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Imprinting of Thymic Glucocorticoid Receptor and Uterine Estrogen Receptor by a Synthetic Steroid Hormone at Different Times after Birth

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ABSTRACT. 1. Single allylestrenol treatment (hormonal imprinting) of 3-day old rats reduced the density of thymus glucocorticoid receptors and increased the density of uterus estrogen receptors at adult age.

2. Similar treatment of 7-, 14-, or 28-day old animals did not alter the binding capacity of the receptors of the adult animals at all.

3. In 3-day-old animals, the direction of imprinting was similar to the prenatal imprinting of the thymus glucocorticoid receptor (reduction), whereas neonatal treatment of uterine estrogen receptors decreased receptor density, and imprinting on the 4th day increased it. This means that the imprintability persists only to the 4th day; its consequence can be changed.

4. The experiments demonstrate that hormonal imprinting can be provoked by allylestrenol not only pre- or neonatally, as was done in previous experiments, but also a few days later. The imprintability was lost between the 4th and 8th day of life. GEN PHARMAC 30;5:685–687, 1998. **© 1998 Elsevier Science Inc.**

KEY WORDS. Glucocorticoids, hormonal imprinting, steroid receptors, thymus, uterus, allylestrenol

INTRODUCTION

The developing hormone receptors require an encounter with the hormone for completing their maturation (Csaba, 1980; 1981). In the presence of the appropriate hormone perinatally, a physiological hormonal imprinting develops, which results in the normal binding of the hormone and the normal response of the receptor-bearing cell for life. However, in that time, molecules similar to the adequate hormone (hormone analogues, members of the same hormone family) also can be bound by the receptor, causing faulty imprinting with a life-long change in its binding capacity (Csaba, 1986, 1991); for example, treatment of rat fetuses or newborns with synthetic steroid hormones (diethylstilbestrol, allylestrenol) durably decreases uterine estradiol binding (Csaba *et al.*, 1986) and, by an overlap on the related receptors, glucocorticoid (dexamethasone) binding of the thymus gland (Inczefi-Gonda *et al.*, 1986). The changes at the receptor level are also manifested in disturbances of genetic, morphological and behavioral characters (Bern *et al.*, 1987; Gibson *et al.*, 1991; Gray Nelson *et al.*, 1994; Iguchi, 1992; Iguchi *et al.*, 1987; Tchernitchin and Tchernitchin, 1992).

In earlier experiments, the treatments mentioned were done prenatally, mostly in the first day of life and sometimes at puberty (Csaba and Inczefi-Gonda, 1993). Prenatal and neonatal treatments were found to cause great effects, and treatment at adolescence also can cause misimprinting. However, the imprintability of receptors between these two time points is not known. In the present experiment, we wanted to fill this gap; that is, to study the possibilities of imprinting later than the first day, but before puberty. Allylestrenol, a synthetic steroid hormone (used for the prevention of abortion in endangered pregnancies), was used as imprinter, and the binding capacity of glucocorticoid receptors of the thymus as well as estrogen receptors of the uterus was measured. We used allylestrenol not only because of its administration for the prevention of abortion in endangered pregnancies of human beings, but also because of its use in our previous animal experiments (Csaba *et al.*, 1986; Inczefi-Gonda *et al.*, 1986).

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MATERIALS AND METHODS

Organs of 85 male and 80 female adult Wistar rats were studied in the experiments. Three-day-old animals (on the 4th day because the day of birth was considered as day 0) received 30 μg; 7-day-old, 40 μg; 14-day-old, 50 μg; and 28-day-old animals, 100 μg allylestrenol (Organon, Oss, Holland) dissolved in 1% benzylalcohol and suspended in sunflower seed oil, in one injection, subcutaneously. Controls received the solvent. When the males were 6 weeks old or the females 3 months old, cytosolic (soluble) receptor fractions were prepared from thymi or uteri (respectively). In females, the study of uteri was done 8 days after ovariectomy. Organs of five animals were used for one measurement.

Preparation of cytosol fraction

All procedures were performed at ice/water temperature. Tissues examined were cut into pieces and homogenized in Tris-HCl containing 1.5 mM EDTA, pH 7.4 (freshly supplemented with 20 mM molybdate and 2 mM dithiotreitol) with a motor-driven glass-Teflon Potter homogenizer 1.5 ml/1 g wet weight. Homogenates were centrifuged at 100,000*g* for 60 min at 4°C, and the supernatants were used for receptor assays. Protein content was estimated by the Coomassie-blue method.

Saturation analysis with ^{3}H-dexamethasone

Increasing concentrations (0.625, 1.25, 2.5, 5, 10, 20, 40 nM) of ^{3}H-dexamethasone (Amersham, Buckinghamshire, England, specific activity 1.55 TBq/mmol) were incubated with thymic cytosol

TABLE 1. Study of thymic glucocorticoid receptor (six weeks old male animals)

Group (age in days)	*n*	K_d (M 10^{-9}) ± SD		B_{max} (M 10^{-9}) ± SD	
Control (untreated)	5	5.08	0.44	3.18	0.16
3	3	4.75	0.80	2.37*	0.25
7	3	6.06	0.70	3.17	0.61
14	3	4.88	1.86	3.21	1.01
28	3	4.41	0.51	2.91	1.01

*Significance in relation to control, $P < 0.01$.

(500 μg protein) in duplicates in a total volume of 100 μl at 0°C. Nonspecific binding was measured in the presence of 100-fold molar excess of dexamethasone acetate (Sigma, St. Louis, MO, USA) at each radioactive concentration.

Saturation analysis with ^{3}H-estradiol

Increasing concentrations of [^{3}H]-estradiol (Izinta, Budapest, 2,4,6,7-^{3}H-estradiol, 3.5 TBq/mmol specific activity, 0.312, 0.625, 1.25, 2.5, 5, 10, 20 nM) were incubated with rat uteri cytosol (300 μg protein) in polypropylene tubes in a 0.1-ml total volume, at 0°C for total binding (*T*). To estimate nonspecific binding (*NS*), another set of tubes contained 100-fold excess of unlabeled estradiol (Organon) for each radioligand concentration. All assays were performed in duplicate.

The reaction was terminated both in ^{3}H-dexamethasone and in ^{3}H-estradiol saturation analysis by adding 200 μl 0.5% dextran coated charcoal suspended in assay buffer into each tube and then pelleting the unbound steroid by centrifugation at 1,500*g* for 15 min; 200-μl aliquots of the supernatants were transferred for scintillation counting into 4 ml Optiphase "HiSafe" (Pharmacia, Lund, Sweden) and counted in a Beckman apparatus (38% efficiency).

Tubes in duplicate containing radioctivity in each concentration plus buffer alone were used to generate both total counts and blanks. The difference between *T* and *NS* was regarded as specific binding (*S*) at each concentration of the labeled ligand: $S=T-NS$.

Analysis of results

Analysis of results was carried out by a computer program written by McPherson (1983) named EBDA and by a nonlinear curve fitting program modified by McPherson (1985) named LIGAND. The relation between EBDA and LIGAND is as follows: EBDA is used to process the raw data, which is then expressed on the appropriate plot. The graphic representation allows initial parameter estimates to be calculated for use by LIGAND. LIGAND is used to obtain final parameter estimates.

RESULTS AND DISCUSSION

Prenatal or neonatal treatment with foreign molecules that are able to be bound to a hormone receptor causes faulty imprinting (Csaba, 1981, 1986, 1991). In earlier experiments, prenatal treatment with allylestrenol of male rats caused a decrease in the thymic glucocorticoid receptors' binding capacity (B_{max}) for life, as did neonatal treatment of females in uterine estradiol receptors (Csaba *et al.*, 1986; Inczefi-Gonda *et al.*, 1986). In the present experiments, the duration of the critical period for imprinting was studied.

The receptor density (B_{max}) of the thymic glucocorticoid receptors of males was significantly reduced only after allylestrenol treatment at the 4th day of life (3-day-old animals). All of the other density values were similar to the control (untreated) animals (Table 1). There was no significant difference at all in receptor affinity (K_d).

The estrogen receptor density of the uterus was significantly elevated only in the animals treated with allylestrenol at the 7th day of life. There was no change related to the controls in the animals treated at the other times. K_d values were unchanged in each treated group in relation to the control (Table 2).

The results demonstrate that the critical period for allyestrenol imprinting in the thymus or uterus is extended to the 4th day of life. The imprintability was lost between this day and the 8th day of life and did not appear up to the 28th day. This means that the period of imprinting is indeed limited to the perinatal period. However, it is known that imprintability returns in puberty (6th and 7th weeks), at least for the estrogen and glucocorticoid receptors of females, when the male glucocorticoid receptor remains refractory for nandrolone imprinting (Csaba and Inczefi-Gonda, 1993). Maybe this phenomenon can be explained by the development of sexuality (secondary sex characteristics) in puberty and the time lag of males in relation to females at this time.

It is important to mention that, in males, the direction of the effect caused by imprinting of the thymus at the 4th day was the same as it was in prenatal treatment (Inczefi-Gonda and Csaba, 1986), and what was observed after different pre- or neonatal steroid (e.g., dexamethasone or benzpyrene) treatments (Csaba *et al.*, 1991; Inczefi-Gonda and Csaba, 1985). Quite the contrary, in females, the treatment at the 4th day caused the binding capacity of uterine estrogen receptors to increase, whereas the effect decreased after earlier neonatal treatment (Csaba *et al.*, 1986). This means that the sensitivity to imprinting remains only up to the 4th day, when the direction of the effect can be reversed. The experiments also strengthen our earlier observations on the stability of K_d, which did not move at all, as in previous experiments (Csaba, 1994).

TABLE 2. Study of uterine estrogen receptor (3-month-old female animals)

Group (age in days)	*n*	K_d (M 10^{-10}) ± SD		B_{max} (M 10^{-10}) ± SD	
Control (untreated)	4	2.73	1.47	7.51	0.13
3	3	2.95	2.32	10.74*	2.09
7	3	5.67	6.10	6.94	0.26
14	3	4.13	2.96	8.50	1.32
28	3	3.69	2.63	7.89	2.01

*Significance in relation to control, $P < 0.05$.

Estrogen receptors appear very early in development. The early appearance was demonstrated in blastocysts independent of the sex of the animal (Gorski and Hou, 1995; Hou *et al.*, 1996); however, in later development, the female's reproductive tract was favored. During development, the estrogen receptor has to encounter estrogenic hormones (ligands), which did not abolish its sensitivity to the imprinting by receptor-affine molecules. The latter happens only a few days after birth.

The loss of imprintability a few days after birth is true in regard to allylestrenol treatment. However, the imprinting window would be open for a longer or a shorter time for other imprinters. When we studied the time dependence of insulin imprinting (unpublished data), we found that only the first postnatal day was suitable for doing it. In contrast, benzpyrene provoked stronger imprinting 3 weeks after birth than it did neonatally (Csaba and Inczefi-Gonda, 1984). Porcine insulin—the imprinter—differs only moderately from rat insulin, whereas benzpyrene is a steroidlike molecule with significantly disparate character from that of hormones. Allylestrenol is in an intermediary position, which could mean that the similarity or dissimilarity of the imprinter with the adequate hormone has to be calculated into the time dependence.

Another problem is the organ specificity of imprinting. In earlier experiments, perinatal triiodothyronine imprinting increased the density of thymus glucocorticoid receptors of adult male and female rats alike (Csaba and Inczefi-Gonda, 1996). However, there was no change at all in the binding capacity of uterine and thymic estrogen receptors. In another case, neonatal treatment with the peroxysome proliferator clofibrate (Csaba *et al.*, 1995) or vitamin D_3 (Mirzahosseini *et al.*, 1996) affected the sexual behavior of adults without changing estrogen or glucocorticoid binding of the uterus or thymus. These findings mean that imprintability by the same molecule is different in different organs.

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