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Differential Perinatal Testosterone Secretory Capacity of Wild House Mice Testes Is Related to Aggressiveness in Adulthood

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Testosterone secretory capacity of testicular Leydig cells was determined in fetal males of an aggressive and a nonaggressive genetic selection line of wild house mice. They were studied at Days 15–18 of gestation and on the first day after birth. A previously described morphometric method was used to quantify 3β-hydroxy steroid dehydrogenase (3β-HSD)-stained Leydig cells in testicular sections to determine testosterone secretory capacity, which may be considered to reflect circulating plasma testosterone in the fetus. The results of this study show that the testosterone secretory capacity of Leydig cells in the testis changes differentially during intrauterine development in males of the aggressive and nonaggressive selection lines. The peak secretory capacity is reached at Day 17 of gestation for the males of the aggressive selection line, while the peak for the nonaggressive males is reached on the first neonatal day. The larger anogenital distance observed in aggressive males suggests a higher prenatal testosterone level in these males. The importance of the difference in timing of the perinatal 3β-HSD peak top individual variation in adult aggressive behavior is discussed.

In the study of male intraspecific aggressive behavior, individual variation is a well-known phenomenon. In a group, levels of aggression often determine the individual's position in the social hierarchy. In a behavior genetic study on populations of wild house mice, it was shown that this individual variation cannot be fully explained by social environmental factors. A distinct genetic component is also involved (van Oortmerssen, Benus, and Dijk, 1985). This notion led to the development of two lines of genetically selected wild house mice. One line has been selected for short attack latencies (SAL), whereas the other line has been selected for long attack latencies (LAL) (van Oortmerssen and Bakker, 1981). These selection lines allow an analysis of the relative contribution of genetic and ontogenetic factors in the development of adult aggressive behavior.

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Several studies clearly indicate that plasma testosterone is an important causal factor in aggressive behavior, not only in adulthood, but also during ontogeny (Albert, Jonik, Watson, Gorzalka, and Walsh, 1990; Schuurman, 1981; Edwards, 1969; vom Saal, 1983a; Rines and vom Saal, 1984). The results of a previous study, in which the relationship of responsiveness to circulating testosterone (T) on adult aggression was studied, pointed already to the importance of the perinatal period (van Oortmerssen, Dijk, and Schuurman, 1987). Furthermore, we found that the neonatal secretory capacity of the testes in the two selection lines are inversely related to the level of adult aggressive behavior, i.e., LAL neonates have a significantly higher percentage of Leydig cells in the testis than SAL neonates (de Ruiter, Koolhaas, Keijser, van Oortmerssen, and Bohus, 1992). Functionally, a higher amount of Leydig cells (T secretory capacity) is related to higher plasma levels of testosterone (see Orth and Weisz, 1980; Weisz and Ward, 1980; Lording and de Kretser, 1972).

The higher percentage of Leydig cells in the testes of the LAL neonates may cause a suppression in the responsiveness to T in adult LAL males. This may not be the case in SAL males, as they show no enhanced neonatal T production capacity. This idea was confirmed by a study by Compaan, Ruiter, Koolhaas, van Oortmerssen and Bohus (1992), in which neonatal T treatment led to a reduction in aggression in LAL males. Surprisingly, the aggressive behavior of SAL males was not affected by such a treatment. This indicates that not only the neonatal T level, but also the sensitivity of the central nervous system to T in this period, differs between the two selection lines. This difference in T secretory capacity and sensitivity of the central nervous system to circulating T at Neonatal Day 1 suggest that an important part of the differentiation process may already have occurred prenatally.

A number of studies on the role of perinatal T in the sexual differentiation of rats emphasize the importance of a prenatal T surge around Day 18 postconception in males (MacLusky and Naftolin, 1981; Gorski, 1991; Ward, 1992). Males are characterized by a high prenatal T surge, whereas an experimentally induced high T surge at Day 18 will permanently masculinize the fetal brain of females (Perakis and Stylianopoulou, 1986; Hoepfner and Ward, 1988). On the other hand the process of defeminization and masculinization can be prevented by blocking prenatal T surge via prenatal stress or administration of anti-androgenic agents that have proved to reduce prenatal T effects (Orth, Weisz, Ward, and Ward, 1983; Ward and Weisz, 1984; Lambert, Mitchel, and Robertson, 1987; vom Saal, 1978). Moreover it has been reported that prenatal enhancement of androgen level sensitizes the male (or female) to testosterone with respect to aggressive behavior in adulthood (Gandelman, Simon, and McDermott, 1979; vom Saal, 1979).

In view of the role of neonatal T in the individual differentiation of

aggressive behavior within the male sex, it is also necessary to know whether and when the two genetic selection lines differ in prenatal T. This paper describes our search for such differences by measuring 3β-hydroxy steroid dehydrogenase (3β-HSD)-stained testes sections of male fetuses. 3β-HSD is considered to reflect fetal T secretion. Because of the small blood volume in prenatal and neonatal mice, a determination of individual plasma T levels is not possible. For this reason we used a previously described method to estimate morphometrically the amount of enzyme-histochemically characterized Leydig cells, as a measure of T secretory capacity (de Ruiter *et al.,* 1992). Since fetal 3β-HSD activity and T production can already be observed around the 14th–15th post-conceptional day (Baillie and Griffiths, 1964), testes were studied from fetal males of both selection lines on the 15th, 16th, 17th, and 18th gestational day and on the first day after birth.

Since vom Saal (1983b, 1978) has shown that the anogenital distance (AGD) in newborn mice (and rats) reflects the prenatal T exposure, the AGD of neonatal mice of both selection lines were measured.

METHODS

Animals and Treatment

Male and female house mice *(Mus musculus domesticus)* of the SAL (39th generation) and LAL line (15th generation) were kept separately as a pair in a double set of Plexiglas cages of 17 × 11 × 13 cm connected with each other by a Plexiglas tube (diameter 6 cm), which was closed by a perforated sliding door, so they could see and smell each other. They were kept in climate-regulated rooms at 20°C with a LD 12:12-hr cycle (light off, 12.30 hr). Food and water were available *ad libitum.*

The females were aged between 4 to 7 months. The fathers of the SAL females showed an average attack latency of 50 (± 25) sec. The fathers of the LAL females did not show attack within 10 min, which is the maximum score used. (The procedure for determining the mean individual attack latency of the males is described in van Oortmerssen and Bakker, 1981.) The number of litters measured per postconceptional day is two or three. The number of males used per litter also varied from two to three.

To obtain an accurate estimate of conception, every 4th day, cages were connected by removing the sliding door between 16:00 and 9:00 hr, in the dark period. Thereafter, every 2nd day bodyweight of the females was evaluated. If they were pregnant with more than two pups, a significant weight increase could be registered at Days 11–12, as determined by pilot studies. After autopsy, when the number of pups was known, the day of conception could be reconstructed. The day on which male and female were separated again was considered to be Day 0 of gestation.

Anogenital distance at 4 days of age was measured according to the method of vom Saal (1978) using a binocular microscope, equipped with an ocular micrometer, with a magnification of $\times 10$.

Autopsy

Pregant females were killed by CO_2 inhalation. Uterectomy was carried out rapidly and the uterus was kept in cold saline. Fetus body weight was measured, and the testes were rapidly frozen, sectioned, and used for histochemistry and subsequent morphometric analysis. Per selection line, per postconceptional day about six fetuses were used, descended from two to three litters.

Histochemistry

For the localization of delta 5-3β-HSD activity, the testes were rapidly frozen with CO_2 and subsequently cut into 15-μm sections. The sections were incubated for 2.5 hr at 37°C in a freshly prepared medium containing 0.12 m*M* dehydroepiandrosterone as a substrate solved in 1 ml DMF, 1.75 m*M* nicotinamide, 0.17 m*M* Nitro Blue Tetrazolium, 0.54 m*M* NAD. Incubation of the sections was carried out according to the method of Baillie, Ferguson, and Hart (1966).

Morphometry

To determine the percentage of Leydig cells in testicular tissue per animal six to eight sections of both testes (about 0.18×10^6 μm^2) were taken for area measurements of the selectivity (3β-HSD) stained Leydig cells by automatic discrimination from the hardly stained background, using the IBAS automatic image analysis system of KONTRON.

Statistical Analysis

For statistical evaluation two-way analysis of variance (ANOVA) was used. Differences between selection line per age group were determined with Student's *t* test (as well as Wald Wolfowitz run test).

RESULTS

In the testes of the males of both selection lines the first 3β-HSD-positive interstitial cells (Leydig cells) could be detected at Day 14 post-conception, but the 3β-HSD staining was too weak for morphometrical analysis.

The results of the morphometric analysis of the testicular Leydig cells are depicted in Fig. 1. Analysis of the data (ANOVA) of the Leydig cell measurements of Days 15–18 postconception and the first neonatal day shows that there is no significant group effect ($F = 0.008$; $df = 1,4$; $P > 0.05$), but a significant effect of time ($F = 34.723$; $df = 1,4$; $P <$

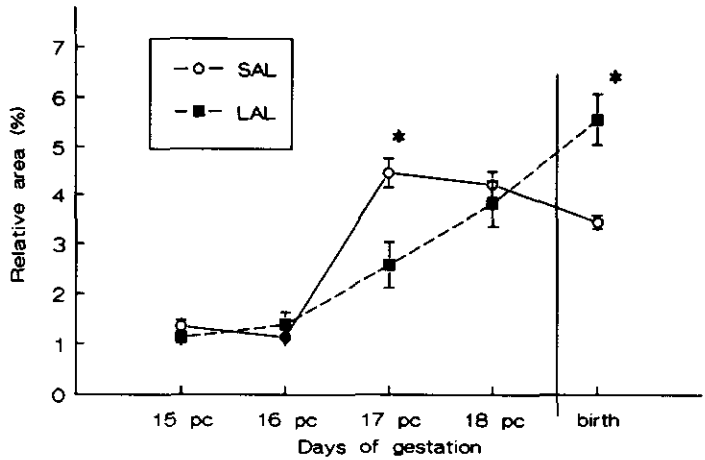

FIG. 1. Percentage of Leydig cells (mean area (%) ± SEM) in testes sections of fetal mice on the 15th, 16th, 17th, and 18th postconceptional days as well as in neonatal males on the first day of life. * $P < 0.05$.

0.05). Moreover there is a significant group × time interaction ($F = 9.899$; $df = 1,4$; $P < 0.01$).

Statistical evaluation of the separate data points shows that at Day 17 postconception, 2 days before birth, in the male fetuses of the SAL line there is a significantly larger amount of Leydig cells ($P = 0.005$, Student's t test) than in the males of the LAL line (Fig. 1).

At Day 18 postconception in the males of both selection lines Leydig cell populations are equal in size, because in SAL males the Leydig cell population is decreasing, while in LAL males this population is (still) growing (Fig. 1).

On the first neonatal day in the LAL males Leydig cell area percentage has increased so far that the (relative) amount of interstitial cells is significantly larger ($P = 0.001$, Student's t test) than in the males of the aggressive SAL line (Fig. 1).

Measurements of the AGD on the 4th neonatal day clearly show that in the males of the SAL selection line the AGD is significantly larger ($P < 0.001$, Student's t and Wald Wolfowitz) than in the males of the LAL selection line (Fig. 2). Interestingly a similar significant difference is observed when the females of both selection lines are compared ($P < 0.001$, Student's t and Wald Wolfowitz; Fig. 2). After correction for the small differences in body weight, the differences between SAL and LAL remained significant for males ($P = 0.014$, Student's t) as well as for females ($P = 0.020$, Student's t).

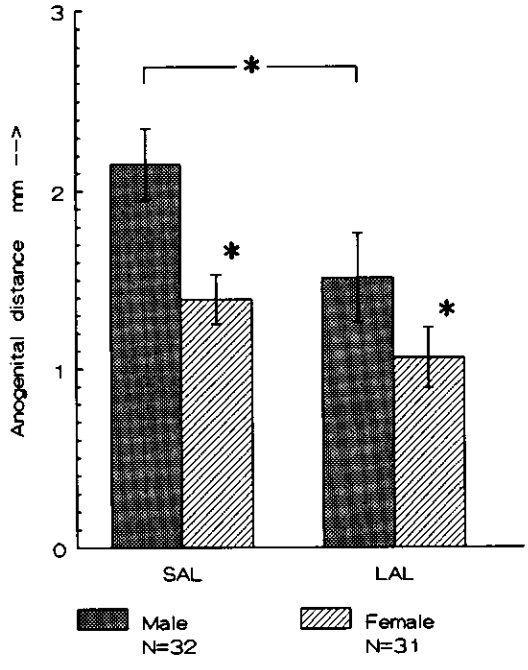

FIG. 2. Mean (± SD) anogenital distance of neonatal males and females of both selection lines at Day 4 of life. * $P < 0.05$.

DISCUSSION

The results of this study show that the amount of 3β-HSD-positive interstitial Leydig cells in the testes changes differentially during perinatal development in the aggressive (SAL) and nonaggressive (LAL) selection lines of wild house mice. The highest T secretory capacity is reached in the SAL fetus on the 17th gestational day. Provided that the amount of 3β-HSD-positive Leydig cells reflect the average level of circulating T (Orth and Weisz, 1980; Weisz and Ward, 1980), the SAL fetus appears to be more androgenized and defeminized than the LAL fetus. The observed significantly larger anogenital distance (which is a reflection of prenatal T; vom Saal, 1978; Hoepfner and Ward, 1988) in the neonatal SAL males is consistent with this idea.

From the work of Rhees, Shryne, and Gorski (1990) it is known that the onset of the hormone sensitive period of the brain, e.g., the sexually dimorphic nucleus of the preoptic area, with respect to sexual differentiation in the rat is Day 18 of gestation, about 3 days before birth. At this time in the developing male a maximal rise in T plasma level (Weisz and Ward, 1980) and subsequently a maximal rise in E_2, due to aromatization in the hypothalamus (Rhoda, Corbier, and Roffi, 1984; Tobet,

Baum, Tang, Shim, and Canick, 1985) and androgen receptor activation (Toyooka, Connolly, and Resko, 1991), takes place. Day 18 of gestation in the rat is probably a developmental stage comparable to Day 17 of gestation in mice in which gestation lasts 19 days instead of 21 days, as in the rat. It seems, therefore, that in the SAL fetuses, the prenatal T surge coincides nicely with the sensitive period of the brain for the masculinizing and defeminizing action of T (see Gorski, 1991; Ward, 1992). The differences in the course of perinatal T between the two selection lines reveal a difference in the timing of the T surge relative to the time of birth, and probably, to the development of the brain.

The process of sexual differentiation of the brain with respect to reproductive as well as nonreproductive behaviors takes place during the perinatal period, during which the brain is more or less irreversibly defeminized and/or masculinized (Gorski, 1991; Ward, 1992; Beatty, 1992). Our present results suggest that the mechanisms involved in sexual differentiation are also involved in the individual differentiation within the male sex. In accordance with the reports of Gandelman *et al.* (1979) and vom Saal (1979), the prenatal enhancement of androgen level sensitizes the adult SAL male to testosterone, leading to an increased capacity to display aggressive behavior. In the house mouse it has been established that postpubertal SAL males are more sensitive to a standard dose of T, as measured by attack latency, than are the nonaggressive LAL males (van Oortmerssen *et al.*, 1987).

It seems that, due to the later occurring T surge in the LAL fetus, the neonatal brain is less masculinized but still sensitive to T. Indeed, neonatal T treatment affects adult aggressive behavior in the LAL line (Compaan *et al.*, 1992). However neonatal T treatment had no effect on adult aggressive behavior in the SAL line, suggesting that the high prenatal T surge in the SAL fetus has fully masculinized the brain in these males.

Presumably this differentiation process is not restricted to social behavior only. A number of experiments have shown that adult levels of aggressive behavior reflect active coping behavior (Benus, Koolhaas, and van Oortmerssen, 1987, 1991). For example, SAL males are good active shock avoiders, whereas LAL males perform poorly in this situation (Benus, Bohus, Koolhaas, and van Oortmerssen, 1989). In rats it has been reported that reducing the effectiveness of the prenatal T surge by prenatal treatment with the anti-androgen cyproterone acetate abolishes the sex differences in avoidance behavior. In the treated fetuses, the anogenital distance has also been reduced (Scouten, Grotelueschen, and Beatty, 1975; Beatty, 1992). These cyproterone acetate-treated males resemble our LAL males with respect to the reduced anogenital distance and a low level of avoidance behavior (Benus *et al.*, 1987, 1989).

The potential causal relationship between the different developmental pattern of perinatal T production in the two selection lines and the dif-

ferences in aggressive and coping behaviors seen in the adult mice will have to be tested further in studies manipulating perinatal testosterone.

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