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Aromatase Activity in the Preoptic Area Differs Between Aggressive and Nonaggressive Male House Mice

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COMPAAN, J. C., A. WOZNIAK, A. J. H. DE RUITER, J. M. KOOLHAAS AND J. B. HUTCHISON. *Aromatase activity in the preoptic area differs between aggressive and nonaggressive male house mice.* BRAIN RES BULL 35(1) 1–7, 1994.— Treatment with testosterone (T) or estradiol (E₂) facilitates intraspecific aggressive behavior in adult rodents. Brain aromatization of T to E₂ appears to be involved in facilitation of fighting behavior. In the present study we measure the in vitro brain aromatase activity (AA) in the preoptic area (POA), amygdaloid nuclei (Am), ventromedial hypothalamus (VMH), and parietal cortex (CTX) from two strains of adult male house mice, which were genetically selected for territorial aggression, based upon their attack latencies (short attack latency: SAL; long attack latency: LAL). The results reveal a higher AA in the POA of nonaggressive LAL males, as compared to aggressive SAL animals. The POA AA is, thus, inversely correlated with aggressiveness. The AA levels in both the VMH and Am do not differ significantly between strains. Furthermore, a differential brain area-specific AA distribution exists: POA > VMH AA in LAL, whereas POA < VMH in SAL. In both selection lines, the Am exhibits the highest levels of AA, as compared to the other investigated areas. Kinetic studies revealed that the aromatase K_m is similar in both strains. The results indicate that the strain difference in AA is specific to the POA, but is not necessarily positively correlated with circulating plasma T levels. Other factors, in addition to androgen, are probably involved in the regulation of POA aromatase. We suggest that a higher neural androgen receptor sensitivity exists in the POA of nonaggressive LAL males, resulting in higher adult POA AA, despite lower concentrations of circulating T.

Aggression	Aromatization	Preoptic area	Ventromedial hypothalamus	Amygdala
Bed nucleus of the stria terminalis		Testosterone		

INTRASPECIFIC aggressive behavior is known to be influenced by the circulating level of the gonadally secreted steroid hormone testosterone (T) (1,10). Castration of adult rats and mice results in a decrease of aggressive behavior, which can be restored after T replacement (5,28). Furthermore, the baseline plasma T concentration is positively correlated with aggression in adult male rats (45). Not only T, but also estradiol (E₂) administration augments aggressive behavior after gonadectomy (35,47,48), whereas a blockade of E₂ action reduces the T-elicited fighting response (12). Moreover, a different genetic predisposition to the aggression-promoting properties of T and E₂ has been shown in wild mice (15,55). These studies have led to the hypothesis that a differential degree of conversion of T to E₂ by the enzyme aromatase may be involved in the expression of aggressive behavior.

The effects of T on aggressive behavior are on specific areas in the central nervous system (CNS) (1). Several brain areas, i.e.,

ventromedial hypothalamus (VMH) (29,36), amygdala (Am) (8,9,57), and preoptic area of the anterior hypothalamus (POA) (2,7), are involved in the expression of aggression. The VMH, Am, and POA are target sites at which T is mainly converted to E₂ (34). Although brain conversion of T to E₂ is not essential to evoke fighting behavior (10), particularly the VMH, Am, and POA exhibit high aromatase activity (AA) levels in various species, including mouse (61), rat (39), hamster (26), dove (24), and Japanese quail (3). Moreover, the activity of brain aromatase is mediated by testosterone (21,22,50) via androgen receptors (40).

In our laboratory two selection lines of wild house mice are available. These lines are genetically selected for territorial aggression, based upon intermale attack latency score (ALS) in a resident–intruder paradigm (54). This paradigm has been chosen because it closely resembles the naturally occurring aggression at the border of a territory (31). The ALS is hardly influenced by crossfostering or isolation during de-

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velopment and showed a negative relationship with the frequency of other aggressive behavioral elements, including attacking, fighting, and chasing (54,56). The males of these strains not only differ in aggression, but also in several T-related parameters. Adult males of the aggressive short attack latency (SAL) line have a higher plasma T level, a higher seminal vesicle weight (55), and a larger testicular T production capacity (17), as compared to nonaggressive males of the long attack latency (LAL) line. Moreover, aromatase activity occurs in androgen target areas of the mouse brain (61).

One important factor in the interindividual variation of aggressive behavior might be the activity of the T-metabolizing enzyme aromatase in the brain. To investigate whether T aromatization is related to the differential expression of aggression, we measured *in vitro* aromatase activity in the POA, VMH, and Am of aggressive SAL and nonaggressive LAL male mice. Because these selection lines also differ in circulating plasma T level, we predicted that the aggressive line would have a higher brain AA level.

METHOD

Animals

Adult wild male mice (*Mus musculus domesticus*), genetically selected for territorial aggression based upon attack latency score (ALS), were used (54). The animals were derived from the long attack latency (LAL) (generations 19–22) or short attack latency (SAL) (generation 44) selection lines. The mice were housed on sawdust bedding in standard mouse breeding Plexiglas cages (17 × 11 × 13 cm) in animal rooms at controlled light:dark cycle (12L:12D; lights off at 1230 h) and temperature (19–21°C). Standard lab chow and water was available *ad lib*. The litters were weaned at 3 weeks. At the age of sexual maturity (6–8 weeks) each male was paired with one female of the same selection line. At the age of 14 weeks 10 males of both selection lines were tested for their attack latency score (ALS) according to a standard procedure (54). The ALS is the mean of the attack latencies scored on three consecutive days, determined between 1300 h and 1500 h, during the early dark phase. Each experimental animal was confronted with a gonadally intact male albino mouse opponent (MAS-GRO). The test was terminated immediately after an attack occurred, or, in the absence of any attack, after 10 min. Thus, for those animals that did not fight at all, the ALS was set at 600 s. To avoid measuring a direct effect of aggressive behavior on aromatase activity, the animals were sacrificed 1 month after behavioral testing.

Brain Sampling

All animals were quickly anaesthetized with ether and killed by decapitation. Subsequently, the brains were removed and immediately frozen at –80°C. Coronal brain slices were dissected according to the method described previously (24,52). The various brain areas, including preoptic area (POA), ventromedial hypothalamus (VMH), amygdala bilaterally (Am l, Am r), and parietal cortex (CTX) were removed using a modified Palkovitz punch technique, and stored at –80°C. Thereafter, the various micropunches were individually homogenized in 100 μ l ice-cold TEK buffer (100 mM Tris-HCl, 50 mM KCl, 1 mM Na₂EDTA; pH 7.4), frozen and stored at –80°C.

Aromatase Assay

The activity of the T-aromatizing enzyme was measured by an *in vitro* microassay in individual brain punch samples using maximal substrate concentration, and in pooled samples ($n =$

9–10) of POA and left Am at several substrate concentrations for kinetic analysis. This assay is based on the stereospecific release of ³H₂O from (1 β -³H)-T as a result of aromatization. We used this tritiated water assay as previously described (51) with the following modifications. Incubation of the homogenates was carried out for 30 min at 37°C in a microplate. Each well contained 30 μ l homogenate, 0.2 mM NADPH, and (1 β -³H)-T (final volume 50 μ l). The substrate (1 β -³H)-T was prepared from (1 β ,2 β -³H)-T (NET-187, specific activity: 40–50 Ci/mMol, New England Nuclear) by overnight hydrolysis, and redissolved in TEK buffer so that 10 μ l would yield approximately 3 × 10⁵ cpm, equivalent to 300 nM. This is approaching a saturating substrate concentration for the mouse brain aromatase (61). Kinetic studies were based on the method of Wozniak et al. used in this paper (61). Pooled POA and Am l samples enabled 6 × 30 μ l aliquots to be incubated at substrate concentrations 6, 12, 25, 50, 100, and 300 nM (1 β -³H)-T. Assay controls used either boiled homogenate, or homogenate containing 10^{–4} M of the specific aromatase nonsteroidal inhibitor Fadrozole (CGS 16949A: 4-(5,6,7,8-tetrahydroimidazo-[1,5-a]pyridin-5-yl) benzonitrile HCl, provided by A. S. Bhatnagar, Ciba-Geigy) (59,60). The reaction was stopped by freezing the microplate in solid CO₂, and adding 150 μ l distilled 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 (51) the eluates were decanted into vials, 2 ml scintillation fluid (Ultima Gold, Packard) was added, and ³H₂O product was counted in a scintillation counter (Model 3255, Packard). Interassay variation ($\leq 10\%$) was checked by including a few aliquots of the same active pooled aromatase sample in all assays.

Protein Assay

Protein concentration was measured by a sensitive modified Coomassie dye binding assay (minimum of 1–2 μ l homogenate is needed; ≥ 0.1 – 0.2 μ g protein detectable) using bovine serum albumin as standard (49). Aromatase activity is thus expressed as fmol ³H₂O formed/h/mg protein.

Statistics

The data on aromatase activity in the various brain areas of SAL and LAL selection lines were evaluated for significance using a multivariate analysis with repeated measures [Pillai's test in SPSS/PC+ MANOVA (20)]. Differences in AA between SAL and LAL selection lines in the corresponding brain areas were analyzed by post hoc Student's *t*-test. Paired *t*-test was used to clarify the differences in distribution pattern of AA within each selection line. Spearman Rank correlation test was used to determine if the correlation between attack latency score and AA in the various brain areas was significant. As a critical region for significance we used $p < 0.05$.

RESULTS

Reaction rates with the ³H₂O assay were linear with incubation time (15–60 min) and sample concentration (up to 2 mg protein/ml; data not shown). The experimental conditions fall within these limits. The mean protein concentration of the incubated samples was 1.7 mg/ml. Extreme differences in protein concentration between individual homogenates were not found. The assay controls showed low counts per minute, which were comparable to background counts.

FIG. 1. Aromatase activity in pooled ($n = 9-10$) preoptic area (POA; left side) and left amygdaloid nucleus (Am l; right side) of males of the aggressive short attack latency (SAL; open symbols) and nonaggressive long attack latency (LAL; closed symbols) selection line. Velocity plots (top) and Lineweaver-Burk transformation (bottom) are shown. Experimental conditions are as described in the text.

Kinetics of AA

The aromatase kinetic analysis of SAL and LAL POA and Am l is shown in Fig. 1. The velocity curves and linear Lineweaver-Burk data transformation are presented. There are differences in aromatization rates between brain areas (Am l > POA). In addition, differences exist between selection lines in POA AA. The K_m of aromatase from both brain areas of these selection lines are similar.

Difference in Brain AA Between SAL and LAL Mice

The mean attack latency scores (ALS) of the nonaggressive LAL ($n = 10$) and aggressive SAL ($n = 10$) males used in this experiment were 581.1 ± 10.5 s and 63.7 ± 16.5 s, respectively. Spearman Rank correlation test reveals a significant positive correlation between POA AA and ALS and, therefore, an inverse correlation with aggressiveness ($r_s = 0.489$, $p < 0.05$; Fig. 2). No correlation exists between ALS and AA in both left and right Am, VMH, or CTX.

MANOVA reveals a significant effect of brain area, $F(3, 42) = 17.73$, $p < 0.001$, and an interaction of brain area with selection line, $F(3, 42) = 3.38$, $p < 0.05$. Therefore, a significant difference between selection lines exists in specific brain areas. Thus, the nonaggressive LAL group has a POA AA (180.68 ± 29.3 fmol/h/mg protein) significantly higher than that of SAL males (80.70 ± 12.5 fmol/h/mg protein; $p = 0.01$). No significant differences in VMH AA were found between LAL and SAL animals. The mean AA of both left and right Am in the LAL males shows a slightly, but not significantly, higher activity than SAL males. Also, the left Am tends to have a higher AA than the right Am, in both selection lines (Fig. 3).

Furthermore, within each selection line an area-specific distribution of aromatase activity exists in the brain. As presented in Fig. 4 in the LAL males the POA AA is significantly higher than VMH AA ($p < 0.05$), whereas the opposite occurs in the SAL males ($p < 0.05$). The CTX shows a very low AA of 29.7 ± 7.5 , and 28.1 ± 8.8 fmol/h/mg protein, in LAL and SAL males, respectively, whereas the Am has the highest AA com-

FIG. 2. Natural logarithms of individual attack latency scores ($\ln(\text{ALS})$) of intact aggressive short attack latency (open circles) and nonaggressive long attack latency males (closed circles) and their corresponding aromatase activities (AA) in the preoptic area of the hypothalamus (POA). A significant correlation exists between ALS and POA AA (Spearman Rank correlation: $r_s = 0.489$, $p < 0.05$).

pared to all other investigated brain areas ($p < 0.05$) in both SAL and LAL animals (Fig. 3).

DISCUSSION

Testosterone and Aromatase Activity

In the present study we demonstrate that nonaggressive male LAL mice have a higher level of aromatase activity in the POA than aggressive SAL males, whereas no significant differences are found between SAL and LAL selection lines in the Am and VMH. Although aromatase activity is generally low in adults of this mouse species, kinetic analyses yield linear plots of the transformed velocity data. The enzyme from both SAL and LAL mice obeys single substrate Michaelis–Menten kinetics using this *in vitro* assay. These kinetically determined activities (Fig. 1) agree well with the mean individual activities determined at single-point substrate concentration (300 nM T; Fig. 2). Both experiments show independently that the AA levels of the Am are higher than those of the POA in both strains, and that the LAL POA AA is higher than that of SAL mice. The similar substrate binding affinities (apparent K_m) of aromatase obtained from brain areas of both SAL and LAL animals, suggests that the genetic selection for aggression has not changed the metabolic properties of the enzyme.

The higher POA AA indicates a higher estradiol formation in the POA of nonaggressive LAL males than in aggressive SAL males, assuming that the aromatase substrate T is equally available in brains of both selection lines. However, we have shown in previous studies that adult nonaggressive LAL males have a lower plasma T level (SAL: 6.3 ± 0.6 and LAL: 4.8 ± 0.4 ng/ml plasma), a lower seminal vesicle weight (55), and a smaller percentage of testicular Leydig cells (SAL: 4.25% and LAL: 3.0%) (17), as compared to aggressive SAL mice. Accordingly, high levels of circulating T are not necessarily positively correlated with brain AA. However, the above-described reciprocal relationship between cir-

FIG. 3. Mean aromatase activities (AA + SEM) of intact aggressive SAL (hatched bars) and nonaggressive LAL males (solid bars) in the preoptic area (POA), ventromedial hypothalamus (VMH), amygdala left and right (Am l, Am r), and parietal cortex (CTX) of the brain. Significant difference between selection lines in POA AA exists ($*p < 0.05$).

culating T and brain AA seems to be at variance with studies providing evidence for the important role of T in inducing AA in the POA, VMH, and Am. Because males exhibit higher plasma T levels than females, several studies have indicated a naturally occurring sex difference in brain AA. Thus, a sex difference in POA AA level has been demonstrated in rat (39,52), dove (24), and quail (43). In the rat levels of AA in the VMH and medial amygdala (MeAm) differ between the sexes (39,52). This sex difference is dependent on circulating plasma T level, because AA decreases in the POA, VMH, and MeAm after castration but can be restored by T replacement (3,39,50,52). The T-dependent induction of AA is apparently mediated by the androgen receptor (40), although a positive feedback of estradiol on its own synthesis also exists (25,38). The neural

FIG. 4. Aromatase activity (AA) in the preoptic area (POA) and the ventromedial hypothalamus (VMH) of aggressive SAL (open circles) and nonaggressive LAL male mice (closed circles). Lines join samples from the same individual. Aromatase activity distribution differs significantly between strains: LAL: POA > VMH and SAL: POA < VMH ($p < 0.05$).

androgen receptor-mediated AA induction is also sexually differentiated in the POA, MeAm, and VMH; i.e., males show higher AA induction after standard T treatment than females (3,37,52). Neonatal gonadectomy and subsequent T treatment attenuates the sex difference in adult rat POA AA (52). Accordingly, a sex difference in adult androgen receptor sensitivity, mediating the AA in the POA, is probably established during ontogeny. Moreover, an inverse correlation has been shown between circulating androgens and POA, VMH, and Am AA in male androgen-insensitive testicular feminized (Tfm) rats, as compared to their normal littermates (41). Hence, the apparent discrepancy between adult circulating T levels and brain AA in the currently investigated mice might be due to a differential organization of neural androgen sensitivity around birth. This hypothesis suggests that during the perinatal period a higher CNS sensitivity to T is established in the nonaggressive LAL, as compared to SAL mice. The increased sensitivity may lead to higher adult AA in the hypothalamus and limbic structures, despite lower levels of adult circulating androgen in LAL than in aggressive SAL males. Indeed, ontogenetic studies on percentage of testicular Leydig cells, plasma T, and brain AA, and neonatal manipulation of T in SAL and LAL selection lines, indicate a differential organization of the CNS around birth (13,14,16). Moreover, preliminary results indicate a higher brain androgen receptor density in LAL males (Compaan et al., unpublished data). Besides the genomic action of T via the androgen receptor system, other factors might regulate the brain AA (6,11,30,33,60).

Distribution of Aromatase Activity

The results of the present study provide a quantitative profile of the distribution of aromatase activity in some hypothalamic nuclei and limbic structures of wild house mice. The highest level of AA is demonstrated in the Am, intermediate levels occur in both the VMH and POA, whereas a very low level of AA exists in the CTX of the brains of both SAL and LAL selection lines. This distribution of AA is in accordance with our earlier study performed on BALB/c and Swiss NIHs mice, in which a relatively high level of AA was also demonstrated in the Am and an intermediate level of AA in the hypothalamus (61). Levels of AA in POA and Am of the wild house mouse are similar to those in BALB/c mice.

Regional differences in brain AA have been shown in rat (39), hamster (26), and dove (24). However, within the genetic male of the currently investigated species of wild mice a differential brain area-specific distribution of AA exists in the brain. In the LAL animals, the AA is higher in the POA than in the VMH, whereas the SAL males have higher VMH than POA AA. Accordingly, a differential regulation of AA in both hypothalamic nuclei might exist. This is also suggested for AA in the anterior hypothalamus and POA of doves (25) and hamsters (26). In addition, the slight, but not significant, difference in AA levels between left and right Am suggests an asymmetry in expression of the enzyme aromatase. This would be in accordance with Von Ziegler and Lichtensteiger (58), who also found lateralization of AA levels in the rat brain during ontogeny.

The results of our study contrast with an immunocytochemical study in brains of Jackson/C57 black and Swiss mice (4), in which no aromatase immunoreactivity was observed in the POA, VMH, or Am. However, it is difficult to compare *in vitro* activity measurements with immunolocalization of the enzyme *in vivo*. In the former method, the activity of aromatase is measured, whereas in the latter a so far unknown epitope of aromatase is recognized by the antibody. This recognition may even change due to enzyme activity-related alterations of the epitope (53).

Testosterone Dependent AA and Aggression

Although aggression is positively correlated with T and E₂ (1,47), we observed an inverse correlation between POA AA and aggression in SAL and LAL mice. We can speculate about the functional significance of the observed inverse correlation between POA AA levels and aggressiveness. That LAL mice are less aggressive than SAL animals, is obviously not due to a lower conversion rate of testosterone to estradiol by aromatase in the various investigated brain areas. Although no correlation was found between whole mouse brain cytosolic fraction AA and aggressiveness, an inverse correlation has been reported between aggression and whole brain nuclear fraction AA (18). However, it is difficult to validate the hypothesis that a higher POA estradiol formation would reduce aggressiveness, because it is known that E₂ facilitates aggressive behavior (35,47,48). Alternatively, it is possible that in LAL males low plasma levels of T are compensated for by a higher E₂ formation in the POA. The compensation is insufficient to reach the attack latency score of the aggressive SAL line, due to either low E₂ formation or neural E₂ insensitivity. Finally, the role of the POA in the expression of aggressive behavior is still unknown. The bed nucleus of the stria terminalis, a region immediately dorsal to the POA, rather than the POA itself, is also important in modulating androgen-dependent aggressive behavior (1). In contrast to mice, a positive correlation between POA AA and aggressiveness has been shown in Japanese quail (42). Although Schlinger and Callard established a method of separately measuring aggressive behavior in terms of a peck order (44), this type of aggression is directly linked to reproductive state, and thereby probably a change in POA AA.

The MeAm is known to be involved in aggressive behavior, because of its crucial role in social learning and memory (9,27,57), as it receives input from the olfactory bulb and projects to the VMH (32). Both the Am and VMH are known to be involved in the expression of aggressive behavior (29). In our study the amygdala exhibits the highest level of AA in both SAL and LAL selection lines, but no significant difference in AA between the two strains was found.

Finally, T is not only converted to estradiol, but is also converted to 5 α -reduced androstanes, including 5 α -dihydrotestosterone (DHT), in the hypothalamus and limbic structures (46). Indeed, DHT induces AA in the POA of castrated rats (38), in contrast to doves, in which no AA is induced by peripherally injected DHT (23). Moreover, a synergistic effect of E₂ and DHT on aggressiveness has been reported (19,47,48). These studies suggest that 5 α -reduction of T in the brain also plays a major role in interstrain, and possible interindividual differences in aggressive behavior. Accordingly, the role of 5 α -reductase in the differentiation of aggression as well as neural sex steroid sensitivity during ontogeny and at adult age of both SAL and LAL selection lines requires further investigation.

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