Characterization of Rat Hypothalamic Progestin Binding by Spheroidal Hydroxylapatite Chromatography

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The progestin-high-affinity-binding components in rat target tissues have been assayed by a simple and precise procedure by using spheroidal hydroxylapatite. The progestin ‘receptors’ in the uterus and hypothalamus of female rats are highly specific for progestins, which they bind with high affinity (K_d for [3H]progesterone in hypothalamus is 1.9 nM and in uterus is 3.7 nM). The dissociation of [3H]progesterone from receptor in vitro is rapid: t_1/2 = 45 min in uterine cytosol; t_1/2 = 160 min in hypothalamic cytosol. The binding is destroyed by proteinase. In the cytosol of hypothalamus and cortex of developing rats, progestin ‘receptors’ were present in both male and female rats by 2–3 days after birth; subsequent changes in concentration of these ‘receptors’ appeared to be independent of sex. Concentrations of progestin ‘receptor’ were close to adult values by 8–9 days, and thereafter changed relatively little.

Oestrogens and progestins have important regulatory roles associated with the development and maintenance of sexual functions in mammals. It is generally accepted that most, though not all, of the actions of these sex hormones are mediated by specific receptor proteins present in the cytosol of target tissues, translating to the cell nucleus where they exert their effects (King & Mainwaring, 1974; Buller & O’Malley, 1976). In the rat uterus, some of the observed physiological changes that occur during the reproductive cycle can be correlated with translocation of the oestrogen specific receptor at pro-oestrus (Clark et al., 1973; White et al., 1978) or during the early critical phases of pregnancy (L. Myatt, G. Chaudhuri, M. G. Elder & L. Lim, unpublished work). Similar relationships with regard to function and oestrogen receptors have also been demonstrated for the hypothalamus (White et al., 1978).

On the other hand, despite the established importance of progesterone in hypothalamic functions (Barraclough, 1973; Feder & Marrone, 1977) and its putative role in sexual differentiation (Dorfman, 1967; Shapiro et al., 1976), there is scant information on the intracellular relationships of the progesterone receptor. This is related to past difficulties in detecting and characterizing the receptor because of the presence in rat plasma and rat tissue preparations of the corticosteroid-binding globulin, which exhibits a high affinity for progestins. The introduction of the synthetic progestin R-5020 (17,21-dimethyl-19-norpregna-4,9,11,12-tetraene-3,20-dione), which has little affinity for corticosteroid-binding globulin, has enabled the detection of progestin-specific cytosol receptors in rat uterus (Philibert & Raynaud, 1973) and in female rat hypothalamus (Kato & Onouchi, 1977). These latter workers reported that without oestradiol priming receptors were not detectable; however, by the combined use of Sephadex LH-20 and [3H]progestin R-5020 we were able to demonstrate specific high-affinity progestin binding in the hypothalamus of unprimed female rats (Thrower & Lim, 1978). Simultaneously, MacLusky & McEwen (1978), by using similar techniques, showed that cytosol progestin receptors were present in the hypothalamus of unprimed ovariectomized female rats, but that its concentration was increased on priming with oestradiol.

We now report a procedure involving the use of [3H]progesterone and chromatography on spheroidal hydroxylapatite (Booth et al., 1977) that considerably simplifies the assay of specific progestin binding in target tissues. By using this procedure we have characterized the progestin binding in hypothalamic cytosol and monitored developmental changes in this cytosol binding in both male and female rats.

Materials and Methods

Animals

Two strains of Wistar rat were used in these experiments: the COBS strain from Charles River,
Margate, Kent, U.K., and the Porton strain (Animal Breeding Unit, Carshalton, Surrey, U.K.) bred in our laboratory. Unless specified, experiments refer to the latter strain.

**Preparation of tissue extracts**

Animals were killed by decapitation, and the preparation of uterine and hypothalamic tissue extracts was as previously described (White et al., 1978). ‘Cortical tissue’ was that removed from the forebrain after the anterior and posterior cuts during preparation of the ‘hypothalamic block.’

Glycerol was included in the standard buffer as follows: 10 mM-Tris/HCl, pH 7.6; 1 mM-EDTA; 1 mM-dithiothreitol; 10% (v/v) glycerol (TEGD buffer). Cytosols were prepared from the tissues as previously described and were used immediately.

**Assay of progestin binding**

(a) *Sephadex LH-20 exclusion chromatography.* Cytosols were extracted with dextran-coated charcoal for a total of 10 min (5 min incubation followed by 5 min centrifugation) at 4°C to remove endogenous steroid. Without this step, estimates of receptor in the tissue were greatly decreased. [3H]Progestin R-5020 (sp. radioactivity 91 Ci/mm; NEN Chemicals, Winchester, Hants. SO21 3HQ, U.K.) was added to the supernatant in the required concentration (1–2.5 nM for routine assays) with 1 μM-cortisol, in parallel assays with and without unlabelled 1 μM-progesterone. The cytosol was then incubated for 3 h (uterine preparations) or 16 h at 4°C. The macromolecular-bound radioactivity was determined after exclusion chromatography on columns of Sephadex LH-20 (White et al., 1978). Total soluble radioactivity in each sample was also measured, as there was appreciable adsorption by the glass tubes of [3H]-progestin R-5020.

Receptor content was calculated from the difference in bound radioactivity in the presence and absence of unlabelled progesterone; this value was subsequently adjusted for the amount of saturation predicted from the concentration of free steroid in the incubation, by using the value for the equilibrium dissociation constant given in the text (see Table 1).

It was also necessary to allow for a rapid loss of progestin-binding capacity in our preparations of uterine cytosol, although the binding in neural tissue preparations was very stable.

(b) *Spheroïdal hydroxylapatite chromatography.* Portions of cytosol (0.25 or 0.4 ml) were run directly into columns (0.6 cm × 2 cm) of spheroïdal hydroxylapatite (BDH Chemicals, Poole, Dorset, U.K.) previously equilibrated in TEGD buffer. After 30 min at 6°C, the columns were washed with 1 ml of TEGD buffer to remove unbound protein and endogenous steroid, and 0.6 ml of TEGD buffer containing 2.5 nM-[3H]progestin R-5020 or [3H]progesterone and 1 μM-cortisol, in the presence and absence of unlabelled 1 μM-progesterone, was run in. Uterine cytosols were then incubated for 2 h, hypothalamic or cortical cytosols for 16 h. The columns were then washed with 3 ml of TEGD buffer, eluting greater than 95% of the unbound ligand; the radioactivity of this fraction was determined and taken as a measure of free [3H]-labelled ligand. After five washes with 1 ml of TEGD buffer (spaced at 15 min intervals for hypothalamus and cortex, but carried out within 25 min for uterus), columns received another wash with 3 ml of TEGD buffer followed by two washes with 3 ml of 0.1 M-NaOH; the radioactivity in these three washes was determined.

The characteristic elution pattern is illustrated in Fig. 1. All the macromolecular-bound radioactivity on the column was eluted in the first NaOH wash. [In the absence of cytosol, this fraction still showed a small (relative to cytosol binding) peak of radioactivity; the size of this peak could be estimated from the pattern of radioactivity in the other washes and allowance was made for this in all calculations of ‘receptor’ content.] Binding data from these

![Fig. 1. Measurement of high-affinity progestin binding with spheroïdal hydroxylapatite](image-url)
columns was then treated in the same way as that from Sephadex LH-20 chromatography, to give estimates of 'receptor' content.

Binding of 'receptor' to spheroidal hydroxylapatite during the preincubation step was complete within 5 min for uterine cytosol, and within 30 min for hypothalamic cytosol. Once the unbound material had been washed off, the column-bound 'receptor' became stable, suggesting that the observed loss of binding in uterine cytosol preparations was due to proteolytic activity.

Measurement of radioactivity

$^3$H-labelled ligand from Sephadex LH-20 chromatography was measured in 20 vol. of BBS3 scintillant (White et al., 1978) at 33% efficiency in a Beckman LS230 spectrometer. $^3$H-labelled ligand from spheroidal hydroxylapatite chromatography was extracted in 8 ml of toluene/5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (Ciba-Geigy) scintillant for 24 h, and the toluene aqueous mixture was counted for radioactivity directly (40% efficiency).

Results

Characterization of $[3^H]$progestin binding in tissues of normal (unprimed) adult female rats

These characterization studies were carried out initially on cytosol extracts from hypothalamic and uterine tissues.

Presence of high-affinity progestin binding. In Table 1, a comparison is given of the amount of progestrone-suppressible binding of $[3^H]$progesterone R-5020 and $[3^H]$progesterone in these cytosols, as estimated by Sephadex LH-20 chromatography and spheroidal hydroxylapatite chromatography. Binding-affinity data are also included to facilitate comparison.

The results (corrected here for dissociation of steroid and/or breakdown of the binding species, as quantified elsewhere in the text) were essentially the same, whichever assay technique was employed. Both tissues contained a species that binds progestins with high affinity [comparable with other steroid-receptor systems (Buller & O'Malley, 1976)], and was present in concentrations considerably higher than those of oestrogen receptors in the same tissues (White et al., 1978).

There was considerable non-suppressible binding of $[3^H]$progesterone R-5020 in our preparations of hypothalamic cytosol (around 50% of the total binding at 1 nM $[3^H]$progesterone R-5020); for $[3^H]$progesterone with spheroidal hydroxylapatite chromatography, the corresponding binding was less than 25% of total binding, even with 2.5 nM $[3^H]$progesterone in the assay. For this reason, most of the subsequent data on progestin binding in brain...
tissues were obtained by using spheroidal hydroxylapatite chromatography with $[^3H]$progesterone.

**Specificity.** The specificity of these binding species for progestins is indicated in Table 2. Oestradiol, testosterone and cortisol at 1 $\mu$m concentration (400-fold excess) did not significantly compete with progestin binding in either tissue. The absence of competition by cortisol for $[^3H]$progesterone-binding to spheroidal hydroxylapatite is compatible with the report by Booth et al. (1977) that corticosteroid-binding globulin does not bind to spheroidal hydroxylapatite.

**Binding characteristics.** Scatchard analysis (Scatchard, 1949) of equilibrium saturation binding curves for the suppressible $[^3H]$progesterone binding in uterine and hypothalamic cytosols (Fig. 2) also indicated that the binding represents a single high-affinity species in each case, with $K_d = 3.7$ nM for uterus and 1.9 nM for hypothalamus. Data for the binding in cortical cytosol are also shown; the binding characteristics were very similar to those of hypothalamus ($K_d = 1.3$ nM).

The dissociation of $[^3H]$progesterone from the high-affinity binding species in hypothalamus and uterus after binding to spheroidal hydroxylapatite is shown in Fig. 3. The short half-life of binding (at 6°C) in both tissues means that greater than 90% of the 'receptor' in the extracts was labelled by our incubation procedures, whether or not it was initially bound to endogenous progesterone, because the duration of incubation employed comprised several half-lives of steroid dissociation.

**Sensitivity to proteinase.** Incubation of hypothalamic cytosol with proteinase (Streptomyces griseus proteinase, 0.2 mg/ml; Sigma) for 30 min at 30°C before or during spheroidal hydroxylapatite chromatography completely destroyed specific $[^3H]$progesterone binding [control values were decreased by 40% under the same conditions (results not shown)].

**Developmental changes in progestin-'receptor' concentrations in rat brain**

Fig. 4 summarizes our data on the amounts of specific high-affinity progestin binding in the cytosol of hypothalamus and cortex of developing rats, measured as binding to spheroidal hydroxylapatite.

At all ages studied, there was no difference between male and female rats in the concentrations of progestin-'receptor' in cortex or in hypothalamus cytosol. Specific progestin binding was already present at 2–3 days after birth, and appeared to

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Table 2. Specificity of the uterine and hypothalamic progestin 'receptor'

The values for progestin R-5020 were determined from a comparison of dissociation constants with those for progesterone. Other values were obtained from repeated experiments measuring the effect on $[^3H]$progesterone binding (at 2.5 nM concentration) of the presence of 0.1 $\mu$m, 1 $\mu$m or 10 $\mu$m concentrations of other steroids.

<table>
<thead>
<tr>
<th>Progesterone</th>
<th>Uterus</th>
<th>Hypothalamus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progestin R-5020</td>
<td>90</td>
<td>95</td>
</tr>
<tr>
<td>17β-Oestradiol</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Testosterone</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&lt;1</td>
<td>&lt;1</td>
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</tbody>
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Relative affinity (%)
increase in concentration as well as absolute content during the first 10 days particularly in the cortex. Thereafter, at 35 days (puberty in the female rats was at around 35–40 days), 60 days and 80 days, there was little change in 'receptor' concentration in either hypothalamus or cortex. Saturation-binding studies on the neonatal binding of progesterone in 5 day hypothalamus (female) showed that the binding was unchanged in the presence and absence of cortisol, and was of the same high affinity as in adults \( K_d = 1.7 \pm 0.1 \text{ nm} \) (mean ± s.d.).

**Discussion**

*A specific progestin 'receptor' in neuronal tissues?*

Attempts to demonstrate a progestin 'receptor' in neuronal tissues of the rat have encountered numerous technical problems, with the result that although a number of groups have now shown the presence of specific progestin binding of high affinity in hypothalamic cytosol (Kato & Onouchi, 1977; Moguilewski & Raynaud, 1977; MacLusky & McEwen, 1978; Thrower & Lim, 1978), it remains premature to refer to this as a receptor, in the sense of the classical steroid-receptor model (Jensen et al., 1968).

The present methodology has certain advantages over previous techniques in addressing this problem of identification. First, it has an intrinsic specificity, in that it identifies only those species that bind both to progesterone (or progestin R-5020) and to spheroidal hydroxylapatite. Conversely, it is important that the putative hypothalamic 'receptor' be one of those species; the likelihood of this is increased by the observation that the uterine progesterone receptor, which is well characterized (Vu Hai & Milgrom, 1978a,b; Walters & Clark, 1978), binds to spheroidal hydroxylapatite (Booth et al., 1977), as do oestrogen receptors from both uterus and hypothalamus (results not shown). Furthermore, we have been able to identify specific progestin binding in ovary and pituitary by spheroidal hydroxylapatite
chromatography (S. Thrower & L. Lim, unpublished work) as well as in uterus: in each tissue this binding shows the same pattern of change in concentration during the oestrous cycle as that reported for the rat uterus by Vu Hai & Milgrom (1978c).

Secondly, the immobilization of the binding species on the spheroidal hydroxylapatite column enables endogenous progestins, as well as much of the non-specific binding (in particular corticosteroid-binding globulin) and perhaps also degradative enzymes, to be washed out before labelling and assay.

The major disadvantage of the assay at present is the inability to recover the binding species from the column, since 3 m-potassium phosphate did not give quantitative elution of binding even for uterine receptor (in contrast with the findings of Booth et al., 1977).

The hypothalamic binding species on spheroidal hydroxylapatite that we describe in the present paper shares a number of receptor-like characteristics with the uterine progestin receptor, accounts for virtually all of the high-affinity progestin binding in vitro and behaves as a single species as indicated by the linear Scatchard plot of the binding.

To date we have been unable to demonstrate translocation of this binding species out of the cytosol in response to administered progesterone, or even to detect a nuclear progestin-binding species in salt extracts of nuclei by spheroidal hydroxylapatite chromatography after desalting, although both these features have been observed in rat uterus (S. Thrower & L. Lim, unpublished work).

Nevertheless, this binding species constitutes the main candidate for mediating the actions of progestins on neural tissues. One possibility is that the neuronal nucleus is not directly involved in these actions, and there is some evidence that this may be the case (Feder & Marrone, 1977; Bennett et al., 1975).

Neuronal progestin binding in neonatal rats

The presence of progestin-binding species in hypothalamus and cortex during the neonatal period raises the question of whether they play a role in sexual differentiation, the pattern of which is determined at this early time (McEwen et al., 1974). The involvement of progesterone has already been postulated by Shapiro et al. (1976), and the presence of this binding species could provide a mechanism for this action.

The striking similarity of male and female in this context suggests that the concentration of these receptors may be a structural feature of brain development, rather than as in other target tissues being primarily regulated by the rate of secretion of oestrogens. On the other hand, there are reports in the literature that progestin binding in ovariectomized rats can be induced by oestradiol administration (Kato & Onouchi, 1977; MacLusky & McEwen, 1978).

The results of many physiological studies on the neural actions of progesterone in regulating sexual development and function (McEwen, 1978) emphasize the complexity of the response, and underline the need for an accurate molecular understanding of its mechanism of action.

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References


Thrower, S. & Lim, L. (1978) Biochem. Soc. Trans. 6, 1012–1014


