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OCCURRENCE AND PROPERTIES OF 17β-OESTRADIOL RECEPTORS IN RAT BRAIN

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Two aspects of the presence in brain and pituitary of material that is presumed to be receptor for 17β-oestradiol (E_2) will be discussed. These are, the occurrence and distribution in brain and pituitary cytosol from rats of oestradiol binding activity and, secondly, comparison of the properties of the oestradiol binding reaction in cytosols from different areas of brain, pituitary and peripheral target tissue.

Our working definition of the putative oestradiol receptor is the property of forming slowly dissociating complexes with [^{3}H]-labelled E_2 in a reaction that is suppressed by excess unlabelled diethyl stilboestrol (DES) and when the dissociation constant of the reaction (K_d) is of the order 10^{-10} M. At the present state of the art, it seems that discrepancies in findings between different laboratories and between our own earlier and later findings lie in methodological differences, and it would be as well to say something of the methods used in the present experiments [1]. For the separation of free E_2 from E_2 in the bound state in incubates of cytosol to which labelled E_2 had been added, we use very small columns of the lipophilic gel, Sephadex LH20. Perhaps the most significant step in our procedure is the interval between the addition of the incubates to the column and elution, when column flow is stopped. During this period E_2 bound in low affinity, rapidly dissociating complexes is removed from the macromolecular fraction and so interference with the measurement of small amounts of high affinity binding is overcome. In all cases parallel incubations are carried out with unlabelled DES present in approximately 20 × molar excess; the difference in the binding of E_2 in the presence and absence of DES estimates the DES suppressible binding and these values when transformed in the conventional Scatchard plot are fitted by straight lines from which the abundance of binding sites and the affinities of the reactions are calculated.

Using this method we have measured the numbers of E_2 binding sites separately in cytosols from anterior, middle and posterior regions of the hypothalamus of adult rats in various states. The dissection limits for anterior hypothalamus extend from 1 mm. anterior to 0·5–1 mm. posterior to the optic chiasma and therefore includes sites in the pre-optic area shown by autoradiography to concentrate E_2. In all female animals, those with low endogenous E_2 (in metoestrus or 3 weeks after ovariectomy) or those with high endogenous E_2 (in pro-oestrus) the greatest concentrations of binding sites were found in anterior hypothalamus, though in pro-estrus the numbers of available sites in all three hypothalamic regions are markedly reduced. The same pattern of distribution is seen in orchidectomized males and the total number of hypothalamic sites is only slightly less than in females with low endogenous E_2. In contrast, the intact males, while the number of sites in mid and posterior hypothalamus are not different from those in castrate males, anterior hypothalamus contains only half as many available sites; it is only in intact males that the number of sites in anterior hypothalamus is less than in the middle region. This distinct pattern of site distribution in intact males is clearly not in itself an expression of male genotype but yet is dependent on testicular integrity. This might be explained if in the adult male conversion of testosterone to E_2 or some other ligand for the E_2 binding system were to occur predominately in anterior hypothalamus/pre-optic area, thus selectively decreasing the availability of E_2 binding sites in the intact male. This explanation is, to a degree, supported by the effect of castration on the abundance of sites in pituitary and amygdala. The concentration of sites in pituitary is about 100 times greater than in amygdala and in both tissues, in the female, the available E_2 binding sites in cytosol are reduced when endogenous E_2 levels are high. The abundance of sites in males tends to be slightly less than in females but after orchidectomy there is, as in anterior hypothalamus, a significant rise in the sites available for E_2 binding in amygdala but not in pituitary, again correlating with the distribution of the aromatizing systems.

Recently, Sheridan *et al.*[2] have shown by autoradiographic methods, that uptake and retention of E_2 occurs in 2-day-old female rats in a pattern of localization in brain and with a limited capacity for E_2 that is similar to that seen in adults. In contrast, in our earlier experiments using Dextran charcoal adsorption [3] to separate bound from free E_2 and in sedimentation studies by Plapinger and McEwen[4] and by Kato *et al.*[5], DES suppressible high affinity binding of E_2 was not detectable in early post natal life but increased rapidly between the 19th and 25th day after birth. Extensive binding

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of E_2 in brain cytosol from neonates in a system that resembles α-fetoprotein in affinity for E_2, in sedimentation properties and in that E_2 binding is unaffected by excess DES could interfere with detection of small amounts of E_2 binding by the oestrogen specific moiety. However, in our method using LH20 the influence of α-fetoprotein-like binding is much reduced, and, indeed, E_2 binding by α-fetoprotein in plasma of 5-day-old rats cannot be detected.

In our experiments with 5-day-old rats we used two blocks of brain, (a) the complex of hypothalamus, pre-optic area, amygdala and midbrain and (b) the rest of the brain anterior to pons-equivalent to cortex.

The ratio of oestrogen specific high affinity binding site concentration in adult cortex–amygdala–hypothalamus are in the order 1:5:11 in mixed cycling females and 1:3:6 in intact males. In 5-day-old rats, the DES suppressible binding sites are distributed in roughly equal concentration in both brain areas and at levels similar to those found in adult hypothalamus or amygdala. Values for the reaction affinities for E_2 obtained in these experiments with cytosol from brain of 5-day-old rats invariably were 3–4 times less than in adult preparations, however, for reasons to be given later, we believe that the difference is artefact.

The neonatal brain E_2 binding activity is, in most other ways, indistinguishable from the binding moiety from adult tissues—by definition, they are broadly oestrogen specific, readily saturable with DES, K_d in the order 10^{-10} M; they are inactivated by treatment with pronase but not by DNAase or RNAase and precipitation by protamine plus inactivation by parahydroxy mercuribenzoate indicate that the binding moiety is a protein of acidic character with functional SH groups.

However, there are some differences in properties of the reaction in cytosol from various sources. In some instances, we believe these differences to be due to the absence or presence of a separate factor modifying the overall binding reaction and such an explanation could apply in all cases.

There are differences in rates of reaction: equilibrium is reached in <5 min at 30°C with uterine or hypothalamic cytosol whereas with pituitary, cortex or amygdala more than 10 min is required. Adsorption of the complexes by Dextran charcoal is negligible with cytosol from adult hypothalamus but occurs readily in cytosol of neonatal brain.

Also, there is a consistent difference in affinity for E_2 between pituitary cytosol and cytosol from brain region or uterus. Values for K_d for pituitary in adults of both sexes are in the range of $0{\cdot}6$–$0{\cdot}7 \times 10^{-10}$ M while for other tissues pooled from the same animals the values are in the range $0{\cdot}9$–$1{\cdot}4 \times 10^{-10}$ M. We suspect that there might be present in pituitary cytosol an additional oestrogen binding moiety of very high affinity but not sufficiently abundant or qualitatively different from the more widely distributed oestrogen binding system as to cause significant departure from linearity in the Scatchard plots.

The classical approach to differential characterization of receptor systems is by the quantitative comparison of the interactions with many substances having agonist or antagonist actions in the system. This we have done by estimating affinity constants for 11 such compounds in the E_2 binding systems of cytosols from brain regions, pituitary and uterine of both male and female adult rats. The values have been obtained from the inhibition of binding of labelled E_2 in the presence of unlabelled test substance.

The affinity constants for the 11 compounds extend over a range of about 4 orders of magnitude but the ranking orders for the affinity constants is the same for each of the tissues. However, inspection of the absolute values of the affinity constants reveals some differences between the tissues. For example, if one compares the affinity in uterine cytosol with those in hypothalamus, pituitary, cortex or amygdala in female and male adults, out of 88 possible contrasts, 19 of the differences are significant at the 1% probability level. The tendency for higher affinities in pituitary is confirmed, significantly so in 7 of the 11 compounds. On the other hand, significant differences between amygdala or cortex and uterus are uncommon although the rates of reaction in these brain areas are very different from that in uterine cytosol. Significant differences between male and female also are comparatively rare, occurring only in 3 out of 44 contrasts. The compounds for which the affinity constants in uterus were most frequently significantly different from other tissues, were the 3α and 3β androstanediols—the lower affinity always being in uterine cytosol. This encourages us in the belief that some of these results could be meaningful since both of these substances have been shown to affect gonadotrophin release at doses that do not have uterotrophic effects.

The affinities for oestrogenic agonists and antagonists in cytosol from brain regions in 5-day-old rats also have been compared with those in hypothalamus and amygdala of adult females. There are no differences between brain regions or between sexes in the neonates and the only significant differences between neonate and adult are found with the two compounds, E_2 itself and estrone, for which the estimated affinities in neonatal brain are always lower than in adult hypothalamus or amygdala. Many of the compounds have been tested for binding with α-fetoprotein and although most of these have little or no affinity for that binding system, α-fetoprotein is selective for both E_2 and estrone. It is possible therefore that the apparently lower affinities for these compounds in the DES suppressible binding system is an artefact due to presence of α-fetoprotein-like material in the incubates.

Sheridan *et al.*[2] drew attention to the conflict of their autoradiographic studies with the negative

results of biochemical investigation of estrogen specific receptor-like binding in neonatal rat brain. It would seem that the answer to the riddle is that there is, indeed, in brain of 5-year-old rats a high affinity E_2 binding system that is indistinguishable from the corresponding systems in adult rat brain in the tests applied by us. However, in reaching that conclusion other thorny problems are raised. For example, why were Kato *et al.*[5] and Plapinger and McEwen[4] unable to detect adult receptor-like binding of E_2 by ultracentrifugation techniques—is it possible that the neonatal binding moiety has different sedimentation characteristics from the substance in adult hypothalamic cytosol? Also, while autoradiographic studies in 2-day-old rats showed localization of E_2 in hypothalamus and amygdala, there was no localization in cortex—what, then, is the significance of the large amounts of E_2 binding moiety we find in the cerebral cortex of 5-day-old rats?

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