Biomedical **Reviews** 1997; 7: 41-50

ISSN 1310-392X

DEVELOPMENTAL PROFILE AND REGULATION OF BRAIN ESTROGEN SYNTHESIS BY AROMATASE

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SUMMARY

• *Aromatase cytochrome P450 enzyme catalyzes the formation ofestrogenfrom androgen in distinct regions of the vertebrate brain. During neural development, local estrogen synthesis is required for the sexual differentiation of male brain characteristics and the differentiation of neural circuits Involved in sex-specific behaviors and neuro endocrine functions. A common phenomenon in all species studied so far is that the male brain displays much higher and. brain region-specific aromatase expression during distinct periods of brain development than the female one. An important question arising from these findings is which factor(s) play a role in regulating estrogen formation in a sex- and brain region-specific fashion. Among the factors investigated, androgens have been found to be the most powerful regulators of brain aromatase. In vitro experiments using primary cell cultures of embryonic mouse brains showed that sex differences in aromatase activity in hypothalamic cells develop, at first, independently of gonadal steroids. Later during embryonic development, aromatase expression is regulated in a region- and sex-specific way by circulating androgens. Hypothalamic aromatase neurons are inost sensitive to androgen exposure whereas cortical and midbnlin,(> H'x are insensitive. Moreover, androgens affect morpliolt\\!'*col. maturation ofaromatase-immunoreactive cells by stimulating neurite outgrowth and dendritic arborization. These findings suggest that androgens function as major rnorphogenetic factors during the differentiation of the mammalian hypothalamic aromatase system. During late embryonic development and perinatally, androgen levels differ between sexes, being significantly higher in males. This time period corresponds exactly to the developmental stage when aromatase activities are highest in the male hypothalamus. It seems plausible therefore that higher androgen concentrations in the male circulation during ontogenesis are causally connected with the observed sex differences in hypothalamic aromatase activity. (Biomed Rev 1997; 7: 41-50)*

Received for publication 5 April 1997 and accepted 7 June 1997.
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INTRODUCTION

• Structural and functional sex differences in the vertebrate central nervous system (CNS) have been described at almost every level (reviewed in 1). Although the way in which brain morphological dimorphisms between sexes contribute to functional sex differences is not well understood, sex-specific behaviors and centrally regulated processes are generally believed to rely on such structural differences (2, 3). Sex differences in the adult CNS are generally confined in terms of volumes of brain nuclei to differences in cell numbers/densities and synaptic morphology (3-6). Most, but not all, sexually differentiated nuclei of the mammalian hypothalamus are larger and contain more -cells in males than in females (7-12). It has to be noted, however, that sex differences are not restricted to the hypothalamus. A number of recent investigations have demonstrated sexually dimorphic structures and functions in almost all brain regions such as the striatum, cortex, and hindbrain (1, 13-16).

An important question which has been intensively studied over the last lour decades concerns the developmental processes which are responsible for a sexually dimorphic organization of CNS structures. As early as 1959, Phoenix and collaborators (17) published a fundamental study on the effects of gonadal steroid hormones during perinatal development on sexual behavior in guinea pigs. It was suggested that gonadal steroids may cause a sex-specific organization of the male CNS during acritical perinatal period of development. Later on it was demonstrated that the testosterone metabolite 17B-estradiol was indeed the biologically active compound controlling sexually dimorphic development ol the male brain (18,19). Direct evidence lor the aromatization of androgens to estrogens by central neural tissues was provided by Naftolin c^o/ (20). These findings led to the ,,aromatization hypothesis" postulating that androgen effects on the differentiation of male-specific brain circuitries and functions in vertebrates are actually mediated by estrogens formed locally in discrete brain areas from androgens by the enzyme aromatase. The aromatase is an enzyme complex comprised of a specific form of microsomalcytochromeP450 and the flavoprotein NADPH-cytochrome P450 reductase (mixed function oxidase) and catalyzes the conversion of testosterone into 17B-estradiol (21). Studies of the avian aromatase system have contributed to an understanding of how this enzyme system develops in relation to a complex sexually dimorphic motor pattern - song. One of the most interesting models is the Zebra finch, in which there is a sexual dimorphism in the size of brain nuclei controlling song. In males, both the volume and cell size of motor nuclei, and higher forcbrain nuclei involved in the control of song, are larger than those in females (25). The development of the song control system depends in part on estrogens acting on the brain in post-hatching development (25). The source of estrogen is still unclear. However, sex-specific circulating levels of estrogen (higher levels in the male) may be due (o extremely high rates of aromati/.ation in the telencephalon at post-hatching periods of development (26,27). .

Over the years, a considerable number of investigations have now confirmed that perinatal estrogen exposure of the CNS permanently changes neuronal/glial morphology and brain functions in males (reviewed in 2, 22-24).The sexually dimorphic nucleus of the medial prcoptic area (SDN-POA) is probably one of the best studied sexual dimorphic structures of the mammalian brain. Originally described in Nissl-stained frontal sections of the rat dicncephalon, this cytoarchitechtonically heterogeneous nucleus was found to be five to eight times larger in male than in female rats (9). Subsequent developmental studies revealed that the differentiation of this nucleus depended on a massive increase in volume in the male due to higher neuronal numbers which did not occur in the female (29,30). Interestingly, sex differences in the SDN-POA are already apparent by postnatal day 2 (29, 30). Prom these and other studies it was concluded that the ontogeny of sex differences in the vertebrate brain takes place during a limited critical period of neural development during late embryonic stages and the first postnatal days (31). Later on, the establishment of the SDN-POA was clearly shown to depend on the perinatal steroid hormone milieu (31,32), and it has been demonstrated that the development of the SDN-POA requires the aromatization of androgens, i.e. is mediated by estrogens (33,34). Itis now generally accepted that estrogens formed within the brain play a decisive role for the development of most of the sex-specific characteristics in the CNS so far described, and that these sexual dimorphisms become established during a hormone-sensitive critical period of development, usually around birth (2,4,6,22). As shown above for the differentiation of the SDN-POA, estrogens arc capable first affecting the neuromorphology by directly regulating cell proliferation and/or cell death. Besides determining the fate of neurons/glial cells, estrogens also modulate functional (35) and neuromorphological properties, such as cell size (33), synapse formation (37-39), axon outgrowth (40, 41), and dendritic arborization (41,42). Finally, it is believed that developmental estrogen effects at the cellular level represent the basis for sexual dimorphisms in brain circuitries responsible for sex-specific behaviors and neuroendocrine functions (2, 3, 28). Notwithstanding the importance of the epigenetic action of estrogens for the generation of sexual dimorphic brain structures and functions as shown above, there is, in addition, emerging evidence that genetic factors, probably coded by Y-chromosomal genes, are involved in the process of sexual differentiation of the CNS (1,3,10-12,24,43-45). It might be concluded that sexual differentiation of the brain requires not only epigenetic, hormone-dependent mechanisms but also the brain-intrinsic realization of the genetic program in particular neuronal populations.

Although the role of estrogens formed in distinct brain areas for the process of sex-specific neuronal development in vertebrates is intensively studied and reviewed by several authors in the past (22,46-48), little is known about the regulation of the key enzyme in estrogen synthesis, the aromatase, during embryonic and early postnatal development in male and female CNS. In this review we will focus on developmental aspects of the regulation of brain aromatase expression and activity in the rodent and avian brain. Thisreviewwill, where appropriate, go into the particulars of the regulation of aromatase in the adult brain. Finally, recent studies will be presented which have utilized molecular biology techniques to investigate brain aromatase.

DISTRIBUTION AND DEVELOPMENTAL PROFILE OF BRAIN AROMATABE

M Aromatase enzyme activity has been detected in several limbic brain tissues of all vertebrate species studied so far (49, 50). Indirect (51,52) as well as direct (53) evidence clearly suggests the aromatase is a neuron-specific enzyme in the rodent CNS, whereas in the avian brain, residual aromatase activity has

also been reported for glial cells (54). Using aromatase-specific antisera and immunocytochemical staining techniques, analysis of the subcellular distribution of aromatase revealed that this enzyme is confined to the cell so ma as well as to axons, dendrites, synaptic boutons (55, 56) and even growth cone-like structures (57). These immunohistochemical findings are in agreement with the previous demonstration of aromatase activity in synaptosomal and microsomal preparations (58,59).

Aromatase activity and aromatasc-immunoreactive nerve cells arc not uniformly distributed throughout the brain but rather arc highly concentrated in discrete limbic and hypothalamic ureas (52, 60-62) whereas only low to moderate levels of aromatase activity can be detected in several other brain regions, such as cortical areas, the hippocampus, the pituitary, and the midbrain (52,53,63-66). Generally, immunocytochemical undm .\Vf((hybridization or poly merase chain reaction studies (65,67-f)9) of the aromatase and the aromatase mRNA, respectively, have confined the distribution and localization of aromatase to CNS structures where aromatase activity has been previously detected. Highest brain aromatase levels in adults are found in dicncephalic structures such as the bed nucleus of .s'fna f<?/'-mmo/As- (BNST) and the medial preoptic nucleus as well as in the medial and cortical amygdala compared to intermediate levels which have been measured in the anterior hypothalamus and the ventromedial nucleus (62)

Consistent with its important role for the sexual differentiation of reproductive brain functions, levels of hypothalamic uromatase activity are significantly greater during the perinatal period in rodents than at any subsequent age. Hypothalamic aromatase activity rises rapidly before birth in rodents (mouse, rat), followed by a sharp decrease during the initial postnatal period (52, 69-71). This is in agreement with findings which demonstrate peak levels in hypothalamic aromatase mRNA expression at this developmental stage in the mouse and rat (69-72). In addition, immunocytochemical screening for aromatase-containing neurons during the development of the rat brain revealed that these cells can be first detected in the preoptic area on the 13th day of gestation, followed later on from day 15th onwards in the BNST and amygdala. As gestation progresses, the number of aromatase-positive neurons and staining intensity for aromatase in the diencephalon gradually increases and peaks around birth (73). During early postnatal development, hypothalamic aromatase decreases to lower levels until the onset of sexual maturation when a further decline in enzyme activity can be observed (47,74,75). An important outcome of studying developmental characteristics of hypothalamic aromatase is the finding that a highly significant sex difference in aromatase activity levels exists throughout development. In general, males show higher enzyme activities than females (47, 62, 63, 70, 75), and more aromatase-immunoreactive cells are found in males (52,53,76). The existence of a sexual dimorphism in estrogen synthesis within the hypothalamus and preoptic region during perinatal development is regarded as the basis for the sexual differentiation of male brain structures associated with reproductive functions (4,25,28,91).

In addition, there is evidence that an developmental profile of aromatase expression, as described above for the hypothalamus, may also exist in the rat midbrain. The major difference between the both brain regions is that, in the midbrain, aromatase expression (enzyme activity and levels of aromatase mRNA) is low around birth and shows a distinct peak postnatally between postnatal day 2 and 10 (65). In contrast to the hypothalamus, sex differences were not observed in the developing midbrain. An important question arising from the sex- and brain region-specific expression of aromatase concerns which developmental factors arc involved in regulating this ontogenetic profile of brain estrogen synthesis in males and females.

DBVELOPMENTAL REGULATION OF BRAIN AROMATA9B

M Although brain aromatase has been investigated for more than two decades, little is known about the regulation of estrogen synthesis, in particular in the developing brain. Despite the fact that the regulation of neural aromatase is still a matter of controversy, this section will focus on the characterization of the effects of steroid hormones, neurotransmitters, and other biologically active compounds on brain aromatase activity/expression during perinatal development.

As shown in the previous section, hypothalamic aromatase activity in the mouse displays a clear-cut developmental profile and highest activity levels just before and around birth. During this period, sex differences in estrogen synthesis capacity are most significant. However, it has to be mentioned that early sex differences in aromatase activity and in numbers of aromatase-immunoreactive cells appear to be already manifested at embryonic day 13 (53, 91). These data imply that hypothalamic aromatase is highly regulated during late embryonic brain development and leads to the following questions: (;) What does the enzyme and is the regulation sex-specific?, and (;V) Is aromatase regulation phasic and do sensitive regulatory periods differ between sexes? A number of studies have demonstrated that the classical neurotransmitter norepinephrine and the B-adrenergic agonist isoproterenol inhibit neural aromatase in rat tissue and cell cultures (77,78). Moreover, factors known to stimulate the formation of cAMP have been shown to be potent inhibitors of aromatase activity (77). These findings contrast with another study using cultured turtle brain cells which has found that dihutyryl cAMP enhances enzyme activity (79). Similarly, cAMP appears to increase aromatase activity in cxtragonadal and gonadal tissues (80, 81). Taken these data, it appears that adrenergic regulation of aromatase is different in

neuronal and non-neuronal cells and between species, and it is likely that adrenergic neurotransmission coupled to the cAMP-dependcnt inlracellular signal transduclion pathway plays to some extent a role in the short-term regulation of aromatase activity, possibly by enzyme phosphorylation. This regulatory pathway cannot, however, explain neither long-lasting changes in aromatase expression, i.e. the developmental profile, nor the observed sex differences, since a higher activity of noradrenergic systems located in the locus coeruleus and projecting to the hypothalamus is generally associated with the male sex (reviewed in 82). It might be assumed that the adrenergic input for the hypothalamic aromatase system reflects rather a short-term modulatory than a long-term regulatory mechanism. Other factors tested for their ability to change hypothalamic aromatase activity such as fibroblast growth factors, epidermal growth factor, and insulin-like growth factors, were ineffective (77). In comparison to these *in vitro* data, *in vivo* studies have shown thai prenatal stvess, alcohol and nicotine exposure affect brain aromatase. Fetuses of stressed mothers displayed reduced aromatase activities in both sexes (83), ethanol exposure increased enzyme activity in males only (84), and nicotine treatment abolished sex differences in hypothalamic aromatase (85). In conclusion, these *in vitro* and *in vivo* studies give some insights in the regulation of perinatal hypothalamic aromatase but are not sufficient to answer the questions raised at the beginning of this section. In particular, the complex regulation of a transient developmental and sex-specific profile of aromatase expression cannot be explained by the above factors. ;,«;'

The failure to find clear-cut regulatory factors, drew the attention of several research groups to gonadal steroids as potential aromatase regulators. In particular, androgens secreted by the fetal testes fullfill several criteria to be regarded as aromatase regulators during development: (/) the onset and profile of androgen synthesis and secretion into the systemic circulation coincides exactly with the profile of hypothalamic aromatase expression (86, 87), (/;') there are sex differences in androgen plasma concentrations during late embryonic and early postnatal development (86, 87), and *(Hi)* embryonic hypothalamic, bul not cortical aromatase-immunoreactive neurons coexpress androgen receptors (57). For the first time in 1981, we reported thai androgens induce aromatase in the adult avian brain (88). In the following years, we have shown that, in addition, embryonic avian aromatase is positively regulated by androgens (89). These studies led to the hypothesis that androgens are not only the substrate lor ihe aromatase bul also function as important regulatory compounds for the developing and adult avian brain aromatase system (90, 91). At about the same time, it has been demonstrated that androgens also regulate hypo-Ihalamic aromatase through a receptor mechanism in the adult rat (47, 92) and in other mammalian species (93), and thai androgen ic stimulation of aromatase activity is due to an transcriplional induclion of aromalase mRNA expression in the hypothalamus (94-96). Mosl surprisingly, subsequent studies on the androgenic regulation of neural aromatase in the developing mammalian brain failed to demonslrate an unambiguous role for androgens. Castration of fetal monkeys, for example, had no effect on hypothalamic aromatase (97), androgens either stimulated aromatase in transplants of fetal rat hypothalamic tissue (98) and in embryonic ferret diencephalic nuclei (99), decreased aromatase in cultured rat neural tissue (77), or had no effect on aromatase in rat hypothalamic explants (77). In order to reexamine the influence of androgens on developing hypothalamic aromatase nerve cells more systematically as well as to focus in these studies on the cellular level, we studied the effects of testosterone on aromatase activity/expression in mouse brain cell cultures (hypothalamus, cortex) prepared from different embryonic stages. Moreover, these primary cultures were raised in a sex-specific way, since we determined the sex of the embryonic donor by inspection of the gonadal anlage prior to cultivation of cells (15), By this experimeatal strategy, we were able to study the influence of androgens and other factors on embryonic aromatase at different developmental stages, in different brain regions, in a sex-specific way, and at ihe cellular level. Our studies revealed that androgens stimulate aromalase aclivity in hypothalamic, but not cortical, cultures of both sexes prepared from embryonic day 15 mouse feluses (100). This effect was dose-dependent and mediated through androgen-receptor activation, since it was completely blocked by the androgen receptor antagonist flu tarn ide. Further analysis of androgen effects have shown that the stimulation of aromatase activity results from an increase in the number of neurons expressing aromatase (100) and aromatase mRNA (Beyer, unpublished observalion), and from an increased aromatase expression per aromatase-immunoreactive cell (100). Interestingly, androgens were only effective during a short developmental window. When cultures were prepared from fetuses on embryonic day 13 or 17, no such an androgen effect was observed (48, 101). These dala strongly imply that aromatase neurons become sensitive to circulating androgens only during a distincl time-window of embryonic development. It remains, however, still unclear which factors determine this sensitivity. At least, the expression of androgen receptors by aromatase-immunoreactive neurons, or by cells which become aromatase-posilive later during developmenl after androgen stimulation, seems to be a decisive event. From these findings we conclude that the failure of previous studies to find a clear-cut role of androgens in developmental regulation of brain aromatase might result from the fact thai Ihe lissues and cell cultures used for these experimenls did nol match the appropriate sensitive period for aromatase induction, and thai none of the sludies listed above used sexed embryos for their culturing experiments (77,97). In addition to the functional regulation of hypothalamic aromatase, our studies further revealed that androgens also regulate the morphological maturation of hypothalamic aromatase cells. Thus, androgen treatment of cell cultures grown in steroid-free synthetic medium stimulates

neurile outgrowth and branching in aromatase neurons; again, these morphogenetic effects depend on androgen receptor activation and are restricted to hypothalamic, but not cortical aromatase cells (57). Taken together, our data clearly demonstrate that androgens function as morphogenetic signals for developing hypothalamic aromatase neurons, thereby being potentially effective in influencing the activity, plasticity, and synaptic connectivity of hypothalamic aromatase systems (48, 101,102).

Our data further suggest that the sex-specific development of hypothalamic aromatase neurons is likely to result from differences in androgen concentration, since testosterone levels during this androgen-sensitive embryonic developmental stage arc higher in males compared to females (86, 87). However, sex differences in androgen levels do not appear to be the only factor responsible for sexual dimorphisms of the hypothalamic aromatase system. First, aromatase activity and the number of uromatase-positive neurons are higher in males well before the onset of androgen synthesis by the fetal testis (52, 53, 100). This strongly implies that sexual differentiation of the hypothalamic aromatase system develops originally independent of gonadal steroids. Second, sex differences of the hypothalamic aromatase system also appear to be further affected and possibly stabilized by androgens and differences in androgen-responsiveness between sexes during late embryonic development (103, 104). Third, there is emerging evidence that sex differences in the mammalian brain involve the sex-specific realization of a cell-intrinsic genetic program (3, 10-12, 15, 24). One candidate gene that might be reponsible for generating male-specific properties of neural cells is the Y-chromosomal gene and teslis-determining factor, *Sry* (105, 106). Recent evidence suggests that Sry, a putative transcriptional activator with a DNA-binding domain, is expressed in mouse brain (107) and is capable of binding to DNA sequences in the promoter region of the gene encoding aromatase (108). Further studies of the genomic control of aromatase expression in male and female developing hypothalamus are required that will permit the molecular basis of this complex developmental pattern of sex-specific aromatase expression to be elucidated.

CONCLUSION

• Estrogen formation from androgens is catalyzed by the aromatase, a neuronal enzyme. This enzyme plays a crucial role in the sexual differentiation of the male brain in mammals during prc- and early postnatal periods of development. Regulation of the brain aromatase is an important step in determining when estrogen is available to act on the differentiating neural cells. Hypothalamic aromatase expression in rodents is characterized by a precisely delimited ontogenetic profile with peak levels of estrogen synthesis just before and around birth. During this time span, males display significantly higher aromatase activities compared to females. Several lines of evidence suggest that hypothalamic aromatase expression is highly regulated perinatally. Besides the influence of classical neurotransmitters such as norepinephrine on the catalytic activity of the enzyme, androgens appear to be the major regulatory factors for the developing hypothalamic aromatase system. Thus, androgens target the functional status of aromatase neurons by regulating the expression of the enzyme. By stimulating the morphological maturation of aromatase cells, the androgenic environment of the hypothalamus also appears to be instrumental in determining sex differences in a network of aromatase-immunoreactive nerve cells which provide estrogen required for the differentiation of male brain structures.

ACKNOWLEDGEMENTS

• This work was supported by the Deutsche Forschungsgemeinschaft (Be 1444/2-1), the European Community (Biomed I), and the Medical Research Council, UK.

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