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Steroid metabolising enzymes in the determination of brain gender ☆

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Abstract

The neurotrophic effects of oestrogen formed in the brain are important in brain sexual differentiation of the central nervous system and behaviour. Aromatase, converting testosterone to oestradiol-17β, is a key enzyme involved in brain development. In primary cell cultures of foetal hypothalamus, we have found that male neurones consistently have higher aromatase activity than in the female. Using a specific antibody to the mouse aromatase, immunoreactivity was localized in the neural soma and neurites in hypothalamic cultures. Additionally more male foetal hypothalamus neurones express aromatase than in the female. Testosterone increases aromatase activity in parallel with a greater number of aromatase-immunoreactive neurones. Testosterone also increases soma size, neurite length, and branching of cultured hypothalamic neurones. The neuronal aromatase activity appears to be sensitive to the inductive effects of androgen only during the later stages of foetal development. Endogenous inhibitors of the aromatase are also likely to have a regulatory role. This work suggests that regulation of a network of aromatase neurones, sensitive to the hormonal environment of the hypothalamus, may determine when oestrogens are available for neurotrophic effects underlying brain differentiation.

1. Introduction

Oestrogens are now known to regulate expression of neurotrophic factors in the brain and influence neuronal growth. Brain oestrogens influence synaptogenesis and dendrite formation [1–3]. There is also increasing evidence that steroids, and particularly oestrogens, have effects on cognition and learning and a protective action in degenerative diseases such as Alzheimer's [4]. Disorders in gender-specific brain development are likely to be caused in part by regulatory disruption of brain oestrogen formation at specific periods of early ontogeny. Clinical disorders such as Turner's syndrome, Klinefelter's syndrome, congenital adrenal hyperplasia, androgen insensitivity syndrome (AIS), and aromatase deficiency syndrome, which all involve defects in sexual differentiation, are likely to affect oestrogen bio-availability and brain development. An important developmental action of steroids including oestrogen, involving neurotrophic effects, concerns the differentiation of sexually dimorphic brain areas and behaviour. Sexual differentiation of the mammalian brain and behaviour occurs during steroid-sensitive phases in the perinatal period [5,6]. Permanent 'organising' effects of steroids during development can be distinguished from reversible 'activational' effects in the adult [7]. Steroids, therefore, play a pivotal role in the early organisation of behaviour [8] as well as in the formation of sexually dimorphic brain structures. However, hormone action that suppresses female behaviour does not necessarily enhance masculine behaviour. Therefore, there are at least two processes involved. Work on mutants, notably testicular feminised male (tfm) rats and mice, which are deficient in androgen receptors, has shown that in the male, separable processes of masculinisation and defeminisation

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exist involving different hormonal conditions [9]. Antioestrogens such as Tamoxifen selectively prevent behavioural differentiation, suggesting a specific action of oestrogen in development which is separable from the masculinising role of androgen. Behavioural work has undoubtedly provided fundamental evidence that oestrogens have potent organising effects in the developing brain.

Oestradiol-17β required for brain differentiation is thought to be formed by the local aromatisation of testosterone in preoptic (POA) and limbic areas [5,6]. Expression of the brain cytochrome P450 aromatase is a key event underlying the organisational effects resulting in male-specific neuronal morphology, control of reproductive behaviour and patterns of gonadotrophin secretion [10]. We have found that an array of metabolites, both biologically active and inactive is formed in brain areas, such as the hypothalamus, which are associated with steroid action in adulthood [11]. These enzyme systems cannot be studied in isolation, because in the intact brain different pathways of androgen metabolism occur in the same areas and compete for mutual substrate. In view of the heterogeneity of brain tissue, it is not known yet to what extent these pathways occur in different cells, or co-exist in the same cell type. However, our radiometric studies of the adult brain in avian and mammalian species have led to four conclusions on the role of androgen-metabolising enzymes [12]. First, these brain enzymes activate androgens to effective steroidal forms such as oestrogen in neurones involved with behavioural control. Second, androgens can be catabolised to biologically inactive forms, and the inactivating enzymes influence the level of substrate, testosterone, available in target cells during sensitive periods of early brain development. Third, steroid metabolites are formed by competing pathways which modulate enzymatic production of the behaviourally effective steroids. Fourth, environmental factors such as social stimuli and stress, known to affect behavioural development, influence the steroid-metabolising pathways that form both active and inactive metabolites from androgen. Therefore, oestrogen formation occurs against a background of other steroidogenic activities.

Marked inductive effects of androgen on POA aromatase activity have been demonstrated by our work, and more recently that of others in birds and mammals, suggesting that steroid regulation of brain aromatase is a widespread phenomenon [5,13]. A crucial question is to what extent genetic and physiological factors regulate aromatase activity in early brain development. Does the hormonal environment of the foetal brain directly modulate the formation of oestrogen? Exogenous steroids, including androgens, introduced into the maternal circulation have relatively little influence on preoptic aromatase activity. Oestrogen formation is much higher in male than female foetal rodents both in the amygdala and hypothalamus [14]. Peripubertally developing male brain aromatase activity is also higher than in the female [15]. Sex differences in brain aromatase activity could be due in part to differentiation of the system involved in steroid regulation of the enzyme in the male. Since it has not been possible to demonstrate a substantial inductive effect of androgen on the embryonic brain aromatase of mammals, initial work has been carried out in birds in view of the accessibility of the avian embryo for experimental alteration of steroid levels. The conclusions from our work [16] on early avian embryogenesis suggest first that testosterone greatly increases oestrogen formation in the developing brain, and second that the steroid sensitivity of the aromatase system is phasic in that cells containing the enzyme do not respond before critical periods of development. There may be more than one sensitive period, but there is a sudden appearance of sensitivity to androgen in early embryonic development which may correspond to androgen receptor differentiation [17]. Our kinetic studies of the avian aromatase also indicate an apparent K_m Michaelis Constant change during development as well as initially a low activity compared to the adult (Wozniak and Hutchison, unpublished data), suggesting either that the enzyme in the developing embryo differs from the adult form, or that regulation of the enzyme changes during ontogeny.

At present, the molecular mechanisms underlying sexual differentiation of the foetal brain aromatase system are not understood. However, in view of the importance of the aromatase system in breast carcinomas, there have been extensive molecular studies of the aromatase gene that have clarified expression in the ovary, placenta and mammary tumours [18]. Recent work has revealed that the aromatase gene has different promoters, enabling it to be expressed tissue-specifically both in the mouse and human. Six leader exons have been identified, each flanked by a promoter, and primary transcripts are tissue-specifically utilised through alternative splicing. There are, for example, both ovary- and brain-specific promoters, and the corresponding different brain and ovarian mRNAs have been characterised [19,20]. From our work, it is apparent that regulation of the aromatase also occurs non-genomically. We have evidence for potent inhibition of hypothalamic aromatase activity by endogenous androstanediones and neurosteroids. Our hypothesis is that a network of cells, probably glial, exists in the developing and adult brain, synthesising 5α/β-reduced steroids and also progestogenic neurosteroids, which participate in the inhibitory regulation of neuronal aromatase and related enzyme activities.

Recent research by our group to investigate the role

of aromatase in mouse brain development using gender-specific cultures, has shown that aromatase is concentrated in hypothalamic neurones, and that there is a sex difference (male > female) in both hypothalamic aromatase activity and aromataseimmunoreactive neurone number [21,22,23]. We have identified a similar sexually dimorphic hypothalamic aromatase system in the Mongolian gerbil, showing that the developmental sexual dimorphism is not restricted to the mouse (Hutchison, R. E., unpublished data).

2. Control of oestrogen formation in the adult brain

The metabolic formation of steroids has been subjected to a more detailed study in the adult brain than during development. Whereas a single 5α-reduced product, 5α-dihydrotestosterone (5α-DHT) is required peripherally for the differentiation of the external genitalia (e.g., in humans), studies of brain cells have revealed, for example, that both 5α- and 5β-reductases are implicated in the behavioural action of androgen [11]. An array of metabolites, both biologically active and inactive, are formed in developing brain areas, such as the hypothalamus, which are associated later with adult behaviour. These developing enzyme systems cannot be studied in isolation, because in the intact brain different pathways of androgen metabolism occur in common brain areas. In view of the heterogeneity of brain tissue, it is not known yet to what extent these pathways occur in various brain cells or co-exist in the same neural type. However, primary cell culture studies have shown that both α- and β-reduced metabolites of testosterone are formed in glial cells. Current hypotheses on the regulation of the brain aromatase system are largely derived from work on adult birds and rodents. Oestrogens are formed locally from androgens in hypothalamic cells of all vertebrates that have been studied so far [24,25]. Human placental, and rat, mouse and chicken ovarian aromatase genes have been cloned and sequenced [20,26,27]. However, the brain enzyme has only been characterised in fish using molecular genetic techniques [28]. kinetic studies in the ring dove reveal differences in aromatase apparent K_m between brain areas and between avian brain and ovary (Wozniak and Hutchison, in preparation), and during development. It is not clear what modulates aromatase expression or whether mRNAs for aromatases in different oestrogen target sites are derived from different gene transcripts. The adult POA aromatase activity can be increased markedly by steroid action. Exogenous testosterone increases the activity of the preoptic enzyme five-fold in the dove without changing the K_m of the enzyme [29]. Activation of the enzyme probably involves induction of aromatase expression but possibly also changes in non-genomic regulation. Formation and action of oestrogen via its receptors may occur in the same brain areas, and even in the same cell types. Aromatase and oestrogen receptors are co-localised in the avian brain, but the degree of cellular co-localisation varies according to brain area [30]. Co-localisation of oestrogen receptors and the aromatase has been shown to occur in developing rat and mouse brain neurones [31–33]. Elimination of circulating testosterone by castration in the male ring dove drastically reduces aromatase activity, but does not affect the number of oestrogen receptor cells in the POA detected by immunocytochemistry [34]. The inductive effects of androgen on brain aromatase activity have been demonstrated in other avian (for a review see [35]) and mammalian species [5,36], suggesting that steroid regulation of brain aromatase is a widespread phenomenon. Androgens have recently been shown to increase aromatase expression in the rat [37] and mouse hypothalamus [38,39]. In both birds and mammals, there appears to be synergism between oestrogens and androgens in the induction of brain aromatase [40,41]. Rapid changes in environmental stimuli derived from socio-sexual interaction have short-term effects on brain oestrogen formation in the male dove. Experimental visual stimuli in courtship interactions rapidly induce an increase in brain aromatase activity which parallels that seen during the normal male reproductive cycle [5]. Socio-sexual stimuli clearly have a major influence on testosterone aromatisation in the brain. Although species differ in the way in which aromatase activity is regulated, environmental stimuli appear to have direct effects on steroid metabolising enzymes in the adult avian brain. Photoperiod also influences oestrogen formation in the mammalian (hamster) brain. The anterior hypothalamus, which includes the photosensitive suprachiasmatic nucleus involved in the photoperiodic control of the testes and circadian rhythmicity, contains an active aromatase system. This system is photoinhibited independently of circulating androgen level in the hamster, whereas the POA, which also contains high aromatase activity, is insensitive to both photoinhibition and reduction in circulating testosterone resulting from castration [42]. In addition to regulation mediated through changes in gene expression, endogenous brain-derived inhibitors of the aromatase (e.g., 5α-reduced androstanes) influence oestrogen formation [43]. Other pathways of androgen metabolism (e.g., formation of 5β-reduced androstanes) regulate brain aromatase through removal of shared substrate, testosterone, and, therefore, reduce the amount of formed oestrogen.

3. Regulation of oestrogen formation in the developing brain

Two crucial questions, still largely unanswered are: to what extent do genetic and physiological factors regulate aromatase activity in early brain development, and does the steroid environment of the brain directly modulate formation of oestrogen? Prenatal sex differences in preoptic aromatase activity have been found in the rat [44,45] and ferret [46]. Similarly, a small sex difference in aromatisation levels has been seen in pooled POA samples of embryonic day (ED) 18 rats [47]. In the foetal rat and ferret [46] exogenous steroids, including androgen introduced into the maternal circulation, do not appear to modify brain aromatase activity. However, aromatase in the foetal guinea pig medio-basal hypothalamus, septum and cortex is induced by testosterone or 5α-DHT given to the pregnant mother [48]. Treatment of pregnant female ferrets with testosterone, 5α-DHT or flutamide (an androgen receptor blocker) does not influence foetal brain aromatase activity. The latter treatment suggests that androgen receptors are not involved in prenatal brain aromatase regulation in ferrets. Neonatally, male rats have higher aromatase activity than females in the amygdala, temporal lobe and anterior hypothalamus, whereas no sex differences have been found in the POA [49]. Aromatase activity is higher in the foetal male than female rat amygdala and anterior hypothalamus than in the female [50], but there are asymmetries in oestrogen formation which complicate interpretation and attempts to localise the enzyme. Aromatase mRNA is expressed very early in prenatal rat brain ontogeny [51]. However, steroid effects at this stage of brain aromatase development have not been tested. Neonatal gonadectomy followed by testosterone treatment attenuates the aromatase activity gender difference in adult rat POA [52]. It can be suggested that changes in aromatase activity are due in part to maturation of the androgen receptor system involved in regulating the enzyme, although other factors such as neurotransmitters are undoubtedly involved. The current view has been until recently that steroids do not directly modify developing mammalian brain aromatase levels until postnatally and also in adulthood, There appears to be a fundamental difference in regulation of the mammalian brain aromatase system between early development and the adult. However, the results of foetal mammalian exposure to hormones via maternal treatment are difficult to interpret, because of exogenous steroid catabolism by the foeto-placental unit, diminishing levels reaching the brain. There are, however, transient changes in the activity of the developing aromatase system at puberty [53], suggesting that regulation of the enzyme can fluctuate according to stage of postnatal development.

Few studies have so far been able to demonstrate an inductive effect of androgen on the embryonic mammal brain aromatase system. Work has been carried out in birds in view of the accessibility of the avian embryo for experimental alteration of steroid levels. The conclusions from work on the embryogenesis of Japanese quail [54,55] are that: (a) testosterone influences oestrogen formation in the developing brain; and (b) the sensitivity of the aromatase system is phasic in that cells containing the enzyme do not respond before a critical period of development. Thus, in quail, there is a sudden appearance of sensitivity to androgen around embryonic days 10 to 12.

To understand the role of the aromatase system in the sexual differentiation of the brain, the following questions have to be answered:

1. Is the enzyme localised in developing brain areas which later participate in the oestrogenic action necessary to regulate the neuroendocrine system and behaviour?
2. Which brain cells contain the aromatase and competing enzyme systems?
3. What regulates the brain aromatase?
4. Does oestrogen formation reach a peak during specific steroid-sensitive periods of brain development?

At present, the best way to begin to answer these questions, as they concern foetal mammalian brain development, is by using gender-specific primary cell culture techniques in which cells containing the enzyme can be distinguished. At the same time a direct comparison has to be made with the intact developing brain using radiometric assay methods which have successfully demonstrated sex differences in the perinatal brain aromatase system previously [44,49]. The mouse is a useful model, because stages of embryogenesis can be recognised following timed matings, and the aromatase gene has been recently cloned and sequenced [20]. The BALB/c mouse strain has an active and regionally localised aromatase system [56]. In addition, brain cell types can be identified using specific probes for neurones and various types of glia. Since there are also well established sex differences in cultured hypothalamic neurones, identified for neurotransmitter systems [57–59], the developing aromatase system can be related to the distribution of neuroactive catecholamines and peptides. For our studies, the activity of the developing aromatase system has been compared between tissue homogenates dissected from intact perinatal mouse brains, and cells derived from primary cell culture. The objective has been to assess development of the enzyme system in conditions matching as closely as possible the in vivo situation. Using this design, we have compared the gender-specific development pat-

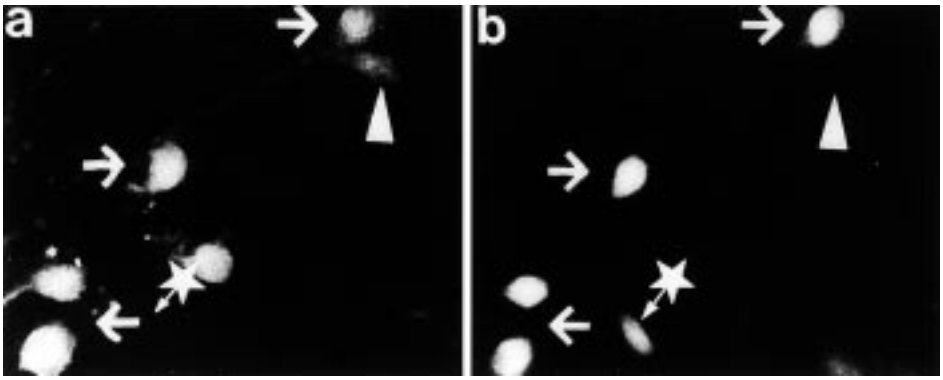

Fig. 1. Immunofluorescence double-labelling for (a) aromatase and (b) androgen receptors in hypothalamic mouse primary cultures. A sub-population of aromatase-positive neurones (arrows) co-express androgen receptors. The arrowhead points at an aromatase-positive cell which does not show immunoreactivity for androgen receptors. The star marks an androgen receptor-positive cell not being labelled for aromatase.

tern of aromatase activities from foetal (ED 13, 15 and 17), and neonatal, mouse hypothalamic and cerebral, homogenates, as well as neuronal cell cultures. There is a regional distribution of aromatase activity which can be identified in samples from primary cell culture and also in micro-punched samples taken from the intact brain. In the cortex, aromatase activity is low and sex differences are not apparent irrespective of whether it is measured in ED 15 cultured cells or dissected cortex from foetuses or neonates [60,61]. However, significant sex differences in aromatase activity are found in hypothalamic cultured cells as early as ED 13. In ED 15 cultured hypothalamic cells from both sexes, aromatase activity is detectable after 3 days in vitro (DIV), but there are no sex differences. However, sex differences appear after 6 DIV, and activity is significantly increased, indicating that maturation of the hypothalamic aromatase system occurs with time in culture and is accompanied by sex differentiation of the aromatase system. Male cultured hypothalamic cells always have higher aromatase activity than females. The aromatase is primarily neuronal, since treatment of ED 15 hypothalamic cultures with kainic acid results in a 70–80% decrease in aromatase activity compared to non-treated cultures, and the sex difference seen in hypothalamic cells is no longer present. Astroglial-enriched postnatal hypothalamic cultures exhibit very low aromatase activity after 6 DIV, and no sex differences are present, providing further evidence that aromatase in the foetal mouse brain is neuronal [61].

Kinetic studies comparing aromatase in ED 17 mouse hypothalamic cell cultures and homogenates of both genders indicate similar testosterone substrate binding affinities (apparent K_m approximately 40 nM), suggesting that cultured and micro-dissected neuronal aromatase are catalytically similar [61]. ED 15 mouse primary hypothalamic cell culture work also demonstrates higher aromatase activity in the male than female, as well as perinatally, and for it to be primarily neuronal rather than astroglial. There are brain area differences in aromatase activity. Since ED 17 hypothalamic neurones show a more marked sex difference in enzyme activity than ED 15 neurones, a rapid maturation of the aromatase system occurs at this stage in development which appears to match a peak in circulating testosterone levels of male foetuses. This developmental profile of aromatase activity in hypothalamic cultures is of particular interest. After 3 DIV in ED 15 cells, aromatase activity does not differ significantly between the sexes but exhibits a different developmental pattern (male greater than female) by 6 DIV. At present, we cannot distinguish whether this developmental sex difference reflects a male-specific, cell-intrinsic programme, or is due to a cell-specific induction of the developing aromatase system before cell culture on ED 15. The developmental increase in aromatase activity, which is neuronal, could result from either more cells expressing aromatase or increased expression within an existing set of aromatase-containing cells. There may be a dichotomy in the source of the enzyme. This possibility depends on whether the aromatase cells seen after 3 DIV are shown to be neuroblasts or developing neuronal soma without processes. Since the cultures are grown in a medium with undetectable amounts of sex steroids, sex differences in aromatase-containing neurones of the early embryonic brain develop in the absence of androgens or oestrogens [62]. However, hypothalamic cells cultured on ED 13 or 15 may be influenced at earlier stages in development by circulating steroids. Work is still in progress to try and determine whether the latter possibility is likely. Recently, cell type and region-specific expression of mRNA has been demonstrated in mouse cultured brain cells [63], but sex differences were not examined in this case.

Testosterone increases the number of aromatase-immunoreactive neurones in hypothalamic cultures of

ED 15 mice. Aromatase positive neurones clearly co-express androgen receptors (Fig. 1). We have now demonstrated using semi-quantitative PCR that there is a significant sexual dimorphism in the expression of aromatase mRNA at birth (Fig. 2) [64]. mRNA levels increase during development of both male and female embryos. This is an effect mediated via the androgen receptor (Fig. 3), However, there is no sex difference in aromatase gene expression. Therefore, the marked sexual dimorphism in aromatase protein demonstrated immunocytochemically, must be due to sex differences in mRNA translation. The factors involved are still unknown.

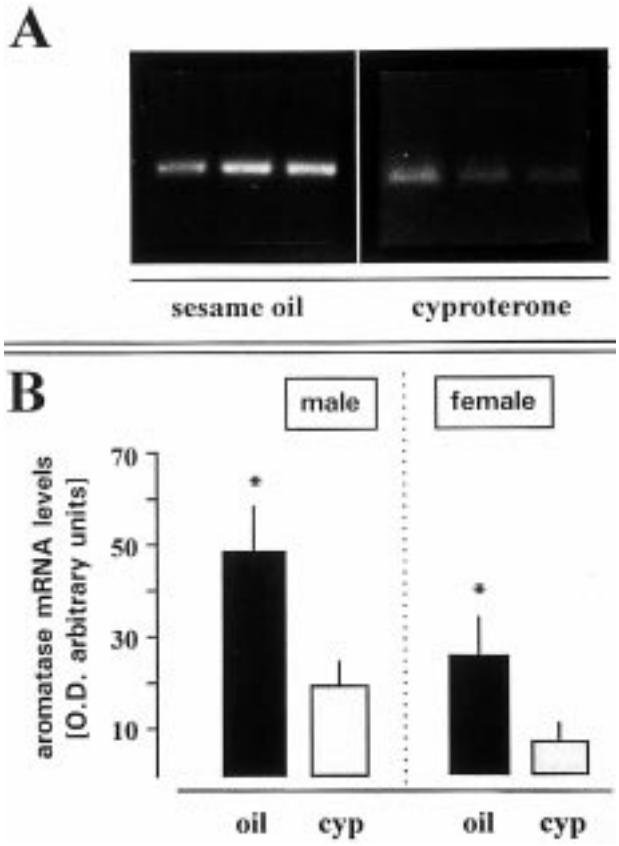

Fig. 2. Developmental pattern of aromatase mRNA expression in the male and female mouse hypothalamus analysed by semi-quantitative RT-PCR. (A) A representative Southern blot showing pre- and post-natal ontogenetic profiles of aromatase mRNA expression in both sexes; ov, ovary. (B) Semi-quantitative analysis of developmental aromatase mRNA expression in the male and female mouse hypothalamus. A clear-cut increase in aromatase expression was found in males, with the highest mRNA levels at birth (PO). Sex differences were present at PO and P15 ($^{*}P \leq 0.01$, $^{**}P \leq 0.005$) with higher levels in the male hypothalamus compared to females. The number of analysed individuals (each from a separate litter) per sex and age are given in [brackets]. E, embryonic, P, post-natal. For details of the amplification protocol and the semi-quantitative analysis see Karolczak et al., J. Neuroendocrinol. 10: 267–274 (1998).

4. Endogenous inhibitors of the brain aromatase

Networks of oestrogen-forming and oestrogen-sensitive neurones developing both before and after birth are important in brain differentiation. These networks interact with other developing neuronal systems influenced by the corticosteroid 'stress' hormones and progestogenic neurosteroids. Such networks containing prospective endogenous inhibitory regulators of the neuronal aromatase system may affect neural differentiation and associated behavioural development. In order to look at the rapid non-genomic inhibitory regulation of this crucial enzyme in the CNS, a comparison has been made between developing and adult steroidogenic systems in mammals, and also birds which have a more active aromatase system. The developmental profiles and kinetics of various steroid metabolising enzymes have been compared in the avian (ring dove) and rodent (mouse) brain. Hypothalamic and POA aromatase activities are measured in this work using our in vitro assay technique which we have developed to enable enzyme kinetics to be performed on individual brain areas. Adult male ring dove hypothalamic aromatase has an activity of approximately 20 pmol 1β-^{3}H-testosterone converted to $^{3}H_2O$/h/mg/prot. There is some range in activity which is probably attributable to individual variation in circulating levels of androgen in the blood since androgen induces the male POA aromatase. Hanes kinetic plots indicate an adult male dove POA apparent K_m POA of ~4 nM. This low aromatase K_m value indicates a strong substrate binding affinity, characteristic of a rate-limiting enzyme. However, the embryonic and post-hatching dove hypothalamic aromatase apparent K_ms are higher. We have found that dilution of developing dove brain homogenate results in a lowering of the measured aromatase apparent K_m. Thus, a 2-fold dilution of post-hatching dove hypothalamus reduced the aromatase apparent K_m from 85 to 40 nM, but there was no similar dilution effect seen in adult POA. Our conclusion is that the decrease in developing POA aromatase apparent K_m with sample dilution is due to the lowering of endogenous aromatase competitive inhibitor concentrations present in the embryonic/post-hatch hypothalamus (Wozniak and Hutchison, unpublished data).

Do these endogenous aromatase inhibitors exist in the developing mouse brain, and can they be identified at critical phases of brain development? Both natural and synthetic aromatase inhibitors have been evaluated in our work. They bind competitively, having no effect on V_{max} which is still achievable at a saturating substrate concentration, but increasing the apparent K_m (the substrate binding affinity is thus reduced). We have also measured dilution-induced lowering of aromatase apparent K_m in the ovary. In addition to

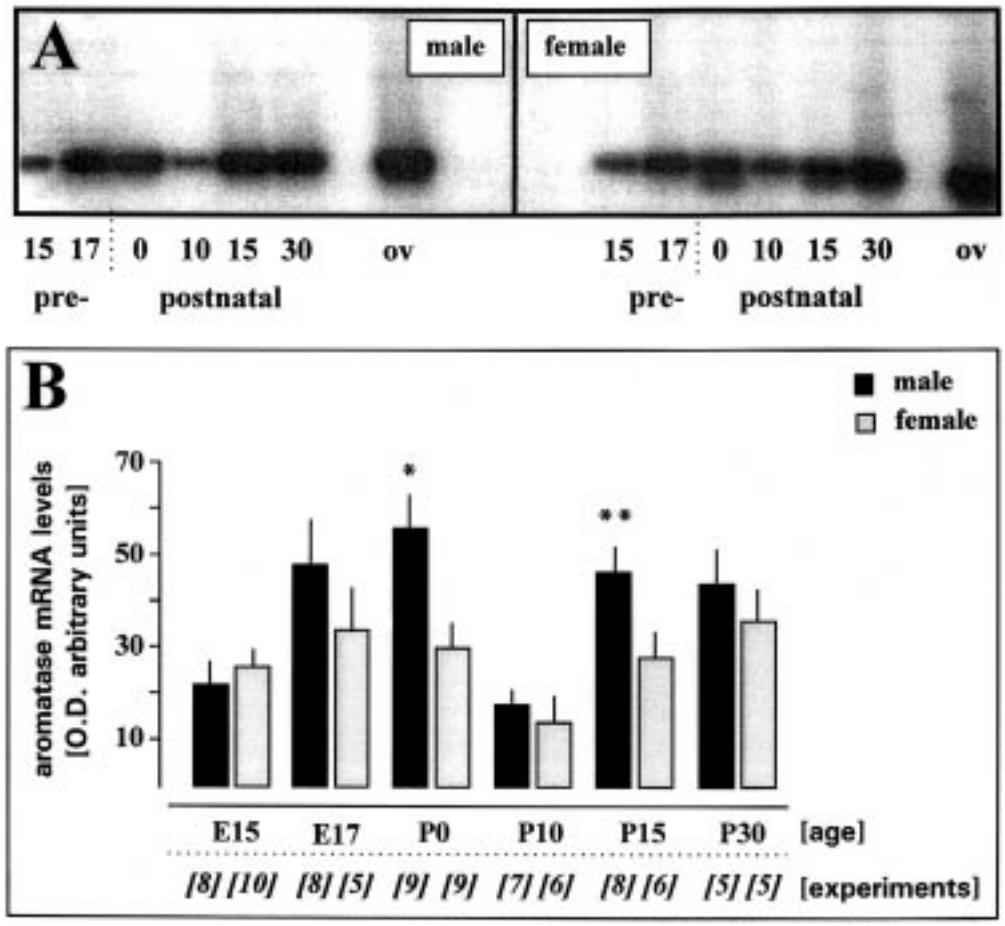

Fig. 3. Effects of in utero-treatment with the androgen receptor antagonist cyproterone acetate (daily from embryonic day 15 until delivery, 10 mg/0.1 ml sesame oil) on hypothalamic aromatase mRNA expression in newborn male and female mice. (A) An ethidium bromide-stained gel shows three representative examples (males) for each of the treatments. Note the low levels of aromatase mRNA after treatment with cyproterone acetate compared to controls treated only with the vehicle sesame oil. (B) Semi-quantitative evaluation of RT-PCR products of treated animals. In both sexes, treatment with cyproterone acetate reduced the levels of aromatase mRNA significantly ($^{*}P \leq 0.01$). In total, six to eight individuals from three different litters were analyzed per sex and age; cyp, cyproterone acetate. For details of the treatment and the RT-PCR protocol see Karolczak et al., J. Neuroendocrinol. 10: 267–274 (1998).

such observations, many of these K_m dilution-sensitive brain tissues contain very high activities of other steroidogenic enzymes (e.g., 5α/β-reductases), providing high levels of potential aromatase-inhibitory metabolites. In particular, 5α-androstanedione (Fig. 4) is a highly potent inhibitor of brain aromatase activity, with a comparable K_i value to synthetic aromatase inhibitors in clinical use or trial (Fig. 5). From Dixon and secondary replot of this data, the K_i values for the embryonic mouse hypothalamic cell culture, and adult dove preoptic area aromatases, are 23 nM, and 6 nM, respectively. Other endogenous androgenic metabolites (e.g., 5β-DHT) also inhibit brain aromatase activity but not as strongly. Even enantiomeric pairs can have very different potencies, with one being inhibitory and the other not at all. Progestogenic neurosteroids with analogous A, B and C ring structures to androgens are currently being investigated for their corresponding inhibitory potencies. In addition to potency, the selectivity of endogenous and synthetic aromatase inhibitors can be compared, by measuring their degree of non-specific inhibition of other brain steroidogenic enzymes, including 5α/βreductases, 17β-hydroxysteroid dehydrogenase, and 5α/β, 3α/β-hydroxysteroid dehydrogenases. Thus, although the synthetic triazole inhibitor Arimidex (Anastrozole, Zeneca Pharmaceuticals) and the imidazole containing Fadrozole (Ciba–Geigy) are both very potent brain aromatase inhibitors, the former is more selective, since 5β-reductase and (5β) 3α-hydroxysteroid dehydrogenase activities are less inhibited in dove brain areas (data unpublished Wozniak and Hutchison). The synthetic steroidal inhibitor 4-OH-androstenedione and the natural 5α-androstanedione are both equally less selective towards these other androgen-metabolising enzymes than the imidazole Fadrozole in the dove. However, in mouse hypothalamus, 5α-androstanedione is less inhibitory towards 5α-reductase than is 4-OH-

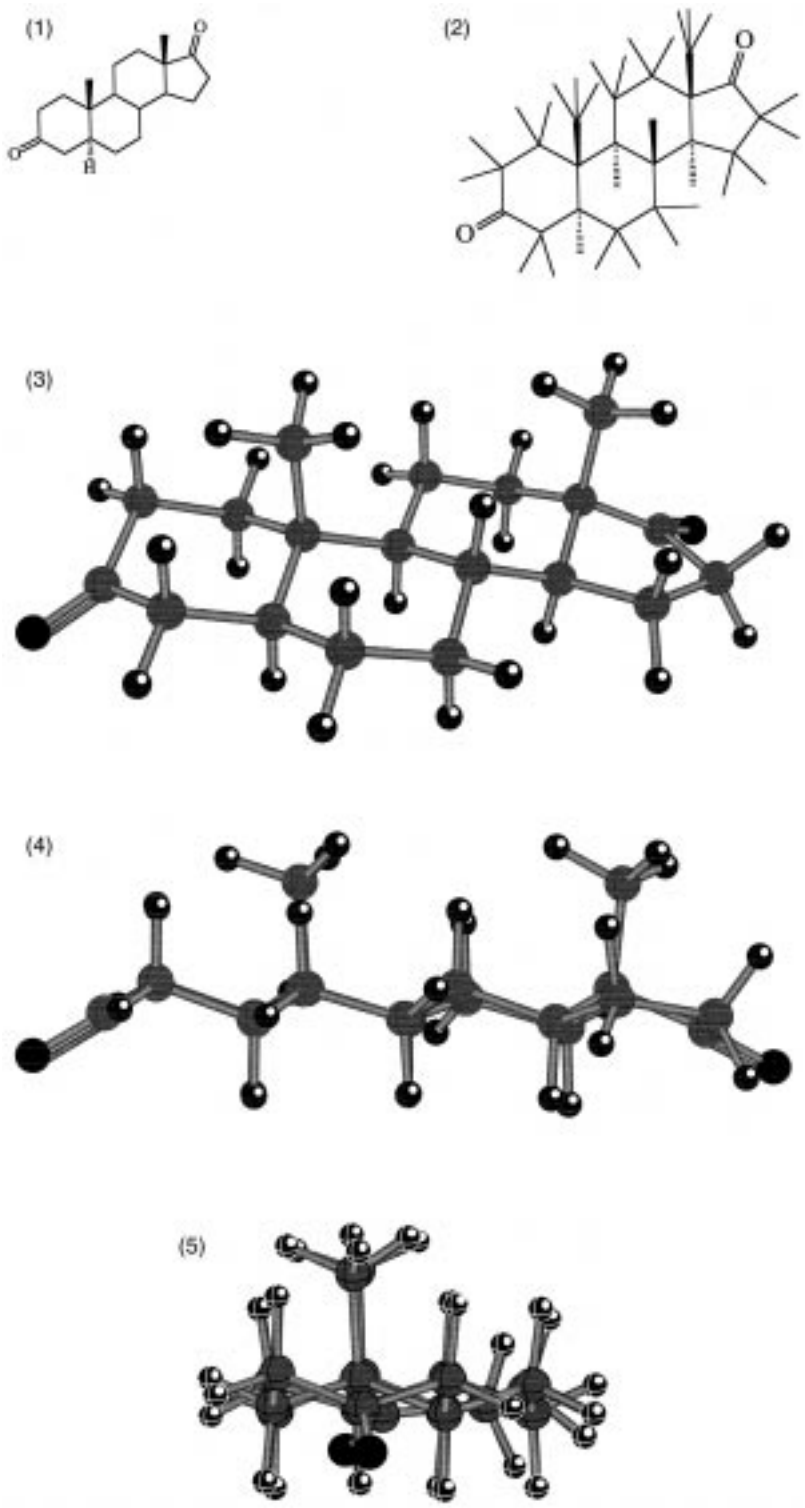

Fig. 4. Five structural representations of the potent endogenous aromatase inhibitor 5α-androstane-3,17-dione. (1) Showing the upward methyls C 18 and C 19; and omitting all hydrogens except for the downward' pointing chiral 5α hydrogen. (2) All the atoms and bonds. Chiral atom-bonds are shown as wedges, solid or dashed if in the α or β conformation, respectively. (3) A 3D representation in 2D, viewed from above the steroid molecule. The oxygen of the two carbonyl (C – O) groups, comprising the dione, shown in red, are important charged groups for hydrogen bonding with amino acid R groups in the aromatase active site. In the human, His 128 and Phe 134, and Asp 309, Lys 473 and His 475, interact with the C3, and C17 oxygen attachments, respectively. (4) Lateral view. Note that the 5α-androstanedione structure is effectively linear, except for the two methyls. (5) Frontal view. The last three structures, although showing artificial 3D representation give an indication of the optimal steric requirements for steroid entry into, and preferential binding, at the enzyme active site.

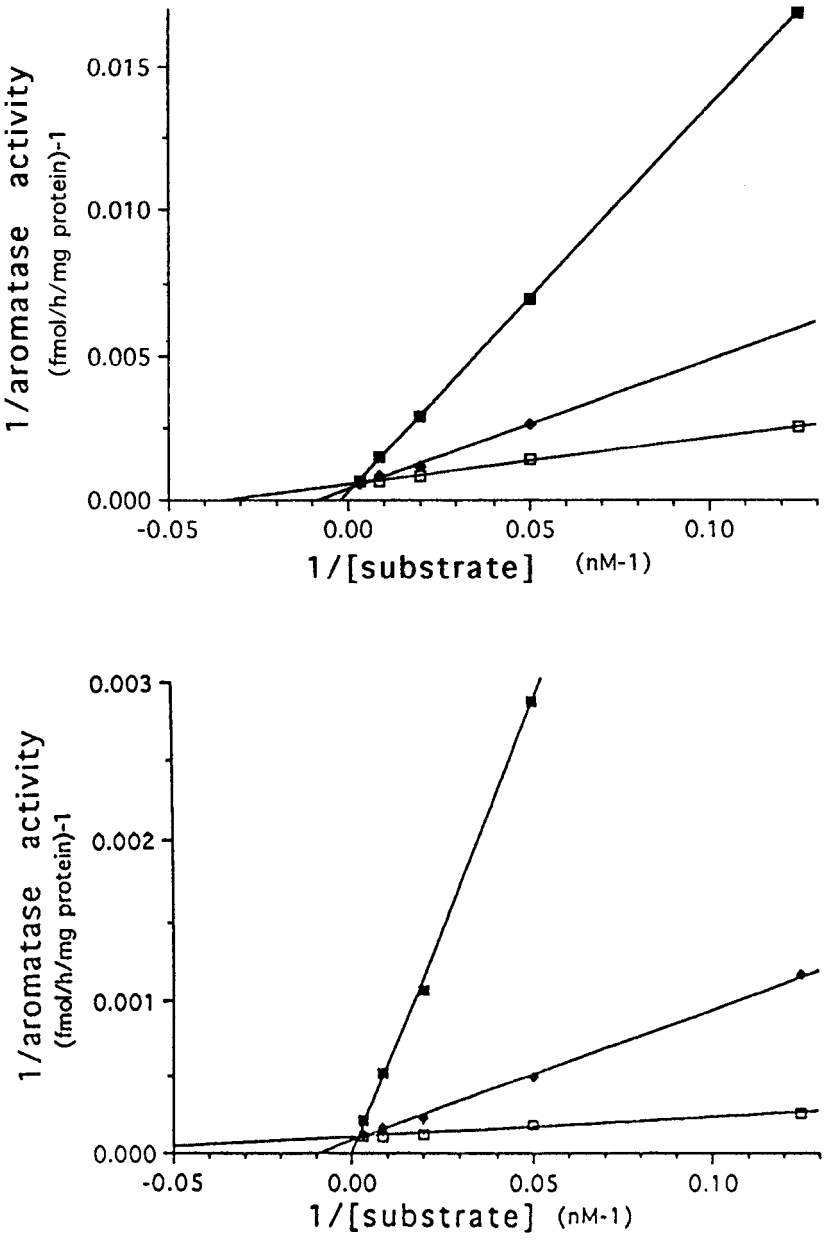

Fig. 5. In vitro brain aromatase inhibitory kinetics with endogenous 5α-androstane3,17-dione, in two species. Upper diagram: male embryonic day 15 Balb/c mouse hypothalamic primary cell culture. Lower diagram: adult male dove micro-dissected preoptic area. Homogenate aliquots of both brain samples were incubated with a concentration range of testosterone as substrate, and 0 M (□), 10^{-8} M (◆) and 10^{-7} M (■) partially inhibitory concentrations of 5α-androstanedione. Lineweaver-Burk plots are shown.

androstenedione, demonstrating differences in aromatase inhibitor selectivity. Interpretation of the effects of synthetic aromatase inhibitors is further complicated because they have different physical chemistries (e.g., some are water soluble, others not), so their physiological fate including metabolic half-lives will differ. Aside from their metabolism, non-polar inhibitors may readily accumulate in fatty tissues; whereas soluble ones should be more easily excreted. Physiological catabolism of these drugs can also generate fragments of unknown reactivity, including free radicals. The major degradation products formed from synthetic inhibitors such as Fadrozole or Arimidex within the brain have not yet been studied. Therefore, in considering both potency and selectivity of aromatase inhibitors in the brain, the breakdown of these compounds is important. The catabolic pathways involved and products in the brain are still virtually unknown.

5. Conclusions

Steroids have functional effects in the differentiation of brain gender in terms of the neuroendocrine system and behaviour. Oestrogens derived from aromatisation of androgen within brain neurones are effective in differentiating behavioural mechanisms in both male birds and mammals. Steroid levels and environmental factors such as socio-sexual stimuli influence the formation of oestrogen by aromatase activity in the brain of the adult male. Two important questions which still have to be resolved are what determines changes in aromatase activity effective for the differentiation of the sexually dimorphic brain, and when does this regulation occur? There is little doubt that in mammals regulation of the aromatase gene expression is important in both foetal and postnatal brain development. The limits of the sensitive periods for brain aromatase regulation are still virtually unknown. In the intact foetal mouse brain, oestrogen formation is neuronal rather than glial. Neurones developing in the embryonic male contain higher aromatase activity than the female, and this sex difference exists at early stages of embryonic development. However, the brain aromatase appears to be regulated by genetic factors during this very early embryonic period. It is only later in development that the brain aromatase system is controlled by androgen and possibly other steroids. Development of the androgen receptor may have a role in determining when the later steroid-sensitive phases of aromatase regulation begin. Although testosterone appears to be important in the control of oestrogen formation in the brain, the exact mechanism of steroid regulation of the aromatase gene is still unknown. Factors that promote the development of connectivity in steroid-sensitive aromatase neurones, and whether there is a steroid-independent phase in neuronal ontogeny, are also unknown. However, developing aromatase-containing neuroblasts probably form processes that connect to other aromatase neurones. Since the sex of individuals can be identified from an early embryonic age using molecular genetic techniques, it should be possible in the future to determine the relative contribution of genetic and hormonal factors in brain differentiation. We conclude that the oestrogen-forming capacity of the brain has the characteristics and plasticity necessary for provision of effective oestrogen for brain differentiation. The aromatase activity required develops at critical stages in prenatal development. It should be possible to identify aromatase-containing neurones in relation to developing neurotransmitter and steroid receptor systems as well as other factors (e.g., growth factors and peptidergic hormones) involved in the development of sex-specific neuronal connectivity.

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