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NEONATAL STERILIZATION OF RODENTS WITH STEROID HORMONES: 5. A NOTE ON THE INFLUENCE OF NEONATAL TREATMENT WITH ESTRADIOL BENZOATE OR TESTOSTERONE PROPIONATE ON STEROID METABOLISM IN THE BRAIN AND TESTES OF ADULT MALE RATS

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SUMMARY

The influence of neonatal treatment with androgens or estrogens in rats was evaluated by measuring in the testes and the brain of adult animals the conversion of 4-androstene-3, 17-dione, 3β-hydroxy-5-androstene-17-one, 3β-hydroxy-5-pregnen-20-one, and testosterone. In the testes, neonatal treatment with estradiol benzoate produced an enzymatic defficiency manifested by decreased utilization of C-21 precursor and 17-oxo reduction. Neonatal treatment with testosterone propionate produced an increase in 17β-hydroxyoxidase activity and decrease in the activity of 17-oxo reductase. In addition, the amounts of testosterone and androstenedione in the brain of treated rats were significantly decreased suggesting an alteration in the permeability of the blood–brain barrier.

INTRODUCTION

It is well established that exposure of animals to androgens or estrogens shortly after birth may produce suppression of gonadal function when the animals reach maturity. It has been suggested that during neonatal development gonadal hormones may alter those centers in the brain which in adults direct gonadal function [1, 2] but results of experiments from our laboratory indicate that other modalities may be involved. We have noted that neonatal treatment with steroid hormones influences in adult males testosterone turnover time [3] and alters the rate of testosterone transformation in plasma [4].

The present studies were undertaken to elucidate some aspects of steroid metabolism in the brain and testes of adult male rats injected at the age of 5 days with estradiol benzoate or testosterone propionate.

MATERIALS AND METHODS

Treatment

Five-day-old male pups (Holtzman strain, Madison, Wisconsin) were injected subcutaneously with 250 μg of estradiol benzoate or 1000 μg of testerone propionate dissolved in 50 μl of sesame oil. Control animals were injected with oil alone. The animals, weaned at the age of 26–28 days were maintained under standard laboratory conditions. Adult animals (200-days-old) were treated as follows (4): in one experiment controls (C) and estradiol benzoate treated males (EB) were injected intraveneously with a mixture of 4·5 μCi of [4-^{14}C]-3β-hydroxy-5-androsten-17-one (S.A. 57·1 mCi/mmol) and 90 μCi of [7-^{3}H]-3β-hydroxy-5-pregnen-20-one (S.A. 14·7 Ci/mmol). The isotopic ration ^{3}H/^{14}C determined in aliquot was 20. In the second experiment C, EB and testosterone propionate treated (TP) animals were injected with a mixture of 4·5 μCi of [4-^{14}C]-testosterone (S.A. 58·2 mCi/mmol) and about 70 μCi of [1,2-^{3}H]-4-androstene-3, 17-dione (S.A. 50 mCi/mmol); the isotopic ratio was 13·7. Twenty min after the injection, the animals were sacrificed, brain, testes and kidneys were excised, weighed and frozen until processed further.

Isolation of radioactive material. Selected tissues were homogenized with 70% aqueous ethanol, the solids removed by centrifugation were washed with the same solvent and the process was repeated twice. The supernatants were combined, the solvent from the pooled extracts was removed by flash evaporation and the residue, taken up in ether was partioned three times between an equal volume of water. A 5 per cent aliquot was taken from each fraction to determine radioactivity present. Aqueous fractions were not processed further.

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Abbreviations used: androstenedione, 4-androstene-3,17-dione; DHA, 3β-hydroxy-5-androstene-17-one; pregnenolone, 3β-hydroxy-5-pregnene-20-one.

Table 1. Body and organ weights of 200 day old male rats injected at the age of 5 days with estradiol benzoate or testosterone propionate

Treatment, (μg)	No. of rats	Body wt. (g)	Organ weights, mg ± S.E. Testes	Brain	Kidneys
0	10	460	3320 ± 124	1350 ± 42	3150 ± 131
EB (250)	11	490	2420 ± 191	1300 ± 61	2465 ± 104
TP (1000)	2	470	2990	1290	2210

EB = estradiol benzoate.
TP = testosterone propionate.

Purification procedures

The solvent from ether extracts was removed under nitrogen, the residue was dissolved in a small amount of methylene chloride and the solution was applied to silica gel plates (no. 6060, Eastman Kodak Company, Rochester, New York). The chromatograms were developed in benzene–chloroform–methanol (7:2:1 by vol.) at room temperature; the solvent front ascended 16–18 cm in about 2 h. 5α-Androstan-3β,17β-diol, 3β-hydroxy-5-androsten-17-one and progesterone were used as standards with each plate to localize the "diols", "monols" and "diones" zones. The standard spots were developed with 1% solution of phosphomolybdic acid. The purification of the crude ether extracts by t.l.c. resulted in a satisfactory separation of steroids with different functional groups; i.e. androstan or pregnan derivatives with two alcohol groups ("diols") from steroids with one alcohol and one ketone group ("monols") and from compounds with two ketones ("diones"). We could not achieve the separation of isomers, such as 3α- from 3β-alcohols, or 5α- from 5β-dihydro compounds. The fractions identified as "triols" represent a zone of slower mobility than steroidal diols. The zones corresponding to steroid standards were cut out, eluted with absolute methanol, the eluates were combined and brought up to a standard volume, and the ^{3}H and ^{14}C activity was determined in an aliquot.

Methods. Measurement of radioactivity, the source of solvents and of labelled steroids, and the general experimental conditions were similar to those described previously [4].

Table 2. Total radioactivity partitioned between ether and water from intravenously injected steroids in testes, brain and kidneys of 200 day old male rats injected neonatally with estradiol benzoate or testosterone propionate

Steroid injected	Radioactivity detected, d.p.m. × 10^3/g of tissue Testes ether	Testes water	Brain ether	Brain water	Kidneys ether	Kidneys water
Controls						
[^{3}H]-Pregnenolone	43·7 (75%)	14·8 (25%)	279 (55%)	227 (45%)	206 (50%)	203 (50%)
[^{14}C]-DHA	6·1 (83%)	1·0 (17%)	24·7 (90%)	2·8 (10%)	21·2 (65%)	11·3 (35%)
[^{3}H]-Androstenedione	80·1 (76%)	24·8 (24%)	133 (83%)	27·3 (17%)	245 (58%)	152 (42%)
[^{14}C]-Testosterone	7·5 (83%)	1·5 (17%)	12·1 (89%)	1·6 (11%)	22·7 (69%)	10·3 (31%)
Estradiol benzoate treated						
[^{3}H]-Pregnenolone	56·4 (68%)	26·5 (22%)	312 (48%)	340 (52%)	230 (56%)	181 (44%)
[^{14}C]-DHA	6·8 (84%)	1·3 (16%)	24·1 (87%)	3·5 (13%)	29·1 (69%)	13·2 (31%)
[^{3}H]-Androstenedione	98·8 (82%)	21·7 (18%)	104 (68%)	48·1 (32%)	229 (48%)	246 (52%)
[^{14}C]-Testosterone	8·6 (86%)	1·4 (14%)	9·8 (76%)	3·1 (24%)	19·5 (54%)	16·3 (46%)
Testosterone propionate treated						
[^{3}H]-Androstenedione	167 (71%)	66·6 (29%)	223 (89%)	27·9 (11%)	399 (49%)	403 (51%)
[^{14}C]-Testosterone	16·2 (89%)	2·0 (11%)	20·0 (92%)	1·8 (8%)	32·1 (57%)	23·6 (43%)

RESULTS

Table 1 shows the number of animals used in the experiments, mean body weights and the average weights of organs found at autopsy. Consistent with many previous observations we again noted decreased testes and kidney weights in animals injected neonatally with androgens or estrogens.

Table 2 provides the basic information on the amount of radioactivity found in aqueous and ether extracts expressed as d.p.m. $\times 10^3$ per g of wet tissue weight. In addition the percentage distribution of radioactive material between ether–water has been listed (figures in parenthesis).

Results of two experiments are included in Table 2. In the first experiment controls and EB males were injected with [^{3}H]-pregnenolone and [^{14}C]-DHA. In the second experiment the mixture consisted of [^{3}H]-androstenedione and [^{14}C]-testosterone.

In the brain, and testes, the steroids were present mainly in the non-conjugated, ether extractable form. An exception was found in the brain of animals injected with pregnenolone and DHA. In this case, about one-half of the radioactivity was associated with water soluble conjugates. As expected, kidney extracts yielded only about 50% of the radioactivity as ether soluble fractions. It is of interest to note that in the brain (and in the kidneys) the amount of radioactivity present was significantly higher than in the testes.

The results obtained by purifying the ether soluble material obtained from animals injected with pregnenolone and DHA are shown in Table 3.

Most significant differences were noted in the material isolated from testes and the brain.

In the testes accumulation of radioactive material was significantly higher in animals sterilized by a neonatal injection of estradiol benzoate as compared to controls. The greatest difference was in the "dione" fraction. Calculated as d.p.m./g wet tissue in EB animals this fraction was 4080 [^{3}H] and 418 [^{14}C] as compared to 507 [^{3}H] and 51 [^{14}C] detected in controls, or 20 and 3% of the total radioactivity, respectively. This indicates that testes of EB animals were unable to utilize 3β-hydroxy-5-pregnen-20-one as well as controls. In both groups the ^{3}H/^{14}C ratio of about 10, indicating increased contribution of DHA toward the common metabolic pool (the injected isotopic ratio was 20).

In the brain, the total radioactivity was likewise higher in EB group as compared to controls. A significant portion of the radioactivity was associated with polar fractions. The ^{3}H/^{14}C ratio was close to 20 indicating that this fraction may represent metabolites of both precursors formed outside the brain which crossed the brain barrier with equal ease. The isotopic ratio was increased to over 40 (both groups) in the "monols" and "diones" fractions suggesting that C-21 steroids penetrated with greater facility the blood-brain barrier than C-19 compounds. No significant differences were noted in the material isolated from kidneys except for a 40% increase of more polar components in the EB group.

Distribution of steroid fractions obtained following purification of ether soluble material from animals injected [^{3}H]-androstenedione and [^{14}C]-testosterone is shown in Table 4. Controls, EB treated and a few TP treated animals were used in the study.

Most significant differences were seen in the material isolated from testes. In the control group 37·4% of androstenedione was converted into testosterone (monols fraction) and more polar metabolites (60·1%). Only 2·1%(630 [^{3}H] d.p.m.) exhibited the same mobility as androstenedione and the isotopic ratio material was 15·2, close to the initial ratio of 13·7. In the EB group about 25% (12745 [^{3}H] d.p.m.) was found in the dione fraction. The ^{14}C contribution was negligible giving an isotopic ratio of 354. Decreased androstenedione con-

Table 3. Total radioactivity isolated from various zones after t.l.c. partition of ether extracts from testes, brain and kidneys

t.l.c. zone	Radioactivity detected, d.p.m. $\times 10^2$/g of tissue					
	Testes		Brain		Kidneys	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Controls						
Triols	77	9	450	26	380	37
Diols	39	4	129	20	226	25
Monols	38	4	619	12	116	5
Diones	5	0·5	33	1	18	1
Estradiol benzoate treated						
Triols	91	10	619	31	536	49
Diols	41	5	187	24	271	36
Monols	28	3	625	15	211	7
Diones	41	4	29	1	22	1

^{3}H radioactivity derived from injected 3β-hydroxy-5-pregnen-20-one.
^{14}C radioactivity derived from injected 3β-hydroxy-5-androsten-17-one.

Table 4. Total radioactivity isolated from various zones after t.l.c. purification of ether extracts from testes, brain and kidneys

t.l.c. zone	Radioactivity detected, d.p.m. × 10^2/g of tissue					
	Testes		Brain		Kidneys	
	^3H	^{14}C	^3H	^{14}C	^3H	^{14}C
Controls						
Triols	134	9	140	9	306	20
Diols	44	3	71	8	225	13
Monols	111	9	227	20	212	12
Diones	6	0·4	87	2	29	1
Estradiol benzoate treated						
Triols	153	10	109	7	180	10
Diols	35	2	80	10	300	18
Monols	196	15	219	16	282	18
Diones	127	0·4	63	1	44	3
Testosterone propionate treated						
Triols	141	10	101	6	463	32
Diols	81	6	82	12	59	34
Monols	137	12	293	19	453	28
Diones	55	5	97	2	68	3

^3H radioactivity derived from injected 4-androstene-3,17,dione.
^{14}C radioactivity derived from injected testosterone.

version was reflected in slower transformation into polar metabolites which accounted for only 36·5%. The reverse reaction, oxidation of 17β-alcohol to 17-ketone proceeded slowly both in controls and EB animals and only about 2% of ^{14}C was found in the dione fraction (41, and 36 d.p.m., respectively). In the TP group the contribution was significant; 518 ^{14}C d.p.m. were detected in the dione fraction. Calculation of the blood-organ ratio (d.p.m./ml of blood : d.p.m./g wet tissue weight) shows a high (12·7) androstenedione ratio for controls indicating that following intravenous injection this steroid is rapidly converted into testosterones (and other compounds). In contrast, the ratio for EB animals was only 0·9, indicating a much more slower conversion. In males exposed neonatally to testosterone propionate, androstenedione blood/organ ratio was 3·2 suggestive of decreased ability of the testes to reduce 17-ketone in the synthesis of testosterone. In addition, we have noted a nine-fold increase of androstenedione fraction arising from testosterone which suggests an increase in 17β-hydroxy oxidase activity.

In the material isolated from the brain the $^3H/^{14}C$ ratio (dione fraction) was between 39 and 46 in all the three groups. The increase of ^3H material may indicate that reduction of 17-ketone to 17-alcohol proceeds more slowly in the brain as compared to the other tissues.

DISCUSSION

The effect of neonatal injection of estradiol benzoate, or testosterone propionate, into 5 day old male rats was evaluated when the animals reached the age of 200 days. Previously we have reported [4, 5] that as a result of EB treatment, the pattern of androgen biosynthesis in the adult animals appeared abnormal and testosterone plasma levels were decreased. The present data are based on information obtained by processing brain, kidneys and testes extracts obtained in these experiments. Since the data are based on evaluation after a single, 20 min interval we have no assurance that the conversion of testosterone precursors was linear, and parallel for the groups studies and all conclusions drawn must be viewed as comparism between controls, and treatment groups. The evaluation of endogenous steroid production is precluded since we have used two isotopes in our studies.

Comparism of the relative abundance, isotopic ratios and blood-organ ratios found in the various fractions after t.l.c. purification provides some information of bio-chemical lessions that apparently result from neonatal steroid treatment. Table 5 summarizes present findings:

Table 5. Deficiencies contributing to decreased androgen biosynthesis in the testes of rats induced by neonatal androgen or estrogen exposure

Treatment	Biosynthetic deficiency
Estradiol benzoate	Decreased utilization of C-21 precursors Decreased 17-oxo reduction
Testosterone propionate	Increased 17β-hydroxy oxidation Decreased 17-oxo reduction

It should be noted, however, that despite the abnormality, the testes of both EB and TP rats conserved to some degree the biochemical potential to synthetize testosterone and an apparently normal metabolizing activity was present.

Neonatal steroid treatment did not prevent steroid hormones from reaching the brain. Most abundant were 3β-hydroxy-5-pregnen-20-one, and its conversion products; from 10 to 40 times more accumulated in the brain as compared to the other three steroids used. Calculation of the blood–brain ratio indicate that androstenedione, and testosterone crossed less readily into the brain of treated rats. The ratio for androstenedione and testosterone in EB rats was 1/2 and 1/3, and in TP group about 1/6 that of controls. It has been reported [7] that the uptake of tritiated testosterone is decreased in the hypothalamus of rats castrated neonatally with estradiol benzoate. Our present data are in support of these findings.

Several workers have pointed out that many enzyme systems directing metabolism of steroid hormones are sex dependent. These include Δ^4-5α reductase activity in the liver, the 3β-, 11β- and 17β-hydroxysteroid oxidoreductases, and the 20-keto reductases and hydroxylases (see Ref. 6 for a review). Denef and DeMoor have shown that the formation of 3β- and 20β-hydroxy metabolites can be enhanced and the activity of -5α reductase activity decreased by neonatal TP treatment of female rats [8]. Ghraf *et al.* [9] have shown that administration of estradiol benzoate to male rats 2 days after birth results in a decrease of 6β-hydroxylase activity, and abolishment of 16α-hydroxylation, which is typical of male adult rats.

We interpret the results of the present study to indicate that exposure of male rats shortly after birth to androgens, or estrogens, induces changes in the biochemical potential of enzymes which in adult life direct the synthesis of gonadal hormones. This would indicate that shortly after birth the information to such biochemical processes has been expressed only imperfectly. It may be that the synthetic gonadal hormones which are used by most investigators to induce "neonatal sterilization", act in some way to disturb the information imprinted in the genetic code. This suggests a closed physiological cycle in which abnormal gonadal steroidogenesis affects the brain which in turn affects the gonadal function. The hypothesis that hypogonadism resulting from exposing animals after birth to androgens or estrogens is mediated by differentiating the hypothalamus into a "plastic-male" or "cyclic-female" type appears to be open to revision. Clearly as the result of neonatal steroid hormone treatment the brain–hypothalama–pituitary–gonadal axis balance and feedback mechanisms have been disturbed but considerable work remains yet to be done to chart the interdependencies of all the modalities involved.

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