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ANDROGEN-INDUCED SEXUAL DIMORPHISM IN HIGH AFFINITY DOPAMINE BINDING IN THE BRAIN TRANSCENDS THE HYPOTHALAMIC-LIMBIC REGION

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1 High affinity binding of [^{3}H]-dopamine and [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) was measured in membrane fractions prepared from cerebral cortex, amygdala, hypothalamus, thalamus and brain stem of rats of either sex and of rats which had been either neonatally castrated or androgenized.

2 Binding was measured in rats of 8, 20 and 30 days old as well as in adults.

3 [^{3}H]-dopamine bound with approximately 30 nM affinity and [^{3}H]-5-HT with approximately 10 nM affinity to all areas of the brain tested. The relative inhibitory effects of haloperidol, apomorphine, *cis*-flupenthixol, unlabelled dopamine, noradrenaline, spiroperone, (+)-butaclamol, fluphenazine, pimozide and 5-HT on [^{3}H]-dopamine binding in the cerebral cortex was consistent with receptor status for the binding components there as were the relative inhibitory effects of methysergide, dopamine, fluoxetine and ouabain on [^{3}H]-5-HT binding in the fore brain.

4 Neither [^{3}H]-dopamine nor [^{3}H]-5-HT binding varied with the state of the sexual cycle in females.

5 There were no sexual differences in [^{3}H]-5-HT binding in any of the brain areas tested nor was it affected by neonatal androgenization or neonatal castration.

6 [^{3}H]-dopamine binding was greater in the cerebral cortex and amygdala of male than of female rats. These differences could be mimicked artificially by neonatal castration of males (female type development) or neonatal androgenization of females (male type development). Sexual dimorphism did not become overt until 20 days of age and did not extend to hypothalamus, thalamus or brain stem.

7 It is concluded that neonatal sex differences in exposure to steroid hormones has permanent effects on the number of dopamine binding sites in the cerebral cortex and is suggested that this sexual dimorphism extends to the amygdala.

Introduction

The sexual differentiation of the rat brain is not solely and directly under genetic control but is brought about by sex hormones acting in infancy (Harris, 1964, and for recent reviews of the literature see Booth, 1979; Goy & McEwen, 1980). It appears that testicular androgens are converted to oestrogens in the hypothalamic-limbic region (MacDonald & Doughty, 1974; Reddy, Naftolin & Ryan, 1974) and that it is these oestrogens, which, when presented at a critical perinatal period, masculinize the hitherto undifferentiated brain, which contains cytoplasmic and nuclear oestrogen receptor systems (Barley, Ginsburg, Greenstein, MacLusky & Thomas, 1974; Salaman, Thomas & Westley, 1976; Westley & Salaman, 1976, Westley, Thomas, Salaman, Knight & Barley, 1976; Thomas & Knight, 1978) through which, and through subsequent RNA and protein biosyntheses (Salaman & Birkett, 1974; Thomas & Knight, 1978) the hormone is believed to act. It is worth noting, that while small amounts of oestrogens (Döhler & Mancke, 1978; Döhler, 1978) and/or progestogens (Shapiro, Goldmann, Bongiovanni & Marino, 1976) may be necessary for the full development of the femal type brain, sexual differentiation is essentially from the (potential) female state to the male.

The sexual differentiation of the brain involves differences in synaptogenesis. Raisman & Field (1973) have shown that in the normal adult female rat the number of synapses of non amygdaloid origin on dendritic spines in the pre-optic area is higher than in the male and that these differences can be mimicked by either neonatal castration or by the administration of androgens to newborn females. That there is a

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morphological basis to sexual differentiation has since been confirmed (Gorski, Gordon, Shryne & Southam, 1978). What is not known, however, are the anatomical limits of central nervous sexual differentiation, and the pharmacological nature of the synapses involved. As the sexual differentiation of the brain is a microcosm of the more subtle aspects of brain differentiation in general, these omissions are important.

In this paper we describe observations on the binding of radiolabelled 5-hydroxytryptamine (5-HT) and dopamine to particulate fractions prepared from different areas of the brains (selected for ease and reproducibility of dissection) of infant and adult rats of either sex, of artificially masculinized (neonatally androgenized) females and of artificially demasculinized (neonatally castrate) males. We decided to study 5-HT and dopamine binding as there is evidence that these two neurotransmitters are involved both in the sexual differentiation of the brain and in sexual function in the adult (see Booth, 1979; Goy & McEwen, 1980) and as oestradiol (E_2)-induced modification of dopamine-dependent adenylate cyclase activity in the newborn is believed to be critical to the sexual differentiation of the brain (Ani, Butterworth & Thomas, 1980).

We decided to use 5-HT and dopamine themselves as the radiolabelled ligands and some of their more 'specific' agonists and antagonists as unlabelled putative inhibitors of the binding reaction because we had no preconceptions about which of individual subclasses of binding site (D_1, D_2 or D_3; 5-HT_1 or 5-HT_2) might be involved and we did not wish to restrict ourselves by choosing radioligands which bound relatively specifically to any particular subclass.

A preliminary account of this work has already been published (Karakiulakis, Knight, Powell & Thomas, 1978).

Methods

Animals

Wistar rats, Carworth-Europe (remote strain, cross bred in our own animal house) were used unless otherwise specified. Animals of the following physiological states were used: (i) Female (a) immature, either normal or neonatally androgenized, (b) adult, either normally cycling (oestrus, dioestrus, metoestrus or pro-oestrus) or else ovariectomized or sham ovariectomized one week before assay. (ii) Male, either normal of neonatally castrate, immature or adult. In addition, some animals were injected, via the lateral ventricle with 100 μg (in 5 μl saline) of 6-hydroxydopamine on the first day of life.

Neonatal androgenization of females was carried out by injecting 30 μg of testosterone proprionate (s.c. in 0.05 ml ethyl oleate) on day 2 of life (dob = 0) or by injecting 0.5 μg of testosterone proprionate, in a similar manner, daily on days 0–4. Both these treatments caused androgenization, as measured by subsequent constant vaginal cornification. Injections of vehicle alone served as controls. States of the oestrous cycle where determined by daily vaginal smearing for at least 3 cycles. Where normal female rats were used only those showing regular 4 day cycles were chosen. Neonatal castrations were carried out immediately after birth on animals anaesthetized by hypothermia.

Tissue preparation

Rats were beheaded between 09 h 30 min and 10 h 00 min and their brains immediately dissected (0–4°C) as described below. The cerebellum was removed (1) and the brain stem separated (2) by slicing along the plane between the corpora quadrigemina and the mammillary bodies. A coronal section (3) was then made just (1 mm in adults) anterior to the optic chiasma and the hypothalamus and amygdalae separated from the rest of the brain by a horizontal section (4) at the level of the rhinal sulci. Hypothalamus and amygdalae were then separated from one another by sagittal sections (5, 6) along the hypothalamic sulci. The cerebral cortex was shelled off (7) distal to the corpus callosum and pooled with the frontal cortex, shelled off, in a similar manner (8) from the tissue lying anterior to section (3). The remainder of the tissue lying anterior to (3) was designated 'corpus striatum' and that lying posterior to (3) designated 'thalamus'. Tissues were homogenized in 40 volumes (ml/g wet wt.) of buffer 'A' using 10 strokes of a Teflon glass homogenizer rotating at 3000 rev/min. The homogenate was centrifuged (50,000 $g \times 10$ min) and the pellet resuspended in another 50 volumes of buffer A, rehomogenized (3 strokes, 3000 rev/min) and recentrifuged as above. The pellet was resuspended in 25 volumes of buffer B and rehomogenized. Buffer A = 50 mM Tris HCl, pH 7.7; B = 50 mM Tris HCl, pH 7.5. The 25 volume homogenate was then prepared for assay. Preparation took place at 0–4°C.

Assays

Assay methods were based on those of Bennet & Snyder (1976).

5-Hydroxytryptamine The '25 volume' preparation was diluted with an equal volume of Buffer C (50 mM Tris HCl, pH 7.35 containing 0.2% (−)-ascorbic acid, 20 μM pargyline and 8 mM $CaCl_2$. The resulting '50 volume' preparation was pre-incubated for 5 min

at 37°C to inactivate endogenous monoamine oxidases (Bennet & Snyder, 1976).

A volume of 500 μl (≡ 10 mg wet wt.) was then incubated in triplicate (routinely for 10 min at 37°C) with 500 μl of a solution containing [^{3}H]-5-HT (7.5 nM unless otherwise specified) and either unlabelled 5-HT (routinely 10 μM) or other putative inhibitors. After cooling to 4°C the incubates were centrifuged (3000 g × 10 min). Pellets were superficially washed three times with 1 ml of ice cold buffer B, dissolved in 100 μl of 0.5 M NaOH and transferred to 10 ml of dioxan naphthalene scintillant.

Dopamine The method was essentially that used for 5-HT except that the '25 volume' homogenate was diluted to '100 volumes' before assay (5 mg wet wt. of tissue being used per incubation) and that the incubation medium contained in addition, 12×10^{-2} M NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$; [^{3}H]-dopamine (routinely 8–10 nM) was added in place of [^{3}H]-5-HT and unlabelled dopamine (routinely 10 μM was added in place of unlabelled 5-HT).

5-Hydroxytryptamine and dopamine The amount of radiolabelled ligand bound to tissue was estimated on a Beckman L230 counting system using [^{3}H]-*n*-hexadecane as in internal standard. High affinity binding was calculated subtracting activity in the presence of excess cold ligand from total activity (Bennet & Snyder, 1976). Binding affinities of radioligands were measured by Scatchard (1949) analysis. Affinities of unlabelled compounds were measured by competition (Creese, Prosser & Snyder, 1978 and for theoretical analysis see Edsall & Wyman, 1958). In all cases at least six concentrations of putative inhibitor were used.

Numbers of sites

In every case, comparisons between the sexes, between different hormonal states and between treated and control groups were made at the same time and were analysed statistically by means of paired t tests. This minimizes artefacts due to day to day variations in experimental conditions. In all cases, a minimum of six separate experiments were analysed and all estimations were made in triplicate. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

Materials

[^{3}H]-dopamine [1, 2-^{3}H], 2.7–10 Ci/mmol and [^{3}H]-*n*-hexadecane (4.86×10^{-6} d min^{-1} g^{-1}) were obtained from the Radiochemical Centre, Amersham. [^{3}H]-5-HT, [5-(1,2-^{3}H(n))], 15–30 Ci/mmol was obtained from NEN.

Unlabelled 5-HT, dopamine, pargyline HCl, apomorphine, *p*-chlorophenylalanine (PCPA), 6-hydroxydopamine, testosterone proprionate (TP) and (−)-ascorbic acid were obtained from Sigma and (−)-noradrenaline from Winthrop Laboratories. Benztropine mesylate was kindly given by Merck, Sharp & Dohme Ltd, *cis*-flupenthixol by Lundbeck Ltd, pimozide and spiroperone by Janssen Pharmaceuticals Ltd, methysergide by Sandoz Ltd, fluoxetine (Lilly 110140) by Lilly Co. Ltd, butacomol from Ayerst Laboratories Ltd, and haloperidol by Searle Pharmaceuticals Ltd.

Dioxan-naphthalene scintillant was made as follows: 200 ml absolute ethanol, 5 g butyl PBD, 80 g naphthalene, to 1 litre with 1:4 dioxan.

All other reagents were of analar grade.

Results

In preliminary experiments we established that the binding of both radiolabelled ligands to membrane fragments had reached equilibrium by 5 min at 37°C and we, therefore, used an incubation time of 10 min for both assays. We also established that binding was rectilinear with respect to tissue concentration up to 15 mg (wet wt.)/ml and that we were therefore justified at working at concentrations of 10 mg/ml (5-HT) and 5 mg/ml (dopamine) respectively. This rectilinearity implies that there is no interference with binding by endogenous substances, but in view of the importance of any such inhibition we investigated the effects of particulate-free 100,000 g supernatant preparations (equivalent in concentration to those used in our assays) as putative inhibitors of high affinity binding. These extracts were prepared from brain areas known to contain the greatest concentrations of the appropriate neurotransmitters (corpus striatum for dopamine; amygdala for 5-HT) and did not interfere detectably with the binding of either ligand under experimental conditions. Even a ten fold excess had no effect.

Binding parameters

The effects of increasing concentrations of three representative putative competitors on the binding of [^{3}H]-dopamine to cortical membrane fractions (adult, male, intact) are shown in Figure 1.

The co-ordinates of the lower asympote obtained in the presence of excess unlabelled dopamine, and (not shown) of [^{3}H]-5-HT binding in the presence of unlabelled 5-HT, justify our routine use of 1 μM concentrations of these compounds in routine incubations. High affinity radioligand binding can be estimated from the differences between the upper

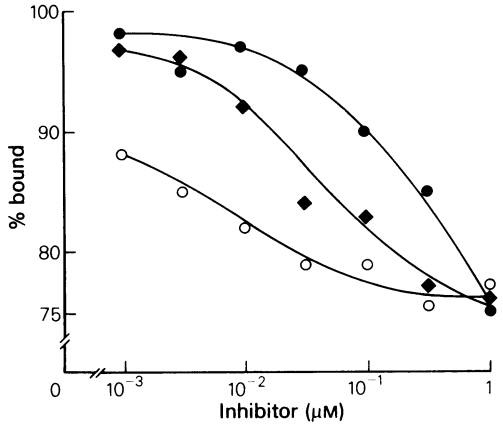

Figure 1 The effects of increasing concentrations of three representative unlabelled compounds on the binding of [^{3}H]-dopamine (10 nM) to cerebral cortical membrane fractions of adult male rats. (◆) Dopamine; (○) apomorphine; (●) (+)-butaclamol. Axes: vertical [^{3}H]-dopamine bound as a percentage of binding in the absence of inhibitor. Horizontal: concentrations of inhibitors (μM).

and lower asymptotes and the concentrations (references: upper, [^{3}H]-dopamine alone; lower [^{3}H]-dopamine in the presence of 1 μM unlabelled dopamine) of inhibitor needed to reduce this by half (IC_{50}) gives a measure of the reciprocal of the affinity. Values for IC_{50} obtained from the above and from similar experiments are shown in Table 1. A parallel series of experiments gave IC_{50} values, of various putative inhibitors against [^{3}H]-5-HT in the forebrain of: unlabelled 5-HT, 12 nM; methysergide, 430 nM; dopamine 10 μM; fluphenazine and ouabain 100 μM.

Table 1 The effects of various unlabelled compounds on the binding of [^{3}H]-dopamine to cerebral cortical membrane fractions of rats

	IC_{50} (nM)	
Drug	*Male cortex*	*Female cortex*
Haloperidol	2 (1)	2 (3)
Apomorphine	2	5
cis-Flupenthixol	10 (2)	12 (3)
*Dopamine	54 (7)	47 (9)
Noradrenaline	—	50 (3)
Spiroperone	—	100 (1)
(+)-Butaclamol	150 (2)	130 (3)
Fluphenazine	750 (2)	700 (3)
**Pimozide	7000 (3)	—
*5-HT	12000 (3)	10000 (3)

IC_{50} values (see text) are concentrations needed to reduce [^{3}H]-dopamine binding by half of that achieved by 1 μM dopamine. Numbers of experiments are shown in parentheses.
*Similar results were obtained in brain stem and **, *corpus striatum.

The inhibitory affinities of unlabelled dopamine and 5-HT are virtually indistinguishable from those of their labelled (see below) analogues. Inhibitors of presynaptic uptake (ouabain, fluoxetine and benztropine) have little effect on the reactions; known inhibitors of receptor binding (haloperidol, apomorphine, *cis*-flupenthixol, unlabelled dopamine, noradrenaline, spiroperone, (+)-butaclamol, fluphenazine, pimozide, dopamine, methysergide, 5-HT have affinities for the binding sites similar to those found by others (Pardo, Creese, Burt & Snyder, 1972; Burt, Enna, Creese & Snyder, 1975; Creese, Burt & Snyder, 1975; Seeman, Chan-Wong, Tedesco & Wong, 1975; Bennet & Snyder, 1976; Burt, Creese & Snyder, 1976; Creese *et al.*, 1978; Kebabian & Calne, 1979; List, Titeler & Seeman, 1980). There is little cross reactivity between 5-HT and dopamine, and noradrenaline had a similar effect to unlabelled dopamine on [^{3}H]-dopamine binding.

In another series of experiments (Figure 2) the binding of increasing concentrations of radioligand (in the presence or absence of excess unlabelled ligand) was used to construct binding isotherms, Scatchard (1949) transformations of which were used to calculate both saturation binding capacity and the equilibrium dissociation constants of reaction (Kd). Kd values were of the order of 30 nM (dopamine) and 12 nM (5-HT). There was no evidence of any variation of affinity with either brain area, sex or age, Kd values (nM) for 5-HT being: whole brain, male 12.5 ± 1.2, female 10.4 ± 1.5, neonate 12.6 ± 1.3; brain stem, adult male 11.0 ± 1.8; amygdala, adult male 13.5 ± 2. Corresponding values for dopamine were: male 30 (5), female 30 (3); brain stem, male 35 (5); amygdala, male 20 (3); corpus striatum, male 22 (7), female 27 (3); whole brain, neonate, male 15 (3). The symbols (±) indicate the standard errors of the means of at least 6 experiments. The numbers in parentheses indicate the numbers of observations. Standard errors were not calculated for dopamine as the numbers of observations were, at times, as low as 3.

Since we found no evidence that the binding affinities we were measuring varied with respect to age, brain area or sex and since we felt it important to measure binding in five areas of the brain in two classes of animal (male vs female; treated vs control) simultaneously (20 separate assays) we felt justified in using a simplified procedure previously adopted by others (Bennet & Snyder, 1975) for measuring amounts of high affinity binding. In routine experiments high affinity binding was measured by subtracting (triplicate values) radioligand bound in the presence of 10 μM unlabelled ligand (lower asymptote Figure 1) from that bound (triplicate values) in the absence of unlabelled.

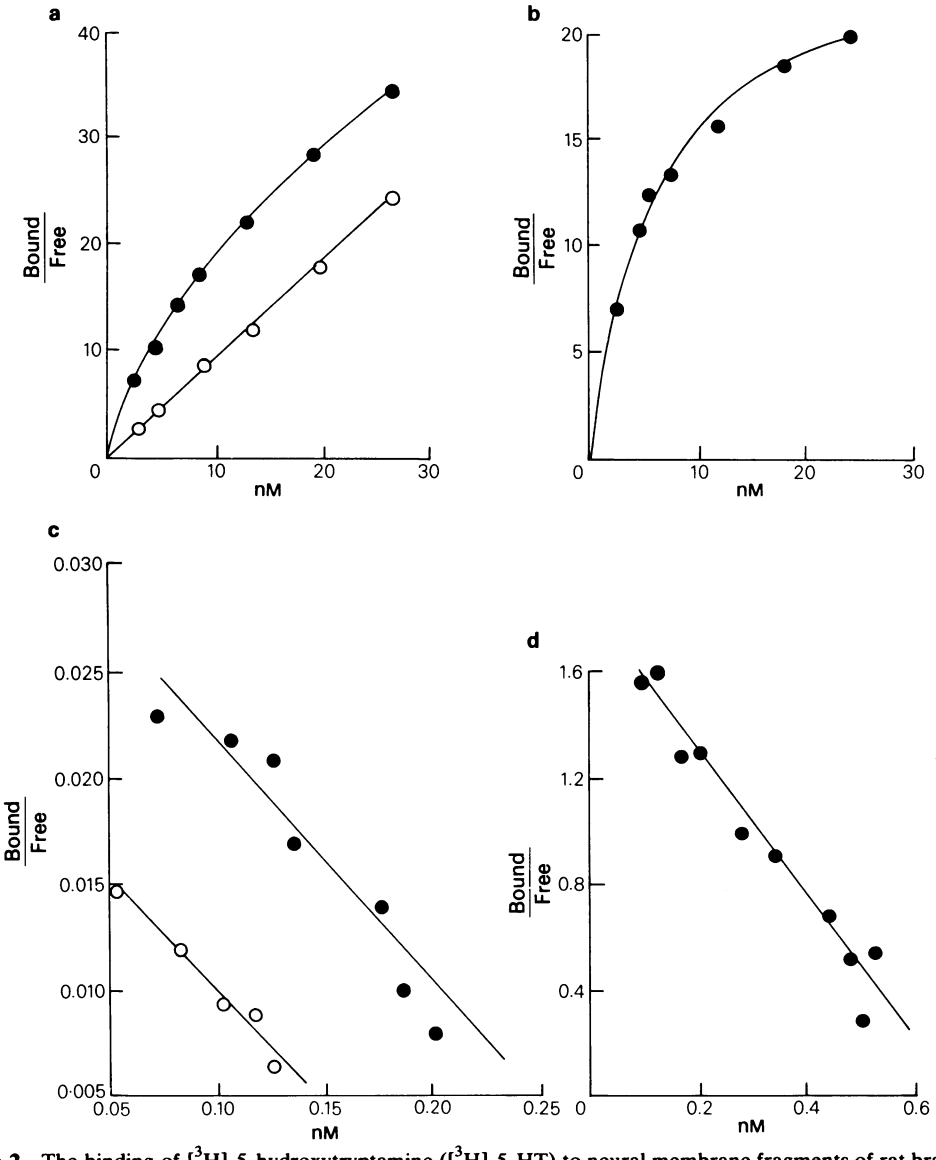

Figure 2 The binding of [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) to neural membrane fragments of rat brain: (a and b) vertical axes, 5-HT bound d/min × 10^{-3}; horizontal axes concentration of free 5-HT. (a) shows total (●) radioactivity bound together with radioactivity bound in the presence of excess 1 μM unlabelled 5-HT (○); (b) is a plot of the difference (●−○) and represents high affinity binding; (c) is a Scatchard transformation of (b), (●): on vertical axis, the ratio (bound/free × 10^3); on horizontal axis, bound 5-HT (nM). These data were obtained with a tissue concentration of 10 mg (wet wt.)/ml as shown in (c) (○). The degree of parallelism between the lines shows that tissue concentration does not affect estimates of binding parameters. (d) A Scatchard transformation of a binding isotherm of [^{3}H]-dopamine to neural membrane fragments of rat corpus striatum. Vertical axis; the ratio (bound/free) × 10^2: horizontal axis; dopamine bound (nM).

Estimates of high affinity binding obtained by this method were in good agreement with those obtained by Scatchard (1949) analysis (for example, male 5-HT: amygdala 270 ± 10, Scatchard, 300 ± 20, simplified assay; brain stem 100 ± 6, Scatchard, 105 ± 7 simplified; dopamine striatum, male 133 (3), Scatchard, 140 (3) simplified). Values are in pmol/g protein. As the simplified assay measured binding at 10 nM radiolabelled ligand rather than saturation binding capacity, estimates from Scatchard plots have been interpolated back to this value for purposes of comparison.

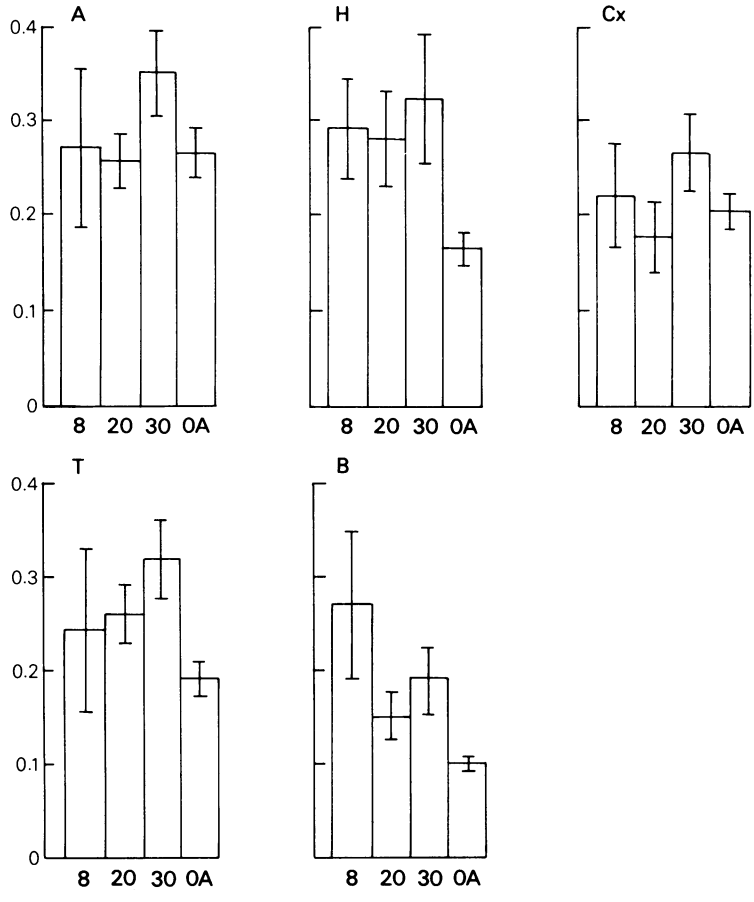

Figure 3 The effect of age on high affinity [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) binding in five areas of female rat brain. Vertical axes; high affinity 5-HT bound (nmol/g protein). Horizontal axes; age in days (OA-ovariectomized adult). A = amygdala, H = hypothalamus, Cx = cortex, T = thalamus, B = brain stem. Adult females were ovariectomized. Values are means of at least six paired experiments, vertical lines show s.e.mean.

The effects of age and of normal and of artificially mimicked gender on 5-HT binding are shown in Figures 3–5. The magnitude of 5-HT binding was amygdala > hypothalamus > cerebral cortex > thalamus > brain stem. In the brain stem, binding capacity decreases with age. In other tissues it increases up to day 30 and thereafter decreases. The anatomical distribution of 5-HT binding is similar to that of tryptophan-5-hydroxylase (Renson, 1973) the rate limiting enzyme in the formation of 5-HT. Neither normal sex nor neonatal androgenization had any effect on 5-HT binding capacity (Figure 4) nor was it affected by the different stages of the oestrous cycle or by ovariectomy (Figure 5).

The magnitude of dopamine binding was amygdala > cortex > thalamus > hypothalamus > brain stem (Figures 6–9). Values increase steadily from birth to maturity.

In contrast to 5-HT binding the effects of both normal and of artificially induced gender on dopamine binding capacity were striking (Figures 6–9). Dopamine was significantly higher in two areas (cerebral cortex and amygdala) of the adult male brain than in the female. Other areas were not affected. These effects did not become apparent until 20–30 days of age (Figure 6) and can be mimicked by neonatal castration and androgenization (Figures 7, 8). Short term changes in hormonal status (oestrous cycle and ovariectomy) had no effect on dopamine binding.

In another series of experiments, designed to test the effects of chemical 'denervation' on binding, adult female rats, neonatally treated with 6-hydroxydopamine (intracerebral injection, 100 μg in 5 μl) were ovariectomized and dopamine binding compared with that of similarly ovariectomized (3

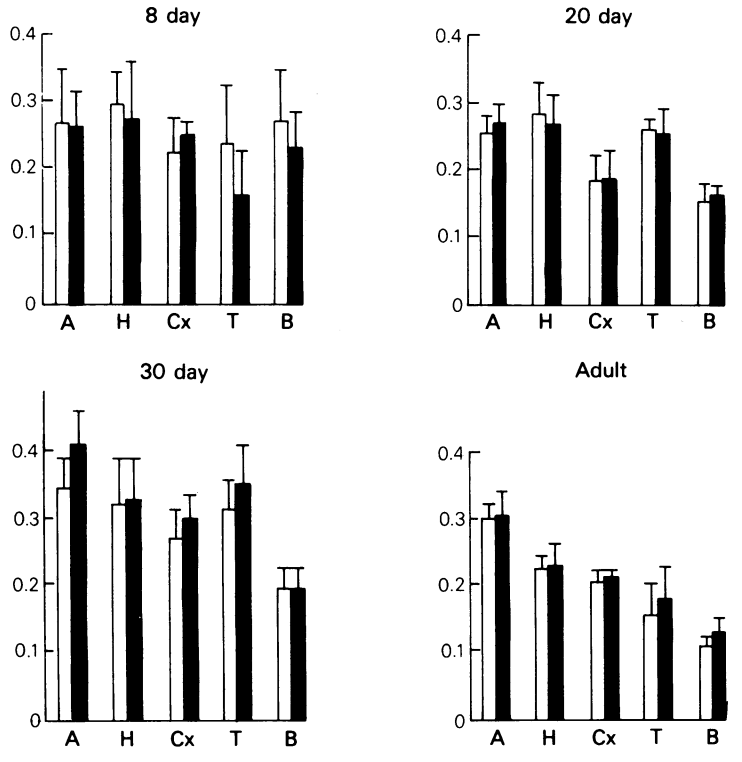

Figure 4 The effect of neonatal testosterone proprionate treatment on high affinity 5-hydroxytryptamine (5-HT) binding in neural membrane fragments from five areas of female rat brain. Vertical axes as in Figure 3. Brain areas as in Figure 1. Shading indicates that animals have received testosterone proprionate (30 μg) on day 2 of life. There are no significant differences between treated and untreated animals.

weeks) controls. Binding (hypothalamus, cortex, amygdala and brain stem) in treated rats was double that found in controls (Table 2). Binding was not measured in the 'thalamus' as that tissue was unidentifiable in the treated animals.

Discussion

Our aim, in carrying out the work described here was to see whether sex steroids had any effects, either short or long term, on the binding of either [^{3}H]-5-HT or [^{3}H]-dopamine in the brain and, if they did, to investigate the nature of that binding.

In the case of [^{3}H]-5-HT our task was relatively simple. We did not detect any differences between the amounts of [^{3}H]-5-HT bound to equivalent brain areas in male and in female rats of any of the ages tested, nor did the oestrous cycle, ovariectomy, neonatal androgenization or neonatal castration have any effect, so we restricted ourselves to making a cursory examination of the nature of the binding sites. The similarity of their distribution to that of tryptophan 5-hydroxylase (Renson, 1973) suggests that we are measuring binding of physiological importance and the inability of even mM concentrations of ouabain and of fluoxetine to inhibit binding shows that we are not measuring active, presynaptic, uptake of the radioligand. The effect of methysergide is consistent with receptor status for the binding sites as is the ~10 nM affinity of [^{3}H]-5-HT itself (Bennet & Snyder, 1976). Having come to this tentative conclusion, and because of the essentially negative results of

Table 2 High affinity specific binding of dopamine (DA) from various regions of brains from control rats or rats treated neonatally with 6-hydroxydopamine (6-OHDA)

	DA binding (nmol/g protein)	
Brain area	6-OHDA	Control
Hypothalamus	0.57 ± 0.06	0.28 ± 0.03
Brain stem	0.14 ± 0.02	0.15 ± 0.01
Cortex	0.41 ± 0.02	0.21 ± 0.01
Amygdala	0.67 ± 0.05	0.32 ± 0.07

Both groups of rats were assayed when adult and ovariectomized 3 weeks before assay.

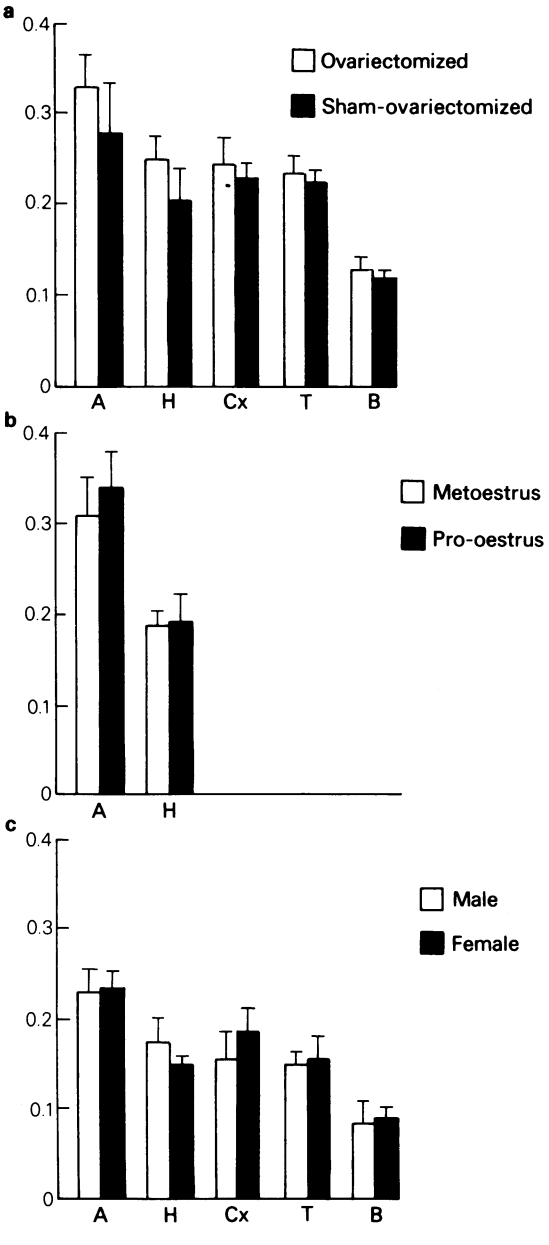

Figure 5 The effects of (a) ovariectomy, (b) the oestrous cycle and (c) sex on 5-hydroxytryptamine binding in different areas of the rat brain. Horizontal axes, brain areas, as defined in Figure 1. Vertical axes, nmol/g protein. There are no significant differences between the different physiological states.

our work with [^{3}H]-5-HT, we decided to characterize the sites no further. This means that we may have overlooked some possibly more subtle effects of sex steroids on 5-HT binding: indeed, it has recently been shown that oestradiol and imipramine, when administered together, depress 5-HT_2 receptor number in the rat cerebral cortex (Kendall, Stancel & Enna, 1981).

Dopamine

The radioligand bound to all areas of the brain tested, including the cerebral cortex, where, although high affinity neuroleptic binding has been detected in rat and man (Fields, Reisine & Yamanura, 1977; Mackay, Doble, Bird, Spokes, Quik & Iversen, 1978; di Paolo, Carmichael, Labrie & Raynaud, 1979) and where dopamine-sensitive adenylate cyclase has been demonstrated (Ahn, Mishra, Demirjian & Makman, 1976; Bockaort, Tassin, Thierry, Glowinski & Premont, 1977) it has been denied that [^{3}H]-dopamine itself binds (List *et al.*, 1980).

The distribution of binding (bearing in mind the limits of our dissections), broadly speaking parallels the distribution of dopamine innervation of the brain (Lindvall, 1979; Palkovits, 1979).

Nowhere did we find any evidence of short term changes in binding capacity with either ovariectomy or with the sexual cycle, but in two areas (cerebral cortex and amygdala) we found a sexual dimorphism which could be mimicked by appropriate neonatal hormonal manipulation. Binding in these two tissues (but not elsewhere) was higher in males than in females: androgenized females bound [^{3}H]-dopamine at male levels and neonatally castrate males in amounts similar to the female. These sexual differences did not become overt until 20 days of age.

The specificity of binding in the cortex had a dopaminergic spectrum. The concentrations of unlabelled competitors needed to inhibit high affinity [^{3}H]-dopamine binding by 50% (IC_{50} values) were, in reverse order of magnitude: haloperidol $<$ apomorphine $<$ *cis*-flupenthixol ($\lesssim$10 nM) $<$ dopamine $\sim$ noradrenaline $<$ spiroperone ($\lesssim$100 nM) $<$ (+)-butaclamol $\approx$ fluphenazine ($<\mu$M) $<$ pimozide $<$ 5-HT ($\sim$10 μM).

This spectrum of inhibitory activities (in particular its combination of relatively high binding affinities for haloperidol, *cis*-flupenthixol, apomorphine with a relatively low binding affinity for spiroperone) makes it hard to assign the binding we have measured to any of the classes of dopamine receptor so far described (Kebabian & Calne, 1979; List *et al.*, 1980). Our binding is not to D_1, but seems to share the properties of D_2 and of D_3 receptors. Since it seems likely that different assay media may affect receptor binding in different ways; since the heterogeneity of our brain membrane fractions with respect to binding components other than those under investigation may induce complex artefacts (different combinations of drugs) and since a new method of classifying

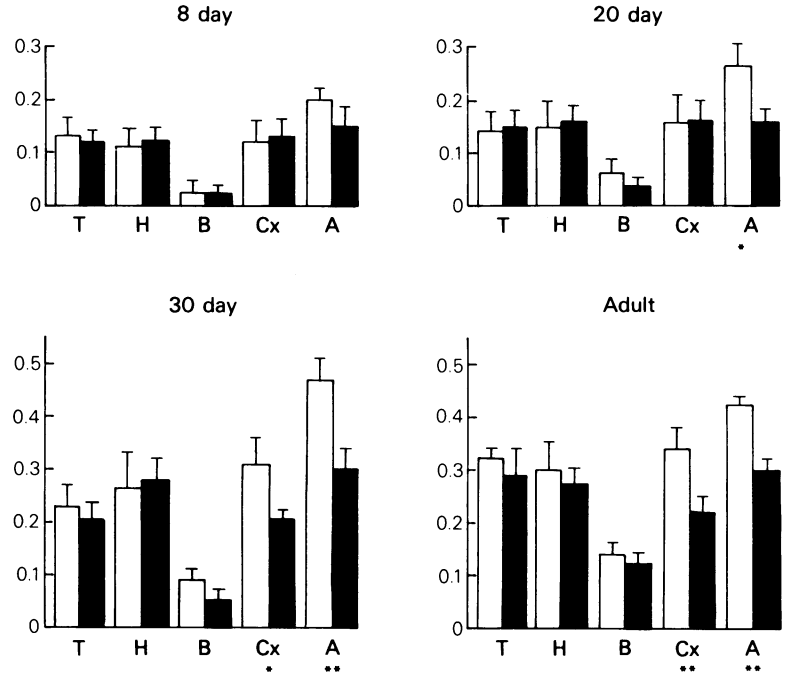

Figure 6 The effects of age and sex on high affinity dopamine binding in rat brain. Vertical axes; nmol/g protein. Horizontal axes; brain areas as defined in Figure 1. Significance of differences (paired *t* tests) are indicated by asterisks: $*P<0.05$; $**P<0.02$; $***P<0.01$. Open columns: male, solid columns: female.

dopamine receptors, using completely different criteria from those used by Kebabian & Calne (1979) and List *et al.* (1980) has recently been proposed (Cools & van Rossum, 1980) we feel it prudent not to try to assign our binding to any particular subclass, but merely to say that it is dopaminergic.

It is clearly not to noradrenaline receptors, which, be they either α or β, have completely different properties (Alexander, Davis & Lefkowitz, 1975; Byland & Snyder, 1976; U'Prichard, Greenberg & Snyder, 1976; Greenberg & Snyder, 1977; Davis, Strittmatter, Hoyle & Lefkowitz, 1977) nor is it due to reuptake into presynaptic nerve terminals since binding is increased in animals neonatally treated with 6-hydroxydopamine, a procedure reported to destroy both noradrenaline (Sachs & Jonsson, 1975) and dopamine (Creese & Iversen, 1975) nerve terminals.

Thus our most important findings are those of sexual dimorphism in number of dopamine, but not of 5-HT binding sites in the brains of adult rats, that this dimorphism can be mimicked by neonatal castration of males and by neonatal androgenization of females, and that sexual differences extend beyond the hypothalamic-limbic region.

That this dimorphism cannot be due to any interference by endogenous dopamine (or any other endogenous agonist or antagonist) is shown by our observation that any such substances would be washed out during the preparation of the tissue. Nor can the sexual dimorphism in dopamine binding that we have shown be due to sexual differences in the activities of either monoamine oxidases (MAO) or catechol-*O*-methyl transferase (COMT) since (1) our pre-incubation with pargyline is reported to destroy endogenous MAO, (2) pargyline (a MAO inhibitor) is present in the assay medium, (3) the direction of any artefacts due to metabolism should be the same for both dopamine and 5-HT, (4) metabolism during the assay would yield curvilinear Scatchard plots and (5) the regional and sexual differences in MAO and COMT activities reported (Vaccari, Brotman, Cimono & Timiras, 1977) are not consistent with any hypothesis linking them artefactually to our results.

Despite the fact that sexual dimorphism in 5-HT levels in the brain has been described (Ladowsky & Gazari, 1970) we found no dimorphism in high affinity 5-HT binding. This strengthens our confidence in our observations on dopamine binding.

At first sight it is hard to understand how sex hormone exposure in infancy can modify dopamine

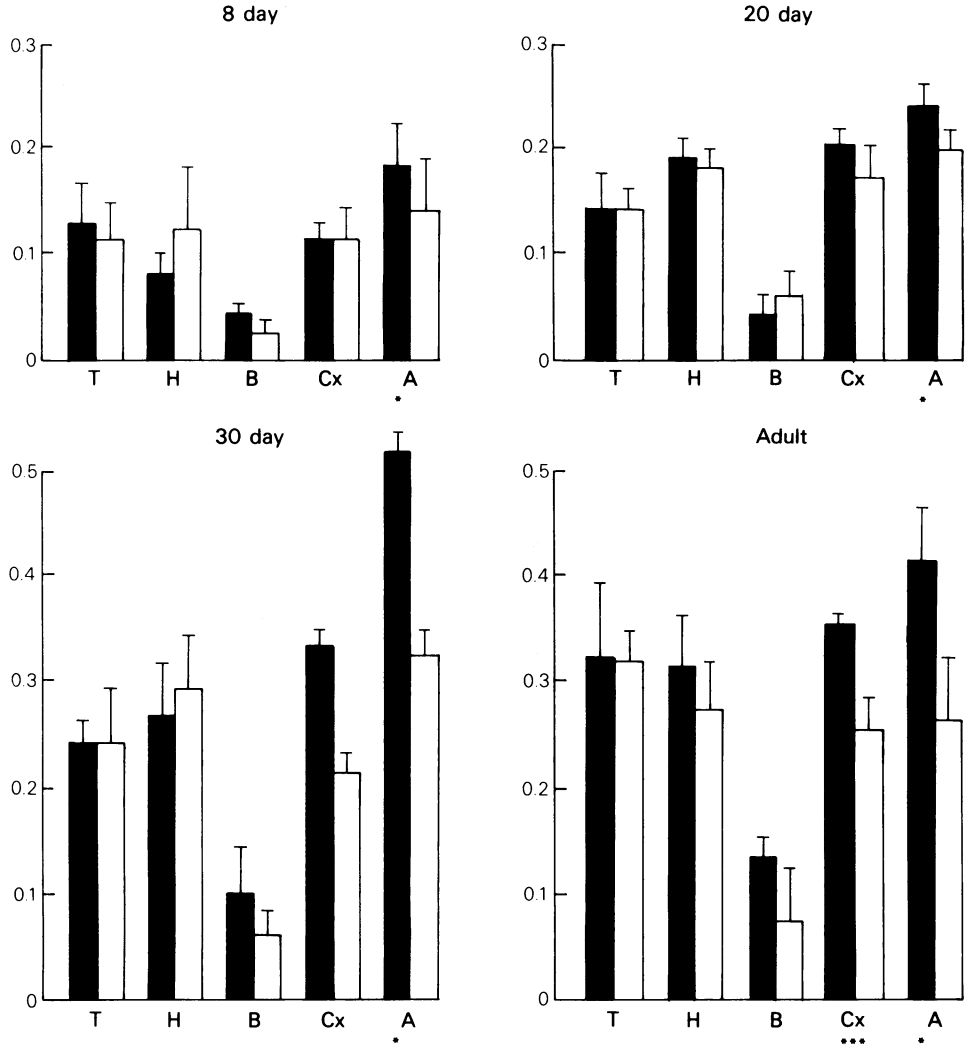

Figure 7 High affinity dopamine binding in different areas of the brain in control and androgenized female rats of different ages. Vertical axes, dopamine binding, nmol/g protein. Horizontal axes; brain areas as defined in Figure 1. Open columns: control; solid columns: rats treated with testosterone proprionate. Asterisks indicate the degree of significance (paired *t* tests) between the same areas in different states: $*P<0.05$; $**P<0.02$; $***P<0.01$.

binding in the adult, particularly as (a) changes do not become overt until 20 days postnatally at the earliest (the critical period for masculinization is from days 0–4) and (b) non limbic areas of the brain are affected (the conversion of testosterone to the masculinizer, oestradiol, is confined to the hypothalamic-limbic region).

Though we have no further direct evidence at present, we feel that the most likely hypothesis is that (1) testosterone is converted to oestradiol in the hypothalamic-limbic region, (2) this induces the synthesis of a protein which suppresses dopamine-dependent adenylate cyclase (Ani *et al.*, 1980) preventing the selective stabilization of synapses in that region, (3) this subsequently influences the development of dopaminergic projections to other areas of the brain and thus the number of dopamine receptors.

Our results show that sexual differentiation affects areas of the brain not classically associated with sexual function and we predict that many more functional differences between male and female brains will come to light in the future. It is worth mentioning, in this context, that it has just been shown

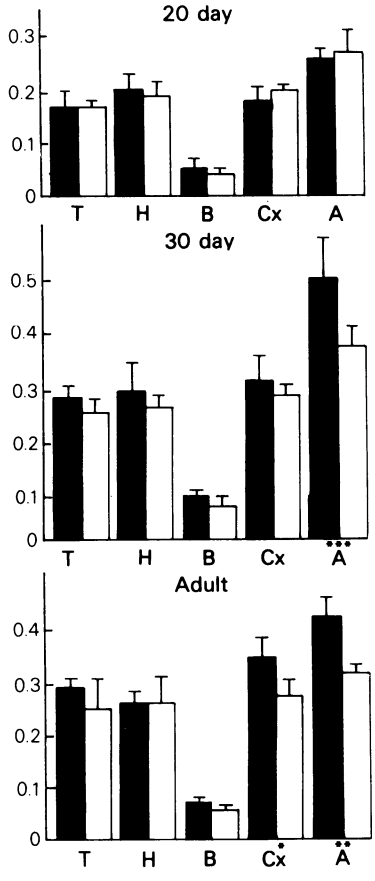

Figure 8 High affinity dopamine binding in different areas of the brains of normal (solid columns) and neonatally castrated (open columns) male rats. Axes, symbols and degrees of significance as in Figure 7.

(Masur, Boerngen & Tufit, 1980) that there are sex differences in the response to apomorphine in rats which, it is suggested, could be due to differences in dopaminergic systems of specific CNS regions.

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