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Non-saturable transport of [^{3}H]oestradiol across the blood–brain barrier in female rats is reduced by neonatal serum*

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Summary. Adult ovariectomized rats were decapitated 5 sec after intracarotid injection of a 200-μl bolus containing [^{3}H]oestradiol plus [^{14}C]butanol, which served as a freely diffusible reference for transport across the blood–brain barrier. When buffered Ringer's solution (with 20% ethanol added to dissolve the oestradiol) was used as the injection vehicle, 83 ± 6% (mean ± s.e.m.) of the injected [^{3}H]oestradiol (0·17 μM) reaching the brain microvasculature was extracted in one circulatory passage. Addition of unlabelled oestradiol (34 or 68 μM) to the injection bolus did not reduce this percentage of extraction. When 0·1% bovine serum albumin rather than ethanol was added to buffered Ringer's solution to ensure solubilization of [^{3}H]oestradiol (0·043 μM), the percentage brain extraction was 82 ± 6%, and again, addition of unlabelled oestradiol (300 μM) did not reduce this value significantly. This finding suggests that oestradiol diffuses into the rat brain passively and is not transported via saturable carrier molecules. When serum from an adult, ovariectomized, female rat was used as the injection medium, the brain extracted 56 ± 4% of the injected [^{3}H]oestradiol (0·17 μM), whereas only 13 ± 1% was extracted from a pool of serum obtained from 9-day-old young rats of both sexes. Brain extraction of [^{3}H]oestradiol (0·043 μM) was significantly lower when the injection vehicle used was serum collected from female rats killed on Days 0, 5 or 20 after birth than on Days 30 or 120. These in-vivo results suggest that neonatal rat serum contains a factor (presumably α-fetoprotein) that restricts oestradiol influx into the developing brain.

Introduction

In the adult female rat, oestradiol of ovarian origin is partly responsible for induction of ovulatory surges of luteinizing hormone (LH) and behavioural oestrus. These oestradiol effects depend on the steroid's action in the central nervous system. In order to affect brain parenchyma, oestrogens, like other plasma constituents, must pass through the blood–brain barrier. Much research has focussed on localization of central neurones that take up oestradiol (Pfaff, 1968; Stumpf, 1968) as well as on the binding kinetics of intraneuronal receptors for this steroid (Anderson, Peck & Clark, 1973). In contrast, very little attention has been paid to the factors that regulate oestradiol transport through the blood–brain barrier. In the present study experiments were performed to determine whether a saturable blood–brain barrier transport system for oestradiol exists in the adult rat.

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Much evidence (reviewed by Baum, 1979) suggests that oestrogenic metabolites of testosterone act perinatally to cause several aspects of brain sexual differentiation in the male rat. Indirect physiological and in-vitro biochemical findings suggest that a blood-borne factor, probably α-fetoprotein, avidly binds circulating oestradiol in the neonatal rat and thus protects the genetic female from what would otherwise be an adverse effect on brain differentiation. Experiments were therefore conducted to determine whether serum from neonatal rats can reduce transport of oestradiol into the rat brain.

Materials and Methods

Animals

Female rats (100–150 g; Charles River Breeding Laboratories, Wilmington, Massachusetts) were maintained in a colony room with lights on between 07:00 and 19:00 h; food and water were provided *ad libitum*. All females were ovariectomized 14–21 days before use in an experiment; all experiments were performed late in the afternoon.

Estimation of oestradiol transport into brain

[2,4,6,7,16,17-^{3}H]Oestradiol (sp. act. 143 Ci/mmol) and *N*-[1-^{14}C]butanol (sp. act. 1·86 mCi/mmol) were purchased from New England Nuclear Corp. (Boston, Massachusetts). The [^{3}H]oestradiol was purified on Sephadex LH-20 columns before use (Wu & Lundy, 1971). Unlabelled oestradiol was purchased from Steraloids (Wilton, New Hampshire) and used without further purification. [^{3}H]Oestradiol, supplied in a benzene–ethanol solution (9:1, v/v), was evaporated to dryness under a stream of nitrogen before being redissolved in the injection vehicle.

Transport of [^{3}H]oestradiol across the blood–brain barrier was studied by the method developed by Oldendorf (1970). Rats were anaesthetized with pentobarbitone sodium (40 mg/kg) and small supplemental doses of ether were given as needed. The left common carotid artery was exposed via a ventral median neck incision and the surrounding fascia and strap muscles were separated by blunt dissection. With a sharp, 27-gauge, 5-cm needle, the left common carotid artery was injected with a 200-μl bolus of either Ringer's solution (0·33 g $CaCl_2$, 0·30 g KCl, 8·6 g NaCl and 2·38 g Hepes per litre) or rat serum containing [^{3}H]oestradiol and tracer amounts of [^{14}C]butanol, which served as a freely diffusible internal reference (Oldendorf & Braun, 1976). The bolus was injected rapidly, minimizing the possibility of its mixing with the blood during its passage through the brain. This technique ensured that only the contents of the injection bolus and the permeability of the blood–brain barrier determined the influx of radioisotopes into the brain. Significant mixing of the bolus with the injected rat's own serum does not occur during passage through the cerebral capillaries and the measurement is therefore of the extraction of the label from the bolus, and not from the bolus mixed with the rat's serum (Oldendorf, 1971). Rats were decapitated 5 sec after injection; this time is sufficient for the bolus to pass through the rat brain once (Pardridge & Oldendorf, 1975).

The brain was quickly removed from the skull; the hemisphere (minus cerebellum) ipsilateral to the injection site was homogenized and then dissolved in 1·0 ml NCS tissue solubilizer (Amersham Co., Arlington Heights, Illinois) by heating to 50°C and shaking for 2 h. Three portions of each injection medium were treated in an identical manner. After addition of a toluene-based scintillation fluor, all samples and standard were counted for 10 min in a Packard liquid scintillation counter; disintegrations per minute (d.p.m.) were calculated using the external standard method. For each brain sample, the Brain Uptake Index (BUI) was

calculated according to the formula (Oldendorf, 1970):

$$\text{BUI (oestradiol)} = \frac{^{3}\text{H d.p.m.}/^{14}\text{C d.p.m. (brain tissue)}}{^{3}\text{H d.p.m.}/^{14}\text{C d.p.m. (injection vehicle)}} \times 100$$

[^{14}C]Butanol penetrates the blood–brain barrier completely, with very little reverse diffusion into the cerebral vasculature occurring during the 5 sec after injection (Oldendorf & Braun, 1976). Therefore, the BUI for [^{3}H]oestradiol essentially equals the percentage extraction of this steroid by the brain from the cerebral vasculature (Oldendorf & Braun, 1976). The results of the present experiments are expressed in this latter form. These results reflect the percentage of [^{3}H]oestradiol actually extracted from the brain vasculature (i.e. percentage of whatever amount reaches the brain capillaries), and not the recovery of injected [^{3}H]oestradiol, since >90% of the carotid injectate never reaches the brain but instead is distributed to the external carotid artery.

Experiment 1

One group of 17 ovariectomized female rats received intracarotid injections of 4 μCi [^{3}H]oestradiol (0·17 μM) and 0·25 μCi [^{14}C]butanol dissolved in Ringer's solution containing 20% ethanol (v/v) to ensure steroid solubility. The solution was buffered to pH 7·4 with 10 mM-HEPES solution (Sigma Chemical Co., St Louis, Missouri). Additional groups of ovariectomized female rats received injections of the same vehicle, with unlabelled oestradiol added in concentrations of either 34 μM (N = 16) or 68 μM (N = 17).

Experiment 2

A group of 4 ovariectomized female rats received intracarotid injections of 1 μCi [^{3}H]oestradiol (0·043 μM) and 0·05 μCi [^{14}C]butanol dissolved in buffered (pH 7·4) Ringer's solution containing only 0·1% (v/v) bovine serum albumin to ensure steroid solubility. A second group of females (N = 6) received the same radioisotopes in the same vehicle but with unlabelled oestradiol (300 μM) also added.

Experiment 3

One group of ovariectomized female rats (N = 7) received intracarotid injections of 4 μCi [^{3}H]oestradiol (0·17 μM) and 0·25 μCi [^{14}C]butanol dissolved in serum collected from an adult, female rat ovariectomized 1 month before. A second group of ovariectomized rats (N = 5) received the same radiolabelled compounds dissolved in serum pooled from several 9-day-old young rats of both sexes.

Experiment 4

Groups of ovariectomized female rats received intracarotid injections of 1 μCi [^{3}H]oestradiol (0·043 μM) and 0·05 μCi [^{14}C]butanol dissolved in serum collected from gonadally intact female rats aged 0 (day of birth) (N = 5), 5 (N = 4), 20 (N = 5), 30 (N = 4) or 120 (N = 4) days.

Results

Experiment 1

When buffered Ringer's solution containing 20% ethanol served as the injection vehicle, the percentage of [^{3}H]oestradiol extracted by the brain averaged 83%. This value was not signifi-

cantly diminished ($F(2, 47) = 2{\cdot}47$; $P = 0{\cdot}0936$) by addition of increasing concentrations of unlabelled oestradiol (Table 1). In this experiment, the d.p.m. counts in individual samples ranged from 805 583 to 11 097 for ^{3}H and from 24 331 to 892 for ^{14}C.

Table 1. Effects of unlabelled oestradiol in Ringer and serum on extraction of [^{3}H]oestradiol by rat brain

	Unlabelled oestradiol			Rat serum	
	0 μM	34 μM	68 μM	Adult	Neonatal (Day 9)
No. of rats	17	16	17	7	5
% extraction	83 ± 6	84 ± 4	85 ± 6	56 ± 4	13 ± 1

Values are mean ± s.e.m. The 200 μl Ringer or serum contained 4 μCi [^{3}H]oestradiol and 0·25 μCi [^{14}C]butanol.

Experiment 2

Ethanol was excluded from the injection vehicle, and only 0·1% bovine serum albumin was added to the buffered Ringer's solution to ensure steroid solubility. Also, the [^{3}H]oestradiol concentration in the injection vehicle was reduced to 0·043 μM. The percentage brain extraction of oestradiol was identical to that found in Exp. 1: 82 ± 6% (mean + s.e.m.) in the absence of unlabelled oestradiol, and 80 ± 3% in the presence of unlabelled oestradiol (t (8) = 0·3274). The d.p.m. counts in individual samples ranged from 88 733 to 1649 for ^{3}H and from 4170 to 843 for ^{14}C.

Experiment 3

The percentage extraction by brain of [^{3}H]oestradiol from ovariectomized, adult rat serum averaged 56% (Table 1), compared to 80–83% from buffered Ringer's solution (Exps 1 and 2). The percentage extraction of [^{3}H]oestradiol from neonatal serum was significantly lower than from the adult serum (t (10) = 8·4, $P < 0{\cdot}001$, two-tailed). The d.p.m. counts in individual samples ranged from 204 068 to 1344 for ^{3}H and from 30 222 to 508 for ^{14}C.

Experiment 4

The results extended those of Exp. 3: brain extraction of [^{3}H]oestradiol differed significantly, depending on the age of the rats from which the serum was collected, for the injection medium ($F(4, 18) = 18{\cdot}16$, $P < 0{\cdot}001$) (Table 2). Individual *a posteriori* group comparison (Scheffe tests) showed that significantly less ($P < 0{\cdot}01$) [^{3}H]oestradiol was extracted from serum collected on Days 0, 5, and 20 after birth than on Day 120, whereas the percentage extraction from serum collected on Day 30 after birth was equivalent to that from serum collected on Day 120. The d.p.m. counts in individual samples ranged from 43 516 to 3155 for ^{3}H and from 5113 to 941 for ^{14}C.

Table 2. Effects of neonatal serum on [^{3}H]oestradiol extraction by adult rat brains

	Age (days) when serum was collected				
	0	5	20	30	120
No. of rats	5	4	5	4	4
% extraction	21 ± 2	12 ± 1	23 ± 2	55 ± 6	57 ± 4

Values are mean ± s.e.m. The serum volume was 200 μl and contained 1 μCi [^{3}H]oestradiol and 0·05 μCi [^{14}C]butanol.

Discussion

The results of Exps 1 and 2 show that the rat brain extracted a high percentage of [^{3}H]oestradiol during a single circulatory passage when Ringer's solution served as the injection vehicle, regardless of whether ethanol or bovine serum albumin was used to solubilize the steroid in the injection bolus. A blood–brain barrier, carrier-mediated transport system has been demonstrated for circulating thyroid hormones (Pardridge, 1978), as well as for several metabolic substrates, including amino acids (Pardridge, 1977) and glucose (Pardridge & Oldendorf, 1975). If carrier-mediated transport of oestradiol across the blood–brain barrier occurred, addition of unlabelled oestradiol to the injected solution (Exps 1 and 2) should have reduced the influx of oestradiol into the brain (Pardridge & Oldendorf, 1977); it did not, which suggests instead that oestradiol enters the brain by passive diffusion due to its high lipid solubility. Pardridge & Meitus (1979) reached the same conclusion, based on results almost identical to those of Exps 1 and 2 which were obtained by using an injection vehicle of buffered Ringer's solution containing 0·1% bovine serum albumin plus 0·45% ethanol.

The percentage brain extraction of [^{3}H]oestradiol was consistently lower from adult rat serum (Exps 3 and 4) than from buffered Ringer's solution (Exps 1 and 2). Savu, Nunez & Jayle (1975) reported that albumin, and to a lesser degree orosomucoid, collected from adult rat serum has appreciable affinity for diethylstilboestrol *in vitro*. Perhaps these proteins also have a small but measurable affinity for oestradiol, which would explain the present findings.

Brain extraction of [^{3}H]oestradiol from the cerebral vasculature was drastically reduced when serum collected from young rats aged 0–20 days served as the injection medium (Exps 3 and 4). Pardridge & Meitus (1979) obtained a similar effect using 67% serum collected from 2- to 3-day-old rats of unspecified sex. In Exp. 4, this capacity to restrict oestradiol transport into brain was greatly reduced in serum collected on Day 30 or later after birth. Previous in-vitro results have demonstrated that serum of fetal and neonatal rats contains a protein that binds oestradiol specifically with an affinity nearly as high as that displayed by intracellular receptors for this steroid (Raynaud, Mercier-Bodard & Baulieu, 1971). The protein is probably α-fetoprotein and is distinct from albumin and sex steroid-binding protein. Nunez *et al.* (1971a, b) demonstrated in-vitro binding of oestradiol and oestrone by serum from male and female rats just after birth. This binding capacity dissipated during the first 3 weeks of life and was gone by Day 28 after birth. This in-vitro finding corresponds well with the in-vivo results of Exp. 4, in which the percentage brain extraction of [^{3}H]oestradiol reached adult levels when serum from 30-day-old rats served as the injection vehicle.

Much evidence suggests that oestradiol, normally formed in the brain from testosterone of testicular origin, acts in the developing male rat brain to reduce or eliminate the capacity to display feminine sexual behaviour and the cyclic release of pituitary gonadotrophins in adulthood (McEwen, Lieberburg, Chaptal & Krey, 1977; Vreeburg, Van der Vaart & Van der Schoot, 1977). The critical period for oestrogenic action extends from the late prenatal period through the first 10 days of life. The concentrations of oestradiol receptors in several brain regions of rats of both sexes peak during the first 15 days after birth (MacLusky, Chaptal & McEwen, 1979). In the female rat, circulating oestradiol levels are elevated towards the end of the same period (Meijs-Roelofs, Uilenbroek, de Jong & Welschen, 1973). Despite the availability of circulating ligand, occupancy of oestradiol receptors was minimal in cell nuclei isolated from the brains of female rats aged 2–26 days; MacLusky *et al.* (1979) suggested that the presence of α-fetoprotein in neonatal rat serum normally protects the developing rat brain from the potential 'defeminizing' action of circulating oestradiol. α-Fetoprotein may also be present in cells that line the brain ventricles and in neurones of the cerebral cortex, hippocampus, thalamus, hypothalamus and amygdala, as well as in serum, circumventricular organs, and cells surrounding brain blood vessels (Benno & Williams, 1978). In the rat, therefore, serum may be only the first line of defence at which α-fetoprotein restricts the access of oestradiol to its neural receptors during development.

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