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Neonatal Sex Reversal of the Brain and the Urinary Excretion of Sex Dependent Proteins (SDP) in the Rat

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With 3 Figures

Summary. In contrast to females adult male rats excrete a variety of low molecular weight sex dependent urinary proteins (SDP). Electrophoretic separation of these proteins yields at least 8 protein bands which are arranged in typical patterns. The present study was performed to investigate the effect of sexual differentiation, which can be influenced by neonatal hormone treatment, on production and excretion of the individual SDP-bands (I–VIII).

Two major groups of rats were studied: one group was neonatally treated with testosterone propionate (TP, females) or cyproterone acetate (males). Another group of rats with or without neonatal TP-treatment were gonadectomized in adulthood and subsequently implanted with TP. The results demonstrated that SDP excretion is mainly related to the circulating plasma testosterone levels. The sexual differentiation of the brain, however, influences the quantity of SDP excreted which is especially evident for bands I and II. Neonatal cyproterone had influence on these two bands only.

The results demonstrate that the hormonal mechanisms regulating the excretion of SDP varies in respect to the different protein bands. The functional role of sexual brain differentiation on the excretion of SDP and the detailed mechanisms by which the brain may control this excretion remain to be determined.

Key words: Rat proteinuria — Sexual dimorphism — Sexual differentiation — Testosterone — Cyproterone

Introduction

Adult male rats normally excrete a variety of sex-dependent urinary proteins (SDP) which are of low molecular weight (LMW). These proteins can be identified by immunological methods — they are then referred to as α2u-globulin and can be resolved into a family of 5 isoelectric variants (Roy et al., 1983). Electrophoretic separation of male rat urinary proteins by micro-disc electrophoresis (Neuhoff, 1973) results in typical

patterns of at least 8 different protein bands (Alt et al., 1980). In the following, the proteins detected by micro-disc electrophoresis will be named by the more general term sex-dependent protein (SDP) because identity with α2u-globulin has not yet been proven. α2u-globulin is synthesized in the liver, circulates in the plasma and enters the urine by glomerular filtration. The typical pattern of SDP is also found in male rat plasma in a concentration similar to that of α2u-globulin (Alt et al., unpublished results). The synthesis of α2u-globulin in the liver is controlled mainly by androgens but also by a variety of other hormones such as oestrogens, thyroxine and glucocorticoids (Kurtz and Feigelson, 1977; Sippel et al., 1975). Studies on mRNA in rat liver demonstrate that it is not expressed at all in female rats (Kurtz and Feigelson, 1977).

As yet, the biological function of these specific proteins is not quite clear. Rat SDP might be associated with pheromonal activity as it is postulated for the sex-dependent urinary proteins of the mouse (Vandenbergh et al., 1975). The present study was performed to investigate the hormonal control of the different SDP-bands of male rats in more detail trying to differentiate between the organizational effects of testosterone postnatally and the activational effect of testosterone in adult life. Similar experiments have been carried out by Vandoren et al. (1978, 1980). However, these authors did not differentiate between the different SDP-species but determined α2u-globulin as a whole.

Postnatal treatment of rats with testosterone is known to influence sexual differentiation of brain structure (Döhler et al., 1986; Döhler et al., 1984) and function (Goy and McEwen, 1980) as well as sexual differentiation of liver, kidney and adrenal steroid metabolism (Ghraf et al., 1975; Hoff and Schriefers, 1973).

Female rats which are treated with testosterone in the neonatal period remain anovulatory because they lose the capacity to release gonadotropins in a cyclic fashion. In adulthood these animals demonstrate male sexual behaviour when treated with an activational dose of testosterone. Male rats treated postnatally with androgen antagonists such as cyproterone acetate, retain the female (cyclic) mode of gonadotropin release and demonstrate female rather than male sexual behaviour when mature (Neumann and Elger, 1969).

Material and Methods

Animals. Male and female inbred rats were bred in the Central Animal Laboratory of the Hannover Medical School and were maintained under SPF-conditions at 21° C, relative humidity of 55 ± 5%, a light-dark cycle of 12:12 hours and standardized rat chow (1314 fortified Altromin). On their first day of life, newborn rats were subdivided with regard to their sex and were raised in groups of 6–10 animals of the same sex per mother until day 25 of age. After day 25 of age the rats were kept in groups of 3–4 animals per cage.

Neonatal treatment of non-gonadectomized rats. In 24 h-intervals from day 1 to 10 after birth 12 male Lewis/Ztm rats received 0.5 mg cyproterone acetate (Schering AG, Berlin, F.R.G) in 0.05 ml sesame oil subcutaneously. 11 female Lewis/Ztm rats received 50 μg testosterone propionate (Serva Feinbiochemica, Heidelberg, F.R.G.) in 0.05 ml sesame oil s.c. from day 1 to 5. Control animals received sesame oil s.c. over the same period of time. Urines were collected when the animals were 130 days of age. Some characteristics of brain and gonadal differentiation are summarized in Table 1.

Gonadectomized rats with and without neonatal treatment. Male and female inbred Sprague-Dawley rats were maintained as described above. Neonatal treatment: 10 females received 20 μg testosterone propionate in 0.025 ml sesame oil from day 1 to 5 s.c. Control females received injections of 0.025 ml sesame oil without testosterone propionate. 12 male animals did not receive any neonatal treatment. Gonadectomy: Female rats were ovariectomized on day 80. The animals were anesthetised with 100 mg/kg BW Vetalar (Parke, Davis & Co, München, F.R.G.) and 10 mg/kg BW Rompun (Bayer, Leverkusen, F.R.G.). The ovaries were removed, weighed and the presence

or absence of corpora lutea was protocolled, On day 140 silicone-tubings (30 mm long, inner diameter 1.5 mm, outer diameter 2.5 mm) were implanted subcutaneously into the ovariectomized rats. The tubing contained 25—30 mg testosterone propionate. 45 days after implantation urines were collected in metabolic cages overnight and plasma samples were taken by puncturing the retroorbital venous plexus under ether anaesthesia for control determinations of serum testosterone levels. The male rats were also gonadectomized on day 80 and received testosterone propionate implantations 6 weeks after gonadectomy. Some characteristics of brain and gonadal differentiation are summarized in Table 1.

Analytical methods and statistics. Total protein was determined by the Lowry method (Lowry et al., 1951). The urinary proteins were separated by micro disc-electrophoresis in 2 µl-capillaries as described by Neuhoff (1973). Albumin, high molecular weight protein with an electrophoretic mobility below that of albumin (HMW proteins) and low molecular weight proteins (LMW proteins, including the different fractions of SDP) were determined by planimetry (see Fig. 1, Alt et al., 1980). Creatinine was determined in the urine by the Jaffe reaction (Bonsnes and Taussky, 1945). Plasma testosterone was determined as described by Wong et al. (1983). All values are calculated as mean ± SD. The t-test was applied to detect differences between groups. Unless otherwise stated the level of significance was chosen as $p < 0.01$.

Table 1 Differentiation of brain and gonads in different experimental groups (see also Dörner, 1976)

	Brain (cyclicity)	Gonads	Sexual behavior	Plasma testosterone	Estrogens
♂ controls	male	male	male	male	basal secretion
♂ + Cyprot. nn	female[a])	male	female[b])	male	basal secretion
♀ controls	female	female	female	female	female
♀ + TPnn	male	infertile female	male	female	reduced female
(♂) + TPImp	male	castrated	male	male	basal secretion
(♀) + TPnn + TPImp	male	castrated	male	male	basal secretion
(♀) + TPImp	female	castrated	female	male	basal secretion

Note. Cyprot. nn = neonatal treatment with cyproterone acetate; TPnn = neonatal treatment with testosterone propionate; TPImp = implantation of testosterone propionate following gonadectomy; [a]) Structural brain differentiation (SDN-POA) is male (Döhler et al., 1986), [b]) female if treated with estrogens.

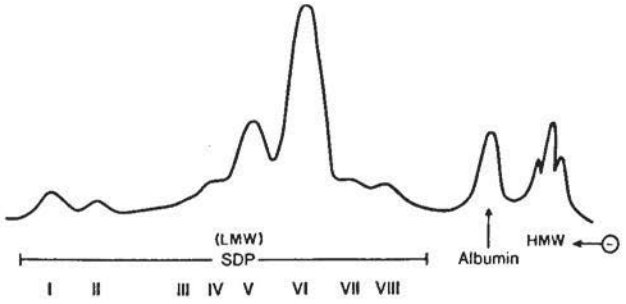

Fig. 1 Electrophoretic diagram of male urinary proteins following densitometry. The sex-dependent protein bands (SDP) are numbered consecutively with Roman numerals. HMW = high molecular weight proteins

Results

Non-gonadectomized rats. The body weight reflects sex-differences (Table 2). Males and females, which had been treated with TP postnatally, had significantly higher body weights than control females. Except for differences in urine volume per 100 g body weight between control males and control females there were no significant differences in urine volume and creatinine excretion irrespective of whether or not the values were related to body weight (Table 2). The results on protein excretion are com-

Table 2 Sex dependence of body weight, urine volume and creatinine excretion

		Body weight (BW) (g)	Urine volume (µl/h/100 g BW)	Creatinine excretion (µg/h/100 gBW)
♂ Controls	(9)	324 ± 15 *	131 ± 34 *	211 ± 65
♂ + Cyprot. nn	(12)	340 ± 28	126 ± 24	190 ± 32
♀ Controls	(9)	207 ± 6 *	191 ± 25	239 ± 41
♀ + TPnn	(11)	275 ± 28	149 ± 62	203 ± 41
(♂) + TPImp	(12)	393 ± 29 * *	155 ± 44	189 ± 25
(♀) + TPnn + TPImp	(10)	339 ± 16 *	211 ± 68	201 ± 61
(♀) + TPImp	(10)	289 ± 12	181 ± 41	198 ± 19

Note. The non-gonadectomized rats were 130 days of age at the time of urine collection and the gonadectomized rats were 185 days of age. TPnn = neonatal treatment with testosterone propionate, Cyprot. nn = neonatal treatment with Cyproterone acetate, TPImp = testosterone implantation after gonadectomy. Differences between following groups were statistically analyzed: ♂ controls ↔ ♂ + Cyprot. nn; ♂ controls ↔ ♀ controls; ♀ controls ↔ ♀ + TPnn; (♂) + TPImp ↔ (♀) + TPnn + TPImp; (♂) + TPImp ↔ (♀) + TPImp; (♀) + TPnn + TPImp ↔ (♀) + TPImp. * $p < 0.01$.

Table 3 Sex dependence of urinary protein excretion

	Total protein (µg/h/100 g BW)	Albumin (µg/h/100 g BW)	LMW (µg/h/100 g BW)	HMW (µg/h/100 g BW)
♂ Controls	258 ± 78 *	9.17 ± 3.36	156 ± 44 *	17.5 ± 6.2
♂ + Cyprot. nn	241 ± 71	8.09 ± 3.29	163 ± 54	17.6 ± 6.4
♀ Controls	89 ± 38 *	8.74 ± 3.43	8.80 ± 5.0	14.8 ± 4.1 *
♀ + TPnn	48 ± 22	5.16 ± 2.98	9.16 ± 4.8	9.10 ± 4.7
(♂) + TPImp	455 ± 166 *	70.5 ± 65.3 *	152 ± 32 *	58.5 ± 38.8 *
(♀) + TPnn + TPImp	351 ± 188	51.9 ± 35.5 *	154 ± 51 *	40.0 ± 14.8 *
(♀) + TPImp	261 ± 71	16.0 ± 15.8	99 ± 41	21.3 ± 12.0

Note. LMW = low molecular weight proteins including specific sex-dependent proteins (SDP), HMW = high molecular weight proteins with an electrophoretic mobility below that of albumin. Non-gonadectomized rats were 130 days of age and gonadectomized rats were 185 days of age at the time of urine sampling. TPImp = testosterone implantation after gonadectomy, TPnn = neonatal treatment with testosterone propionate, Cyprot. nn = neonatal treatment with cyproterone acetate. Differences between following groups were statistically analyzed: ♂ controls ↔ ♂ + Cyprot. nn; ♂ controls ↔ ♀ controls; ♀ controls ↔ ♀ + TPnn; (♂) + TPImp ↔ (♀) + TPnn + TPImp; (♀) TPnn + TPImp ↔ (♀) + TPImp, * $p < 0.01$.

piled in Table 3, and the results of measuring the different bands of male SDP are compiled in Table 4. Significant differences were observed between males and females not only in respect to the excretion of low molecular weight proteins (LMW) and total protein but also in the excretion of albumin and HMW proteins. The differences in albumin and HMW excretion, however, disappeared when the values were related to body weight whereas the difference in LMW protein excretion remained statistically significant (Table 3). Significant differences between control females and TP treated females in the excretion of total protein and HMW protein appeared only if the values were related to body weight. Typical electrophoretic diagrams for the urinary proteins of male and female, treated and untreated, rats are shown in Fig. 2. Postnatal treatment of male rats with cyproterone acetate did not influence body weight, urinary volume, creatinine or protein excretion in adulthood. The differentiation of SDP demonstrates differences between control males and cyproterone treated males in bands I and II (Table 4). The excretion of SDP, located in bands I and II, was lower in cyproterone treated rats whereas the other bands were the same or slightly higher.

Table 4 Individual sex-dependent urinary proteins in relation to neonatal treatment (Excretion rates in µg/h/100 g body weight)

	band I	band II	band III	band IV	band V	band VI	band VII	band VIII
♂ Controls	18.8 ±4.3 *	7.81 ±3.1 *	3.30 ±2.6	6.11 ±2.8	14.1 ±6.5	78.7 ±29.6	12.1 ±6.1	6.14 ±1.6
♂ + Cyprot. nn	14.3 ±4.4	5.53 ±1.7	3.12 ±1.8	6.85 ±3.1	19.1 ±8.5	78.2 ±14.7	13.7 ±6.3	7.21 ±3.1
(♂) + TPImp	11.6 ±3.4 ***	5.62 ±2.2 **	4.45 ±1.3	9.44 ±3.1	26.5 ±6.6	68.7 ±18.1	11.0 ±3.4	12.1 ±4.9
(♀) + TPnn + TPImp	12.4 ±4.2 ***	5.07 ±1.6 *	5.10 ±2.6	10.7 ±4.0	31.0 ±10.0	67.0 ±24.5	11.4 ±4.0	9.90 ±5.0
(♀) + TPImp	3.90 ±2.1	3.46 ±1.7	3.70 ±1.8	7.90 ±4.0	23.4 ±8.1	55.4 ±20.4	8.40 ±3.4	8.60 ±5.7

Note. TPImp = testosterone implantation after gonadectomy, TPnn = neonatal treatment with testosterone propionate, Cyprot. nn = neonatal treatment with cyproterone acetate. Differences between following groups were statistically analyzed: ♂ Controls ↔ ♂ + Cyprot. nn; (♂) + TPImp ↔ (♀) + TPnn + TPImp; (♂) + TPImp ↔ (♀) + TPImp; (♀) + TPnn + TPImp ↔ (♀) + TPImp. * $p < 0.025$; ** $p < 0.01$; *** $p < 0.0005$.

Gonadectomized rats with testosterone implantation. The body weight of TP implanted male rats was higher than that of females. Those female rats which had been treated with TP in the neonatal phase had body weights intermediate to the body weights of neonatally untreated male and female gonadectomized rats. Differences in urine volume were not significant. Male rats excreted more creatinine than TP implanted females without neonatal TP treatment, but this difference became insignificant if the values were related to body weight (Table 2).

Plasma testosterone levels were the same in all three groups (Table 5). The relative ovarian weights of the females treated with TP in the neonatal period were significantly lower than those of untreated females. In untreated females the vagina opened on day 35

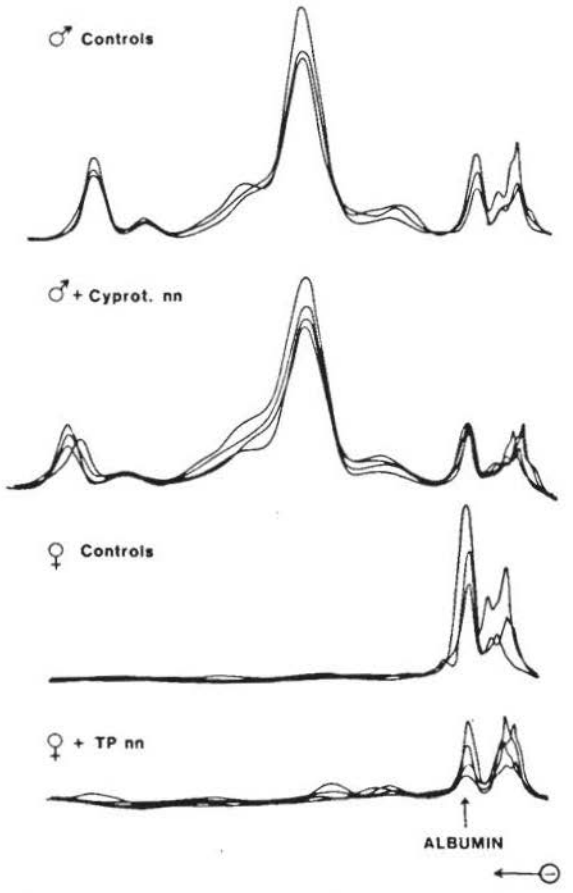

Fig. 2 Typical electrophoretic diagrams of urinary proteins of non-gonadectomized rats. The diagrams were superimposed to demonstrate the variability. The direction of migration is from right to left. The proteins on the right side of albumin are high molecular weight proteins, the proteins migrating faster than albumin are low molecular weight proteins. The sex dependent proteins belong to the low molecular weight proteins

Table 5 Plasma testosterone and relative ovarian weight of gonadectomized rats with testosterone implantation

	Body weight (g)	Relative ovarian weight (mg/g BW)	Plasma testosterone (ng/ml)
♂ + TPImp	354 ± 37 * *	—	5.04 ± 2.81
♀ + TPnn + TPImp	242 ± 44	0.123 ± 0.043 *	4.92 ± 3.47
♀ + TPImp	239 ± 12	0.306 ± 0.039	5.34 ± 2.03

Note. The rats were 80 days of age. TPnn = neonatal treatment with testosterone propionate, TPImp = testosterone implantation after gonadectomy, * $p < 0.01$.

to 37 whereas in the females with neonatal TP treatment the vagina remained closed. On the day of gonadectomy 100% of the untreated females demonstrated corpora lutea but none of the TP treated females.

The excretion rates of albumin, LMW and HMW proteins of female rats with neonatal TP treatment were the same as those of males (Table 3). The protein excretion rates of female rats without neonatal TP treatment, however, were significantly lower than the protein excretion rates of male rats and of females treated neonatally with TP. These differences remained statistically significant when the excretion rates were calculated per 100 g body weight. The electrophoretic diagrams of urinary proteins (Fig. 3) demonstrate typical male patterns of all three groups of gonadectomized rats with testosterone implantation. The diagrams also show the variation of the degree of masculinization of castrated female rats with TP implantation.

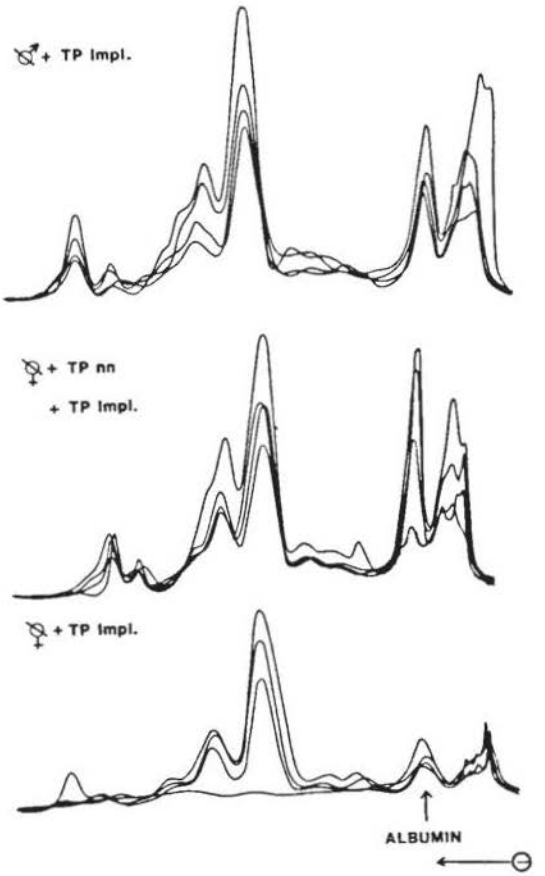

Fig. 3 Typical electrophoretic diagrams of urinary proteins of gonadectomized rats with testosterone implantation. The direction of migration is from right to left. The proteins on the right side of albumin are high molecular weight proteins, the proteins migrating faster than albumin are low molecular weight proteins. The sex dependent proteins belong to the low molecular weight proteins

The selective determination of the different SDP-bands demonstrates the effect of neonatal testosterone treatment (Table 4): bands I and II are significantly lower in those females not treated neonatally with testosterone. The other SDP bands were also lower but these differences were not significant.

Discussion

A variety of hormones such as androgens, oestrogens or glucocorticoids control synthesis and renal excretion of a sex-dependent urinary α2u-globulin (Kurtz and Feigelson, 1977; Sippel et al., 1975; Roy, 1973; Vandoren et al., 1978). α2u-globulin is encoded by a multigene family and demonstrates considerable heterogeneity. The electrophoretic separation of male rat sex dependent protein (SDP, which corresponds to α2u-globulin determined with immunological methods) allows quantitative determination of different SDP-bands (Alt et al., 1980).

The urinary concentration of SDP is closely related to plasma concentrations because the glomerular filtration is almost complete and only about 70% of the filtered load is reabsorbed by the tubulus system (Alt et al., unpublished results). The plasma concentration in turn should be closely related to the rate of synthesis unless the renal clearance is greatly changed.

The postnatal influence of hormones on sexual differentiation of the brain and their permanent effects on various body functions in rats has been reported in a great number of studies (Döhler et al., 1984; Goy and McEwen, 1980; Ghraf et al., 1975; Hoff and Schriefers, 1973; Neumann and Elger, 1969; Dörner, 1976; Gorski et al., 1978). Our results on urinary SDP excretion indicate that circulating testosterone is necessary for acute production of SDP. The quantity of SDP excretion, however, is determined by the mode of sexual differentiation during the neonatal period. After implantation of testosterone propionate, adult ovariectomized rats, which had been treated with TP neonatally, excreted as much SDP as male rats and excreted significantly more SDP than ovariectomized rats, which had not been treated neonatally, even though plasma concentrations of testosterone were the same in all three groups. The imprinting effect of a neonatal androgenization on the induction of α2u-globulin in adulthood had already been described by Vandoren and coworkers (1978, 1980). The present data show that this imprinting is only partly inhibited by cyproterone affecting mainly 2 out of 8 SDP-bands. That means, that there are different mechanisms inducing the individual sex-dependent proteins. Consequently methods should be preferred with which the heterogeneous bands can be determined separately.

In contrast to adult male rats in which cyproterone acetate causes superinduction of α2u-globulin (Vandoren et al., 1980) neonatal treatment of male rats with cyproterone acetate reduces the imprinting effect of testosterone for two of the sex-dependent proteins. The ineffectiveness of cyproterone acetate on the rest of SDP corresponds with the ineffectiveness to interfere with male differentiation of the sexually dimorphic nucleus in the rat brain (Döhler et al., 1986) which indicates a possible relationship between these two parameters.

Our results further indicate, that estrogens, which are known to inhibit testosterone induced α2u-globulin synthesis (Sippel et al., 1975), are evidently not responsible for the reduced urinary excretion of LMW proteins of gonadectomized females with TP implants, not treated with TP neonatally, since their estrogen levels are as low as in gonadectomized males.

Another remarkable finding is the sex difference of albumin and HMW protein excretion. These differences are only in part attributable to body weight but seem to be mainly due to differences in kidney function. Possible explanations may be differences in hemodynamics or filter area.

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