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Published in:
Developmental Brain Research

DOI:
10.1016/0165-3806(94)90162-7

ELSEVIER

Developmental Brain Research 82 (1994) 185–192

DEVELOPMENTAL BRAIN RESEARCH

Research report

Brain aromatase activity and plasma testosterone levels are elevated in aggressive male mice during early ontogeny

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Accepted 31 May 1994

Abstract

Testosterone (T) and estradiol (E_2) are involved in intraspecific aggressive behavior. Both steroids exert their effects on behavior via the hypothalamus and the amygdala (Am) of the central nervous system (CNS). In these brain areas T is converted to E_2, by the enzyme aromatase. Both the levels of brain aromatase activity (AA) and the effects of T and E_2 on aggressive behavior in adulthood depend on steroidal organization of the CNS during ontogeny. In this study we measured plasma T and in vitro brain AA of male fetuses and neonates derived from two strains of wild house mice, which had been genetically selected for aggression, based upon attack latency. There were no differences in preoptic area (POA) AA levels between selection lines on either embryonic day (E) 17 or 18, or the day after birth (day 1). In the non-aggressive long attack latency (LAL) males the POA AA increases with age, i.e. was higher on E18 than on E17, which is correlated with brain weight (BrW). This was in contrast to aggressive short attack latency (SAL) fetuses, which only showed a slight, but not significant difference between embryonic days or a correlation with BrW. Neonatally, the POA AA of LAL males tended to decrease in contrast to SAL males. However, SAL neonates had a higher AA in the amygdala (Am) than LAL neonates, whereas no differences exist in the anterior hypothalamus. Thus, a differential brain AA distribution exists in SAL and LAL pups. At day 1 SAL males show higher AA in the Am than in the hypothalamus (POA + AH), whereas in the LAL strain the AA did not differ between these brain areas. In the LAL males plasma T levels decreased from E17 to day 1, whereas the SAL neonates (day 1) exhibited higher circulating T concentrations than LAL neonates. These results suggest a T-independent aromatase induction prenatally in both selection lines, whereas neonatally the higher plasma T level in the SAL line coincides with higher AA levels in the Am. Accordingly, a differential pattern of E_2 formation exists in the brains of the two selection lines during ontogeny. The variation in circulating T and maximal brain E_2 formation around birth might result in a differential organization of adult CNS sensitivity to sex steroids and accordingly differences in aggressive behavior.

Keywords: Aromatase; Testosterone; Estradiol; Hypothalamus; Preoptic area; Amygdala; Cortex; Ontogeny; Aggression

1. Introduction

It is generally accepted that the gonadal steroid hormone testosterone (T) facilitates intraspecific aggressive behavior via specific areas of the central nervous system (CNS) [1,4,23]. Not only T, but also the aromatized product, estradiol (E_2), facilitates aggressive behavior in mice [32,39,40], whereas a blockade of neural E_2 receptors reduces the T-elicited fighting response [7]. Genetic differences in the aggression-eliciting properties of T and E_2 have been demonstrated between CF-1, CD-1, and CFW mice strains [40], and between two selection lines of wild house mice [10,49]. These wild mice were genetically selected for inter-male territorial aggression, based upon attack latency [47,48]. In adulthood these selection lines differ in several T-related parameters. Thus, males of the aggressive short attack latency (SAL) line have higher plasma T levels, higher seminal vesicle weights [49], and larger testicular Leydig cell percentages [13], as compared to males of the non-aggressive long attack latency line (LAL). Although T is reported to induce

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SSDI 0165-3806(94)00105-9

AA in the adult dove [20–22] and rat brain [35,44], the aggressive SAL males show lower levels of aromatase activity (AA) in the preoptic area (POA) of the brain, despite higher circulating T [8]. Hence, in adult mice T is not the only factor determining the brain AA.

Both adult CNS sensitivity to sex steroids and brain AA are known to be dependent on circulating androgens around birth [15,44]. Androgens and estrogens play a crucial role in the sexual differentiation of the CNS during ontogeny [28,51]. Higher plasma T levels occur in male rats prenatally and immediately after birth, as compared to females [11,42,59]. T treatment around day 17–18 of gestation sensitizes the CNS to T circulating after birth [2,19], whereas high prenatal circulating E_2 reduces inter-male aggressiveness [54]. Neonatally, both androgens and estrogens enhance the aggression-promoting properties of T in adulthood [5,9,14,15,53,55]. Together with the elevated plasma T level in males, the brain AA increases in the hypothalamus and limbic structures at birth [16,25,29,45,58].

In view of the role of perinatal androgen and its metabolites in sexual differentiation, it is possible that similar mechanisms are involved in the differentiation of aggressive behavior and T related parameters within the male sex. Therefore we determined the brain AA and plasma T levels of male fetuses and neonates of aggressive SAL and non-aggressive LAL mice.

2. Materials and methods

2.1. Animals

Wild mice (*Mus musculus domesticus*), which were genetically selected for territorial aggression based upon attack latency, were used [47]. Long attack latency (LAL) animals were derived from generations 18–20 and short attack latency (SAL) animals from generations 42–43. Parents were housed in standard perspex cages (17 × 11 × 13 cm) on a sawdust bedding, in animal rooms with a controlled light/dark cycle (12:12; lights off at 12.30 h) and temperature (19–21°C). Standard lab chow and water was available ad libitum. On the day of birth (day 1), blood samples were taken from complete litters.

In order to control the conception date of fetuses, male and female mice were housed individually in standard cages. The cages of these male–female pairs were connected to each other by a perspex tube, blocked by a sliding door [12]. The sliding door was perforated to allow the animals to see and smell each other. Each day vaginal smears were taken; if in oestrus, the female was mated with a male mouse of the same selection line during the middle of the dark phase between 17.00 and 21.00 h by opening the sliding door in the tube. The day after mating was considered to be day 1 of gestation. The females were weighed until pregnancy was established, or mated again if necessary. Total length of pregnancy was 19 days.

2.2. Blood sampling

To measure the plasma testosterone level, blood samples were taken from male fetuses and their mothers of both selection lines between 14.00 and 17.00 h on embryonic days 17 and 18 (E17 and E18). In addition, blood samples were taken from new born males on the day of birth (day 1) according to Weisz and Ward with slight modifications [59].

Each pregnant female was mildly anaesthetized by ether. Prior to hysterectomy a blood sample was taken by cardiac puncture. This sample was kept on melting ice in tubes containing heparin (10 μl of 500 IU/ml), and centrifuged (2,600 × *g*, 10 min, 4°C). The uterus was then dissected and placed in cold saline to slow down metabolism. Fetuses were removed and sexed by identifying the gonads. After cutting the neck area, trunk blood was collected in heparinized capillary tubes (Hawksley & Sons, England; Cat. no. 01603) and centrifuged (Microhaematocrit centrifuge; 15,000 rpm, 6 min). Plasma samples of male animals were pooled until a volume of at least 100 μl was obtained. Several fetuses were necessary to obtain the volume of one pooled sample: 10–14 for E17 and 8–11 for E18. Blood sampling of neonates (day 1) followed the same procedure as described for the fetuses, minus placement in cold saline. Plasma of 5–8 neonates was required for one pooled sample.

This procedure yielded pooled fetal blood samples (n = 5–7) per selection line on each embryonic day, pooled neonatal blood samples (n = 4–6) per strain on day 1, and blood samples of SAL (n = 26–28) and LAL (n = 12–14) mothers on days 17 and 18 of pregnancy. All plasma samples were stored at −20°C until assayed.

2.3. Brain sampling

Immediately after blood sampling, the brains were removed from the SAL and LAL male fetuses (days E17 and E18; n = 9–12), and neonates (day 1; n = 8–11), immediately frozen on solid CO_2, and stored at −80°C. Coronal brain slices were dissected according to the method described previously [8,44]. Neonatal preoptic area (POA), anterior hypothalamus (AH), bilateral amygdala (Am), and parietal cortex (CTX) were removed using a modified Palkovitz punch technique and stored at −80°C. For fetal brains only POA and CTX were obtained. Thereafter the various micropunches were individually homogenized in 100 μl ice-cold TEK buffer (100 mM Tris, 50 mM KCl, 1 mM

Na_2EDTA; pH 7.4), frozen and stored at −80°C until assayed.

2.4. Testosterone radioimmunoassay

Plasma concentrations of testosterone (T) were determined using a standard radioimmunoassay (RIA) kit (Medgenix), without extraction of the samples. The minimal detectable concentration of T was 0.04 ng/ml and the intra- and inter assay variation coefficients were 4.7 and 8.1%, respectively. Cross-reactivities (percentages estimated from the concentration yielding 50% inhibition) with 5α-dihydrotestosterone, androstenedione, 17β-estradiol, dehydroepi-androsterone, and progesterone were 1.0, 1.2, < 0.001, 0.001, 0.053%, respectively. This commercial RIA kit revealed plasma T concentrations which were similar to those obtained by a T RIA after extraction and chromatography of the plasma samples according to Pratt et al. [33].

2.5. Aromatase assay

The activity of the T-aromatizing enzyme aromatase was measured by an in vitro microassay in each individual brain punch homogenate. This assay is based on the stereospecific release of ^3H_2O from [1β-^{3}H]T as a result of aromatization. We used this tritiated water assay as previously described and validated [8,43]. Incubation of the homogenates was carried out for 30 min at 37°C in a microplate. Each well contained 30 μl homogenate, 0.2 mM NADPH, and [1β-^{3}H]T (final volume 50 μl). The substrate [1β-^{3}H]T was prepared from [1β,2β-^{3}H]T (NET-187, specific activity: 40–50 Ci/mMol; New England Nuclear) by overnight hydrolysis and redissolved in TEK buffer, so that 10 μl would yield approximately 3×10^5 cpm, equivalent to 300 nM. This is a saturating substrate concentration for the mouse brain aromatase [8,62]. Assay controls used either boiled homogenate or homogenate containing 10^{-4} M of the non-steroidal-specific aromatase inhibitor Fadrozole (CGS 16949A: 4-(5,6,7,8-tetrahydroimidazo-[1,5-a]-pyridin-5-yl)-benzonitrile HCl; provided by A.S. Bhatnagar) [60,61]. The reaction was stopped by freezing the microplate in solid CO_2 and adding 150 μl water. The thawed samples were transferred onto microcolumns containing 30 mg C-18 sorbent (Bondesil; Jones Chromatography, UK) to bind steroids. After a further extraction with charcoal [43], the eluates were decanted into vials, 2 ml scintillation fluid (Ultima Gold, Packard) was added, and the ^3H_2O product was counted in a scintillation counter (Model 3255, Packard). Interassay variation (≤ 10%) was checked by including a few aliquots of the same active aromatase sample in each assay. The aromatase activity was expressed as pmol ^3H_2O formed/h/mg protein.

2.6. Protein assay

Protein concentration was measured by a sensitive modified Coomassie dye binding assay (minimum of 1–2 μl homogenate volume needed; ≥ 0.1–0.2 μg protein detectable), adapted from Simpson and Sonne [41], using bovine serum albumin as a standard.

2.7. Statistics

Plasma testosterone, brain aromatase activity (AA) levels, and body and brain weights were normally distributed. Brain AA, body and brain weights, and plasma T levels of fetuses, their mothers, and neonates were evaluated using an analysis of variance (ANOVA) of a two (SAL and LAL selection line) × three (days E17, E18, and day 1) factorial design. Significant differences between SAL and LAL selection lines in plasma T levels and AA in the various brain areas, were analyzed by post-hoc Student's *t*-test. Distribution of AA in the various investigated brain areas of neonatal SAL and LAL selection lines were evaluated for significance using a multivariate analysis with repeated measures (Pillais test in SPSS PC + MANOVA [17]). A paired *t*-test was used to clarify the differences in brain AA distribution. A Spearman Rank correlation test was used to determine significant correlations between body weight (BdW), brain weight (BrW) and AA in the various brain areas within each selection line. No individual correlation test could be performed between brain AA and plasma T level in fetuses and neonates, because the T values were derived from pooled plasma samples.

3. Results

3.1. Brain aromatase activity

Paired *t*-test reveals a significant higher POA AA in both selection lines, as compared to CTX, on E17, E18, and day 1 ($P < 0.05$; Fig. 1); but no significant differences between SAL and LAL males exist in POA and CTX AA on these days. The non-aggressive LAL line shows a significant increase in POA AA on E18 as compared to E17 (*t*-test: $P = 0.02$), whereas the POA AA of SAL fetuses tends to increase (not significantly). After birth the LAL selection line shows a slight, but not significant decrease in POA AA, as compared to day E18.

Evaluation of the neonatal brain AA distribution by a MANOVA reveals a significant selection line effect ($F_{1,16} = 5.67$; $P = 0.03$) and an interaction between brain area and selection line ($F_{2,32} = 5.01$; $P = 0.021$). The males of the aggressive SAL line exhibit significantly higher AA levels in the amygdaloid nuclei than

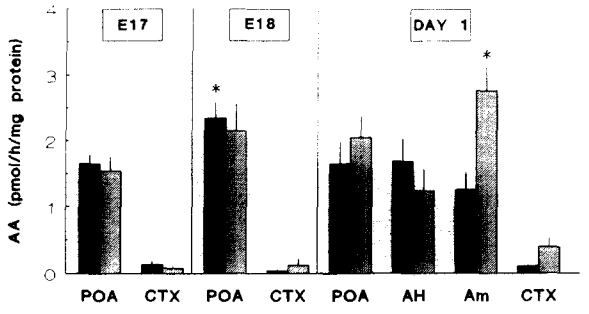

Fig. 1. Mean aromatase activity levels (AA + SEM) in brain areas of male fetal (E17, E18) and neonatal (day 1) mice of the aggressive short attack latency (SAL, hatched bars) and non-aggressive long attack latency (LAL, solid bars) selection line. POA, preoptic area; AH, anterior hypothalamus; Am, amygdala, both left and right; CTX, parietal cortex. In the LAL line a significant increase in POA AA level exists on E18, as compared to E17 (*$P < 0.05$). A significant difference exists in Am AA between selection lines at day 1 (*$P < 0.01$).

neonatal LAL males (t-test: $P < 0.01$), whereas no significant differences are found in POA and AH AA between strains (Fig. 1). Moreover, SAL and LAL neonates show a differential distribution of brain AA. Male neonates of the SAL selection line show a higher AA in the Am, as compared to the averaged POA/AH AA (paired t-test: $P < 0.05$), whereas the LAL neonates show no differences between hypothalamic and Am AA (Fig. 2). The mean protein concentration of individual neonatal and fetal brain area homogenates was approximately 0.6 mg/ml. Assay controls show low counts per min, near background levels.

As presented in Table 1, the mean BdW and BrW increase during development in both SAL and LAL selection lines ($P < 0.001$). No significant difference

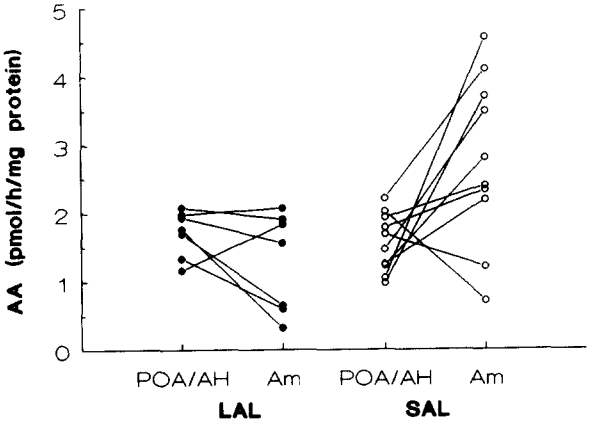

Fig. 2. Aromatase activity (AA) in the hypothalamus, including the preoptic area and anterior hypothalamus (POA + AH), and the amygdaloid nuclei (Am) of male neonatal mice (day 1) of the aggressive SAL (open circles) and non-aggressive LAL strain (closed circles). Lines join related samples from the same individual. AA distribution differs significantly between strains. LAL: Am = POA + AH. SAL: Am > POA + AH ($P < 0.05$).

Table 1
Mean body and brain weights of male fetal (embryonic days (E) 17, 18) and neonatal (day 1) mice from the aggressive short attack latency (SAL) and non-aggressive long attack latency (LAL) selection lines

		Body weight [a] (mg)	Brain weight [a] (mg)
SAL	E17	589.82 ± 17.51	49.73 ± 1.78
	E18	886.78 ± 30.82	65.44 ± 1.66
	day 1	1482.39 ± 34.98	–
LAL	E17	551.58 ± 18.73	51.44 ± 1.85
	E18	815.24 ± 24.76	60.33 ± 2.58
	day 1	1419.80 ± 48.71	–

[a] Significant developmental effect.

exists between selection lines in either mean BdW or BrW on any of the investigated days. As presented in Fig. 3, Spearman Rank test reveals a positive correlation between POA AA and BrW ($r_s = 0.6896$; $P < 0.05$) within the LAL fetuses. A significant correlation exists between POA AA and BrW of SAL males only on E17 ($r_s = 0.6336$; $P < 0.05$). No significant correlation exists between POA AA and BdW of either selection line during development.

3.2. Plasma testosterone

ANOVA reveals a significant interactive effect between selection line and developmental stage ($F_{2,40} = 3.21$; $P = 0.05$) on plasma T levels. LAL fetuses (E17 and E18) tend to have higher plasma T levels than SAL fetuses ($P = 0.06$; Fig. 4), whereas SAL neonates

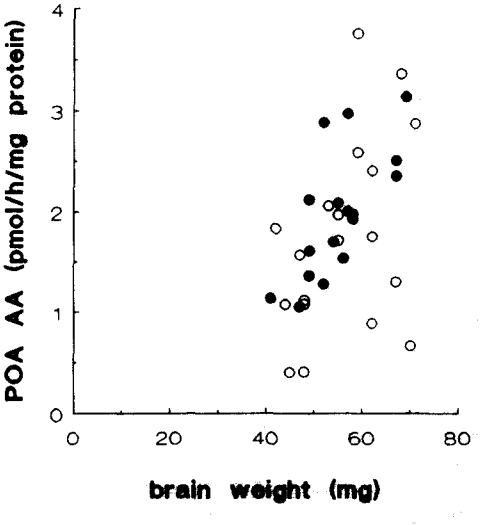

Fig. 3: Brain weights of male fetal mice (E17 and 18) of the aggressive SAL (○) and non-aggressive LAL strain (●) vs. their corresponding aromatase activities (AA) in the preoptic area of the hypothalamus (POA). A significant positive correlation exists within the LAL selection line between POA AA and BrW ($r_s = 0.6896$; $P < 0.05$). For the SAL males a significant correlation only occurred between POA AA and BrW on embryonic day 17 ($r_s = 0.6336$; $P < 0.05$).

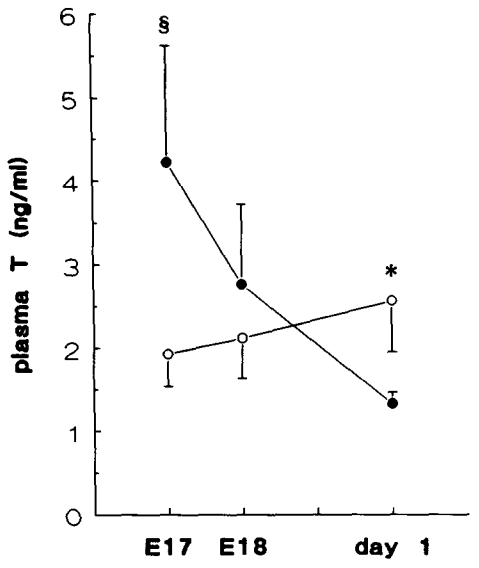

Fig. 4. Mean (+S.E.M.) plasma testosterone (T) levels of male fetal (E17, E18), and neonatal (day 1) mice of the aggressive short attack latency (SAL) (○) and non-aggressive long attack latency (LAL) selection line (●). Note difference in plasma T levels between SAL and LAL strains prenatally (mean of E17 and E18; $P = 0.06$) and neonatally (day 1; $^{*}P \leq 0.05$) and significant decrease of plasma T level in the LAL males from E17 until day 1 ($^{\S}P < 0.05$).

exhibit significantly higher plasma T values than LAL males on day 1 ($P < 0.05$; Fig. 4). In contrast to the SAL line, a significant decrease in plasma T concentration is shown in the LAL line from E17 until day 1 (t-test: $P = 0.05$).

Plasma T levels of pregnant females do not differ between selection lines or between days 17 and 18 of pregnancy (pregnancy day 17, SAL 3.00 ± 0.25, LAL 2.64 ± 0.36; pregnancy day 18, SAL 2.39 ± 0.34, LAL 2.81 ± 0.44).

4. Discussion

4.1. Distribution of brain aromatase activity

The present study reveals a differential pattern of brain aromatase activity levels during ontogeny of aggressive and non-aggressive male house mice. Males of both selection lines exhibit high POA AA on embryonic days 17 and 18, and neonatally on day 1, as compared to CTX. Around birth, POA AA levels do not differ between SAL and LAL selection lines. Prenatally, however, the POA AA levels of LAL fetuses show a significant increase from E17 to E18, whereas the increase in SAL POA AA levels failed to reach significance. In the LAL strain, the increase in POA AA correlates positively with brain weight, in contrast to SAL males (Fig. 3). Accordingly, brain maturation during gestation might be an important factor in determining AA. Therefore it is important to emphasize that the brain and body weights do not significantly differ between SAL and LAL mice on each developmental day (Table 1).

Neonatally, the POA AA of LAL males tends to decrease in contrast to SAL males. Moreover, SAL neonates have higher Am AA levels than LAL neonates, whereas no differences exist in the AH. Indeed, on the day after birth a completely different brain AA distribution exists in SAL and LAL pups. Day 1 SAL males show higher AA in the Am than in the hypothalamus, whereas in the LAL strain the AA does not differ between these brain areas. Overall, this developmental pattern of brain AA is in accordance with previous studies in rodents. In rat AA increases in hypothalamic and limbic structures around 5 days before birth, peak levels are reached 3 days later, after which the enzyme activity declines rapidly until day 4 postnatally [16,25,29,45,58]. The developmental pattern of aromatase mRNA differs from the enzyme activity pattern with respect to reaching peak levels. Aromatase mRNA increases prenatally until a peak is reached a few days after birth, whereas the AA starts to decrease before birth [18,25]. Postnatally, AA declines less rapidly, and by 3 weeks after birth its levels are comparable to those of the adult [16]. Indeed, in the currently investigated mice, the perinatal POA AA and neonatal Am AA are approximately 10-times higher than adult AA levels in equivalent brain areas [8]

4.2. Plasma testosterone and brain aromatase activity

Prenatally, in particular on day E17, LAL males tend to have higher plasma T levels than SAL fetuses. A large variation in T level occurred within the LAL line. This might be due to a small variation in sampling time in relation to the exact appearance of the fast T surge at this stage of development. In addition, the variation in fetal plasma T level is neither related to maternal circulating T level nor to fetal body weights (Table 1).

Although T is reported to induce aromatase in the adult dove [20–22] and rat brain [35,44] through a receptor-mediated mechanism, [36] the higher circulating T levels in the LAL fetuses does not result in higher POA AA, as compared to the SAL animals. This suggests that induction of the male aromatase is not mediated by T prenatally in mice. This is in accordance with literature on sexual differentiation. Thus male rats [59] and mice [52] show higher plasma T levels than females before birth; [59] however, no sex differences are found in POA AA levels prenatally in rat [16,58], ferret [24,57], and monkey [37]. One study in rats reported a very small sex difference in AA levels in a pooled sample of POA and hypothalamus

on day E18 [45]. Treatment of pregnant female ferrets with T, 5α-dihydrotestosterone or flutamide does not influence fetal brain AA [24,46,57]. Even castration or T treatment of fetal rhesus macaques does not affect brain AA [37].

The study on administering flutamide to block the androgen receptors [24], in particular, indicates that the prenatal availability of androgenic substrate determines sex differences independently of an androgen receptor-mediated activation of AA, as occurs in adults [36]. In contrast to mammals, in avian species (Japanese quail) the hypothalamic AA does increase after a T injection into the eggs [38]. Nevertheless, the currently investigated mouse strains show differences in endogenous plasma T levels prenatally, which are not paralleled by changes in POA AA. Hence in mammals, prenatal brain AA probably develops independently of circulating plasma T.

Neonatally, however, SAL males have a higher plasma T level, which coincides with higher AA in the amygdala, as compared to LAL animals of the same age. Accordingly, brain AA is probably mediated by plasma T at this stage of development at least in the amygdala. This is in accordance with studies on sexual differentiation. As compared to females, neonatal male rats show both higher plasma T levels [11,31] and AA levels in the Am, temporal lobe, or AH, whereas no sex differences are found in the POA [29,45]. In rabbit a sex difference was found in AA levels of limbic structures on the day before birth and also in the hypothalamus on day 5 postnatally [34]. In another study of rats, on the day before birth the AA in Am and AH is higher in males than in females, in contrast to AA in the POA, which does not differ [56]. Accordingly, from the day before birth until adult age, brain AA can be affected by circulating T, whereas at earlier stages AA develops independently of T. The increase in T influence on aromatase induction might be due to maturation of the CNS androgen receptor system [27,50].

Although the regulation of AA during ontogeny is complex and depends upon a variety of non-genomic factors [3,6,26,30], the data of the present study are consistent with earlier reports about the time relationship between circulating T and brain AA levels during ontogeny. However, a differential time–course between plasma T and brain AA levels exists in LAL and SAL males around birth. Moreover, as a result of the aromatization of T, a differential pattern of brain E_2 formation must occur in these selection lines as well. The present results suggest a higher E_2 formation in brains of LAL males prenatally, due to more substrate availability. Neonatally, E_2 formation is higher in brains of SAL pups due to increased circulating T levels and higher AA levels in the amygdala. Accordingly, not only plasma T levels differ, but also a differential pattern of E_2 formation exists in the brains of the two selection lines during ontogeny. It is for further research to determine to what extent this variation in T and E_2 peak occurrence around birth might result in a differential organization of adult CNS sensitivity to sex steroids in the genetically aggressive and non-aggressive mice.

Acknowledgements

The investigations were supported in part (J.C.C.) by the Foundation for Biological Research (BION: 426.061), which is subsidized by the Netherlands Organization for Scientific Research (NWO). The aromatase activity studies were carried out by J.C.C. while visiting the AFRC Babraham Institute at Cambridge, UK (supported by NWO/BION: SIR 14–776 (J.C.C.) and a MRC grant to J.B.H.). The authors wish to thank Mrs. G. Korte and Dr. J. Pratt for performing T RIA after extraction and chromatography.

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