates may result from decreased release of DA, resulting from a loss of NOS. Gonadal steroid hormones bind to their receptors in the MPOA and may increase the amount of NOS and other factors. The production of NO then probably facilitates both basal and female-stimulated DA release there, which in turn facilitates copulation.

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