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Distribution and Postnatal Changes of Aromatase mRNA in the Female Rat Brain

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Distribution and postnatal changes of aromatase mRNA in the Wistar strain female rats brain were investigated to elucidate the mechanism of the region-specific and stage-related regulations of brain aromatase activity. Total RNAs extracted from the hypothalamus–preoptic area (HPOA), amygdala, cerebral cortex, cerebellum and anterior hypophysis were subjected to a quantitative reverse transcription-polymerase chain reaction-Southern blotting (RT-PCR-SB) assay. The levels of aromatase mRNA were as follows: amygdala > cerebral cortex ≒ HPOA ≫ cerebellum ≒ anterior hypophysis. These results roughly paralleled the distribution of the aromatase activity and the aromatase protein reported previously, with the exception of that of the cerebral cortex. The postnatal developmental patterns of aromatase mRNA in the HPOA and cerebral cortex were further studied. The levels of aromatase mRNA in the HPOA tended to be high around birth (day 0) and to decrease at days 4–8 followed by no remarkable change to an adult. The developmental pattern in the HPOA was essentially similar to that of aromatase activity. On the other hand, in the cerebral cortex where very low or no aromatase activity was reported throughout developmental stages, aromatase mRNA was low but detectable level around day 0, thereafter gradually increased to an adult level. From these results, it is inferred that synthesis of the aromatase protein by the level of the mRNA seems to mainly regulate the aromatase activity in a region-specific and stage-related fashion in most parts of the rat brain, except for the cerebral cortex where the posttranscriptional regulation may play an important role.

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INTRODUCTION

The formation of estrogens from androgens, which is catalyzed by aromatase *P*-450 enzyme, is believed to mediate the sexual differentiation of neural structures and reproductive behavior [1–4]. Aromatase activity has been detected in several regions of the rat brain. The level of aromatase activity is highest in the hypothalamic and limbic areas but there is little or no activity in the cerebral cortex, cerebellum and anterior pituitary [5–8]. Furthermore, previous reports on ontogeny of brain aromatase activity [9, 10] revealed that the activity in the hypothalamus was the highest at late embryonic stage and high at early postnatal stage followed by decreasing to an adult level with very low or no activity in the cerebral cortex throughout all developmental stages. The mechanism of these region-specific and stage-related regulations of the brain aromatase activity has not been clarified yet. Recently, the aromatase protein was demonstrated by immunohistochemical studies in a majority of the brain areas where aromatase activity was present (with a few exceptions) [11–14], suggesting that the level of aromatase protein is strongly associated with the aromatase activity in the rat brain. Therefore, investigation of the distribution of the aromatase mRNA in the brain was necessary to elucidate the region-specific and stage-related regulations of aromatase activity. However, little information on the mRNA was available so far. In this context, we attempted to conduct biochemical detection and comparison of aromatase mRNA in the rat brain. Since intensity of the signal of aromatase mRNA detected by Northern blotting in our preliminary study was very weak in the amygdala (data not shown), which contained the greatest amount of aromatase activity in the rat brain [6–10], it indicated the inadequate sensitivity of Northern blotting for analyzing the level of the message in the various brain regions. In the present study, we employed a quantitative reverse transcription-polymerase chain reaction-Southern blotting (RT-PCR-SB) assay to investigate distribution and postnatal changes of aromatase mRNA in the rat brain.

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MATERIALS AND METHODS

Animals and tissues

To analyze the intracerebral distribution of aromatase mRNA, three groups of 8-week-old diestrous Wistar strain female rats, consisting of 3 rats per group, were killed by decapitation and then the hypothalamus and preoptic area (HPOA), amygdala (AMY), cerebral cortex (CC), cerebellum (Ce), anterior hypophysis (AP) were dissected and removed, as described previously [15]. To analyze the postnatal changes of aromatase mRNA, female Wistar strain rats at −2, 0, 2, 4, 8, 12 and 18 days of age, and at 8-week-old (diestrous) were used. The rats were divided by ages, and then subdivided into 3 subgroups per age (n = 5–6/subgroup for ages: −2, 0 and 2 days, n = 3/subgroup for ages: 4, 8, 12, 18 days and 8 weeks). The CC and the HPOA were dissected from the rats. In addition, the ovary from 8-week-old diestrous rats was removed for use as a positive control because it has the greatest activity of aromatase.

Chemicals and laboratory equipment

All reagents used in these studies were the highest grade available. Water was double distilled and autoclaved, or treated with an additional 0.1% diethylpyrocarbonate and reautoclaved. All equipment was autoclaved and/or sterilized at 200°C to eliminate RNase activity. Microtubes, pipette tips and centrifugation tubes were used only once to avoid contamination with amplified or cloned genes.

RNA extraction

The tissues were frozen in liquid nitrogen and kept at −80°C until RNA extraction. Total RNA was extracted from each tissue by the guanidium–cesium chloride ultracentrifugation method [16] and the RNA concentration was determined by UV absorption.

Reverse transcription (RT)

Total RNA extracted from each tissue was reverse transcribed to synthesize single stranded cDNA. Briefly, various amounts from five brain tissues (to analyze the intracerebral distribution, see below), 20 ng from the HPOA and the CC at each developmental stage (to analyze the ontogeny), and graded diluted ovarian total RNA were incubated at 42°C for 60 min with 5 U of RAV-2 reverse transcriptase (Takara, Kyoto, Japan) in a 25 μl reaction volume containing 50 mM Tris–HCl (pH 8.3), 100 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM for each dNTP and 10 μM random hexadeoxynucleotide primer (Takara, Kyoto, Japan).

Oligonucleotide primers

The sequences of the oligonucleotide primers for aromatase mRNA are as follows; rAMs: 5′-CTGAACATCGGAAGAATGCACAGGC-3′, and rAMas: 5′-ATTTCCACAATGGGGCTGTCCTCAT-3′. The primer set flanked the rat aromatase cDNA sequence from base 1261 to base 1544, as numbered by Hickey *et al.* [17], which was considered to contain a splicing site judging from the human aromatase gene structure [18]. The sequences of the primers for β actin mRNA are as follows; βAs: 5′-ATCGTGGGCCGCCCTAGGCA-3′, and βAas: 5′-TGGCCTTAGGGTTCAGAGGGG-3′. The primer set flanked the rat β actin cDNA sequence from base 100 to base 343, as numbered by Nudel *et al.* [19], which consisted of the first and second exons.

Polymerase chain reaction (PCR)

The single stranded cDNA was subjected to PCR. Briefly, 1 μl of cDNA (1/25 of obtained cDNA) was amplified in a 25 μl reaction volume containing 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 10 mM Tris–HCl (pH 8.3), 200 μM of each dNTP, 10 μM of each primer and 1.25 unit of *Taq* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT, U.S.A.). The reaction was performed for 26 cycles (to analyze aromatase mRNA) or 18 cycles (to analyze β actin mRNA). Each cycle consisted of an incubation period of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C with the last phase of 72°C of the last cycle extended to 11 min.

Direct nucleotide sequencing

The RT-PCR product generated from either ovary or HPOA, purified by electrophoresis, was subjected to direct nucleotide sequencing using *Taq* DNA polymerase according to the dideoxy method of Sanger *et al.* [20] with modifications.

Southern blotting (SB)

One microliter of the RT-PCR product (1/25 of obtained product) from each tissue was electrophoresed in a 2.0% agarose gel. After the electrophoresis, the product was transferred onto a nylon membrane (Hybond N+, Amersham) using 400 mM NaOH as a transfer solution for 3 h. The membrane was prehybridized in the prehybridization buffer [6 × SCC (1 × SCC: 0.15 M sodium chloride–0.015 M sodium citrate), 150 μg/ml yeast RNA, 1% sodium dodecil sulfate (SDS)] at 42°C for 3 h. Then the membrane was hybridized with a ^{32}P labeled rat aromatase or β actin cDNA probes in the same buffer at 65°C for 12 h. The probes were generated from the respective RT-PCR products by the random priming method (random priming DNA labeling kit, Takara). After the hybridization, the membrane was rinsed twice in 2 × SSC, 1% SDS at room temperature for 10 min and then washed twice in 0.1 × SSC, 1% SDS at 65°C for 20 min. The hybridization signal was analyzed by a bio-image analyzer, BAS2000 (Fuji Film, Tokyo, Japan).

Comparison of the levels of aromatase mRNA and β actin mRNA

In order to compare the levels of aromatase mRNA and β actin mRNA, total RNA of 1280 ng from AP and

Ce, 160 ng from AMY and CC, 40 ng from HPOA (to analyze the intracerebral distribution of aromatase mRNA), 20 ng of the total RNA from five brain regions (to analyze the level of β actin mRNA), 20 ng from the HPOA and the CC at each developmental stage (to analyze the ontogeny of both mRNAs), and graded diluted ovarian total RNAs; 20, 10, 5, 2.5, 1.3, 0.63, 0.32, 0.16, 0.08 ng; were simultaneously subjected to RT-PCR-SB. Using the standard curve which was generated from the amount of ovarian RNAs, the level aromatase mRNA in each tissue was calibrated.

RT-PCR blank

When distilled water, used as a RT-PCR blank, was simultaneously subjected to the RT-PCR-SB with the same reagents, no specific signal could be obtained, indicating that no contamination of any reagents occurred in these experiments.

Statistics

Data were expressed as the mean $\pm$ SD of the 3 groups ($n = 3$/group). A paired Student's t-test was used to test for statistical significance of difference between tissues.

RESULTS AND DISCUSSION

The RT-PCR product from the rat brain

The RT-PCR product of 284 bp, which corresponded in length to the distance between the 5′-ends of the two primers on the rat aromatase cDNA, was generated from the five regions of the brain and ovary. Since the amplified region contained a splicing site (which was predicted by comparison with the aromatase gene structure of the human [18]), the product of 284 bp could originate from aromatase mRNA in these tissues and not from genomic DNA. Two slightly different sequences of the rat aromatase cDNAs were reported; the cDNA sequence derived from the ovarian granulosa cells was reported by Hickey *et al.* [17], and the sequence from the Leydig cell tumor line was reported by Lephart *et al.* [18]. The nucleotide sequence of the RT-PCR product in this study was identical to the former one, implying that mutations of the aromatase gene might occur in the tumor cells. Furthermore, it was reported that three different aromatase mRNAs were expressed in the rat ovary; only the largest mRNA species (at 2.7 kb) appeared to be functional and the two smaller species could not encode a functional aromatase as they lacked the sequence corresponding to the heme-binding domain [21]. However, the RT-PCR product in this study could only originate from the "functional" mRNA and not from the "non-functional" mRNA species because the primers for aromatase mRNA flanked the region including the heme-binding domain corresponding sequence of the cDNA.

A standard curve for comparison of the levels of aromatase mRNA

A standard curve was prepared from the radioactivity of the signals of the RT-PCR products generated from the graded diluted control RNAs (data not shown). Duplicate assays, which resulted in a highly reproducible response pattern, showed a linear correlation between the logarithmic value of the radioactivities of the product signals and that of the weight of the template RNA within the range between 10 and 0.32 ng of its starting weight. Using the standard curve, the level of rat aromatase mRNA could be estimated in different tissues and developmental stages.

The intracerebral distribution of rat aromatase mRNA

The levels of aromatase mRNA were as follows: AMY > CC $\fallingdotseq$ HPOA $\gg$ Ce $\fallingdotseq$ AP (Fig. 1), which roughly paralleled the level of aromatase activity and aromatase protein with the exception of CC [5–8, 11–14]. These findings suggest that the level of the aromatase mRNA mainly regulated aromatase activity in a region-specific fashion through the regulation of synthesis of aromatase protein, in the most of the rat brain regions. It should be noted that a relatively high level of aromatase mRNA was detected in CC where the level of aromatase activity and aromatase protein were reported to be little or absent [5–8, 11–14].

The postnatal changes of the aromatase mRNA in the rat brain

The postnatal developmental patterns of aromatase mRNA in the HPOA and CC were further studied to elucidate the mechanism of the stage-related

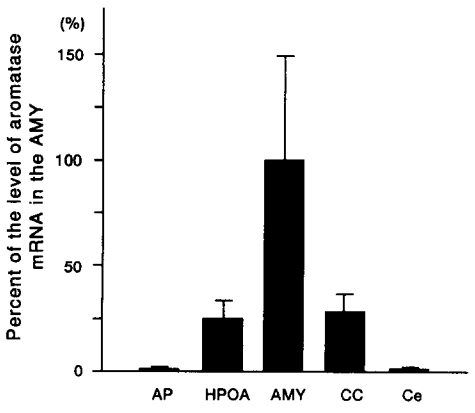

Fig. 1. The intracerebral distribution of rat aromatase mRNA. Total RNAs of 1280 ng from AP and Ce, 160 ng from AMY and CC, 40 ng from HPOA were subjected to a quantitative RT-PCR assay. The level of aromatase mRNA was calculated using the standard curve and the mean level of aromatase mRNA obtained in the AMY was assigned a value of 100%. Each value represents the mean and SD (vertical bar) of 3 groups. HPOA: hypothalamus and preoptic area AMY: amygdala, CC: cerebral cortex, Ce: cerebellum, AP: anterior hypophysis, RT-PCR: reverse transcription-polymerase chain reaction.

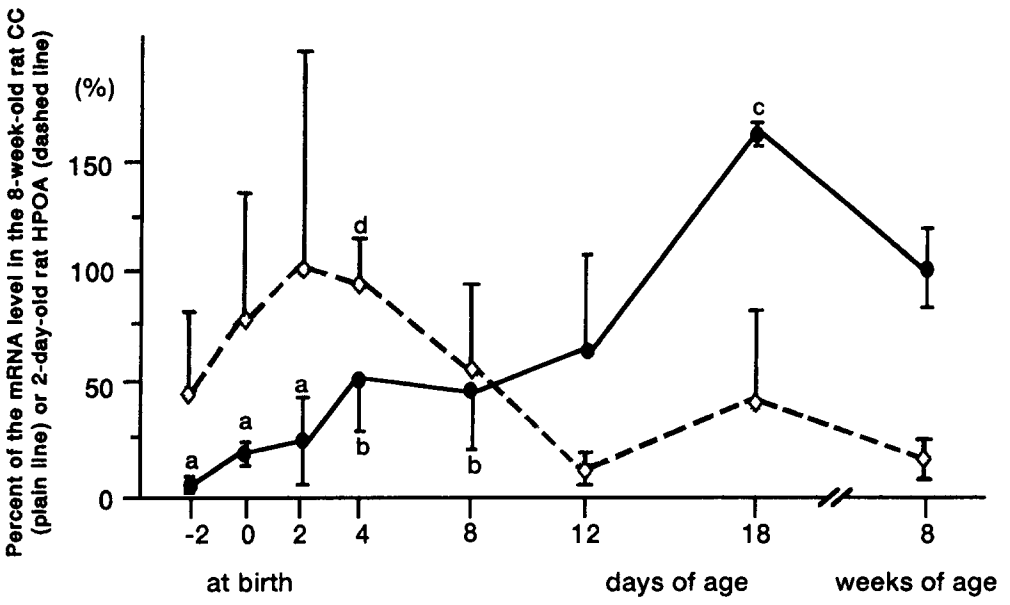

Fig. 2. The ontogeny of aromatase mRNA in the HPOA (dashed line) and the CC (solid line). 20 ng from the HPOA and the CC at each developmental stage were subjected to a quantitative RT-PCR assay. The mean level of aromatase mRNA obtained in the HPOA of the 2-day-old rat (dashed line) and CC of the 8-week-old rat (solid line) was assigned a value of 100%, respectively. Each value represents the mean and SD (vertical bar) of 3 groups. HPOA: hypothalamus and preoptic area, CC: cerebral cortex. a: significantly lower ($P < 0.01$), b: significantly lower ($P < 0.05$), and c: significantly higher ($P < 0.01$) than the level of the mRNA in the CC of the 8-week-old rat. d: significantly higher ($P < 0.01$) than the level of the mRNA in the HPOA of the 8-week-old-rat.

regulations of brain aromatase activity, especially to analyze the developmental changes in dissociation between the levels of the message and protein in the CC. The levels of aromatase mRNA in the HPOA tended to be high around birth (day 0) and to decrease at days 4–8 followed by no remarkable change to an adult (Fig. 2, dashed line). The aromatase activity in the hypothalamus was reported to be the highest at late embryonic stage (embryonic days 18–20) and high at early postnatal stage followed by gradually decreasing to an adult level [9, 10]. Our data on the postnatal changes in the mRNA in the tissue was essentially similar to that of aromatase activity. On the other hand, in the CC where very low or no aromatase activity was reported throughout all developmental stages [9, 10], a very low but detectable level of aromatase mRNA was found at 2 days before birth. Then the cortical massage level was gradually increased to an adult level (Fig. 2, solid line). In order to validate the RNA concentrations which were determined by UV absorption, the levels of β actin mRNA in the same RNA were analyzed. When the levels of aromatase mRNA were calibrated by the respective levels of β actin mRNA, the postnatal changes of the calibrated aromatase mRNA in both tissues were essentially similar to those without calibration (data not shown). Thus the postnatal developing patterns of aromatase mRNA indicated in Fig. 2 were considered to reflect true changes in the level of the message.

From these results, it was indicated that the postnatal developmental changes in the level of aromatase activity in the HPOA might be caused by the changes in its message level. On the other hand, in the CC, mismatch between the level of aromatase activity and aromatase mRNA increased with development, implying that the posttranscriptional regulation might play an important role in the tissue.

Aromatase mRNA in the CC

Dissociation of the levels between aromatase mRNA and aromatase protein may not be only specific to CC, because mismatch of the levels of the mRNA and the protein has been also reported in the rat corpus luteum at mid-gestation [22–24]. Possible explanations for the mismatch in CC may be; (1) "non-functional" mRNA species containing heme-binding domain corresponding sequence are specifically expressed in the CC, (2) specific inhibitory factors of translation of aromatase mRNA is present in CC [25–32], (3) aromatase proteins are inactivated by other enzymes [33]. In addition, it was reported that a mismatch of the levels between aromatase activity and aromatase protein was found in several brain regions, such as the medial preoptic area and the bed nucleus stria terminals [14]. Although reasons for the divergent relationship among the level of the mRNA, the protein and the activity remain unclear, these mismatches indicate that the region-specific and stage-related regulation of aromatase activity may also involve multiple factors which are not yet identified especially in the CC. Further study on the mechanism of gene expression of aromatase is essential to elucidate biophysical role of a gradual

increase in the level of aromatase mRNA in the CC from a neonate to an adult.

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