Specificity of Neonatal, Androgen-Induced Imprinting of Hepatic Steroid Metabolism in Rats

Abstract. *The specificity of the neonatal, androgen-induced, irreversible programming of hepatic steroid metabolism in the rat was investigated. 5α-Dihydrotestosterone propionate and estradiol benzoate were as efficient as testosterone propionate in inducing a male type of liver metabolism in the adult animal, whereas epitestosterone propionate, etiocholanolone propionate, and* o,p'-*DDT were practically inactive in this respect. These findings indicate that different mechanisms are involved in neonatal imprinting of hepatic steroid metabolism and in the well-known neonatal androgenic and estrogenic induction of persistent estrus and acyclic gonadotropin secretion.*

Hepatic metabolism of steroid hormones in the rat is characterized by large sexual differences (*1*). Part of these differences are predetermined at birth by irreversible "imprinting" or "programming" by testicular androgens (*2, 3*). Recent findings indicate that sexual differences in hepatic metabolism are regulated by the hypothalamico-hypophyseal axis (*4*).

Differentiation of the neural mechanisms that regulate gonadotropin secretion and sexual behavior in the adult rat also occurs during the neonatal period. In the neonatal male rat, it is thought that testicular secretions are responsible for the suppression of cyclicity whereas, in the female rat, the absence of gonadal activity during this period allows the hypothalamus to remain in the undifferentiated or cyclic state (*5*). The results of all previous studies have shown the important role of testosterone (*6*) in the process of sexual differentiation of the hypothalamus. In contrast, it has also been demonstrated that exposure of newborn female rats to exogenous estrogens induces permanent sterility with polycystic ovaries, anovulation, persistent vaginal estrus, and absence of female mating behavior (*7*). Similar effects were also seen with the *o,p'*-isomer of DDT (*8*) whereas treatment of the neonatal animals with the 5α-dihydrotestosterone did not inhibit cyclicity in the female rat (*9*). Our findings on neonatal programming of hepatic steroid metabolism led us to investigate whether this type of imprinting resembled the mechanism of induction of persistent estrous syndrome with regard to the nature of active agents.

Male Sprague-Dawley rats were chilled on ice and castrated on day 1 of life. On day 2 the experimental animals were injected subcutaneously with 1.45 μmole of testosterone propionate, estradiol benzoate, 5α-dihydrotestosterone propionate, etiocholanolone propionate, epitestosterone propionate, or vehicle only (20 μl of propylene glycol). The drug *o,p'*-DDT was administered in a higher dose (2.82 μmole on days 2, 3, and 4 after birth) and control animals were given vehicle only [20 percent ethanol (by volume) in propylene glycol on days 2, 3, and 4]. Each experimental group of animals contained at least four rats.

The animals were killed at 56 days of age by cervical dislocation, the liver was excised, and a homogenate was prepared at 0° to + 4°C in a modified Bucher medium (*10*). Microsomes and cytosol were isolated by differential centrifugation (*3*). Microsomes were resuspended in Bucher medium. An NADPH (nicotinamide adenine dinucleotide phosphate, reduced) -regenerating system was added prior to incubation in sufficient amounts to ensure maximal enzyme activities. Samples of the preparations were heated first to 37°C, and then the incubations were started by the addition of 5α-[4-^{14}C]androstan-3α,17β-diol or 4-[4-^{14}C]androsten-3,17-dione dissolved in acetone. The incubation conditions were designed to provide saturation of the enzymes responsible for the formation of primary metabolites. Linear conversions with time and enzyme concentration were obtained. The incubations were stopped by the addition of a mixture of chloroform and methanol (2 : 1, by volume), and the steroids were extracted (*3*). The products were partially separated by silica gel thin-layer chromatography and localized by radioautography. The radioactive zones were scraped off the plates, and portions were taken for measurement of radioactivity in a liquid scintillation counter. Products that did not separate by thin-layer chromatography were resolved by radioisotopic gas-liquid chromatography of the trimethylsilyl ether derivatives with SE-30 as stationary phase. Metabolites were identified by gas chromatography–mass spectrometry. Enzyme activities were calculated as nanomoles of products formed per milligram of protein (*11*). Student's *t*-test was used for the statistical evaluation of the results. The significance was set at $P < .05$.

Only the enzyme activities regulated by neonatal androgenic programming are given in Table 1. Testosterone propionate administration on day 2 led to significantly higher activities of 2α-hydroxylase, 5β-reductase, 3β- and 17α-hydroxysteroid reductases, and 16α-hydroxylase in adult liver. Similar effects were obtained when 5α-dihydrotestosterone propionate and estradiol benzoate were administered; with these two agents, however, 3β-hydroxysteroid reductase was not imprinted. The remaining agents, namely, epitestosterone propionate, etiocholanolone propionate, and *o,p'*-DDT, were much less efficient in affecting hepatic steroid metabolism than were testosterone propionate, 5α-dihydrotestosterone propionate, and estradiol benzoate. The only effects were seen with 3β-hydroxysteroid reductase that was imprinted after administration of epitestos-

Table 1. Long-term effects of neonatally administered testosterone propionate (TP), 5α-dihydrotestosterone propionate (5αdHTP), estradiol benzoate (E_2B), epitestosterone propionate (EpiTP), etiocholanolone propionate (EchP), and *o,p'*-DDT on hepatic metabolism of 5α-[4-^{14}C]androsten-3,17-dione in adult rats. All values are given relative to the control values which are set to 100 percent. The values represent mean values ± standard error.

Enzyme activity	TP	5αdHTP	E_2B	EpiTP	EchP	*o,p'*-DDT
			5α-[4-^{14}C]Androstan-3α,17β-diol			
2α-Hydroxylase	498 ± 93*	273 ± 10†	405 ± 64‡	94 ± 13	168 ± 29	139 ± 28
2β-Hydroxylase	178 ± 45	215 ± 18*	256 ± 30§	40 ± 3‡	152 ± 22	116 ± 40
18-Hydroxylase	106 ± 27	141 ± 16	159 ± 27	73 ± 10	130 ± 29	142 ± 18
			4-[4-^{14}C]Androsten-3,17-dione			
5α-Reductase	58 ± 7‡	56 ± 5‡	51 ± 7‡	110 ± 12	84 ± 3	81 ± 12
5β-Reductase	294 ± 35†	362 ± 43†	400 ± 22†	85 ± 9	100 ± 15	74 ± 7
17α-Hydroxysteroid reductase	Imprinted†	Imprinted†	Imprinted†	Not imprinted	Not imprinted	Not imprinted
3β-/5α-Reduction	Imprinted†	Not imprinted	Not imprinted	Imprinted†	Imprinted	Not imprinted
16α-Hydroxylase‖	Imprinted†	Imprinted†	Imprinted†	Not imprinted	Not imprinted	Not imprinted

*$P < .02$ when compared to control value. †$P < .001$ when compared to control value. ‡$P < .01$ when compared to control value. §$P < .05$ when compared to control value. ‖In the case of the activities of 17α-hydroxysteroid reductase and 16α-hydroxylase and of 3β- and 5α-reduction, the control values are zero. In these cases, this table therefore only states whether imprinting (induction) has occurred.

terone and etiocholanolone propionate.

Our results show that neonatally administered 5α-dihydrotestosterone propionate and estradiol benzoate are almost as efficient as testosterone propionate in programming hepatic steroid metabolism. The relatively specific nature of this imprinting phenomenon is obvious from the fact that epitestosterone propionate and etiocholanolone propionate were almost without effect. The fact that the nonaromatizable androgen 5α-dihydrotestosterone is active as an imprinting agent indicates that the mechanism involved is different from that behind the androgen-induced development of persistent estrous syndrome and acyclic gonadotropin secretion. The involvement of different mechanisms is further supported by the fact that *o,p'*-DDT, administered in doses similar to those inducing persistent vaginal estrus (*8*), did not have a masculinizing effect on hepatic steroid metabolism. Further studies are needed to determine whether different regions of the hypothalamus are involved in control of hepatic metabolism and estrus, respectively.

The question may be raised as to why the hepatic metabolism of steroid hormones in the rat is sex-differentiated by specific neonatal programming of the hypothalamus by testicular androgens. It seems reasonable to assume that the specific regulation of the hepatic enzymes is associated with a specific function of the steroid metabolites that are formed by the action of these enzymes. Thus, the male-specific products of hepatic steroid metabolism (for example, 3β- and 17α-hydroxy-C_{19} steroids) may act as androgen effectors in target organs yet to be defined.

JAN-ÅKE GUSTAFSSON, ÅKE STENBERG
Departments of Chemistry and *Germfree Research, Karolinska Institutet, S-104 01, Stockholm 60, Sweden*

References and Notes

1. E. Forchielli and R. I. Dorfman, *J. Biol. Chem.* **223**, 443 (1956); E. Forchielli, K. Brown-Grant, R. I. Dorfman, *Proc. Soc. Exp. Biol. Med.* **99**, 594 (1958); F. E. Yates, A. L. Herbst, J. Urquhart, *Endocrinology* **63**, 887 (1958); K. Leybold and H. Staudinger, *Biochem. Z.* **331**, 389 (1959); A. H. Conney, K. Schneidman, M. Jacobson, R. Kuntzman, *Ann. N.Y. Acad. Sci.* **123**, 98 (1965).
2. P. DeMoor and C. Denef, *Endocrinology* **82**, 480 (1968); I. Kraulis and R. B. Clayton, *J. Biol. Chem.* **243**, 3546 (1968); J.-Å. Gustafsson and Å. Stenberg, *ibid.* **249**, 719 (1974).
3. K. Einarsson, J.-Å. Gustafsson, Å. Stenberg, *J. Biol. Chem.* **248**, 4987 (1973).
4. J.-Å. Gustafsson and Å. Stenberg, *Endocrinology* **95**, 891 (1974); J.-Å. Gustafsson, A. Larsson, P. Skett, Å. Stenberg, *Proc. Natl. Acad. Sci. U.S.A.*, in press; J.-Å. Gustafsson, M. Ingelman-Sundberg, Å. Stenberg, *J. Steroid Biochem.* **6**, 643 (1975).
5. R. A. Gorski, *J. Reprod. Fertil. Suppl.* **1**, 67 (1966); in *Frontiers in Neuroendocrinology*, L. Martini and W. F. Ganong, Eds. (Oxford Univ. Press, New York, 1971), pp. 237–290.
6. Steroid nomenclature: testosterone (17β-hydroxy-4-androsten-3-one), epitestosterone (17α-hydroxy-4-androsten-3-one), 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one), etiocholanolone (3α-hydroxy-5β-androstan-17-one), and estradiol [1,3,5(10)-estratriene-3,17β-diol].
7. R. A. Gorski, *Am. J. Physiol.* **205**, 842 (1963); R. E. Whalen and R. D. Nadler, *Science* **141**, 273 (1963); G. Dörner, F. Döcke, G. Hinz, *Neuroendocrinology* **7**, 146 (1971); L. P. Coniglio, D. C. Paup, L. G. Clemens, *J. Endocrinol.* **57**, 55 (1973).
8. W. L. Heinrichs, R. J. Gellert, J. L. Bakke, N. L. Lawrence, *Science* **173**, 642 (1971).
9. W. G. Luttke and R. E. Whalen, *Horm. Behav.* **1**, 265 (1970); K. Brown-Grant, A. Munck, F. Naftolin, M. R. Sherwood, *ibid.* **2**, 173 (1971); P. G. McDonald, *J. Reprod. Fertil.* **25**, 309 (1971); ——— and C. Doughty, *ibid.* **30**, 55 (1972); *J. Endocrinol.* **61**, 95 (1974).
10. S. Bergström and U. Gloor, *Acta Chem. Scand.* **9**, 34 (1955).
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
12. Supported by grants from the Swedish Medical Research Council (grant 03X-2819) and from the World Health Organization.

15 April 1975; revised 8 September 1975

Specific Effects of Neurotransmitter Antagonists on Ganglion Cells in Rabbit Retina

Abstract. *Directionally sensitive ganglion cells in rabbit retina lose their directional sensitivity when picrotoxin, an antagonist of the inhibitory neurotransmitter γ-aminobutyric acid, is infused into the retinal blood supply. Strychnine, an antagonist of glycine, does not produce this effect. Other receptive field types are affected by strychnine but not picrotoxin. Inhibitory transmitters therefore have specific functions in information processing in the retina.*

The rabbit retina contains ganglion cells with a great variety of receptive fields. As well as cells with center-surround receptive fields, there are directionally sensitive cells, which respond most vigorously to stimulus motion in a particular direction (the preferred direction), and local edge detectors, which respond to small moving stimuli without any preference for direction of motion (*1*). Other types of receptive field are found in smaller quantities.

A number of synaptic transmitters are found in the rabbit retina, as in most retinas, and it seems likely that each performs a different function (*2*). Our purpose in this work was to find out whether particular transmitters might be implicated in the functions of known receptive field types.

Responses of ganglion cells in the right eye were recorded extracellularly with tungsten-in-glass electrodes inserted through the sclera (*3*, *4*). A particular re-

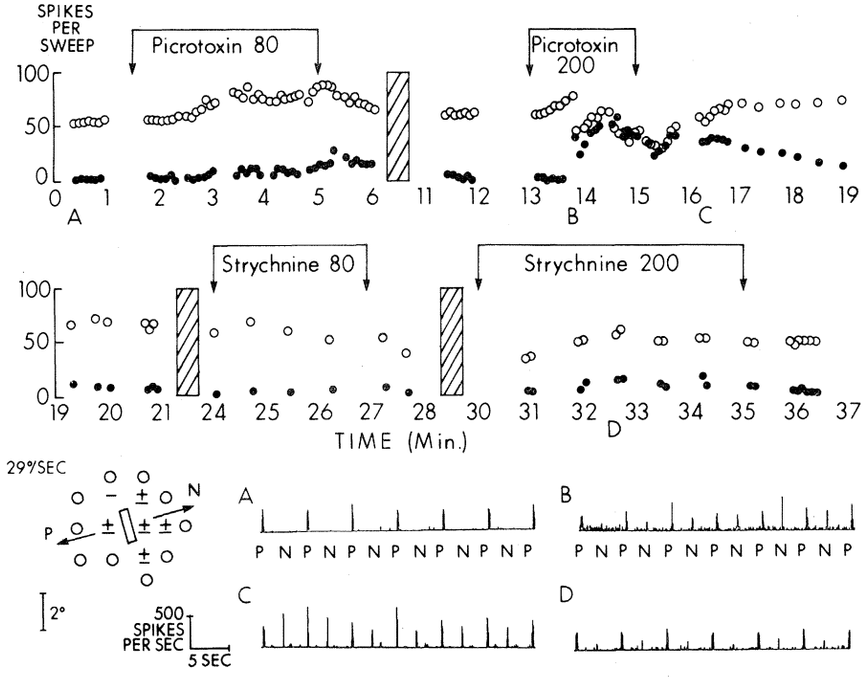

Fig. 1. Effect of picrotoxin and strychnine on an "on-off" directionally sensitive cell. Inset at lower left shows the preferred (*P*) and null (*N*) directions and the size of the stimulus in relation to the receptive field. The graphs at the top show the total number of action potentials per sweep for movement in the preferred direction (○) and null direction (●). Infusion rates are given in micrograms per minute. (A to D) Some of the data from which the graphs were obtained (number of action potentials per bin of 66-msec width): (A) before application of drugs; (B) about 1 minute after starting infusion of picrotoxin at 200 μg/min; (C) about 1 minute after ending infusion of picrotoxin at 200 μg/min; and (D) about 2½ minutes after starting infusion of strychnine at 200 μg/min.