Pharmacology Biochemistry & Behavior, Vol. 23, pp. 439–444, 1985. 0091-3057/85 $3.00 + .00

Prenatal Nicotine Affects Fetal Testosterone and Sexual Dimorphism of Saccharin Preference[1]

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Received 10 December 1984

LICHTENSTEIGER, W. AND M. SCHLUMPF. *Prenatal nicotine affects fetal testosterone and sexual dimorphism of saccharin preference.* PHARMACOL BIOCHEM BEHAV **23**(3) 439–444, 1985.—In order to study effects of nicotine on fetal gonadal axis and sexually dimorphic behavior, time-pregnant Sprague Dawley rats were implanted on gestational day (GD) 12 with an osmotic minipump containing either nicotine tartrate, tartaric acid or saline. Others were sham-operated on GD 12 or left untreated. Male fetuses of all control groups displayed the characteristic rise in plasma testosterone at GD 18 (as compared to GD 17 and 19); this was abolished by nicotine. Adult offspring of untreated or tartaric acid-treated dams exhibited a marked sexual dimorphism in their preference for saccharin-containing drinking water at 0.06–0.25%. No such sex difference was seen in offspring of nicotine-treated rats. In controls, the sexes differed with respect to the proportion of rats with high saccharin preference. In the group of males prenatally exposed to nicotine, the proportion of animals with high preference increased to the female level. These data indicate that prenatal exposure to nicotine can interfere with the development of the male gonadal axis and with the organization of sexually dimorphic behavior.

Nicotine Testosterone Saccharin preference Sex difference Fetus Development Prenatal drug effects

DEVELOPING neuroendocrine systems of the fetus may represent sensitive targets for environmental influences. This is exemplified by the effects of prenatal stress on the fetal gonadal axis [10, 31, 32]. The delicate interaction between hormones and the central nervous system may also be disturbed by drugs, either through direct action on the fetus or indirectly by an alteration of maternal metabolism. In recent years, several centrally active compounds have been reported to affect the developing—fetal or perinatal—gonadal axis, such as cannabinoids [4,5] phenobarbital [3, 8, 9] and morphine [1,33]. Ethanol depresses neonatal testosterone levels in the rat [12] and causes lasting changes in the adjustment of the adrenal axis [28].

Animal experiments have demonstrated that prenatal exposure to nicotine can cause behavioral disturbances [2, 11, 21, 22, 24]. Analogous observations were made in children; in that case, other components of tobacco smoke may have contributed to the syndrome [7,25]. So far, the emphasis has been on alterations in spontaneous activity and cognitive functions. It appears that typical conditions which might reveal changes in neuroendocrine regulation such as sexually dimorphic behaviors, have not been studied. Since nicotine affects adult neuroendocrine systems [14], we investigated its action on the gonadal axis of the male rat fetus. After preliminary experiments had revealed an action on fetal testosterone [15], a sexually dimorphic behavior, i.e., the preference of rats for saccharin solution [26, 29, 30], was analyzed in offspring of nicotine-treated rats.

METHOD

Animals

Zivic Miller Sprague Dawley rats were bred in our colony under controlled light and temperature conditions (lights on 06.00 to 20.00, 22±1°C). Food (NAFAG 850) and water were available ad lib. All experiments were conducted with time-pregnant rats: receptive females were mated with experienced males between 20.00 and 21.30. Sperm-positive females were weighed and caged in groups of two until one day before delivery, in order to prevent unwanted effects from isolation. Gestational day (GD) 1 was defined as the stage 24 hr after the onset of the mating period.

Fetal Plasma Testosterone

Three groups of rats were implanted with an osmotic minipump (Alzet 2001) in light ether anesthesia at GD 12. The pump was filled with either nicotine hydrogen tartrate

[1]Preliminary reports were presented at the International Wenner-Gren Center Symposium on Steroid Hormone Regulation of the Brain, Stockholm, 1980 [15], at the IBRO Satellite Symposium on Drugs and Hormones in Brain Development, Zürich, 1982 [19] and at the Fifth International Meeting of the International Society for Developmental Neuroscience, Chiety, 1984 [20].

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(British Drug House Chemicals), tartaric acid (analytical grade Pharmacopoea Helvetica VI; both compounds dissolved in water, pH 2.5–3.0), or sterile saline. Nicotine was delivered at a rate of 25 μg/100 g × hr (1 μl/hr). The study further included dams sham-operated at GD 12 (skin incision and suture in ether anesthesia) and untreated rats. The dose of nicotine corresponded to body weight at GD 12, i.e., it diminished progressively with weight gain of the dams. A deterioration of nicotine during the week of treatment seems improbable in view of the low pH of the preparation.

Dams were anesthetized with chloral hydrate (600 mg/kg, SC) on either GD 17, 18, or 19 (18.00–20.00). Fetuses were taken out and decapitated (after determination of crown rump length) while still connected to the placenta. Trunk blood was collected into heparinized microhematocrit tubes (Brand) and centrifuged at 3000 g (4°C). Sex was determined by inspection of the gonads. Plasma from male and female fetuses of the same litter was pooled separately and stored at −30°C.

Plasma testosterone was assayed by RIA in duplicate or triplicate by the Hormone Laboratories of the Department of Obstetrics and Gynecology of the University Hospital of Zürich (Prof. Paul J. Keller), using a kit provided by the Federal Institute for Reactor Research, Würenlingen, Switzerland (Dr. R. Andres). The antiserum was raised in rabbits against BSA-testosterone-3-CMO-conjugate (titer 1:150,000; crossreaction with dihydrotestosterone 29%, with progesterone and estrogen less than 0.01%). Mean values of experimental groups were calculated from values of individual litters. When more than one pool of plasma had been obtained for a given sex in the same litter, the mean value of the litter was used for further calculations. Mean values were compared with two-tailed Student's *t*-test.

Saccharin Preference

Saccharin preference was tested in adult offspring of untreated rats (5 litters, 18 males and 23 females) and of rats treated with either nicotine (4 litters, 19 males and 23 females) or tartaric acid (3 litters, 15 males and 11 females). The total amount of nicotine contained in the Alzet pump type 2001 can be expected to be exhausted after 8–9 days, i.e., at around GD 20. All dams delivered spontaneously on GD 23. Littersize was reduced to 8–11 pups. In some litters, daily weight gain and anogenital distance were recorded. The pups were weaned at 28 days; thereafter, male and female littermates were raised in separate groups until the age of 3–5 months. In view of the rapid metabolism of nicotine, the dams could be expected to be drug-free at birth, therefore, no foster mothers were provided. In a recent investigation of effects of prenatal nicotine on central catecholamine systems, offspring raised by their own or by foster mothers exhibited the same concentrations of catecholamines and metabolites ([20] and in preparation). Previous behavioral studies on prenatal nicotine effects did not use foster mothers [11,22] or detected no or only minor effects of cross-fostering [24]. However, we cannot completely rule out minor effects from biochemical sequelae of the prenatal treatment that might have persisted in the lactating rats during the postnatal period.

The preference for saccharin-containing drinking water was tested essentially according to Valenstein and coworkers [29] and Shapiro and Goldman [26], but with an extended concentration range. Rats were individually caged for the experiment. Two bottles of plain water were presented during the first 3 days, then the rats were given a choice between plain water and saccharin-containing water. Each out of 4 concentrations (0.06%, 0.125%, 0.25%, 0.50%) was tested for 3 days (the sequence of concentrations was found to be of no major influence); fluid consumption was measured daily at 15.00. Values were analyzed in terms of the absolute volumes of water and saccharin solution consumed daily by each rat. In addition, the consumption of saccharin solution of each rat was expressed as a percentage of total daily fluid intake. Mean absolute and percentage values of experimental groups were compared with two-tailed Student's *t*-test, differences between individual animals (mean consumption in 3 days) by analysis of variance followed by Scheffe's method of linear contrasts. Proportions of rats exhibiting high or low preference (cf. the Results section) were compared in 2×2 contingency tables.

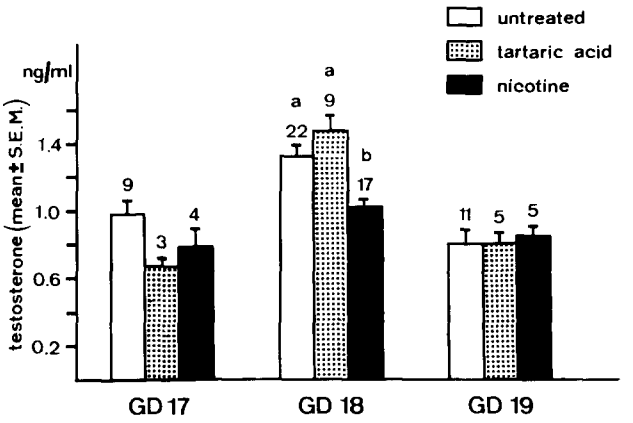

FIG. 1. Testosterone concentration in plasma of male rat fetuses from untreated dams or dams implanted at gestational day (GD) 12 with an osmotic minipump Alzet 2001 containing either nicotine hydrogen tartrate or tartaric acid. Plasma from males of the same litter was pooled (n=number of litters). a=different from GD 17 and GD 19 for $p<0.001$, b=different from untreated group and tartaric acid-treated group for $p<0.005$ and $p<0.001$, respectively.

RESULTS

Fetal Plasma Testosterone

In line with the previous observations of Weisz and Ward [34], plasma testosterone was found to be elevated in male fetuses at GD 18 as compared to both, GD 17 and 19. Nicotine clearly prevented this rise, whereas it did not affect the testosterone levels of GD 17 and 19 (Fig. 1). Since an irritation by the low pH of the solution delivered by the minipump (1 μl/hr) could not be completely excluded, controls were run with both, tartaric acid and saline. The GD 18 levels of these two groups are identical (tartaric acid 1.48 ± 0.28 ng/ml (9 litter, Fig. 1), saline 1.41 ± 0.27 ng/ml (9)) and correspond to the values of untreated rats. No significant change in the GD 18 testosterone value was seen after sham-operation at GD 12 (1.28 ± 0.26 ng/ml (7), different from nicotine for $p<0.01$).

In a number of litters, testosterone was also determined in plasma of female fetuses. The steroid concentration was 0.23 ± 0.24 ng/ml (9) at GD 17 and 0.30 ± 0.11 ng/ml (7) at GD 18; nicotine did not significantly affect the DG 18 level (0.19 ± 0.07 ng/ml (10)).

Sexually Dimorphic Preference for Saccharin Solution

In confirmation of earlier observations [26,29], female offspring of *untreated dams* consumed huge quantities of sweet solution; a clear-cut but significantly lower preference was seen in their male littermates (Fig. 2a). The different functional states could be characterized more precisely when the consumption of saccharin solution was expressed as a percentage of total daily fluid intake (Fig.2b). In our strain, the discrimination between sexes was best at 0.125 and 0.25%, while a number of males showed considerable interest for the 0.06% saccharin solution. An examination of the daily fluid intake of individual rats revealed that it was necessary to study more than one day in order to clearly assess the preference of an animal. Three days was found to be sufficient. In terms of the group average, habituation was negligible during this period (e.g., percentage consumption of 0.125% solution in untreated group, males 1st day (mean±S.E.M.) 69.7±7.0% of daily fluid intake (18 rats), 3rd day 70.1±8.4%; females 1st day 84.1±5.0% (23), 3rd day 88.5±3.8%). Offspring of rats implanted with a *tartaric acid*-containing minipump exhibited an analogous sexual dimorphism (Fig. 2). In the litters studied, the difference between sexes was even more obvious than in the untreated group.

An interesting picture emerged when data from *individual rats* were further analyzed: animals with low as well as high saccharin preference were found in both sexes, but in different proportions. For this analysis, we considered the preference of an animal for two saccharin concentrations, 0.125 and 0.25%, i.e., those which discriminated best between sexes, since rats sometimes exhibited an increased intake of saccharin solution for one or two days without showing a consistent preference for saccharin. The analysis was based on the consumption of saccharin solution expressed as percentage of total daily fluid intake. In the untreated or tartaric acid-treated groups, two thirds of the females demonstrated a high degree of preference (Table 1). In these rats, the saccharin consumption was very stable for a given concentration on different days. The amount of 0.125 and 0.25% saccharin solution they consumed represented 80% or more of their daily fluid intake. A considerable proportion of females consumed over 90%. The preference values of males were much more scattered, but a very high, female-like preference was found in approximately one third of the males. The sex difference in the proportion of animals with high or low saccharin preference (>80% vs. <80%, Table 1) is significant in the tartaric acid-treated group and in the combined control groups. The two types of animals were seen in every litter. No significant differences existed between litters.

In offspring of *nicotine-treated* rats, the sex difference in saccharin preference was completely abolished (Fig. 2). Male mean values approached the female degree of preference, while the behavioral pattern of females seemed unaffected. From the analysis of individual rats, it appears that the disappearance of sexual dimorphism is due to a change in the proportion of animals with high saccharin preference (Table 1). The proportion of males with high and low saccharin preference (>80% and <80% consumption) differs between the tartaric acid-treated group or the combined control groups on one hand and the nicotine-exposed group on the other hand.

The dose of nicotine administered in the present study did not noticeably affect the dams, although at times they appeared to be slightly more alert. At GD 18, the proportion of male to female fetuses was the same in nicotine-exposed (7.6/6.6 per litter) and untreated litters (7.0/5.7). Up to the stage testosterone samples were taken, growth was not markedly affected, but a slight, significant reduction was seen in crown rump length at GD 18: untreated males 21.54±1.09 mm (112 fetuses), females 21.53±1.12 mm (91); nicotine-treated males 21.22±1.19 mm (130; $p<0.05$), females 20.77±1.19 mm (113; $p<0.001$). In offspring from two nicotine-treated and two tartaric acid-treated dams the daily gain in body weight was recorded until weaning. Body weight of prenatally nicotine-exposed male and female pups remained lower by about 8% (Postnatal day 2: tartaric acid group, males 9.44±0.77 g (11), females 8.84±0.81 g (8); nicotine group, males 7.95±0.94 g (11), females 7.82±0.70 g (11). Postnatal day 27: tartaric acid group, males 103.89±6.91 g, females 96.53±5.82 g; nicotine group, males 98.45±7.22 g, females 92.90±5.05 g). The mean number of littermates was somewhat higher in the nicotine-exposed litters. This may have contributed to the postnatal growth difference.

DISCUSSION

Our investigation revealed marked alterations in plasma testosterone levels of the male fetus and in a sexually dimorphic behavior of adult offspring as a result of the administration of nicotine to pregnant rats. An osmotic minipump was used because this principle allowed to avoid repeated handling of the animals and to deliver the drug at a moderate dose over extended periods of time. This is of special importance in the case of nicotine whose transient action would otherwise require multiple injections. Similar considerations underlie the administration of nicotine to pregnant rats in the drinking water [13,23]. The dose used in our studies was extrapolated from earlier electrophysiological data on substantia nigra and locus coeruleus neurons [17, 18, 27]; we tried to take into account the duration of action after SC or IV injection of a given dose. Total daily doses similar to the present one, i.e., 6 mg/kg × day at GD 12 and approximately 4.5–5 mg/kg × day at GD 18, have previously been applied in behavioral studies ([23]: 6 mg/kg × day). This dose did not noticeably change the general state of the dams. Effects on fetal growth were marginal when checked at GD 18; postnatally, offspring of nicotine-treated rats showed a slight growth retardation until weaning. In contrast to observations by Peters and Tang [23] who noted a reduction in birth weight only in males, growth was retarded in both sexes in our animals. The proportion of male and female offspring was unaffected.

In spite of these comparatively mild general effects of drug treatment, we noted marked changes in the fetal gonadal axis, in confirmation of our preliminary observations [15,19]: nicotine treatment resulted in a complete suppression of the rise in male plasma testosterone which is characteristic of GD 18 [34]. Steroid levels of GD 17 and 19 were in the control range which indicates that the drug-induced condition differs from that encountered by Ward and Weisz [32] after stress, when the testosterone peak is shifted to GD 17. The suppression of the testosterone peak is clearly related to drug-treatment, as steroid levels remained unchanged in the various control groups. The question of the site of drug action remains unanswered, however. In principle, effects of nicotine on peripheral or central sites of the fetus as well as actions on the maternal organism could play a role. As pointed out in the introduction, several other psychoactive drugs have recently been reported to interfere with

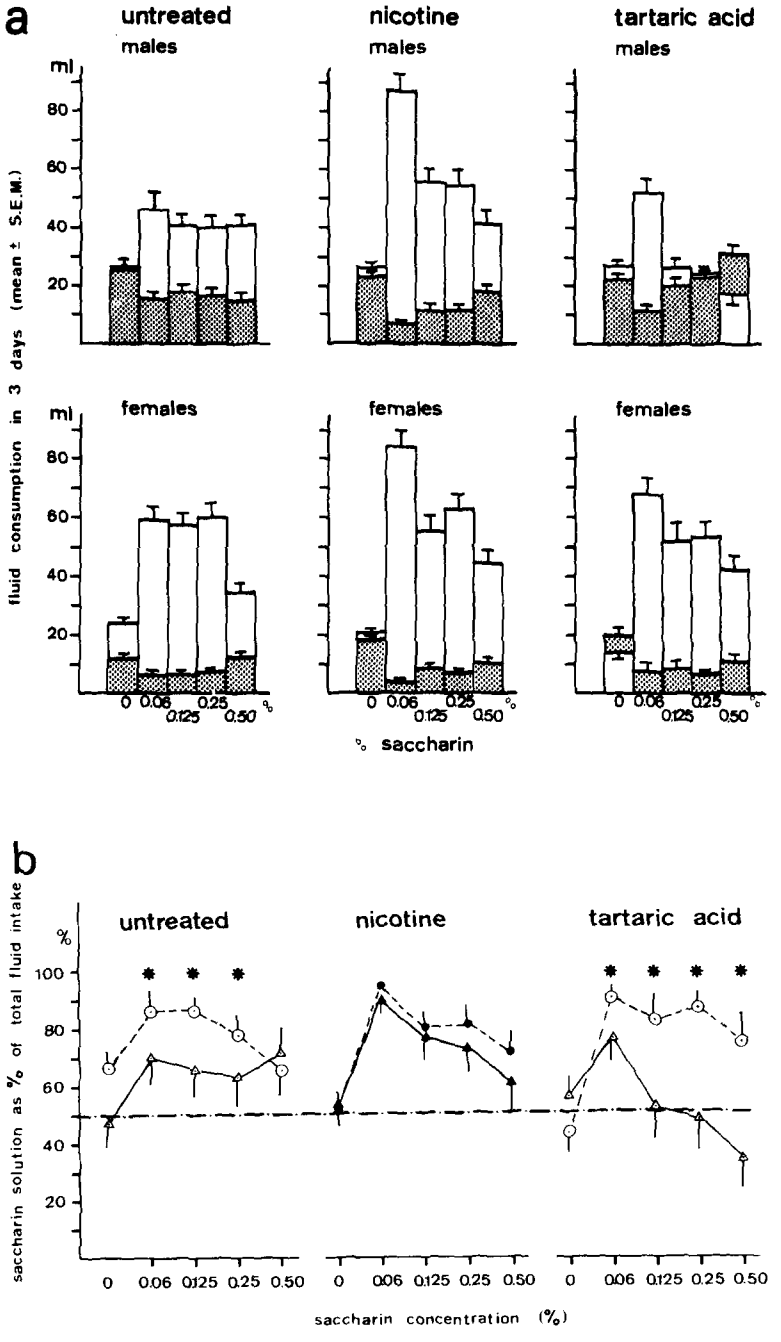

FIG. 2a. Intake of saccharin solution (white columns) and tap water (hatched columns) by 3–5 month old offspring of untreated, nicotine-treated or tartaric acid-treated rats (absolute volumes). Each concentration was presented for 3 days (0=2 bottles of tap water). Mean daily intake±S.E.M. Number of rats (males/females): untreated 18/23, nicotine 19/23, tartaric acid 15/11. The consumption of saccharin solution differs between sexes ($p<0.05$ or better) in untreated rats at 0.125 and 0.25%, in tartaric acid-treated rats at all concentrations; this difference is lost after nicotine. Nicotine-exposed males show higher intake of saccharin solution than untreated males (0.06–0.25%, $p<0.02$ or better) or tartaric acid-treated males (0.06–0.5%, $p<0.001$). Fig. 2b. Consumption of saccharin solution as percentage of total daily fluid intake. Mean±S.E.M. of 3 days per concentration. The mean percentage consumption of saccharin solution is similar in female offspring (circles) of the 3 treatment groups. It is higher than that of untreated males (triangles; 0.06–0.5%, $p<0.02$ or better) and tartaric acid-treated males (0.06–0.5%, $p<0.01$ or better), but almost identical with that of nicotine-exposed males.

TABLE 1

SACCHARIN PREFERENCE IN INDIVIDUAL MALE AND FEMALE OFFSPRING: PROPORTION OF RATS WITH INTAKE OF 0.125 AND 0.25% SACCHARIN SOLUTION REPRESENTING >50%, >80% OR >90% OF TOTAL DAILY FLUID INTAKE

% of Total Daily Fluid Intake	Number of Rats					
	Untreated		Tartaric Acid		Nicotine	
	Males	Females	Males	Females	Males	Females
>50%/<50%	10/8	21/2	6/9	9/2	13/6	19/4
>80%/<80%*	6/12	14/9	3/12	9/2	11/8	13/10
>90%/<90%	5/13	9/14	2/13	7/4	10/9	12/11

*Proportion of males and females with saccharin intake >80%/<80% different in tartaric acid group ($p<0.005$) and in combined control group (untreated + tartaric acid; $p<0.001$). Proportion of males with saccharin intake >80%/<80% differs between either one of these control groups and nicotine group ($p<0.05$).

the male gonadal axis during fetal or perinatal life. A common denominator of such drug actions seems to be the depression of fetal testosterone levels (for review, see [16]). At GD 18 in the rat or GD 16 in the mouse, testosterone was reduced in plasma after cannabinoids and opiates [5,33], in brain after phenobarbital [9]. Reduced steroid levels were further seen in plasma at GD 20 after phenobarbital [9] and in the neonatal rat brain after ethanol [12]. These drugs may affect testosterone through different mechanisms. In the case of cannabinoids, data from adult rats point to central as well as peripheral sites of action [6]; a non-psychoactive compound, cannabinol, also affected fetal testosterone [5]. Altogether, the observations on prenatal stress and drug actions indicate that the developing fetal gonadal axis is more sensitive to environmental influences than might have been expected.

Adult offspring of nicotine-treated rats showed a loss of sexual dimorphism in saccharin preference, specifically, male offspring exhibited the high degree of preference typical of normal females [19]. This represents a new type of behavioral disturbance induced by prenatal exposure to nicotine. There appear to exist sex differences in the sensitivity of certain behaviors, i.e., rearing and locomotor activity in the open field, to prenatal nicotine exposure; in one study [23], these parameters were affected only in males. However, this condition seems to differ from the present one where a sexual dimorphism in behavior was found to be abolished. The type and site of interaction between drug and hormones may not be the same in the two cases.

The results with tartaric acid demonstrate that nicotine rather than non-specific factors was responsible for the loss of sexual dimorphism. The difference between sexes seems even more obvious in the tartaric acid-exposed group. However, untreated and tartaric acid-treated males differ only in their preference for one (0.25%, $p<0.05$) out of the three saccharin solutions with clear-cut sex differences (0.06, 0.125, 0.25%). When the performance of individual rats is studied (cf. below), the two groups again are essentially similar. Moreover, neither tartaric acid nor sham-operation in ether anesthesia on GD 12 did affect plasma testosterone on GD 18. Thus, there is little so far to support the idea of a difference between untreated and tartaric acid-treated males, rather, we would emphasize the common features of the two groups.

When we analyzed our data in more detail, we found two classes of animals with different degrees of saccharin preference in each sex. This classification was based on the consumption of saccharin solution expressed as a percentage of total daily fluid intake, a value that showed less interindividual variation than the absolute volumes of fluid consumption and was found to provide a more clear-cut characterization of the degree of saccharin preference. In order to be classified as animals with high saccharin preference, rats were required to consume 80% or more of their daily fluid intake as saccharin solution with both, 0.125 as well as 0.25% saccharin solution (=6 test days, cf. above). The two sexes turned out not to be homogenous but rather, appeared to be characterized by the proportion of animals with high preference present, which was about $^1/_4$ to $^1/_3$ in males and $^2/_3$ in females (Table 1). As there were no differences between litters in any one treatment group, it seems to be possible to draw conclusions in spite of the relatively small number of litters.

Prenatal nicotine shifted the proportion of animals with low vs. high saccharin preference in male offspring to the female pattern. This situation is reminiscent of observations by Ward [31] made after prenatal exposure to stress: prenatal stress also resulted in a change in the proportion of males showing a typical behavior, i.e., complete male sex behavior. If the behavior was displayed, it was quantitatively normal. Both observations could be explained by the existence of some threshold for steroid action on the developing brain. Such a threshold might be of practical importance, as it would tend to prevent the manifestation of hormone changes induced by drugs or other environmental factors as long as the steroid concentration remained above a critical level.

It seems conceivable that the suppression of the GD 18 testosterone peak and the absence of sexual dimorphism in saccharin preference after nicotine are related to each other, though the available data do not provide direct evidence for such a link. The biochemical basis of both drug effects remains unknown. We recently observed changes in catecholamine metabolism at 2 weeks of age after prenatal treatment with nicotine [20]. Some of the changes were sex-dependent. However, these alterations may be linked as well with other types of behavioral disturbances encountered after prenatal nicotine exposure such as those reported by Peters and Tang [23].

ACKNOWLEDGEMENTS

We thank Prof. Paul J. Keller and Miss C. Gerber (Zürich) for the determination of testosterone. The study was supported by the Swiss National Science Foundation (grants No. 3.547-0.79 and 3.170-0.81).

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