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EFFECTS OF CHANGES IN GONADAL HORMONES ON THE AMOUNT OF AROMATASE MESSENGER RNA IN MOUSE BRAIN DIENCEPHALON

Kazuyo Yamada*, Nobuhiro Harada, Masao Tamaru** and Yasuyuki Takagi

Division of Molecular Genetics, Institute for Comprehensive Medical Science, Fujita Health University, School of Medicine, Toyoake, Aichi 470-11, Japan

**Department of Medical Information Sciences, Faculty of Medical Engineering, Suzuka University of Medical Science and Technology, Suzuka,Mie 510-02, Japan

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Aromatase in brain is known to play a crucial role in development and maintenance of androgen dependent sexual behavior of males. Biochemical studies have shown that the aromatase activity in brain is influenced by androgen. In this study, we measured the aromatase mRNA content of mouse brain quantitatively to gain a deeper insight into this phenomenon. We found that in the diencephalic area it is sexually dimorphic, as reported for aromatase activity. The amount of aromatase mRNA in this area in males was 150% higher than that in females and decreased to the level in females after castration. The content of aromatase mRNA in castrated males was elevated by 2-fold by injection of testosterone and restored to the level observed in intact males. Injection of testosterone also affected the level of aromatase mRNA in normal mice of both sexes, though to lesser extent. On the contrary, injection of estrogen decreased the amount of aromatase mRNA in gonadectomized mice of both sexes. These results show that transcriptional control of the aromatase gene is involved in the mechanism of testosterone to affect sexual behavior.

Aromatase cytochrome P-450(P-450_{AROM}) (EC11.14.14.1) catalyzes the conversion of androgen to estrogens, a rate limiting step in estrogen biosynthesis. This enzyme has received considerable attention because of the essential roles of its reaction products estrogens, in reproductive as well as metabolic processes. In humans, this enzyme activity is found mainly in the ovary and placenta, but has also been detected in some extra-gonadal sites, such as adipose tissue, muscle and liver. The regulation of aromatase expression is complex, involving multiple factors and is coordinated in a tissue-specific manner(1,2).

*To whom correspondence and reprint requests should be addressed. Fax:81(Japan)562-93-8833.

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Naftolin et al.(3) first reported the existence of aromatase activity in the brains of rats and mice, and since then this activity has been found in the brains of all major vertebrate groups (4). *In vivo* and *in vitro* studies have shown that brain aromatase is concentrated in the hypothalamus-preoptic area and limbic structures, which are associated with reproductive functions(5-7). The biological significance of aromatase activity in the brain is its involvement in the action of androgen (8,9). Expression of normal sexual behavior in males is known to depend on androgens: castration diminishes sexual behavior, and administration of testosterone, a major androgen produced in the testis, restores normal sexual behavior. Interestingly, administration of androgen with an aromatase inhibitor or a certain antiestrogen abolishes this effect of androgen. Thus some effect of androgen on sexual behavior is believed to be exerted after its aromatization. Furthermore, estrogens formed *in situ* are considered to be important for this effect(the aromatization hypothesis)(10). These locally produced estrogens in the brain are also suggested to play crucial role in the sexual differentiation of the fetal brain towards the development of male patterns of control of gonadotropin secretion and sexual behavior (8).

Brain aromatization of androgen and its involvement in male sexual behavior and brain development have been studied extensively in rats and some avian species(8,9,11,12). These studies showed that the aromatase activity in the brain is influenced by testosterone, being decreased by castration and restored to the level of intact mature males by testosterone treatment. In this paper, we used a new technique to quantitate small amounts of specific mRNA(13) and measured the content of aromatase mRNA in mouse brain, to determine whether expression of aromatase is regulated at the pretranslational transcriptional level.

Materials and Methods

Reagents. RAV-2 reverse transcriptase and Taq DNA polymerase (AmpliTaq) were purchased from Takara Shuzo Co., (Kyoto). SeaPlaque GTG agarose was obtained from FMA Bioproducts. All other reagents were of the highest quality available.

Animals. Seven-week-old ICR mice were obtained from Nippon SLC Co. (Hamamatsu, Japan). They were kept in an animal room at 23-25°C and given free access to tap water and chow pellets. Animals were gonadectomized at eight weeks old, and kept for another four weeks under the same conditions. Three weeks after the operation, some animals received a daily injection of testosterone(400μg) or β-estradiol(8μg) for four days. Treated and untreated animals were sacrificed the day after the last injection at twelve weeks old.

The diencephalic area was obtained by making a coronal cut anterior to the optic chiasm and a second cut at the caudal edge of the mammillary bodies. The excised region extended dorsally to the superior border of the third ventricle and laterally to the edge of the tuber cinereum.

Preparation of RNA. Tissues were excised and immediately frozen in liquid nitrogen. They were homogenized in 5M guanidine isothiocyanate containing 5mM

sodium citrate and 0.5 % sodium sarcosyl, and total RNA was precipitated by centrifugation through 5.7M CsCl as described by Chirgwin *et al.*(14).

Assay of aromatase mRNA. Details of the method used for quantitative analysis of aromatase mRNA by a combination of reverse transcription and PCR with a fluorescent primer are described elsewhere(13). Briefly, two attomoles(2×10^{-18} moles) of internal standard RNA, aromatase mRNA with an insert of 44 bases to give a PCR product of different size from that of native aromatase mRNA, was added to 10μg of the total RNA fraction and subjected to reverse transcription with a specific primer for aromatase mRNA. The resulting cDNAs were amplified by PCR with a specific fluorescent primer, and the PCR products were analyzed in a 2% SeaPlaque GTG agarose gel with a Gene Scanner 362 Fluorescent Fragment Analyzer(Applied Biosystems). Twenty-one cycles of PCR amplification were performed in the assays described in this paper.

Results and Discussion

In humans, the ovary and placenta are the well recognized sites of estrogen production, but aromatase activity has also been found in various extra-gonadal tissues, and an association of estrogen synthesized in these extra-gonadal tissues with the development of certain types of cancer in women after menopause has been suggested. As we reported previously(13), in mice no aromatase mRNA was detected in non-gonadal tissues other than brain by 21 cycles of PCR amplification of cDNA, which allowed detection of as little as 0.01 attomoles of aromatase mRNA/10μg RNA. The amount of aromatase mRNA in the gonads was high in both females and males. To examine whether gonadal hormones affect the expression of aromatase in other tissues, we gonadectomized both male and female mice and measured their aromatase mRNA contents. Four weeks after gonadectomy, however, we detected no expression of aromatase in various tissues other than brain including adipose tissues(Table I).

On the contrary, in the brain we detected measurable amounts of aromatase mRNA in both normal and gonadectomized mice. The brain aromatase mRNA in male mice decreased 45% from 21.0×10^{-3} attomoles /μg RNA to 11.5×10^{-3} attomoles/μg RNA after castration, the resultant lowered level being similar to that in normal females(13.5×10^{-3} attomoles/μg RNA). Ovariectomy did not cause significant change in the aromatase mRNA content in the brain.

Next we examined the effects of administration of gonadal hormones on the level of brain aromatase mRNA(Fig 1). Four consecutive daily injections of testosterone (400μg/animal/day) increased the content of aromatase mRNA by 200% in gonadectomized males, to the level in intact males. Similar increase of the aromatase mRNA content by administration of testosterone was seen in ovariectomized female. The effect of testosterone was also observed in intact control animals of both sexes, but to a lesser extent(150%). Thus the highest level of aromatase mRNA was detected in normal male mice given testosterone.

Table I. Effect of gonadectomy on aromatase mRNA in various mouse tissues

Tissue	Aromatase mRNA($\times 10^{-3}$attomoles /mg of total RNA)			
	males	castrated males	females	ovariectomozed females
Brain diencephalic area	21.0	11.5	13.4	11.9
Liver	<1	<1	<1	<1
Adrenal grand	<1	<1	<1	<1
Adipose tissue	<1	<1	<1	<1
Kidney	<1	<1	<1	<1
Lung	<1	<1	<1	<1
Testis	1170			
Ovary				810-3080

Total RNA fractions from various tissues, prepared as described in the text were reverse-transcribed with internal standard RNA and amplified by 21 cycles of the PCR with fluorescent primer. The PCR products were quantitated fluorometrically. The analysis was repeated three times for each sample and the aromatase mRNA content was determined by comparison with an internal standard RNA.

Regulation of aromatase activity in the brain has been investigated extensively in rats (5,6) and some avian species(11,12,15). In studies involving microdissection and microassay techniques, aromatase activity was shown to be localized in the hypothalamus-preoptic area and amygdala of rat brain. The activity in hypothalamus-preoptic area was sensitive to the androgen level of animals. Namely, the activity in this area of male rats was higher than that in females, was decreased after castration to the level in females, and was restored to that of normal sexually mature males by administration of testosterone. In quail brain, androgen dependent regulation of aromatase was demonstrated not only by measuring enzyme activity but also by immuno-cytochemical staining with a specific anti-P-450_{AROM} antibody against purified human placental aromatase(16). In the study, the number of aromatase-positive cells increased in the preoptic area(17), where elevation of the enzyme activity was observed. On the other hand, the activity in the amygdala is considered to

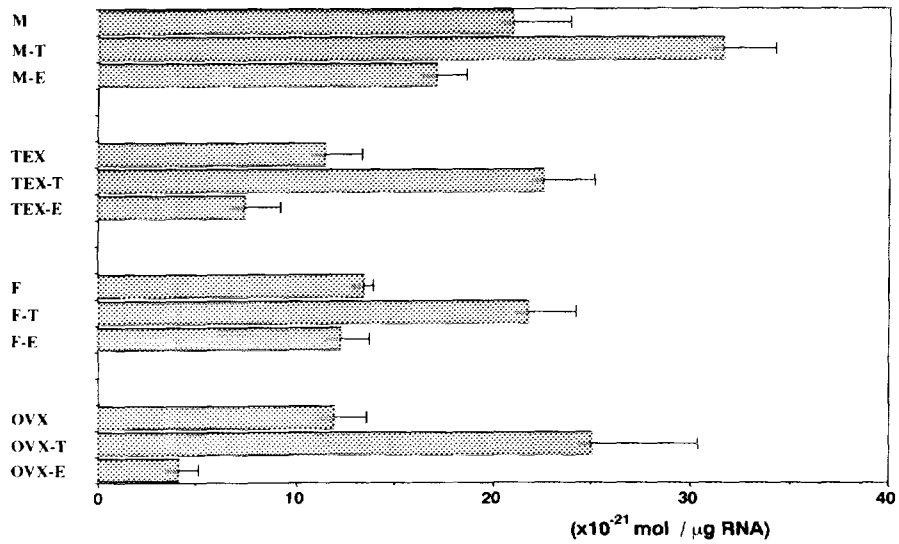

Figure 1. Effects of changes in gonadal hormones on the amount of aromatase mRNA in mouse brain diencephalon. Total RNA fractions were prepared as described in the text. M; males, F; females, TEX; castrated males, OVX; ovariectomized females, -T; treated with testosterone, -E; treated with β-estradiol. The contents of aromatase mRNA were quantitated as described. Each sample was assayed separately three times. Bars are means ±SE for four samples.

be insensitive to androgen: it is not significantly different in males and females and is not affected by administration of testosterone. The change of aromatase mRNA contents shown in our study corresponded well to the changes in activity described in these previous studies, since the diencephalon preparation we used included the hypothalamus-preoptic area but was not likely to include a significant portion of the amygdala.

Androgen receptor is suggested to be involved in induction of aromatase activity by testosterone, because only androgens are effective and concomitant administration of antiandrogens blocks the effects(18). Moreover, testosterone did not induce aromatase activity in the hypothalamus-preoptic area of testicular feminized rats, congenitally deficient in the number of intracellular androgen receptors(19). In this study we showed that expression of the aromatase gene in the diencephalon is regulated by testosterone, and that this regulation of mRNA level is most likely to be responsible for the change in enzyme activity. The major question raised by this *in vivo* study is whether this transcriptional stimulation is exerted directly through transcriptional control of the aromatase gene by androgen receptor in target cells, or indirectly through neural networks, involving other neurotransmitters.

In our final experiment, injection of β-estradiol was found to decrease the level of aromatase mRNA in gonadectomized mice of both sexes, and especially in females(57% decrease). It had no significant effect, however, in normal animals. Suppression of aromatase activity by estrogen has not been reported. In several species, β-estradiol was shown to stimulate brain aromatase activity (15,20) in castrated males, and its synergistic stimulation with 5α-dihydrotestosterone was described, whereas estradiol alone was reported to have no effect on the enzyme activity(6,21). At present, the mechanism of decrease of aromatase mRNA by estrogen is not known. Other gonadal hormones may participate in the regulation of brain aromatase expression especially in females, since the suppression by β-estradiol was observed only in gonadectomized mice. There may also be species differences in this effect of estrogens.

The expression of aromatase has been shown to be regulated by various factors, including cAMP, phorbol ester, glucocorticoids and growth factors in tissues such as the ovary(22,23) and adipose tissues(24). cDNA probes encoding aromatase have been successfully used in Northern blot analysis to determine steady state levels of aromatase mRNA in these tissues, and in most cases, changes in activity of aromatase induced by these factors were found to parallel changes in the levels of aromatase mRNA. The regulation of aromatase seems to be tissue specific. Brain aromatase is unique in its regulation that androgen is the only factor found so far to affect its activity. In this paper, we applied a quantitative method of PCR-mediated amplification of reversely transcribed mRNA for analysis of aromatase mRNA in mouse brain which is not detectable by Northern blot analysis. We showed for the first time that the aromatase mRNA content of rodent brain is regulated pretranslationally by testosterone. The change in mRNA level observed here was in good agreement with the reported change in aromatase activity in rat brain(5,6).

The physiological importance of brain aromatase has also been established in the determination of sexual differences in brain functions during the neonatal stage. As we showed previously(13), aromatase expression in neonatal mouse brain is sexually dimorphic, the expression being highest in the critical period for determination of sex differences in brain function. Together with our previous findings in neonatal brain, the results described here provide evidence that aromatase expression is regulated at the mRNA level in the brain. Recently, Lephart *et al.* examined aromatase activity and the content of aromatase mRNA in pre- and neonatal brains of rats and showed that increase in P-450 $_{AROM}$ mRNA levels determine, at least in part, enzymatic activity during these periods(25). The regulatory factors other than androgen, and the implications of these controls on brain function remain to be elucidated.

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