Endocrine, *vol. 5, no. 1, 59–65, August 1996* 1046–3976/5/1:59–65/$6.75

Sex Differences in Androgen-Regulated Cytochrome P450 Aromatase mRNA in the Rat Brain

Charles E. Roselli, Salah E. Abdelgadir, Emile Jorgensen, and John A. Resko

Department of Physiology and Pharmacology Oregon Health Sciences University, Portland OR

The conversion of testosterone to estradiol by cytochrome P450 aromatase ($P450_{AROM}$) in the medial preoptic area is required for full expression of male sexual behavior in rats. Preoptic $P450_{AROM}$ activity is stimulated by androgens through an androgen-receptor mediated mechanism that regulates $P450_{AROM}$ gene expression. The mechanism of enzyme induction appears to be sexually dimorphic in several species leading to greater testosterone-stimulated $P450_{AROM}$ activity in males than in females. The present study was designed to determine whether the sex difference in androgen-regulated $P450_{AROM}$ activity is manifested at the levels of mRNA expression. We compared the concentrations of $P450_{AROM}$ mRNA and enzyme activity between five different treatment groups: intact males, castrated males (CX), ovariectomized females (OVX), CX males treated with dihydrotestosterone (CX + DHT), and OVX females treated with DHT (OVX + DHT). We found that unstimulated levels of $P450_{AROM}$ mRNA and enzyme activity in both the preoptic area and medial basal hypothalamus were similar in the CX and OVX groups. However, when treated with equivalent doses of DHT, the levels of $P450_{AROM}$ mRNA and enzyme activity in both brain regions were significantly higher in males than in females (i.e., CX +DHT group > OVX +DHT group). These results demonstrate that sex differences in the regulation of $P450_{AROM}$ in brain are exerted pretranslationally by androgen and suggest that gender differences in androgen responsiveness play an important role in regulating gene expression in the adult rat brain.

Key Words: Sex difference; aromatase mRNA; preoptic area; medial basal hypothalamus.

Received March 21, 1996; Revised May 13, 1996; Accepted May 13, 1996.

Author to whom all correspondence and reprint requests should be addressed: Charles E. Roselli, Department of Physiology and Pharmacology, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, OR 97201-3098. E-mail: rosellic@OHSU.edu

Introduction

Adult gonadectomized male and female rats differ in their behavioral response to testosterone (T) treatment in that males show higher levels of androgen-dependent mounting behavior than females (Gerall and Ward, 1966; Ward, 1969). This difference in behavior, in part, is because of sex differences in brain anatomy and function that are established during perinatal development (Gorski, 1985; De Vries, 1990). However, it may also reflect sex differences in the cellular mechanisms by which androgens act. One important pathway of androgen action in the brain involves the conversion of T to estrogen by cytochrome $P450_{AROM}$ (Naftolin et al., 1974). Several studies have demonstrated that aromatization of T in the medial preoptic area is required for the full expression of male sexual behavior in rats (Bonsall et al., 1992; Christensen and Clemens, 1975; Clancy et al., 1995). Preoptic aromatase activity is stimulated by androgens through an androgen-receptor mediated mechanism that regulates aromatase gene expression (Roselli and Resko, 1993; Abdelgadir et al., 1994). Recent experiments have demonstrated that T induces preoptic aromatase to a greater extent in males than in females suggesting that the mechanism of aromatase induction is sexually dimorphic in several species (Schumacher and Balthazart, 1986; Steimer and Hutchison, 1990; Roselli, 1991a; Beyer et al., 1994). The present study was initiated to determine whether the sex difference in androgen-stimulated $P450_{AROM}$ induction is manifested at the level of mRNA expression.

Materials and Methods

Animals

Age-matched 50- to 60-d old male and female Sprague-Dawley rats (Simonsen Breeders, Gilroy, CA) were used in this study. The rats were given free access to food and water, and maintained on a 12-h light/12-h dark lighting schedule. To study sex differences in the effect of androgen on $P450_{AROM}$ mRNA levels, 72 males and 48 females were assigned to the following five treatment groups: intact males (INT), castrated males (CX), ovariectomized females (OVX), CX + dihydrotestosterone (DHT), and OVX + DHT. Gonadectomies were performed on rats anesthetized

with a ketamine (55 mg/kg)/xylazene (5 mg/kg) cocktail. DHT was administered in 30-mm Silastic capsules (id 0.062 in., od 0.125 in., Dow Corning, Midland, MI) placed subcutaneously between the scapulae. One week after the treatments were initiated, the rats were decapitated and the preoptic area (POA) and medial basal hypothalamus (MBH) were dissected according to previously published coordinates (Handa et al., 1986). Brain areas from four animals/treatment were pooled for the RNase protection assay ($n = 6$ replicates). The effects of the five experimental treatments on aromatase activity were studied in an additional group of 18 males and 12 females ($n = 6$ treatment group). Trunk blood samples were collected for radioimmunoassay (RIA) of steroid hormones.

RNA Isolation

Total cellular RNA was prepared according to Chirgwin et al. (1979). After dissection, the brain was immediately frozen on dry ice and stored at –85°C until homogenized in a 4*M* guanidine isothiocyanate lysing buffer containing 50 m*M* Tris, 10 m*M* EDTA, 2% sodium N-lauryl sarcosine, 1% β–mercaptoethanol, and 10 m*M* vanadyl-ribonuclease complexes, pH 7.6. The homogenates were centrifuged through a 5.7*M* CsCl gradient (100,000*g* for 16 h at 20°C). The resulting pellet was suspended in buffer (10 m*M* Tris, 5 m*M* EDTA, and 1% SDS, pH 7.4) and extracted with phenol:chloroform:isoamyl alcohol (24:24:1) and concentrated by ethanol precipitation. Sample RNA concentration was obtained by UV spectrophotometry at 260/280 nm. The RNA samples were stored at –85°C until assayed for $P450_{AROM}$ mRNA by the RNase protection assay.

RNase Protection Assay

A 484-bp fragment of the rat $P450_{AROM}$ cDNA was cloned into the pBluescript KS^+ vector (Lephart et al., 1990). The construct was linearized with *Nco*I and used to generate a ^{32}P-labeled antisense RNA with T3 RNA polymerase and ^{32}P[CTP]. The resulting antisense transcript contained 430-bp of $P450_{AROM}$ 5'-coding sequence and 90-bp of vector sequence. This probe protects two RNA fragments in brain tissue; a 430-bp full-length fragment and a shorter 300-bp fragment. The full-length protected RNA fragment was used to quantify levels of $P450_{AROM}$ gene expression, since we demonstrated previously (Abdelgadir et al., 1994) that the full-length RNA fragment represents mRNA that encodes functional $P450_{AROM}$ enzyme. Sense strand $P450_{AROM}$ used for generating standard curves was synthesized using T7 RNA polymerase and *Bam*HI linearized plasmid. In vitro transcription was performed according to the manufacturer's instructions (Promega, Madison, WI).

The RNase protection assay was conducted using a modification of the Gilman method (Gilman, 1990), as previously described (Abdelgadir et al., 1994). The total RNA (15 μg) was hybridized with 500,000 cpm of gel-purified ^{32}P-labeled antisense probe in 30 μL buffer containing 80% deionized formamide, 40 m*M* piperazine-N-N'-bis (2-ethanesulfonic acid), pH 6.4, 0.4*M* NaCl, and 1 m*M* EDTA for 16 h at 45°C. All samples were also hybridized to rat cyclophilin cRNA probe (Danielson et al., 1988). This 221-bp antisense RNA served as an internal control to which data were normalized. After hybridization, the samples were digested with ribonuclease T1 (900 U/380 μL; Gibco-BRL, Grand Island, NY) for 1 h at 37°C. The digestion reaction was terminated by the addition of 10 μL 20% sodium dodecyl sulfate and 50 μg proteinase-K, after which the samples were incubated for 20 min at 37°C. Protected hybrids were phenol-chloroform extracted, precipitated with ethanol, denatured, and electrophoresed on a 5% polyacrylamide gel containing 7*M* urea. The gels were dried and exposed to X-ray film for 8 h (cyclophilin) or 18 h ($P450_{AROM}$) to generate autoradiograms. The autoradiograms were analyzed by densitometry using a computer-based video-imaging system (Imaging Research, St Catherines, Canada). For each standard curve, a first order polynomial regression was used to relate the autoradiographic signal (i.e., relative optical density x pixels) to known amounts of protected $P450_{AROM}$ sense mRNA. The amount of $P450_{AROM}$ mRNA in each sample was then determined from the assay standard curve and normalized to the internal cyclophilin control value.

$P450_{AROM}$ Activity Assay

Freshly dissected brain tissues from individual rats (n = 5–6/treatment) were homogenized in Dounce tissue grinders in 30 vol of phosphate buffer (10 m*M* KH_2PO_4, 100 m*M* KCl, 1 m*M* EDTA, and 1 m*M* dithiothreitiol, pH 7.4). Aliquots of the microsomes were incubated for 1 h at 37 C with 0.3 μ*M* [1β–^{3}H]androstenedione (NEN Research Products, Dupont Co., Boston, MA; specific activity =27.5 Ci/mmol) in the presence of an NADPH generating system. Aromatase activity was estimated by quantifying the amount of ^3H_2O generated by the stereospecific loss of the C-1β tritium that is proportional to the amount of estrogen formed (Roselli and Resko, 1991). Protein concentrations were determined by the Lowry method (Lowry et al., 1951). The results were corrected for procedural losses and nonspecific conversion, normalized for sample protein concentration, and expressed as fmol/h/mg protein. All samples were analyzed in a single assay.

Steroid Assays

Trunk bloods were allowed to clot at 4 C overnight before they were centrifuged (1500*g* for 30 min) and the sera harvested. Serum steroids (T. DHT, and Diol (5α–androstane-3α-17β-diol) were measured by RIA after ether extraction and chromatography on Sephadex LH-20 using previously described methodologies (Resko et al., 1973; Roselli and Resko, 1984). All samples were assayed in a single RIA. The percentage of recovery, water blanks, and

intra-assay coefficient of variation for each steroid were respectively; T. 78%, 13 pg, and 5%; DHT, 64%, 22 pg, and 10%; Diol, 62%, 3 pg, and 12%.

Statistical Analysis

The mRNA and aromatase activity data were analyzed by Levene's test for homogeneity of variance and, when necessary, the data were transformed to $\log_{10}$ values to correct for heterogeneity of variance. Differences between treatment groups were analyzed by one-way analysis of variance (ANOVA) followed by a post hoc Newman-Keuls multiple range test. The serum steroid data were analyzed by a nonparametic Kruskall-Wallis one-way ANOVA followed by Mann-Whitney U-tests to assess differences between treatments. *P* values less than 0.05 were considered significant for both parametric and nonparametric tests. All statistical analysis was performed using GB-STAT software version 5.4 (Dynamic Microsystems, Inc., Silver Spring, MD).

Results

The basal concentrations of $P450_{AROM}$ mRNA and enzyme activity in the POA (Fig. 1) were not different between CX males and OVX females, but were significantly less than in intact males ($p < 0.05$). One week after hormone treatment, both $P450_{AROM}$ mRNA levels and enzyme activity were significantly greater in CX + DHT males than in OVX + DHT females ($p < 0.05$). The concentrations of $P450_{AROM}$ mRNA and enzyme activity were maintained at intact male levels in CX + DHT males. However, $P450_{AROM}$ mRNA concentrations in OVX+ DHT females were significantly lower than in intact males, and there was a trend for enzyme activity to also be lower in the OVX + DHT group.

In general, the regulation of $P450_{AROM}$ in the MBH (Fig. 2) paralleled regulation in the POA (Fig. 1). Androgen-stimulated concentrations of $P450_{AROM}$ mRNA and enzyme activity were greater in CX + DHT males than in OVX + DHT females ($p < 0.05$) and no sex differences were observed in basal concentrations of mRNA and enzyme activity between CX males and OVX females. There also appeared to be less correlation between androgen-stimulated $P450_{AROM}$ mRNA and enzyme activity in MBH. $P450_{AROM}$ enzyme activity was restored to intact male levels in CX + DHT males, whereas $P450_{AROM}$ mRNA were greater in CX + DHT than in intact males. In OVX + DHT females, $P450_{AROM}$ enzyme activity was significantly lower than in intact males, whereas there appeared to be no difference in $P450_{AROM}$ mRNA between these treatment groups.

Serum T, DHT, and Diol concentrations in CX males and OVX females were significantly lower than in intact male rats ($p < 0.05$ for all; Table 1). In DHT-treated rats, DHT and Diol concentrations in serum were significantly greater than in untreated gonadectomized rats ($p < 0.05$). Serum DHT concentrations were equivalent in DHT-treated

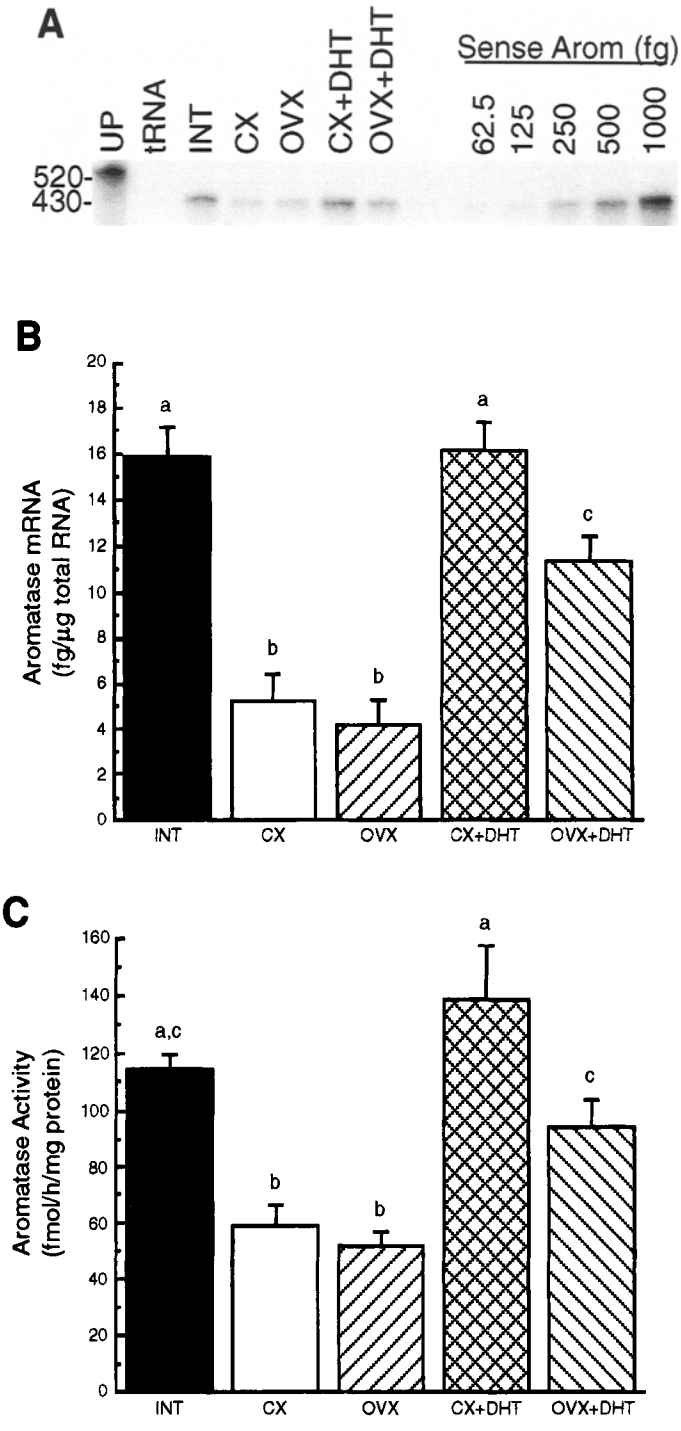

Fig. 1. Sex differences in androgen-regulated $P450_{AROM}$ in the POA. **(A)** Representative RNase protection assay showing the effect of gonadectomy and T treatment on $P450_{AROM}$ in POA (25 µg total RNA/lane). The UP (undigested probe) lane illustrates the $P450_{AROM}$ mRNA probe (520 nt) that was incubated in the assay but not digested. The tRNA lane illustrates $P450_{AROM}$ probe that was incubated with tRNA and digested with RNase (negative control). The band at 430 nt in each of the treatment and the standard curve lanes is the protected $P450_{AROM}$ mRNA fragment. **(B)** Mean (+SEM) levels of the 430 nt protected RNA fragment determined from the standard curve of $P450_{AROM}$ sense RNA and normalized to cyclophilin mRNA levels (not shown); $n = 6$. **(C)** Mean (SEM) concentrations of $P450_{AROM}$ activity; $n = 5$–6. Means with different superscripts differ significantly ($p < 0.05$).

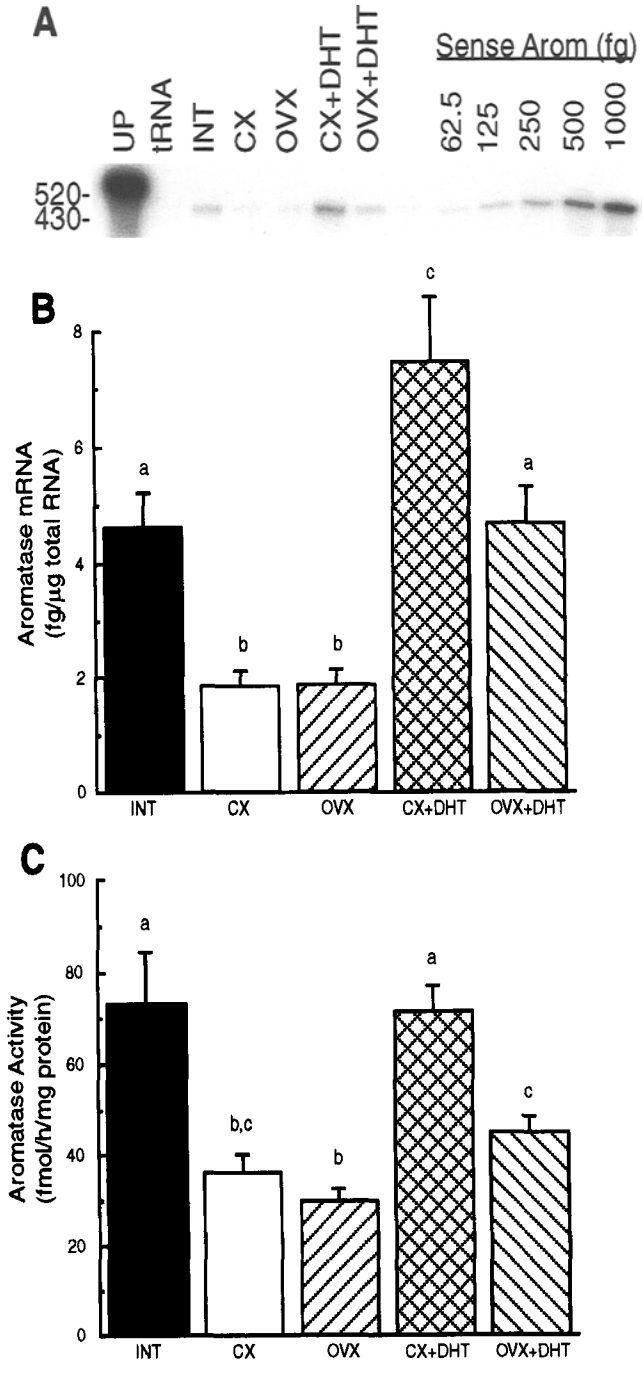

Fig. 2. Sex differences in androgen-regulated $P450_{AROM}$ in the MBH. **(A)** Representative RNase protection assay (25 µg total RNA/lane). **(B)** Mean (±SEM) levels of the 430 nt protected RNA fragment; $n = 6$. **(C)** Mean (±SEM) concentrations of $P450_{AROM}$ activity; $n = 4$–6. Means with different superscripts differ significantly ($p < 0.05$). *See* Fig. 1 for further details.

gonadectomized rats and intact males, whereas serum Diol concentrations in DHT-treated gonadectomized rats were significantly higher than in intact males ($p < 0.05$). As we intended, equivalent serum concentrations of DHT and Diol were achieved in CX males and OVX females treated with DHT. Serum T concentrations in DHT-treated rats were not different from levels in gonadectomized rats.

Discussion

The results of this study demonstrate a clear-cut sex difference in androgen-stimulated induction of $P450_{AROM}$ activity in both the preoptic area and the medial basal hypothalamus confirming previous reports in several species (Schumacher and Balthazart, 1986; Steimer and Hutchison, 1990; Roselli, 1991α; Beyer et al., 1994). This difference in enzyme activity corresponded to a sex difference in $P450_{AROM}$ mRNA concentrations under equivalent conditions of DHT treatment. These results support the suggestion that androgenic stimulation of $P450_{AROM}$ activity in brain results from the pretranslational regulation of $P450_{AROM}$ mRNA (Harada et al., 1993; Yamada et al., 1993; Abdelgadir et al., 1994) and further suggest that this process is sexually dimorphic in rats. In contrast to our results, Yamada et al. (1993) did not observe a sex difference in T-stimulated levels of $P450_{AROM}$ mRNA concentrations in mice. This discrepancy suggests that in addition to obvious experimental differences, species differences may exist in the extent to which the regulation of $P450_{AROM}$ is sexually dimorphic in adults.

In order to make relevant sex comparison of androgen-regulated $P450_{AROM}$, our treatment paradigm was designed to produce equivalent serum levels of DHT in gonadectomized DHT-treated male and female rats. DHT, unlike T. is not aromatizable, and so permits us to examine androgen-stimulated regulation independent of any estrogenic contribution. This is an important consideration since androgen- and estrogen-dependent mechanisms both appear to be involved in the control of $P450_{AROM}$ activity in the POA (Roselli, 1991b). However, it has been reported that whereas treatment with DHT-filled Silastic capsules produces physiological levels of circulating DHT, it also produces increased serum levels of Diol, a weakly androgenic metabolite of DHT (Roselli and Resko, 1984; Roselli et al., 1987). Diol acts as an interconvertable pool for DHT for tissues that contain 3α-hydroxysteroid dehydrogenase, e.g., brain and prostate (Noma et al., 1975; Van Doorn et al., 1975). For this reason, both DHT and Diol were measured in the present study. We found that 1 wk after gonadectomy and DHT treatment, serum DHT and Diol concentrations were equivalent in age-matched male and female rats, thus making it possible for us to compare the levels of $P450_{AROM}$ mRNA and enzyme activity under conditions where males and females were exposed to the same androgenic stimulus.

Treatment of CX males with DHT maintained both $P450_{AROM}$ mRNA concentrations and activity at levels identical to intact males suggesting that this dose provided a physiological androgen stimulus. However, treatment of OVX females with this dose did not increase $P450_{AROM}$ mRNA concentrations in POA to levels observed in intact males, and resulted in a trend toward lower enzyme activity in OVX + DHT females as well. These results are consistent with the hypothesis that a sex difference in androgen

Table 1
Steroid Hormone Concentrations in Sera

Treatment	No. of observations	DHT, ng/mL	Diol, ng/mL	T, ng/mL
Intact (M)	12	$0.14 + 0.02^a$	$0.36 + 0.04^a$	$3.76 + 0.44^a$
CX (M)	12	ND	$0.03 + 0.01^b$	$0.02 + 0.02^b$
OVX (F)	12	ND	$0.01 + .006^b$	$.005 + .003^b$
CX (M) + DHT	12	$0.14 + 0.04^a$	$0.71 + 0.07^c$	$.004 + .003^b$
OVX (F) + DHT	12	$0.16 + 0.03^a$	$0.70 + 0.11^c$	$0.02 + 0.01^b$

See Materials and Methods for details of hormone treatments. CX, Castrate; DHT, dihydrotestosterone; Diol, 5α-androstane-3α-17β-diol; ND, not detectable; OVX, ovariectomized; T, testosterone; M, male; F, female.
^{a,b,c,d}Means with dissimilar superscripts within columns differ significantly by MannWhitney U-Test ($p < 0.05$, two-tailed).

responsiveness in POA, and consequently the capacity for aromatization, is responsible, in part, for gender differences in the expression of androgen-stimulated sexual behaviors. In the MBH, the concentrations of $P450_{AROM}$ mRNA were significantly greater in CX + DHT-treated males than in intact males, but enzyme activity was not different. Moreover, $P450_{AROM}$ mRNA concentrations were not different between intact males and OVX + DHT-treated females, but activity levels were significantly lower in females. The lack of correlation between $P450_{AROM}$ mRNA concentrations and enzyme activity in the MBH suggests that additional factors, either posttranslational or allosteric, may contribute to regulation and should be studied further.

Our results show that the basal levels of $P450_{AROM}$ activity and mRNA concentrations were not different in gonadectomized males and females, indicating that the fractional response to DHT treatment differs between the sexes. Any of several factors could contribute to the greater androgen responsiveness observed in males. Compared to females, males may have greater numbers of aromatase-containing cells or greater amounts of aromatase-coupled androgen receptor per target cell. Alternatively, the coupling mechanism between androgen receptors and aromatase gene transcription may quantitatively differ in males and females. Recent attempts to distinguish between these possibilities have been inconclusive. Beyer et al. (1994), using hypothalamic cultures derived from fetal mice, concluded that sex differences in hypothalamic aromatase activity are a result of higher percentage of neurons expressing aromatase in males rather than to higher $P450_{AROM}$ activity in individual male hypothalamic aromatase-immunoreactive cells. Likewise, a sex difference in the number of immunocytochemically identified aromatase-containing cells in the preoptic area has been found in adult Japanese quail (Foidart et al., 1994) and shrew (Rissman et al., 1996). However, after T treatment, significant sex differences in enzyme activity are present, but gender differences in the numbers of aromatase-immunoreactive neurons are no longer evident (Beyer et al., 1994; Foidart et al., 1994). Furthermore, we reported previously that in microdissected brain nuclei, sex differences in androgen-induced $P450_{AROM}$ activity is generally correlated with gender differences in androgen receptor concentrations, with the medial preoptic nucleus being an important exception (Roselli, 1991a). Taken together, these studies suggest that sex differences in $P450_{AROM}$ are a result of higher proportion of neurons expressing aromatase, and possibly androgen receptors, in males than in females, but do not preclude the possibility that gender differences in androgen regulation of $P450_{AROM}$ also exist at the cellular or subcellular level.

It is generally recognized that aromatization of T to estrogens is required for androgen-stimulated expression of male sexual behaviors in rats and a number of other vertebrate species (Balthazart et al., 1990; Hutchison, 1990; Sachs and Meisel, 1994). Moreover, local aromatization is the primary determinant of regional estrogen receptor occupation in the male rat brain (Yuan et al., 1995). In contrast, the role of aromatization in the adult female brain is not well understood. Females will display components of male copulatory behavior such as mounting, but rarely exhibit the motor patterns characteristic of intromission and ejaculation unless they were treated with androgens perinatally (Sachs et al., 1973). It is not known to what extent aromatization in brain is involved in the expression of mounting behavior in female rats, although it is required in female guinea pigs (Roy and Goy, 1988). Recent evidence suggests that local aromatization may contribute to estrogen receptor occupation in the brain of the female rat during estrus, and could serve to maintain estrogen responsiveness at this stage of the female reproductive cycle (Yuan et al., 1995). In another study, it was shown that T can prime OVX female rats to exhibit lordosis behaviors and elevated LH levels in response to progesterone (Krey et al., 1982). These reports suggest that T and/or its locally synthesized estrogenic metabolites may contribute to behavioral expression and neuroendocrine cyclicity in females.

Regulation of $P450_{AROM}$ activity in the brain by androgens has been extensively studied in rats (Lephart and Ojeda, 1990; Jakab et al., 1993; Roselli and Resko, 1993; Abdelgadir et al., 1994). An androgen receptor-mediated

mechanism is thought to be involved, because induction is specific to androgens, blocked by antiandrogens, and not observed in androgen receptor-deficient rats (Roselli and Resko, 1993). It now *seems* likely that androgen acts, for the most part, by regulating the expression of the $P450_{AROM}$ gene (Harada et al., 1993; Yamada et al., 1993; Abdelgadir et al., 1994). However, a major question which has yet to be resolved is whether this effect is exerted directly by androgen receptors in target cells, or indirectly through growth factors or effects secondary to activation of neurotransmission. A recent report by Honda et al. (1994) demonstrated the presence of a novel exon 1 and promoter of the aromatase gene in human brain. Analysis of the promoter region revealed the presence of both a putative androgen response element and steroidogenic factor-1 (SF-1) binding sequence. The presence of an androgen response element suggests that androgens may exert direct transcriptional control over the aromatase gene in brain. Meanwhile, the consensus SF-1 site, also known as the Ad4 site (Bureau et al., 1995), could confer cAMP inducibility in the regulation of brain aromatase as it does in granulosa cells (Fitzpatrick and Richards, 1993). However, the effect of cAMP on brain $P450_{AROM}$ is controversial because compounds that increase intracellular cAMP concentrations have been reported to induce (Callard, 1981), suppress (Canick et al., 1987; Lephart et al., 1992), or have no effect (Black, 1994), depending on the species, age of the animal, and other experimental conditions. Moreover, although the orphan nuclear receptor SF-1 (also called Ad4-binding protein) is expressed in the rat brain, it is found exclusively within the medial basal hypothalamus (Ikeda et al., 1995) and, for this reason, is probably not involved in $P450_{AROM}$ regulation in other brain areas.

In summary, our results demonstrate that sex differences in the regulation of $P450_{AROM}$ are exerted pretranslationally by androgen in the POA and MBH. Thus, this sex difference in androgen responsiveness appears to play an important role in regulating gene expression in the adult rat brain.

Acknowledgments

The authors wish to thank Edwin Lephart for providing the $P450_{AROM}$ cDNA. This work was supported by NSF grant IBN 94-21759 (CER) and NIH grants HD 18196 (JAR) and HD 18185 (P30).

References

Abdelgadir, S. E., Resko, J. A., Ojeda, S. R., Lephart, E. D., McPhaul, M. J., and Roselli, C. E. (1994). *Endocrinology* **135,** 395–401.

Balthazart, J., Foidart, A., and Hendrick, J. C. (1990). *Physiol. Behav.* **47,** 83–94.

Beyer, C., Green, S. J., and Hutchison, J. B. (1994). *Endocrinology* **135,** 1220–1226.

Black, V. H. (1994). *Endocrinology* **135,** 299–306.

Bonsall, R. W., Clancy, A. N., and Michael, R. P. (1992). *Horm. Behav.* **26,** 240–254.

Bureau, M., Laschet, J., Bureau-Heeren, M., Hennuy, B., Minet, A., Wins, P., and Grisar, T. (1995). *J Neurochem.* **65,** 2006–2015.

Callard, G. V. (1981). *Brain. Res.* **204,** 461–464.

Canick, J. A., Tobet, S. A., Baum, M. J., Vaccaro, D. E., Ryan, K. J., Leeman, S. E., and Fox, T. O. (1987). *Steroids* **50,** 509–521.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979). *Biochemistry* **18,** 5294–5298.

Christensen, L. W. and Clemens, L. G. (1975). *Endocrinology* **97,** 1545–1551.

Clancy, A. N., Zumpe, D., and Michael, R. P. (1995). *Neuroendocrinology* **61,** 98–111.

Danielson, P. E., Forss-Petter, S., Brow, M. A., Calavetta, L., Douglass, J., Milner, R. J., and Sutcliffe, J. G. (1988). *DNA* **7,** 261–267.

De Vries, G. J. (1990). *J. Neuroendocrinol.* **2,** 1–13.

Fitzpatrick, S. L. and Richards, J. S. (1993). *Mol. Endocrinology* **7,** 341–354.

Foidart, A., De Clerck, A., Harada, N., and Balthazart, J. (1994). *Physiol. and. Behav.* **55,** 453–464.

Gerall, A. A. and Ward, I. L. (1966). *J. Comp. Physiol. Psychol.* **62,** 370–375.

Gilman, M. (1990). In: *Current Protocols in Molecular Biology.* Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds). Green and Wiley-Interscience: New York. pp. 4.7.1–4.7.6.

Gorski, R. A. (1985). *Can. J. Physiol. Pharmacol.* **63,** 577–594.

Handa, R. J., Reid, D. L., and Resko, J. A. (1986). *Biol. Reprod.* **34,** 293–303.

Harada, N., Abe-Dohmae, S., Loeffen, R., Foidart, A., and Balthazart, J. (1993). *Brain. Res.* **622,** 243–256.

Honda, S., Harada, N., and Takagi, Y. (1994). *Biochem. Biophys. Res. Comm.* **198,** 1153–1160.

Hutchison, J. B. (1990). *Comp. Phys.* **9,** 27–44.

Ikeda, Y., Luo, X., Abbud, R., Nilson, J. H., and Parker, K. L. (1995). *Mol. Endocrinology,* **9,** 478–486.

Jakab, R. L., Horvath, T. L., Leranth, C., Haracla, N., and Naftolin, F. (1993). *J. Steroid Biochem. Molec. Biol.* **44,** 481–498.

Krey, L. C., MacLusky, N. J., Davis, P. G., Lieberburg, I., and Roy, E. J. (1982). *Endocrinology* **110,** 2159–2167.

Lephart, E. D., Peterson, K. G., Noble, J. F., George, F. W., and McPhaul, M. J. (1990). *Mol. Cell. Endocrinol.* **70,** 31–40.

Lephart, E. D., Simpson, E. R., and Ojeda, S. R. (1992). *J. Neuroendocrinol.* **4,** 29–35.

Lephart, E. D. and Ojeda, S. R. (1990). *Neuroendocrinology* **51,** 385–393.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). *J. Biol. Chem.* **193,** 265–267.

Naftolin, F., Ryan, K. J., Davies, I. J., Reddy, V. V., Flores, F., Petro, Z., Kuhn, M., White, R. J., Takaoka, Y., and Wolin, L. (1974). *Rec. Prog. Horm. Res.* **31,** 295–319.

Noma, K., Sato, B., Yano, S., and Yamamura, Y. (1975). *J. Steroid. Biochem.* **6,** 1261–1265.

Resko, J. A., Malley, A., Begley, D., and Hess, D. L. (1973). *Endocrinology* **93,** 156–161.

Rissman, E. F., Harada, N., and Roselli, C. E. (1996). *J. Neuroendocrinology* **8,** 199–210.

Roselli, C. E., Horton, L. E., and Resko, J. A. (1987). *Biol. Reprod.* **37,** 628–633.

Roselli, C. E. (1991a). *Endocrinology* **128,** 1310– 1316.

Roselli, C. E. (1991b). *Neuroendocrinology* **53,** 79–84.

Roselli, C. E. and Resko, J. A. (1984). *Endocrinology* **114,** 2183–2189.

Roselli, C. E. and Resko, J. A. (1991). In: *Neuroendocrine Research Methods.* Greenstein, B. (ed.). Harwood Academic Publishers: Reading, UK, pp. 937–951.

Roselli, C. E. and Resko, J. A. (1993). *J. Steroid. Biochem. Molec. Biol.* **44,** 499–508.

Roy, M. M. and Goy, R. W. (1988). *Horm. Behav.* **22,** 315–323.

Sachs, B. D., Pollak, E. I., Krieger, M. S. and Barfield, R. J. (1973). *Science* **181,** 770–771.

Sachs, B. D. and Meisel, R. L. (1994). In: *The Physiology of Reproduction.* Knobil, E. and Neil, J. (eds.). Raven: New York, pp. 3–105.

Schumacher, M. and Balthazart, J. (1986). *Brain. Res.* **370,** 285–293.

Steimer, T. and Hutchison, J. B. (1990). *J. Neurobiol.* **21,** 787–795.

Steimer, T. H. and Hutchison, J. B. (1989). *Brain. Res.* **480,** 335–339.

Van Doorn, E. J., Burns, B., Wood, D., Bird, C. E., and Clark, A. F. (1975). *J. Steroid. Biochem.* **6,** 1549–1554.

Ward, I. L. (1969). *Horm. Behav.* **1,** 25–36.

Yamada, K., Harada, N., Tamaru, M., and Takagi, Y. (1993). *Biochem. Biophys. Research Comm.* **195,** 462–468.

Yuan, H., Bowlby, D. A., Brown, T. J., Hochberg, R. B., and MacLusky, N. J. (1995). *Endocrinology* **136,** 96–105.