# Ontogeny of the sex steroid and prolactin receptors in the male rat adrenal gland

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*Summary.* Cytosolic estrogen and androgen receptors and membrane prolactin-binding sites in the male adrenal glands showed a definite pattern during sexual development. The level of sexual steroid receptors paralleled adrenal growth, whereas prolactin binding reached its maximum value in mature rats.

*Key words.* Male rat; adrenal gland; estrogen receptor; androgen receptor; prolactin receptor; ontogeny; sexual development.

It is accepted that sexual steroid hormones can influence adrenal gland function. Estradiol increases corticosterone (B) production both in normal and hypophysectomized animals stimulated with ACTH[3], suggesting a direct action on the gland. Also, estrogens influence the activity of the hypothalamus-pituitary-adrenocortical system[4]. Although testosterone (T) effects are related to the administered dose, in hypophysectomized castrated rats the injection of this androgen increases B production without ACTH replacement[5,6], and T administration prevents adrenal atrophy after hypophysectomy[6]. In agreement with these findings, estrogen and androgen receptors (ER, AR) have been identified in the rat adrenal gland[7–10]. We have demonstrated that their steroid specificities and physicochemical properties (sedimentation rate, electrophoretic mobility, isoelectric point, stability and sulfhydryl dependence) are very similar to those described in other classical target tissues[11–13]. Previously, we studied the influence of sex and gonadectomy on cytosolic ER and AR binding sites in the adrenal[14]. It is well known that prolactin (PRL) can stimulate in vitro B production by diminishing adrenal 5α-reductase activity[15] and hyperprolactinemia also stimulates progesterone secretion in vivo[16]. Coincidently, PRL receptors (PRL-R) were described in rat adrenal glands and their regulation by steroid hormones studied[17].

The aim of the present study was to elucidate the ontogenic pattern of adrenal receptors and then attempt to correlate it with physiologic events in the male rat.

*Materials and methods.* Male albino THOM rats (15 to 90 days of age) were maintained in a temperature and light-controlled (12 L:12 D) room and fed Purina rat chow ad libitum.

Tissue preparation: Rats were sacrificed by decapitation and the adrenal glands removed. Tissues were cooled on crushed ice, dissected and weighed. Adrenals from rats of each specific age were pooled for each experiment and homogenized in buffer A (10 mM Tris-HCl, pH 7.4 containing 0.25 M sucrose, 1.5 mM EDTA, 3 mM $MgCl_2$ and 0.5 mM DTT) in a ratio 1:6 (w/vol), using an Ultraturrax apparatus (Janke and Kunkel, IKA Werk, Staufen, West Germany) at 0–4 °C in a LS-50 Beckman ultracentrifuge. The supernatant (cytosol) was used for the binding studies. The pellet was washed with buffer B (0.14 M NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$, 1.15 mM $KH_2PO_4$, 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$, pH 7.4), and then treated with 2.5 vol. 4 M $MgCl_2$. After 5 min on ice, they were diluted 10-fold with ice-cold buffer B and centrifuged at 20,000 × g for 20 min. The pellets were washed twice and finally resuspended in the same buffer in the ratio 1:5 (wt/vol) for the mesurement of total PRL-R.

Steroid receptor assay: The assay for available ER and AR has been described elsewhere[14]. Briefly, aliquots of 0.1 ml cytosol, containing between 0.30 and 0.45 mg protein, were incubated in triplicate for 16 h at 0–4 °C with a saturating concentration (5 nM) of (2,4,6,7-$^3$H)-Estradiol-17β (Sp. act. 90 Ci/mmol) or (6,7-$^3$H)-hydroxy-17α-methyl-estra-4,9,11-trien-3-one (R 1881) (Sp. act. 89 Ci/mmol), (from New England Nuclear Corp., USA), in the presence or absence of a 500-molar excess of unlabelled estradiol-17β or R 1881 respectively to determine nonspecific binding. It has been shown that ($^3$H)-R 1881 binds to AR as well as to progesterone receptor (PR), however, in a previous report[14] we indicated that the use of triamcinolone acetonide, in order to mask the PR, did not change the levels of AR in the adrenal gland. At the end of the incubation period, the free and bound ($^3$H)-steroids were separated by the dextran-coated charcoal method[14].

Prolactin binding assay: The measurement of total PRL-R has been described in detail previously[18]. Ovine PRL (oPRL, NIH-PS14, kindly supplied by NIAMDD, Bethesda, USA) was iodinated with $^{125}$I by the lactoperoxidase method[19] (Sp. act. 25–30 μCi/μg) and purified weekly by Ultrogel ACA-54 (LKB, Bommen, Sweden) chromatography[20]. Aliquots (0.1 ml) of the pellet obtained after $MgCl_2$ treatment containing 0.35–0.50 mg protein were incubated in triplicate at room temperature for 6 h with a saturating concentration of iodinated oPRL ($^{125}$I-oPRL) (1.8 nM: 270,000 cpm) in the presence or absence of unlabelled hormone for the assessment of nonspecific binding. Bovine serum albumin (BSA) 0.2% was added to the plastic tubes to reduce nonspecific binding. Steroids and PRL binding

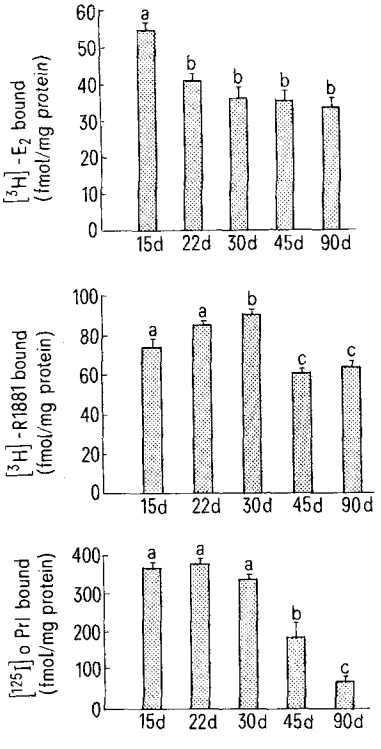


Figure 1. Ontogeny of the estrogen and androgen cytosolic and prolactin binding sites concentration (fmol/mg protein) in the male rat adrenal gland (15, 22, 30, 45 and 90 days of age). Upper graph: estradiol ($^3$H-$E_2$) binding sites; middle graph: androgen ($^3$H-R 1881) binding sites and lower graph: prolactin ($^{125}$I-oPRL) binding sites concentrations (fmol/mg protein), according to rat age in days. The height of the bars represents the mean (± SEM) for 2 pools of each age. The data were analyzed by Tukey's non-parametric multiple test, all groups were compared; identical top letters indicate no difference between them and different letters indicate significant difference ($p < 0.05$).

data are expressed as either fmol/mg protein or fmol/adrenal and the experiments were repeated twice.

Other methods: Protein concentration was assayed by the method of Lowry et al.[21], using BSA as standard. Data were analyzed statistically by Tukey's test[22].

*Results and discussion.* In the male rat available ER expressed per mg adrenal cytosol protein was found to be maximal at 15 days of age (fig. 1, upper graph) and reached a plateau between 1 and 3 months of age. If specific binding of $^3$H-estradiol is expressed as a function of age, the number of binding sites from a low content at 15 days increases between 22–30 days of age, with a step further at 45 days, reaching a peak level at 90 days of age (fig. 2, upper graph). This rise was parallel to the adrenal growth described during development. A similar gradual increase in the ER content in male rat pituitaries has been observed by Barbanel and Assenmacher[23] from 14 to 28 days of age. In our findings, the level of ER may have been underestimated in the 15-day-old rats due to the competing effect of alpha-fetoprotein (AFP), which would bind to the ligand. Such a binding component has been described in the female rat brain pituitary system[24] and the testis[25], and is not longer detectable after 3 weeks of age[25]. However, even in the presence of AFP, ER levels showed a remarkable increase with age from 15 to 22 days, indicating that there was a real increase in this binding sites at the time when AFP levels were decreasing. Available AR concentration showed a moderate but gradual increase onward, being significant by 30 days of age, with a clear diminution when animals reached adulthood (fig. 1, middle graph). Again, when the specific binding of $^3$H-R 1881 is expressed as fmol per adrenal as a function of age (fig. 2, middle graph), the binding capacity, starting from low values at 15 days, rises significantly between 22–30 days with a step increase up to 90 days of age. Attardi and Ohno[26] studying male mice brain cytosol, described a progressive increase of AR concentration up to 23 days of age, but a similar value in adult mice. Coincidently, Moger[27] found at 45 days of age a distinct rise of serum testosterone plus dihydrotestosterone levels.

Total adrenal PRL-R concentration remained approximately constant from 15 to 30 days of age in comparison to values obtained in older animals. As depicted in figure 1 (lower graph), a significant fall occurs at 45 days, which is more evident when animals reach maturity. PRL-R content rose dramatically between 22 and 30 days of age, returning the PRL-R content values in the adult rat to the levels detected at 15 days of age (fig. 2, lower graph). In previous work[18], we demonstrated a similar pattern for available PRL-R concentration although the greatest fall was observed between 20 and 40 days of age. This fall in available receptors without any change in total binding sites could be due to the rise in serum PRL in the circulation described by Negro-Vilar et al.[28]. This decrease in adrenal PRL-R concentration in the male with increasing age is consistent with the negative effect of testosterone reported in castrated male rats by Marshall et al.[17] and dihydrotestosterone propionate in intact immature male rats by our own group (unpublished data). However, total PRL-R content showed a significant and marked rise at 22–30 days, coincidently with a serum PRL peak occuring during sexual development. Reaching maturity, a continuous PRL-R decrease was seen, coincidently with a further increase of PRL levels[28]. More recently, under chronic hyperprolactinemia[29,30], we have observed a down-regulation of total PRL binding activity in the rat male and female adrenal.

When binding data for all receptors is expressed as fmol/mg adrenal tissue, it equals, with the single exception of estrogen binding, the concentration pattern (fmol/mg protein).

These data demonstrate the ontogenic appearance of adrenal estrogen, androgen and prolactin binding sites. Previous studies[4,6,31] have given evidences that these 3 hormones may alter adrenal function. The biological role during male development of the adrenal binding proteins described in this communication still has to be defined.

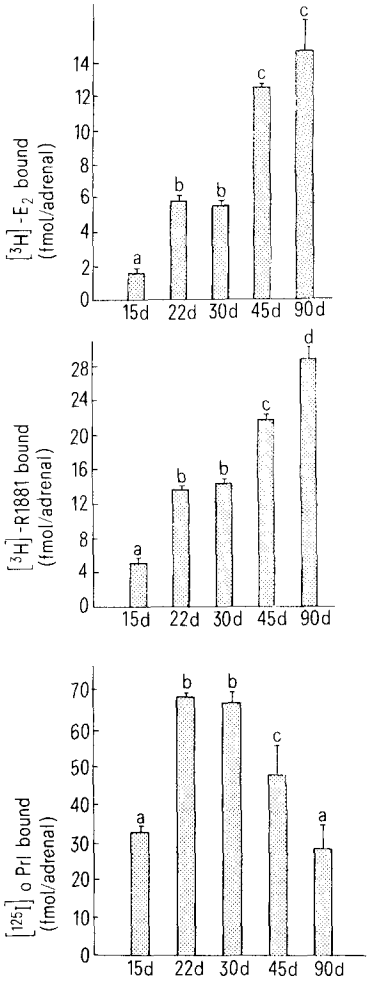


Figure 2. Ontogeny of the estrogen and androgen cytosolic and prolactin binding sites contents (fmol/adrenal) in the male rat adrenal gland (15, 22, 30, 45 and 90 days of age). Upper graph: estradiol ($^3$H-$E_2$) binding sites; middle graph: androgen ($^3$H-R 1881) binding sites and lower graph: prolactin ($^{125}$I-oPRL) binding sites contents (fmol/adrenal), according to rat age in days. The height of the bars represents the mean ( ± SEM) for 2 pools for each age. The data were analyzed by Tukey's non-parametric multiple test, all groups were compared; identical top letters indicate no difference between them and different letters indicate significant difference ($p < 0.05$).

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## Corticotropin releasing factor increases the adrenocortical responsiveness to adrenocorticotropin[1]

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*Summary.* In the course of studying the plasma adrenocorticotropic hormone (ACTH) and corticosterone responses to synthetic corticotropin releasing factor (CRF), we noted some disparity in the responses. A higher dose (20 µg compared with 5 µg per rat i.a.) produced an equal plasma ACTH but greater plasma corticosterone response in adult male rats. Thus, we examined the possibility that CRF increases adrenocortical responsiveness to ACTH. CRF significantly ($p < 0.0005$) increased the plasma corticosterone response to ACTH in rats pretreated with dexamethasone. Thus, synthetic CRF increases corticosterone secretion in rats not only by stimulating ACTH secretion, but also by increasing the adrenocortical responsiveness to ACTH.
*Key words.* Rat; adrenocortical responsiveness; ACTH, plasma; corticosterone, plasma; corticotropin releasing factor (CRF).

Glucocorticoid secretion from the adrenal cortex is regulated by ACTH which in turn is controlled by hypothalamic corticotropin-releasing factor (CRF). The primary role of CRF in regulating pituitary-adrenocortical secretion has been well established, and recently, a 41-amino acid peptide that fulfills many of the criteria of a physiological CRF has been purified from ovine hypothalamic extracts, sequenced[2,3] and subsequently synthesized[3-5]. Although the effects of this synthetic CRF to increase plasma ACTH are readily apparent[4,6-8], we noted some disparity in the effects of CRF to increase plasma ACTH and corticosterone. Thus, we examined the possibility that CRF increased adrenocortical responsiveness to ACTH.

*Materials and methods.* Adult male Sprague-Dawley rats (Charles River, CD) weighing 280–320 g were caged individually in an environmental room at 23 °C with lights on from 09.00–21.00 h for 7 days before use in an experiment. Rats were cannulated[9] 2 days before experimentation to allow systemic administration of drug and blood sampling without stress. Blood (1.5 ml for ACTH plus corticosterone or 0.25 ml for corticosterone) was collected on ice and replaced after each sampling with an equal volume of heparinized saline solution. Blood was centrifuged at 4000 × g for 20 min at 4 °C, and plasma was stored at −70 °C for subsequent determination of ACTH[10] and corticosterone[11]. Synthetic human ACTH, kindly provided by the National Pituitary Agency, NIAMDD, was used for standard and iodination; the antibody used (R1543 raised against porcine ACTH and not cross-reacting with αMSH) was kindly provided by D. Orth. Human plasma was used as the source of corticosteroid-binding globulin in the corticosterone assay. Data were analyzed using 2-way analysis of variance to compare the effects of drug, time, and interaction between drug and time[12].

A study was carried out to examine the effect of synthetic CRF (Peninsula, San Carlos, CA) on plasma concentrations of ACTH and corticosterone. Animals ($n = 6$/group) received CRF, 5 or 20 µg/rat i.a.; blood was withdrawn immediately before and 5, 20 and 30 min after drug administration. The plasma ACTH and corticosterone responses are shown in figure 1. There were significant increases in plasma concentrations of both ACTH and corticosterone after intraarterial administration of both doses of CRF compared to either the basal concentrations or the corresponding hormonal responses after intraarterial injection of saline. The plasma corticosterone responses after i.a. administration of 5 and 20 µg of CRF were further compared using a 2-way analysis of variance. No differences were noted in the effects of the 2 doses of CRF on plasma ACTH; however, the plasma corticosterone response to 20 µg of CRF was significantly ($p < 0.025$) potentiated when compared to the plasma corticosterone response after intraarterial administration of 5 µg of CRF.

A second study was carried out to determine the hypothesis that the potentiated plasma corticosterone response to 20 µg of CRF seen in the previous study resulted from an effect of CRF to increase the adrenocortical responsiveness to ACTH. This