ONTOGENY OF 17β-ESTRADIOL-BINDING PROTEIN IN THE FEMALE RAT HYPOTHALAMUS AND ANTERIOR PITUITARY

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1. Introduction

A line of evidence suggests that estrogen exerts a feedback on the hypothalamus–pituitary-axis. Experiments in the human indicate that two sets of estrogen sensitive neurones exist in the hypothalamus [1], of which one set is stimulated by estrogen to secrete LH-RH, whereas the other set of neurones secreting LH-RH is postulated to be sensitive to estrogen deficiency. Similar mechanisms have been postulated for the rat [2–4]. Previous observations [5,6] led to suggest that estrogen was an important factor involved in the maturation process of the brain–pituitary unit and in the regulation of the onset of puberty. Binding of 17β-estradiol to the pituitary and brain was studied by autoradiographic techniques [7] and a specific, limited-capacity 17β-estradiol-binding protein of the cytoplasm of the rat hypothalamus was described recently [8–13]. Maximal responses to LH-RH were reported during pre-pubertal ages in rats [14,15] and changes were found in the in vitro responsiveness to LH-RH of pituitaries of rats at different ages [16,17]. Furthermore, it was shown that 17β-estradiol and other steroid hormones modulated LH-RH stimulated gonadotropin release [18,19].

The interaction of 17β-estradiol with intracellular receptor proteins is an acknowledged primary event, which presumably mediates physiological activities in estrogen-responsive tissues. This process has been extensively studied in the uterus. Little is known, however, about the role of 17β-estradiol–receptor interaction in the hypothalamus and adenohypophysis during the process of modulating LH-RH stimulated goandotropin release. This knowledge is of importance for the understanding of the mechanisms involved in the alteration of reproductive functions such as onset of puberty and ovulation.

The present experiments were designed to study, 17β-estradiol–receptor interactions in the hypothalamus and anterior pituitary of rats at different ages and after castration to obtain more information on the regulatory function of estrogens at the hypothalamus–pituitary-axis.

2. Materials and methods

Female rats of the Sprague-Dawley strain (Him: OFA (SD) SPF, Forschungsinstitut für Versuchstierzucht, University of Vienna) were used throughout these experiments. The animals were housed in a temperature-controlled room, lighted for only 12 h a day and unrestricted access was provided to food and water. The animals were used at 13, 20, 30, 40, 60, 80 and 100 days of age. The rats were killed by decapitation, hypothalami and pituitaries were excised and placed in chilled TMK-buffer (0.01 M Tris, 0.0015 M $MgCl_2$, 0.01 M KCl, pH 7.2). The posterior lobes of the

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pituitaries were removed and discarded. A total of 30–100 anterior pituitaries and hypothalami, respectively, were used depending on the ages of the animals. All procedures were carried out at 0–4°C. In TMK-buffer, 6–20 glands/ml were homogenized by 10 strokes at 1000 rev/min in a glass-Teflon Potter Elvehjem type homogenizer.

Cytosol was prepared by centrifugation of the homogenate at 110 000 × *g* (av.) for 60 min. Two 0.3 ml aliquots of the cytosol were incubated with 17β-[^{3}H]-estradiol (spec. act. 40 Ci/mmol, Radiochemical Center, Amersham, UK) at a concentration of 2×10^{-9} M in the absence and presence of a 1000-fold excess of unlabeled 17β-estradiol except where otherwise stated. After 30 min the incubation mixture was layered on a 5–22% linear sucrose-gradient. The sucrose-gradient was prepared with TE-buffer (0.01 M Tris, 1.5 M EDTA, pH 7.4) in polyallomer tubes and spun in a SW-65 rotor at 145 000 × *g* (av.) for 18 h (Beckmann Spinco LS 2-65 B). Fractionation of the density-gradients was done by puncturing the bottom of the centrifuge tubes in a Beckmann Fraction Recovery System. The effluents were collected manually. Sedimentation coefficients were determined by methods reported previously [20,21].

Quantitative estimation of the receptor–hormone complex was performed by saturation analyses. The 17β-[^{3}H]-estradiol binding assay was carried out by incubating 0.4 ml cytosol samples with 17β-[^{3}H]-estradiol at concentrations ranging from 2.6×10^{-9} – 3.4×10^{-11} M. Unspecific binding was accounted for by using a 1000-fold excess of unlabeled estrogen. After 18 h 0.2 ml of a charcoal suspension (0.6% charcoal, Norit A, 0.06% Dextrane T-70 in TMK-buffer) was added. The samples were mixed and incubated for 15 min. The incubation mixtures were centrifuged at 3000 × *g* for 10 min. The number of 17β-estradiol-binding sites and apparent affinity constants were estimated by Scatchard plot analysis [22] of binding assays. Protein was estimated by the method of Lowry et al. [23].

Ovaries of 15 animals in each age group were excised and tissue fixation was done in 10% formaldehyde. Tissue was embeded in paraffin and cut at approx. 4 μm thickness. Tissue staining was performed with hematoxineosin.

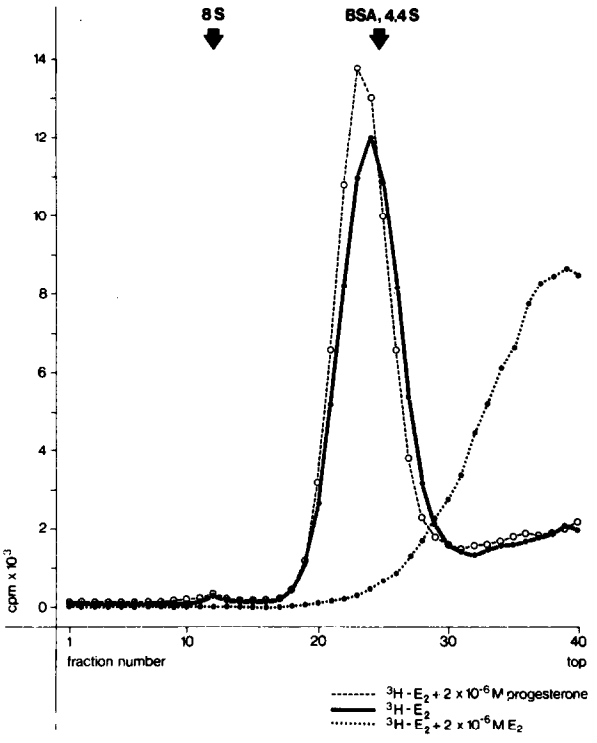

Fig.1. Sucrose-gradient centrifugation of pituitary cytosol of 13-day-old female rats incubated with 2×10^{-9} M 17β-[^{3}H]-estradiol in the absence and presence of 2×10^{-6} M nonradioactive progesterone and 17β-estradiol, respectively.

3. Results and discussion

Analysis of the sedimentation behaviour of the 17β-estradiol-binding protein derived from both hypothalamic as well as pituitary tissue of 13-day-old rats revealed a small peak of labeled estrogen in the 8 S region and a large peak in the 4–5 S region (fig.1). Data shown in fig.1 were obtained with pituitary cytosol samples. Similar results were observed for hypothalamic cytosols, and are therefore not shown. Steroid specificity of the two moieties sedimenting at 4–5 and 8 S, respectively, was examined by using a 1000-fold excess of unlabeled 17β-estradiol and progesterone (fig.1). Unlabeled progesterone did not suppress the peaks of labeled estrogen in the 8 S and 4–5 S region, respectively. On the other hand, unlabeled 17β-estradiol at a concentration 1000-fold over the ^{3}H-labeled estrogen completely abolished

the peak in the 8 S region. In addition, the peak of radioactivity appearing in the 4–5 S region was almost completely eliminated by a 1000-fold excess of unlabeled 17β-estradiol. Furthermore, a 10- and 100-fold excess of unlabeled estrogen was added to incubation mixtures, and the sedimentation patterns were recorded (data not shown). A 10-fold excess of unlabeled estrogen abolished the 8 S peak but did not affect the peak of radioactivity appearing in the 4–5 S region. On the other hand, a 100-fold excess of unlabeled estrogen abolished the 8 S peak and reduced total labeled 17β-estradiol bound in the 4–5 S peak by 96% as compared to the control. Additionally, a 1000-fold excess of testosterone did neither affect the 4–5 S nor the 8 S peak.

It is intriguing to note that the moiety sedimenting at 4–5 S seems to exhibit a similar steroid specificity as the 8 S binding component [24], the latter being generally acknowledged as macromolecular species functioning as 17β-estradiol-receptor in the uterus, hypothalamus and anterior pituitary [25–30]. Previously it was reported that a 12.6-fold excess of unlabeled estrogen did not abolish the 4–5 S peak obtained by gradient-centrifugation of cytosols derived from hypothalami of 14-day-old female rats and the moiety sedimenting in the 4–5 S region was claimed to be unspecific [31]. This is in contrast to data reported in the present communication, which combine to suggest specificity for 17β-estradiol of the 4–5 S binding component.

A small residual amount of the 4–5 S binding component was detected upon gradient centrifugation of both hypothalamic and pituitary cytosols derived from 20-day-old rats. At this age a large peak of radioactivity was recorded in the 8 S region. At all other ages studied only a 8 S binding component was recorded (data not shown). Preliminary experiments indicate, however, large amounts of 4–5 S binding components and rudimentary peaks in the 8 S region for 7-day-old animals, respectively (our unpublished data). Scatchard plot analysis of binding assays also reveal two specific 17β-estradiol binding sites for hypothalami and anterior pituitaries of female rats at 13 days and 20 days of age.

One has a high affinity for 17β-estradiol (table 1) and low binding capacity (figs 2 and 3) and corresponds to the 8 S binding component. The other class of binding sites has a much lower affinity for estrogen

Table 1

Days	Hypothalamus		Pituitary	
	K_1	K_2 (10^{-10} M)	K_1	K_2
13	1.0	42.9	3.5	61.4
20	1.7	41.7	2.5	7.9
30	0.8	–	4.0	–
40	0.8	–	4.0	–
60	1.2	–	2.3	–
80	0.6	–	2.7	–
100	2.1	–	4.0	–

Apparent affinity constants of hypothalamic and pituitary 17β-estradiol-binding sites at different ages of female rats.

(table 1) but a much higher binding capacity (figs 2 and 3) and seems to be consistent with properties recorded for the 4–5 S binding compenent. The number of high affinity hypothalamic binding sites is low at 13 days and 20 days of age and increases in the 30-day-old rat. Thereafter essentially constant levels of high affinity binding sites are observed (fig.2). The decrease in the number of low affinity binding sites at 13 and 20 days is consistent with data registered in the gradient centrifugation experiments. Similarly, low

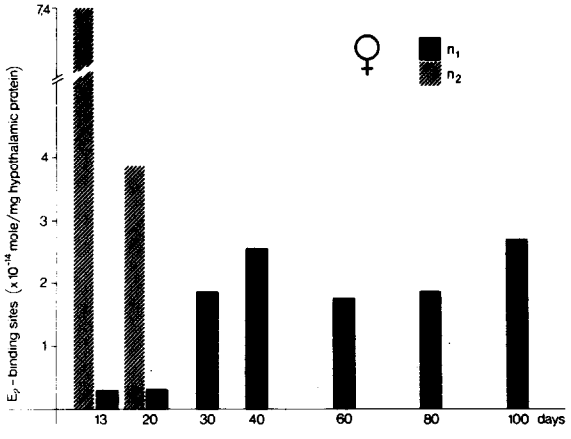

Fig.2. Number of hypothalamic 17β-estradiol-binding sites as function of the age.

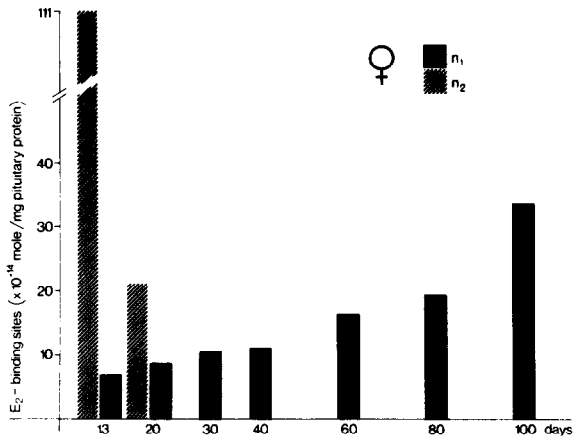

Fig.3. Levels of pituitary estrogen-binding sites at different ages of female rats.

levels of high affinity pituitary binding sites are recorded in 13- and 20-day-old rats, and an increase is seen at 30 days (fig.3). In addition, a drop of the levels of low affinity binding sites at 20 days is observed. Consistent with data on gradient centrifugation no low affinity binding sites are detectable by binding assays at ages 30–100 days. The number of high affinity binding sites is approximately ten-times greater in the pituitary than in the hypothalamus (figs 2 and 3).

The present experiments show that maturation of a high affinity hypothalamic and pituitary 17β-estradiol-receptor sedimenting in the 8 S region takes place at early ages of the female rat. Maturation is complete after 30 days of age. The present data are consistent with previous experiments on the LH-RH stimulated gonadotropin release. At 13 days and 20 days of age the greatest stimulation by LH-RH of gonadotropic hormones was observed in vivo [14,15] as well as in vitro [16,17] indicating a positive feedback control of 17β-estradiol at the hypothalamic and pituitary level. Histological examinations of ovarian tissue at different ages of animals corroborate the data on the number of binding sites (figs 2 and 3). Regression of tertiary follicles was observed in ovaries of 30-day-old animals and corpora lutea were recorded at 40 days.

At present it is generally acknowledged that 17β-estradiol attaches to an 8 S binding protein in the cytoplasm. The complex crosses the nuclear membrane either before or after conformational change of the protein to a 5 S species [32]. The biological role of the 4–5 S binding component found at early stages of development is not fully understood at present. A 4–5 S 17β-estradiol-binding protein distinct from the usual 8 S or salt-derived 4–5 S form was found in uteri of mature rats [33] and in mammary carcinoma [34]. The 4–5 S class of pituitary binding sites is not found in adult ovariectomized animals. In addition, the number of high affinity binding sites remains constant and no changes in the apparent affinity constant can be observed up to 80 days after castration (our unpublished data).

The present experiments suggest that the 4–5 S estrogen-binding component is involved in the maturation of the hypothalamus–pituitary-axis and onset of puberty, but further work is needed to elucidate this mechanism.

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