Pergamon

0960-0760(94)00192-8

J. Steroid Biochem. Molec. Biol. Vol. 52, No. 5, pp. 469–477, 1995
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0960-0760/95 $9.50 + 0.00

Subcellular Localization and Kinetic Properties of Aromatase Activity in Rat Brain

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The conversion of testosterone to estradiol is catalyzed by cytochrome *P*450 aromatase. *In situ* aromatization is required for the full expression of the effects of testosterone in the brain. This study examined the subcellular distribution and reaction kinetics of aromatase in the adult rat brain. Preoptic area, hypothalamus and amygdala were homogenized in isotonic sucrose buffered with potassium phosphate. Tissue homogenates were fractionated by ultracentrifugation. Aromatase activity was measured using a previously validated ^3H_2O assay. Marker enzymes were measured to identify organelles in the different subcellular fractions. Aromatase activity in all 3 tissues was enriched 10-fold in microsomes, but not in other subcellular fractions. The addition of either a NADPH-generating system or 1 mM NADPH to the reaction mixture stimulated aromatase activity in all subcellular fractions, whereas NADH was only minimally effective. In general, substrate affinity constants were equivalent in all brain areas and subcellular fractions (~10 nM) suggesting that one predominant catalytic form of the enzyme is present in the rat brain. One week after castration, aromatase activity was significantly reduced in all subcellular fractions of preoptic area and in the whole homogenate and microsomal fraction of the hypothalamus. Castration did not significantly alter aromatase activity in any subcellular compartment of amygdala. To more critically evaluate its subcellular localization, aromatase activity was measured in purified synaptosomes. Aromatase activity was not enriched in these preparations suggesting that it is not substantially associated with nerve terminals in rat brain.

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INTRODUCTION

The conversion of androgens to estrogens in brain tissues is catalyzed by cytochrome *P*450 aromatase. Aromatization is required for both the sexual differentiation of the brain and the display of adult male sexual behaviors [1, 2]. In adults of several species, the highest levels of aromatase activity are found in the bed nucleus (n) of the stria terminalis and cortical and medial amygdaloid nuclei. Substantial enzyme activity is also found within the medial preoptic n, periventricular preoptic area, and ventromedial n of the hypothalamus [3–6]. These nuclei are reciprocally interconnected [7–10] and generally regarded as a neural circuit associated with reproductive functions in both sexes [2, 11].

Testosterone stimulates aromatase activity in the bed n of the stria terminalis, medial preoptic n, periventricular area, ventromedial n, and medial amygdala, but not in other hypothalamic nuclei or the cortical amygdaloid n [3]. These observations suggest that androgens regulate brain aromatase activity in a region-specific fashion [12]. In addition, androgen-regulated aromatase activity is significantly greater in males than in females [13]. Regional variations and sex differences in the control of brain aromatase are probably important determinants of androgen action since they could modulate the responsiveness of aromatase-containing neurons to circulating androgens.

Several groups have detected aromatase-like immunoreactivity in the rat and mouse brain [14–17]. These studies generally failed to observe aromatase-immunopositive cells in brain areas that typically exhibit moderate to high levels of aromatase activity, in particular the corticomedial amygdala, periventricular preoptic area, medial preoptic n, and ventromedial n. The reason for the lack of a direct correlation between

Received 29 Aug. 1994; accepted 24 Nov. 1994.

the distribution of aromatase activity and immunoreactivity in rat brain is not understood. One explanation is that aromatase could be located in nerve terminals and as such may be difficult to detect immunocytochemically [15, 17]. The support for this argument is derived largely from a study in quail demonstrating that aromatase is found in nerve terminals, i.e. synaptosomes [18]. However, the question of whether aromatase is a synaptosomal enzyme in rats remains speculative.

The present study was undertaken to determine the subcellular distribution of aromatase activity in the rat brain. In addition, we wished to determine whether the kinetic properties, cofactor requirements, and regulation of aromatase are the same in all cellular compartments.

METHODS

Animals

Thirty-eight adult male Sprague–Dawley rats were used in this study. They were group housed, given free access to food and water, and maintained on a 12 h light–dark lighting schedule. Experiments and animal care were conducted in accordance with the principles and procedures outlined by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with the approval of the Institutional Animal Use and Care Committee at Oregon Health Sciences University.

Preparation of subcellular fractions

The preoptic area (POA), hypothalamus (HYP) and amygdala (AMYG) were obtained as described previously [9]. Freshly dissected tissues were pooled from 2–4 male rats and homogenized 1/10 (w/v) in ice-cold potassium phosphate buffered 0.32 M sucrose (10 mM potassium phosphate, 100 mM KCl, 1 mM dithiothreitol and 1 mM EDTA, pH 7.4). The homogenizations were performed with Potter–Elvejham tissue grinders using 10 complete up and down strokes. All subsequent procedures were carried out at 0–4°C. An aliquot of the homogenate was reserved for determination of enzyme activity and the remainder was centrifuged at 800 *g* for 5 min. The resulting pellet was resuspended in a volume of phosphate buffered sucrose equal to the initial homogenization volume and again centrifuged at 800 *g* for 5 min. This step was repeated once. The resulting pellet is referred to as the P_1 pellet. The 800 *g* supernatants were combined and sequentially centrifuged at 11,000 *g* and 100,000 *g* for 20 min each in a Beckman TL100 ultracentrifuge. The 11,000 *g* and 100,000 *g* pellets are referred to as the P_2 and P_3 pellets, respectively. The final 100,000 *g* supernatant is referred to as the S_3 supernatant. Based on observations by Gray and Whittaker [20], the cell subfractions were designated as follows: P_1, unbroken cells, cell debris and cell nuclei; P_2, mitochrondia and synaptosomes; P_3, microsomes; and S_3, cytosol.

Purified synaptosomes were prepared using the method of Löscher *et al.* [21]. For this procedure, tissues pooled from 2 rats were homogenized in 0.32 M sucrose buffered with 10 mM Tris (pH 7.4) and centrifuged at 1000 *g* for 10 min. The low speed supernatant was layered onto 1 ml of 1.2 M sucrose in 11 × 17 Ultra-Clear tubes (Beckman) and centrifuged in a swinging bucket TLS-55 rotor at 220,000 *g* for 15 min (acceleration setting = 3; deceleration setting = 2). The resultant pellet (mitochondria) was discarded, whereas the gradient interface (synaptosomes, myelin and some microsomes) was carefully collected and diluted with 1 ml 0.32 M sucrose. The diluted suspension was then layered onto 1 ml of 0.8 M sucrose and centrifuged at 220,000 *g* for 15 min. The pellet from this centrifugation step is comprised of synaptosomes and was reserved for enzymatic assays.

Aromatase and marker enzyme assays

Aromatase activity was assayed in homogenates and subcellular tissue fractions by using a radiometric assay that measures the stereospecific loss of hydrogen from the C-1β position of [1β-^{3}H]androstenedione (NEN Research Products, Boston, MA) and its incorporation into ^3H_2O. In this assay, the production of ^3H_2O is proportional to the amount of estrogen formed. The details of our assay procedure have been described previously and validated against a product-isolation assay for use with rat brain tissue [22]. Aromatase activity is expressed as fmol ^3H_2O produced/h·mg protein. Protein was measured by the method of Lowry [23].

We measured the relative amounts of biological marker enzymes to assess the distribution of various cellular organelles in the different subcellular fractions. Succinate dehydrogenase (SDH) was measured to identify mitochondria [24]; and NADPH cytochrome C reductase (NCR) was measured to identify microsomes [25]. Each assay was performed in duplicate on two or more separate preparations.

The relative specific activity is expressed as the percent of total enzyme activity recovered in a subcellular fraction divided by the percent of total protein in that fraction. Relative specific activity values >1 indicate an enrichment of enzyme in a subcellular fraction relative to the activity in the homogenate. The relative specific activity was plotted on the ordinate and the percent total protein on the abscissa so that the area of each block is proportional to the percentage distribution of the component in each fraction (deDuve diagram [26]).

Electon microscropy

Purified synaptosomes were examined morphologically. Pellets were washed and then incubated in 200–300 μl Krebs' buffer for 10 min at 37°C. They were fixed overnight at 4°C in glutaraldehyde (3% w/v) and formaldehyde (2% w/v) buffered in 0.1 M sodium cacodylate (pH 7.4) in 1.5 ml polypropylene microcentrifuge tubes. After a rinse with 5% sucrose buffered in

0.1 M sodium cacodylate (pH 7.4), the pellets were dislodged and postfixed at room temperature for 1 h in buffered 2% osmium tetroxide. They were then incubated for 30 min in a filtered, saturated solution of uranyl acetate (~8% w/v) in water. The samples were dehydrated with an ethanol series (10, 30, 55, 75, 95 and 100% v/v in water) and then 100% ethanol : toluene (1:1) and finally twice in pure toluene. The pellets were infiltrated with resin by immersing them sequentially in toluene:expoxy 1:1 by vol for 1 h followed by 1:2 by vol for 12 h. They were embedded in fresh epoxy heated at 65°C for 3 days and then sectioned with an ultramicrotome. Sections were viewed on a JES-100 electron miscroscope (JEOL) and micrographs were taken at random levels throughout the pellets and printed at 40,000 × magnification.

Statistics

Changes in the aromatase activity after castration in the different subcellular fractions were analyzed by two-way analysis of variance, followed by Scheffe's test [27], where appropriate. Data was log transformed when variances were found to be unequal. Comparisons of aromatase activity between homogenates and purified synaptosomes were performed with Student's *t*-tests.

RESULTS

Exp. 1. Subcellular distribution and pyridine nucleotide requirement of brain aromatase

The relative distribution of aromatase activity in the primary subcellular fractions from the POA, HYP and AMYG of male rat brain is shown in Fig. 1. The largest portion of total cellular aromatase (45–53%) was found in the P_1 fraction, probably because this represents an incompletely homogenized fraction. Equivalent percentages of the total cellular aromatase activity were associated with P_2 and P_3 fractions (18–24%), whereas the S_3 fraction contained the lowest proportion (8–10%) of aromatase activity. When compared to the whole homogenate, aromatase activity was enriched approx. 10-fold in the P_3 pellet but not in the other subcellular compartments. As expected [28], the P_3 fraction was enriched in microsomes as indicated by the relative specific concentration of NCR, whereas the mitochondrial marker enzyme SDH was enriched in the P_2 pellet.

Our routine aromatase assay uses an NADPH-generating system. To determine whether this provides sufficient reducing equivalents and whether the pyridine nucleotide requirement is the same in all subcellular fractions, we examined the effect of different pyridine nucleotide cofactors on the measurement of aromatase activity. We found that aromatase activity in all subcellular fractions and brain tissues was stimulated by the additon of either an NADPH-generating system of 1 mM NADPH (Table 1). Although a minimal stimulation was observed when 1 mM NADH was used as cofactor, aromatase activity, corrected for activity observed when no cofactor was added, was generally less than 10% of that observed when NADPH was provided to the incubation.

Exp. 2. Kinetic properties of aromatase activity in different subcellular tissue fractions

Prior to this study, we found that the propylene glycol (⩽5% v/v), routinely used to solubilize [1β-^{3}H]androstenedione, caused a 3–4-fold increase in the apparent K_m determined from saturation experiments, but did not alter the estimate of V_{max} (our unpublished observations). For this reason, we did not add propylene glycol to our substrate mixture in the present studies.

Saturation experiments were performed to measure the apparent K_m and V_{max} of aromatase activity in different subcellular fractions of POA, HYP and AMYG and determine if they showed the same kinetic values as whole homogenates. Aromatase activity was detectable and displayed typical Michaelis–Menton kinetics in all subcellular fractions examined (Table 2). In the POA and AMYG, the apparent K_m values in all subfractions were comparable to those observed in the whole homogenates and, as expected, the V_{max} was highest in the P_3 pellet (microsomes). This was represented by a 4- and 2.5-fold increase in the specific activity of aromatase in POA and AMYG, respectively. In the HYP, the apparent K_m values in both the crude nuclear pellet (P_1 pellet) and cytosol (S_3 supernatant) tended to be higher (i.e. lower affinity) than the values determined in the homogenate and other subfractions, whereas the V_{max} in the microsomal fraction represented a 3-fold increase compared to the specific activity of aromatase in the homogenate.

Exp. 3. Effect of castration on aromatase activity in different subcellular tissue fractions

To further understand the localization and regulation of aromatase, we examined the effect of castration on activity in each cell subfraction. Aromatase activity in all three tissues was highest in the microsomal fraction from both intact and castrated rats (Fig. 2). In POA, aromatase activity was significantly reduced in all subcellular fractions after castration ($F[1,48] = 56$; $P < 0.001$); no significant interaction between cell fraction and treatment was observed ($F[4,48] = 2$; $P = 0.08$). In HYP, there was a significant effect of treatment ($F[1,46] = 30$; $P < 0.001$) as well as a significant interaction ($F[4,46] = 9$; $P < 0.001$). Scheffe's test revealed that aromatase activity was significantly reduced in the whole homogenate and P_3 pellets after castration. In contrast, aromatase activity in the AMYG was not significantly altered after castration ($F[1,75] = 4$; $P = 0.06$), nor was the interaction between cell fraction and treatment significant ($F[4,75] = 0.18$; $P = 0.95$).

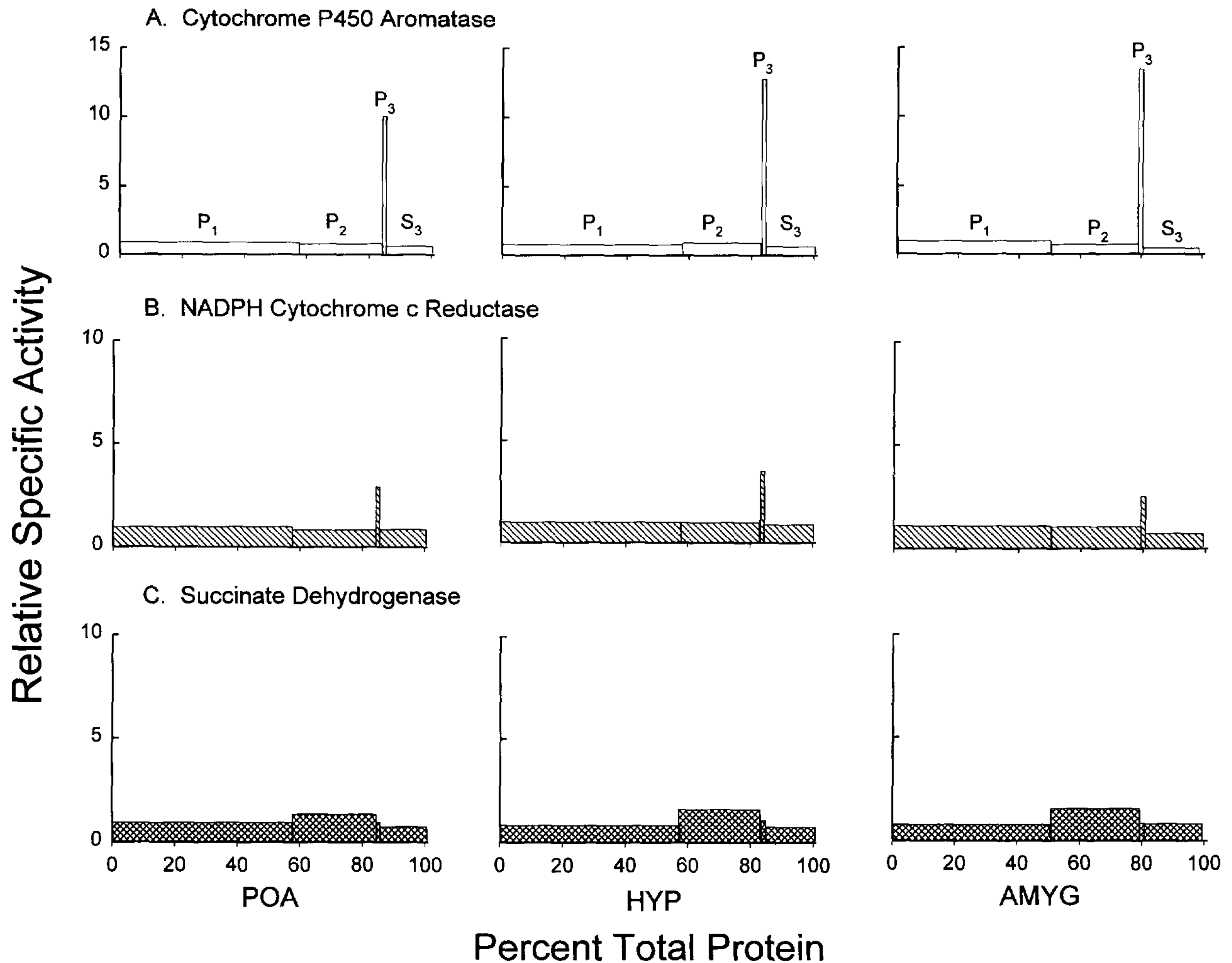

Fig. 1. Subcellular distribution of aromatase activity in the adult male rat brain in relation to established marker enzymes for microsomal NADPH cytochrome *c* reductase and mitochondrial succinate dehydrogenase. Tissue fractionations and incubations were performed as described in "Methods". The data represent the means of two independent experiments. The subcellular fractions are: P_1 (800 *g* pellet), cell debris and nuclei; P_2 (11,000 *g* pellet), mitochondrial and synaptosomes; P_3 (100,000 *g* pellet), microsomes; and S_3 (100,000 *g* supernatant), cytosol. The relative specific activity is expressed as the percent of total enzyme activity recovered in a subcellular fraction divided by the percent of total protein in that fraction. Relative specific activity values >1 indicate an enrichment of enzyme in a subcellular fraction relative to the activity in the homogenate. The relative specific activity was plotted on the ordinate and the percent total protein on the abscissa so that the area of each block is proportional to the percentage distribution of the component in each fraction (deDuve diagram [26]). Mean recoveries of enzyme activities in subcellular fractions relative to homogenates was: aromatase, 80%; NADPH cytochrome *c* reductase, 123%; and succinate dehydrogenase, 86%.

Exp. 4. Measurement of aromatase activity in synaptosomes isolated by sucrose density gradient centrifugation

In the previous experiments synaptosomes were sedimented in the P_2 pellet together with mitochondria [28]. To more critically evaluate the subcellular location of aromatase, we purified synaptosomes by sucrose gradient centrifugation according to the method of Löscher *et al.* [21].

The morphology of the synaptosomal preparation is shown in Fig. 3. Electron microscopic examination revealed the presence of membrane-bound particles that were densely packed with synaptic vesicles and contained well-preserved intrasynaptosomal mitochondria.

Aromatase activity was significantly reduced in synaptosomal preparations from all three brain regions (Fig. 4). Saturation analyses revealed an apparent K_m of approx. 10 nM that was not different between homogenates and synaptosomal preparations (data not shown).

DISCUSSION

In this study, we found that aromatase activity was enriched approx. 10-fold in microsomes prepared from

Table 1. The effect of reduced pyridine nucleotides on male rat brain aromatase activity

Subcellular fraction	Cofactor: None	NADPH generating system	NADPH	NADH
POA				
Homogenate	8.82*	124.83	120.56	16.10
P_1 pellet	13.44	122.20	109.36	20.42
P_2 pellet	9.76	116.50	112.21	17.84
P_3 pellet	3.33	699.66	747.81	21.10
S_3 supernatant	ND	68.83	67.14	5.90
HYP				
Homogenate	5.58	42.93	40.60	6.45
P_1 pellet	2.67	24.00	17.03	1.23
P_2 pellet	4.84	37.64	35.33	6.07
P_3 pellet	ND	244.52	201.36	1.88
AMYG				
Homogenate	10.18	317.84	422.94	26.08
P_1 pellet	11.12	305.60	280.58	31.01
P_2 pellet	10.14	246.42	258.98	22.92
P_3 pellet	5.98	ND	2090.60	78.20

*Aromatase activity expressed as fmol ^3H_2O/h·mg protein. Values are the means of 2 independent experiments that each used tissues pooled from 4 male rats.
ND, not determined.

rat brains. Aromatase activity was found in other subcellular fractions, but the relative specific activity of the enzyme in these other fractions was less than one. These results suggest that aromatase activity is specifically associated with microsomal membranes in the rat brain and not with other cellular compartments or organelles. In addition, we did not observe regional differences between the POA, HYP and AMYG in the pattern of the subcellular distribution of aromatase activity. Thus, the subcellular location of aromatase does not appear to be regionally specified in rat brain.

Ryan [29] was the first to demonstrate that aromatase is a microsomal enzyme. Other reports have described aromatase activity associated with mitochondria in placenta [30, 31], but based on cofactor requirements and physico-chemical properties this activity appears to be due to microsomal contamination of the mitochondrial fraction [31]. In contrast to our results in rats, aromatase activity is enriched in both microsomes and P_2 pellets (11,000 *g* pellet) derived from quail brains [18]. The P_2 pellet contains a mixture of mitochondria and

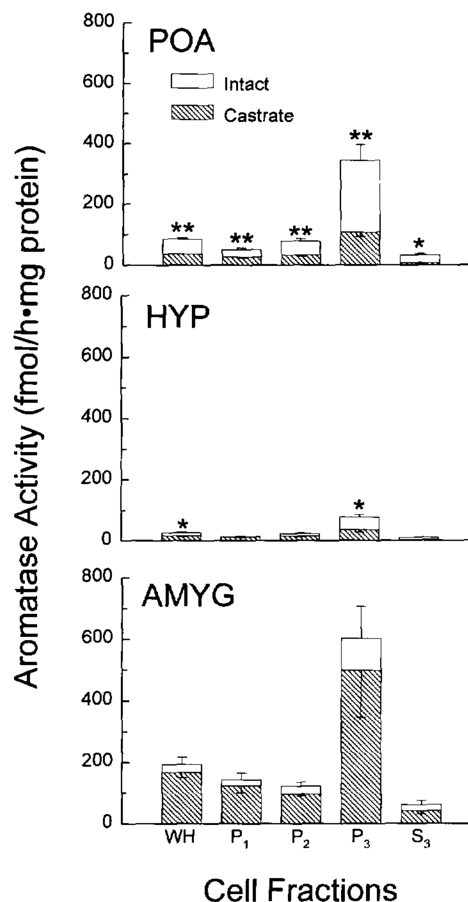

Fig. 2. Effects of castration (1 week) on subcellular compartmentation of aromatase activity in POA, HYP, and AMYG. Data are presented as means ± SEM of 4–9 independent replications. Each replication was performed on tissues pooled from 2 rats. Abbreviations for cell fractions are as in Fig. 1 except for WH, i.e. whole homogenate. Data were analyzed by 2-way ANOVA, and treatment effects were tested by Scheffe's test (*$P < 0.05$ intact vs castrate; **$P < 0.001$ intact vs castrate).

Table 2. Kinetic properties of aromatase activity in subcellular fractions of rat brain

Fraction	Preoptic V_{max}*	Preoptic K_m†	Hypothalamus V_{max}	Hypothalamus K_m	Amygdala V_{max}	Amygdala K_m
Homogenate	98 ± 3	10 ± 1	26 ± 1	8 ± 2	380 ± 2	11 ± 2
P_1 pellet	55 ± 0.5	12 ± 0.4	21 ± 2	21 ± 5	264 ± 7	9 ± 1
P_2 pellet	75 ± 5	12 ± 3	23 ± 1	11 ± 2	190 ± 4	8 ± 8
P_3 pellet	380 ± 11	11 ± 2	77 ± 8	8 ± 3	978 ± 88	11 ± 3
S_3 supernatant	35 ± 2	10 ± 2	8 ± 1	21 ± 9	122 ± 4	7 ± 1

Kinetic values are the means of two independent experiments and were determined using non-linear regression analysis (ENZFITTER computer program, Biosoft, Cambridge, U.K.). Statistics are standard errors of the estimate.
*V_{max} = fmol/h·mg protein.
†K_m = nM.

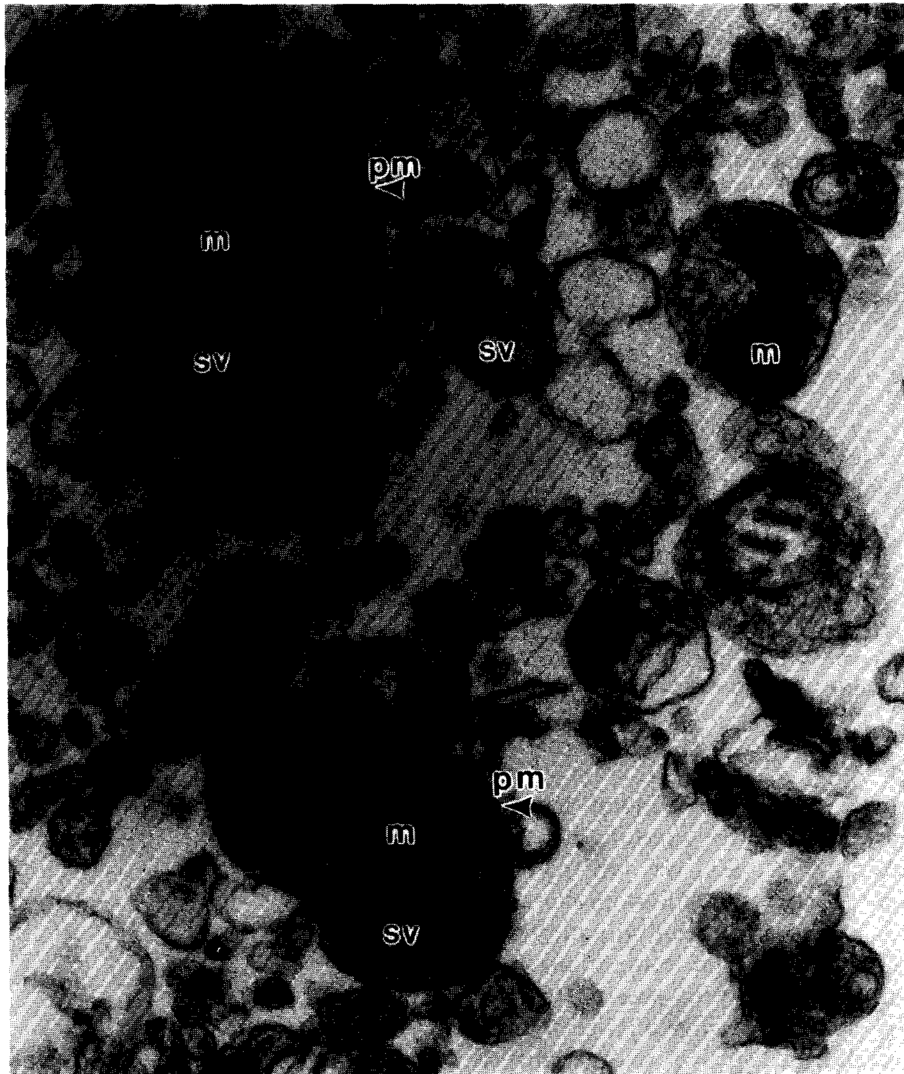

Fig. 3. Morphological appearance of sucrose-purified preoptic synaptosomes. Note the presence of synaptic vesicles (sv) and mitochondria (m) enclosed within plasma membrane (pm) to form a synaptosome. Photographed at 40,000× magnification.

synaptosomes [20]. When this fraction was further subjected to hypotonic lysis and ultracentrifugation, aromatase activity was associated with small particles which were enriched with the microsomal marker NCR, whereas little activity was associated with larger particles identified as mitochondria. These results were interpreted as indicating that aromatase activity is enriched in nerve terminals, i.e. synaptosomes. The preliminary report of the existence of aromatase-immunopositive synaptic vesicles in the medial POA of the quail brain further supports this conclusion [32]. That we did not find aromatase activity enriched in P_2 pellets from rat brain suggests that there are species differences in the subcellular compartmentation of aromatase in brain. Moreover, our results do not support the view that the discrepancies between the distribution of aromatase activity and immunoreactivity in the rat brain are attributable to differences in the subcellular localization of aromatase in certain brain areas.

Aromatase activity exhibited an absolute requirement for NADPH in all brain areas. Our results confirm previous studies using placenta [29, 33] which clearly demonstrated that the aromatase reaction requires NADPH and cannot be supported by NADH. NADPH-cytochrome $P450$ reductase is a ubiquitous protein of the endoplasmic reticulum and is responsible for transferring reducing equivalents from NADPH to any microsomal form of cytochrome $P450$ with which it comes into contact, including cytochrome $P450$ aromatase [34]. Thus, the cofactor requirement displayed by brain aromatase further supports the conclusion that it is predominantly a microsomal enzyme in neural tissue.

Kinetic studies revealed that brain aromatase had a high substrate binding affinity, apparent $K_m \approx 10$ nM.

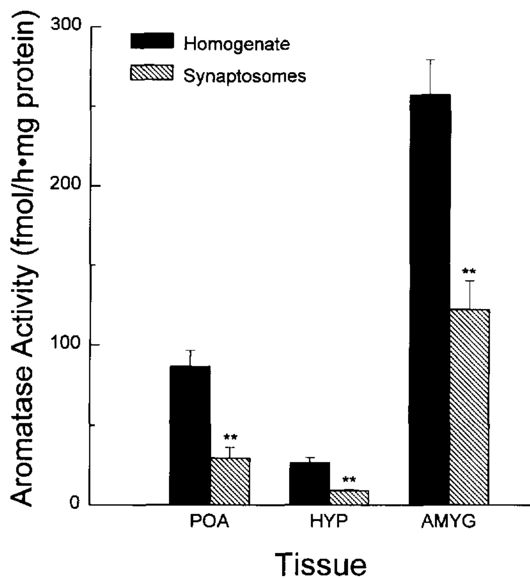

Fig. 4. Aromatase activity in homogenates and sucrose-purified synaptosomes from adult male rats. The data are presented as the means ± SEM of 4 independent preparations. Comparisons of aromatase activity between homogenates and synaptosomes were performed with Student's *t*-tests ($P < 0.001$ homogenate vs synaptosome).**

This value is substantially lower than we reported previously [22, 35], and is directly attributable to the elimination of propylene glycol from the incubation mixture. Substrate affinity constants of this magnitude have been reported for several other species [36–39]. In general, the apparent K_m was equivalent in different brain regions and subcellular compartments suggesting that aromatase exhibits uniform catalytic properties throughout the limbic rat brain. The HYP may be an exception since K_m values in the nuclear and cytosolic fractions were approx. 2-fold greater than in the other compartments. The reason for this difference in enzyme affinity is not known. It could represent a real difference in enzyme characteristics or be due to the release of endogenous competitive inhibitors during the preparation of these subcellular fractions. Compartmental differences in aromatase substrate affinity have also been observed in doves [38].

The greatest levels of aromatase activity were associated with microsomal membranes in both intact and castrated males. Thus, the subcellular pattern of distribution for aromatase activity was not changed after castration. As expected, the response to castration was regionally specific. Aromatase activity was significantly decreased in the POA and HYP, but not in the AMYG. These results confirm our previous studies demonstrating that aromatase is differentially regulated in rat brain [35]. Subsequently, we found that aromatase activity is not uniformly regulated within the anatomical nuclei comprising these brain regions [3]. For instance, aromatase is regulated by androgens in the medial nucleus of the amygdala, but not in the cortical nucleus. Previous kinetic studies demonstrated that testosterone stimulates aromatase activity in POA and HYP by increasing the absolute quantity of enzyme, not by changing its catalytic efficiency [40]. The present results suggest that this effect is achieved without a significant change or shift in the compartmentation of aromatase. Recent evidence from our laboratory [41] demonstrated that aromatase in the rat brain is regulated pretranslationally, presumably at the level of gene expression. The mechanism underlying region-specific regulation of aromatase in brain is not known, but may involve the use of alternative promoters. An abundance of evidence suggests that this mechanism is a major factor in the differential expression of aromatase in human tissues [42]. More recently, preliminary evidence in rats suggests that brain aromatase mRNA transcripts are derived from a different promoter than in rat ovary or Leydig tumor cells [43].

Aromatase activity was not enriched in purified synaptosomes of any brain region. These results independently confirm the conclusion that aromatase is not substantially associated with presynaptic elements in the rat brain. However, they do not preclude the possibility that a minor fraction of all nerve terminals in any given region contains aromatase. Recently immunoelectron microscopic techniques were employed to localize aromatase in rat brain [44]. Aromatase-immunoreactivity was found in both cell bodies and neuronal processes and appeared to be associated with the membranes of the endoplasmic reticulum and nuclear envelope. However, it has not been determined *per se* whether synaptic terminals are immunoreactive.

The effect of androgen-derived estrogen on the expression of adult male copulatory behavior appears to be mediated by classical intracellular estrogen receptors since it requires the synthesis of new mRNA and protein and is inhibited by antiestrogens [45–47]. In addition, the areas of the rat brain that mediate copulatory behavior contain high levels of both aromatase activity and intracellular estrogen receptors [3, 48]. However, we do not know whether estrogens act within the same neurons that synthesize them. Dual-label immunohistochemical studies in quail found that fewer than 20% of all aromatase-positive cells contained estrogen receptors in the medial POA [49]. These results suggest that estrogens, synthesized in certain areas of the brain, exert behavioral effects by acting in a paracrine fashion to influence neighboring neural cells. This action of estrogens may be achieved by acting through classical intracellular receptors. However, it is also possible that locally-synthesized estrogens have non-genomic actions which could operate together with or instead of classical genomic actions [50]. The present results suggest that the synthesis of estrogen in rat brain is not localized within synaptic nerve terminals, but do not preclude the possibility that androgen-derived estrogens have non-genomic actions in brain.

In summary, the present study shows that cytochrome $P450$ aromatase in brain is a microsomal enzyme which displays a high affinity for its substrate and an absolute requirement for NADPH. Our results also indicate that aromatase exhibits similar affinity characteristics throughout the limbic brain indicating that there is one predominant catalytic form of the enzyme. Finally, we found that the specific activity of aromatase in purified synaptosomes was significantly lower than in tissue homogenates suggesting that aromatase is not substantially associated with nerve terminals in the rat brain. These results suggest that there are species differences in the subcellular localization of brain aromatase.

Acknowledgements—The author would like to recognize the technical assistance of Taiwo Fasasi in the completion of this work. This work was supported by NIH Grants HD-23293 and P30-HD-18185.

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