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Rat Occipital Cortical Synapses after Ovariectomy

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Our laboratory reported an increase in the thickness of the occipital cortex in 90-day-old female Long–Evans rats ovariectomized on day 1 compared with sham-operated controls. The present experiment studied the effects of ovariectomy on cerebral cortical synapses. Tissue blocks from the dorsal medial occipital cortex, area 18, of the right hemisphere were examined with a Zeiss 9S-2 electron microscope, magnification 34,125. Significantly more discontinuous postsynaptic densities were observed in the ovariectomized animals than in the sham-operated rats. The ovariectomized group also showed more positively curved synapses and fewer negatively curved synapses than the sham-operated animals, according to the scheme of synaptic curvature outlined by R. M. Devon and D. G. Jones (1979, *Cell Tissue Res.* **203,** 189–200) and S. E. Dyson and D. G. Jones (1980, *Brain Res.* **183,** 43–59). These data may represent the presence of more mature, active, functioning synapses in animals that have been ovariectomized compared with their littermate controls.

INTRODUCTION

Devon and Jones (5) and Dyson and Jones (10) analyzed the curvature of synapses in the molecular layer of the rat occipital cortex as a function of maturation and degree of anesthesia. They reported some interesting observations leading to the hypothesis that synaptic curvature may be an indicator of the level of maturation and function of a synapse. Those researchers distinguished between positively curved, negatively curved, and flat junctions. Positively curved synapses were defined as those in which the postsynaptic density (PSD) curved into the presynaptic terminal; neg-

Abbreviations: PSD—postsynaptic density, SSPP—subsynaptic plate perforation, AFP—α-fetoprotein.

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atively curved synapses were those in which the postsynaptic density curved toward the postsynaptic process (Fig. 1).

An increase in the frequency of flat junctions was noted with age. A larger proportion of the population was negative at days 15, 20, and 28; whereas the proportions of negatively and positively curved junctions were equivalent by days 75 and 225 (10).

Taking the suggestion from Peters and Kaiserman-Abramof (21) that the edges of the PSD represent sites of functional activity, Greenough *et al.* (12) investigated the frequency of cortical "split" synapses (those with a discontinuous PSD) in rats reared in differential environments. The gaps were termed by the authors "subsynaptic plate perforations (SSPPs)." They found that the relative frequency of SSPPs increased dramatically between 10 and 60 days of age. They also observed that rats reared in more complex environments had a significantly greater number of SSPPs in the occipital region than rats reared in isolation.

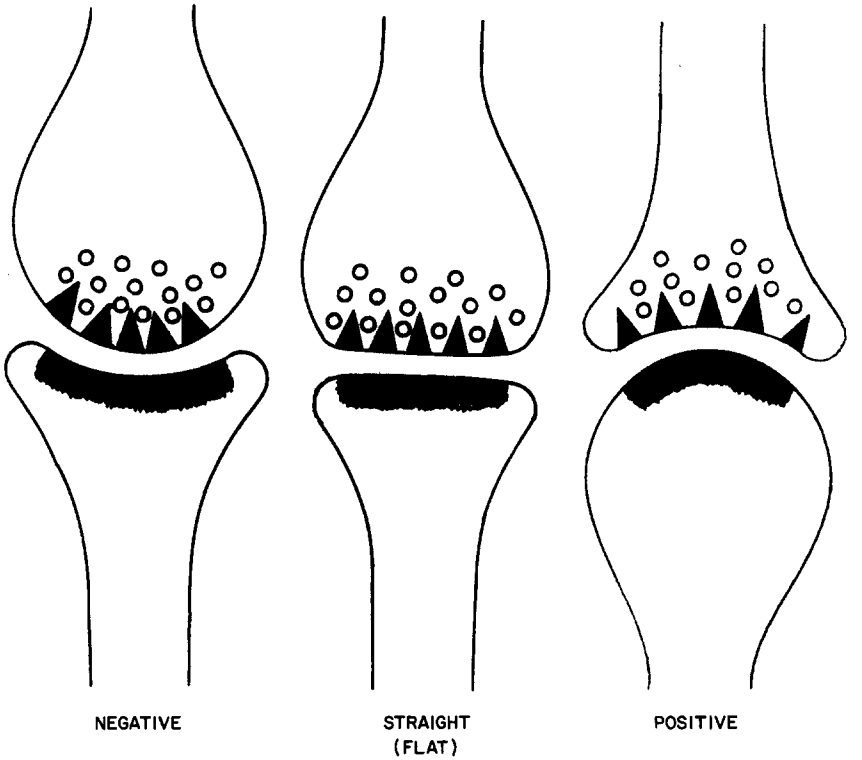

FIG. 1. Drawings illustrating the three types of curvature a synapse may demonstrate [Adapted from Devon and Jones (5)].

That female sex steroid hormones are related to cortical morphology was previously demonstrated. Estrogen was shown to alter cerebral cortical structure (4, 14, 19, 20) and estrogen receptors were localized in the cortices of young animals (11, 16, 17, 22, 23, 26). The neuroanatomic plasticity manifest in increased cortical thickness, a result of both hormonal (19) and environmental (7, 13) influences, was correlated by our laboratory with an increase in the number of glia (8), larger neurons (6), an increase in the length of the PSD (9, 18), and a decrease in neuron density (9). Given that ovariectomy has been shown to increase cortical thickness as well as increase perikarya and nuclei size and decrease neuron density (19), the present experiment was designed to determine if ovariectomy affects synaptic morphology. Parameters examined were the number of synapses, the length of the PSD (synaptic length), number of split synapses, and synaptic curvature.

MATERIALS AND METHODS

Seven littermate pairs of Long–Evans female rats from the Physiology–Anatomy colony were divided randomly and coded at day 1: one of a pair was bilaterally ovariectomized under cryogenic anesthesia and the other was sham-operated, i.e., the ovary was exposed but not removed. Surgery was carried out as described by C. T. E. Pappas (19). The pups were then placed 3 h in a dish within a water bath at 37°C. Before returning them to their mothers, their wounds and their mothers' noses were painted with a gentian violet solution (consisting of 20 ml 10% nitrocellulose, 30 ml amyl acetate, and 0.5 g gentian violet) to mask any foreign odors and thus prevent rejection by the mother. The pups were weaned at day 21 and placed three or four per cage (47 × 26 × 21 cm) until day 90. The rats were coded throughout the complete procedure, from killing to completion of measurement of synapses, when the codes were deciphered.

The animals were killed on day 90. At autopsy, they were weighed, anesthetized with sodium pentobarbitone (0.1 ml/100 g body weight), and perfused with glutaraldehye. The procedure was described by Pappas (19).

The brains were removed and a small tissue block was excised from the dorsal medial occipital cortex, area 18, of the right hemisphere according to the procedure of Bennett *et al.* (2), utilizing a precisely ruled plastic T square to insure uniform removal. The tissue blocks were immersed 2 h in a solution of 4% glutaraldehyde and 2% formaldehyde in a 0.1 M sodium cacodylate buffer (*p*H 7.4) at 4°C. The tissues were washed 5 to 10 min in the buffer at 4°C before being postfixed in 1% osmium tetroxide in the cacodylate buffer for 2 h at 4°C. After dehydration in graded ethanol solutions, the tissues were transferred to propylene oxide, then placed over-

night in one part propylene oxide and one part Spurr's at 4°C. The following day, one additional part Spurr's was added. The tissues were left 4 h at 4°C. Finally all liquid was replaced with fresh 100% Spurr's solution and left overnight. The next morning the tissues were placed in fresh Spurr's in prepared BEEM capsules and placed in a 70°C oven for 12 to 18 h.

After checking for suitable hardness, the blocks were trimmed to eliminate excess resin. Layer II was initially chosen because of the certainty of its identity. To locate layer II, thick sections (0.5 μm) were cut of the entire depth of the cortex for orientation by light microscopy. The block was then retrimmed to include part of layer I, all of layer II, and part of layer III. (This was made easier by the fact that the tissue had been originally cut before embedding in the shape of a trapezoid with the longer of the two parallel edges approximating the pial surface.) Thin sections were cut on an MT-2 Porter–Blum microtome. Section thickness was between 600 and 800 Å as determined by the silver interference color. Ribbons were taken ca. 400 nm apart so as to prevent the same synapses being included (the range of the length of postsynaptic thickening is from 0.25 to 0.45 μm).

The sections were picked up on uncoated 300-mesh copper grids and stained with uranyl acetate and lead citrate, and were examined in a Zeiss 9S-2 electron microscope. Photomicrographs were taken at 10,500 × magnification in a clockwise direction, beginning in the grid squares beneath layer I (i.e., those showing an increase in the number of cell bodies). Somas and blood vessels were excluded. Ten to fifteen pictures were taken per animal from two or three different grid squares. The micrographs were enlarged 3.25 times, giving a total magnification of 34,125 in the 19.4 × 20.5-cm prints.

Only axodendritic synapses were analyzed, due to evidence by Dyson and Jones (10) that axodendritic synapses appear prior to axosomatic synapses. The criteria for all synapses for measurement of the PSD were a clear synaptic cleft and postsynaptic density as well as at least six synaptic vesicles in the presynaptic terminal. All synapses measured were asymmetrical, corresponding to Gray's Type 1. If the vesicles were uniformly distributed in the presynaptic terminal, the postsynaptic thickening was measured both as one intact synapse from its outermost edges, as well as each of the smaller constituent portions of the PSD being measured separately.

The length of the PSD was measured with a clear flexible metric ruler and recorded in millimeters. The lengths of the PSDs of those synapses (or portions thereof) that were too curved to be measured with the ruler were calculated after being fitted to a circle with the same curvature according to the fraction of its circumference that it represented.

The number of synaptic terminals containing at least six clear synaptic vesicles were counted in each micrograph, regardless of whether or not a synaptic cleft or postsynaptic element was visible. To insure that no duplication occurred in the counting of these synapses in successive micrographs, those seen along the borders of the print were included in the count only if they occurred along the upper or left-hand edge. The profiles were marked with a number as they were counted so as not to be included more than once.

Student's *t* test was used in the statistical analysis of the synaptic profile counts and the PSD lengths. For the frequency of occurrence of split synapses, chi-square tests were used to compare both groups and individuals.

Synapses that were sufficiently clear were assigned to one of three categories relating to curvature: positive, negative, or flat, according to the definitions of Dyson and Jones (10) and Devon and Jones (5) (see Fig. 1). Any ambiguous synapses were excluded from the data. These included synapses in which the pre- and postsynaptic membranes were not parallel; synapses which were curved positively in one part and negatively in another; and synapses in which only the edges were curved. The assignment to the various categories did not depend on the degree of curvature (Fig. 2). Because the number of synapses examined differed for each animal, the values were converted to percentages. The percentages were analyzed using three separate Student's *t* tests.

RESULTS

Synaptic Number. Table 1 presents the average number of synaptic profiles per micrograph taken in layer II of the medial occipital cortex for each animal and the averages for each group with standard deviations and standard errors. In five of the six pairs there were fewer synaptic profiles in the ovariectomized animal, but the 11% difference between the groups was not significant. Note that the standard deviations were large.

Synaptic Length. Values for the lengths of the postsynaptic densities measured are presented in Table 2. Four of the six pairs had shorter overall PSDs in the ovariectomized animal, but no significant differences were observable (difference = 5%) with or without the split synapse data. Note again that the standard deviations were large.

Discontinuous Postsynaptic Densities. Table 3 presents the number of discontinuous PSDs found for each animal. The chi-square test revealed that ovariectomized animals had significantly more synapses (20%) with discontinuous PSDs than the sham-operated rats ($\chi^2 = 4.5$, $df = 1$, $P < 0.05$). Discontinuous PSDs occurred in both groups but more frequently in the ovariectomized one.

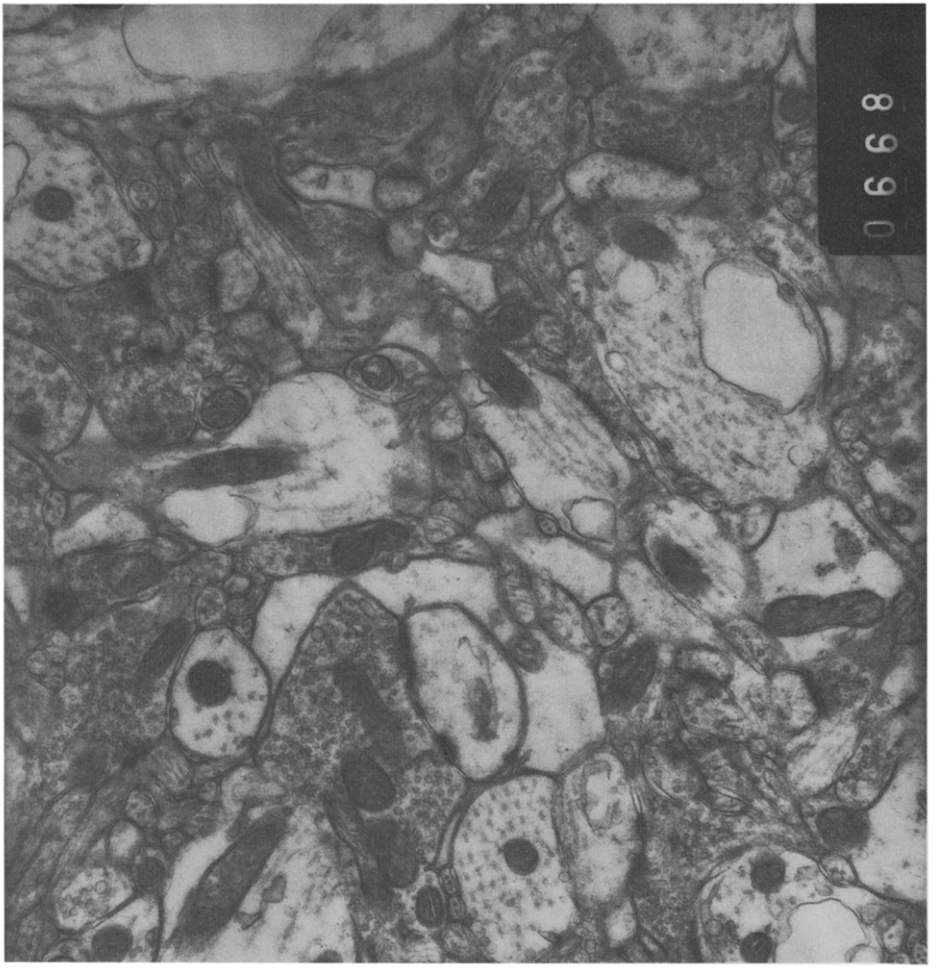

FIG. 2. Electron micrograph of neuropil of rat occipital cortex (layer II), including a synapse with a discontinuous postsynaptic density (left). Note that the majority of the synapses are positively curved. The upper right corner shows a "flat" synapse. Magnification ×20,475.

Table 4 gives the complete data for synapses with discontinuous PSDs (split synapses). It should be noted that there were more discontinuous PSDs with a greater number of pieces in the ovariectomized group than in the sham-operated group. There was no correlation between the number of pieces and the length of the synapse. However, the synapses with discontinuous PSDs were significantly longer (in both groups) than the continuous synapses (compare Tables 2 and 4).

TABLE 1

Synaptic Profile Counts for Layer II, Occipital Cortex

Littermate pair	Number of micrographs	Mean ± SD profiles/micrograph
Control group		
I	10	23.2 ± 5.9
II	12	24.6 ± 6.2
III	16	23.1 ± 5.4
IV	11	21.6 ± 4.2
V	14	23.9 ± 4.2
VI	13	18.5 ± 3.8
Mean ± SD (SE)		22.5 ± 2.19 (±0.89)
Ovariectomized group		
I	16	22.1 ± 3.8
II	12	23.7 ± 6.4
III	12	15.4 ± 3.5
IV	12	19.3 ± 6.3
V	13	17.2 ± 4.5
VI	14	22.9 ± 5.4
Mean ± SD (SE)		20.1 ± 3.34 (±1.36)

Synaptic Curvature. A significant difference was observed in the frequency of occurrence of positively and negatively curved synapses between the sham-operated and ovariectomized animals (see Table 5). The ovariectomized group had significantly ($P < 0.05$) fewer negatively curved synapses (63% fewer) and more positively curved synapses (19% more) than the sham-operated group. There was no difference between the groups in numbers of flat synapses.

DISCUSSION

Given that no significant difference was found in the length of PSD for ovariectomized compared with sham-operated rats, it is pertinent that Dyson and Jones (10) also found no consistent trend in the length of the PSD throughout maturation. In addition, Devon and Jones (5) observed a wide range of postsynaptic lengths in unanesthetized adult rats. In light of these facts, these researchers comment that since the length of the PSD demonstrated such variability, "care needs to be exercised in removing it from the context of the synaptic terminal and its possible relationship to other parameters such as the curvature and area of the terminal" (5). Any significance attached to plasticity in the length of the PSD awaits further work to determine if this parameter, representing the length of the synaptic

TABLE 2

Lengths of Postsynaptic Densities

Littermate pair	Number of synapses measured	Average ± SD length per animal (mm)[a]
Control group		
I	35	13.9 ± 5.7
II	66	10.8 ± 4.2
III	29	11.8 ± 4.6
IV	46	12.4 ± 4.5
V	44	10.0 ± 3.9
VI	30	10.3 ± 4.2
Total	250	
Mean ± SD (SE)		11.5 ± 1.47 (±0.60)
Ovariectomized group		
I	39	12.9 ± 6.4
II	57	10.5 ± 3.3
III	36	9.9 ± 3.7
IV	31	11.0 ± 3.6
V	37	10.4 ± 4.2
VI	46	10.5 ± 4.2
Total	246	
Mean ± SD (SE)		10.9 ± 1.06 (±0.43)

[a] Values are measurements taken from micrographs. To convert to actual length, divide these values by the magnification factor of 34,125.

contact zone, is a measure of synaptic efficacy (or maturity). Furthermore, analysis of the average length of the PSD for a given population of synapses is made more obscure by the fact that changes may be due either to the appearance of newly formed synapses, or to changes in already existing terminals. Distinguishing between these two modes of plasticity in the neural network has yet to be accomplished.

The fact that the ovariectomized animals had more positively and fewer negatively curved synapses than the sham-operated group is interesting in light of the hypothesis proposed by Jones *et al.* (5, 10) concerning synaptic curvature. Applying their hypothesis, the ovariectomized animals have more functioning synapses (positively curved) and fewer nonfunctioning synapses (negatively curved) than their sham-operated littermates. This possibility bears further investigation.

It should be mentioned that the negatively curved synapses observed in this study seemed to be localized together: most of the negatively curved synapses for an animal in either group occurred in only a few micrographs.

TABLE 3

Number of Discontinuous Postsynaptic Densities

Littermate pair	Total number of split synapses	Number of micrographs
Control group		
I	2	10
II	1	12
III	1	16
IV	3	11
V	0	14
VI	2	13
Total	9	76
Mean ± SE	1.5 ± 0.4	
Ovariectomized group		
I	2	16
II	5	12
III	0	12
IV	5	12
V	5	13
VI	3	14
Total	20	79
Mean ± SE	3.3 ± 0.8	

Furthermore, no correlation was demonstrated in this study between the length of the PSD and the curvature of the junction, as was found by Dyson and Jones (10). Those researchers stated that the longest junctions were flat. The flat synapses we observed were of random lengths.

This study demonstrates that ovariectomy increases the frequency of occurrence of discontinuous postsynaptic densities in layer II of the rat occipital cortex. The significance of these results awaits further experimentation concerning the possible functional role of split synapses, or SSPPs. However, given the findings by Pappas *et al.* (20) that ovariectomy causes increased cortical thickness, and evidence by Dyson and Jones (10) that the number of synapses with discontinuities in the PSD increases during maturation, one might speculate that discontinuous PSDs represent larger, more mature synapses. The fact that Pappas (19) found larger perikarya and nuclei after ovariectomy leads to the hypothesis that larger cells produce more split synapses. Pappas' finding that neuron density decreased with ovariectomy (19) further indicates that there would be fewer neurons per unit area, but more mature synapses per neuron in an increased mass of cortex. Therefore, an increase in cortical thickness may represent

TABLE 4

Lengths of Postsynaptic Densities and Their Constituent Pieces in Split Synapses

Littermate pair	Synapse number	Number of pieces	Length of pieces (mm)	Total length of pieces (mm)	Length of pieces + spaces (mm)
Control group					
I	1	2	17.6, 8.7	26.3	35.6
	2	2	6.8, 10.2	17.0	26.1
II	1	2	4.1, 4.8	8.9	14.7
III	1	2	6.1, 5.4	11.5	15.5
IV	1	2	7.5, 10.0	17.5	21.5
	2	2	12.3, 7.1	19.4	25.2
	3	2	6.8, 5.6	12.4	15.4
V	—	—	—	—	—
VI	1	3	7.0, 6.0, 3.8	16.8	21.0
	2	2	6.5, 9.8	16.3	18.3
Ovariectomized group					
I	1	2	9.3, 13.2	22.5	29.5
	2	3	14.0, 4.9, 3.6	22.6	26.6
II	1	3	7.9, 4.8, 5.0	17.7	19.9
	2	3	7.0, 4.7, 16.7	28.4	36.2
	3	2	6.7, 9.5	16.3	18.0
	4	2	4.5, 9.5,	14.0	17.7
	5	2	5.0, 8.1	13.1	15.1
III	—	—	—	—	—
IV	1	2	7.0, 7.0	14.0	19.1
	2	3	9.3, 6.6, 16.8	32.7	42.5
	3	2	5.0, 4.5	9.5	12.0
	4	2	7.8, 6.0	13.8	16.5
	5	2	5.7, 7.5	13.2	15.7
V	1	2	8.5, 6.3	14.8	18.3
	2				29.1
	3				13.2
	4	2	3.0, 5.8	8.8	14.8
	5	2	10.4, 6.9	17.3	21.6
VI	1	2	16.3, 6.1	22.4	30.5
	2	2	5.6, 7.6	13.2	17.2
	3	2	15.2, 6.1	21.3	26.2

an increase in the number of mature synapses. These synapses may have a greater capacity of facilitating transport across the postsynaptic membrane and/or increased reuptake of degradation products as suggested by Greenough *et al.* (12). According to a hypothesis proposed by Siekevitz

TABLE 5

Synaptic Curvature

	Number of synapses							
	Control				Ovariectomized			
Littermate pair	Positive	Negative	Flat	Total	Positive	Negative	Flat	Total
I	21	11	4	36	24	5	8	37
	(58.3)[a]	(30.6)	(11)	(100)	(64.9)	(13.5)	(21.6)	(100)
II	22	23	12	57	33	12	12	57
	(38.6)	(40.4)	(21.1)	(100)	(57.9)	(21.1)	(21.1)	(100)
III	21	5	7	33	28	1	7	36
	(63.6)	(15.2)	(21.2)	(100)	(77.8)	(2.8)	(19.4)	(100)
IV	25	13	10	48	25	3	7	35
	(52.1)	(27.1)	(20.8)	(100)	(71.4)	(8.6)	(20.0)	(100)
V	23	8	15	46	19	12	5	36
	(50.0)	(17.4)	(32.6)	(100)	(52.8)	(33.3)	(13.9)	(100)
VI	25	7	4	36	27	5	7	39
	(69.4)	(19.4)	(11.1)	(100)	(69.2)	(12.8)	(17.9)	(100)
$\bar{X}$	(55.3)	(25.0)	(19.6)	(65.7)	(15.3)	(19.0)		
SE	4.4	3.9	3.2	3.7	4.3	1.1		

[a] Percentage of total synapses given in parentheses.

and collaborators (3), the discontinuous PSDs would represent the "open state," where the PSD proteins have moved apart, perhaps affecting the opening or closing of the Na^+ and/or K^+ channels. Furthermore, those authors suggest that such a mechanism may be involved in the establishment of a memory circuit. Convincing evidence for such a dynamic model of PSD function is unlikely to come from the inherently static information obtainable from electron microscopy, but rather necessitates experiments designed to reflect such dynamic changes.

The findings of Macluskey *et al.* (16) that cerebral cortical estrogen receptor concentrations attain a peak at day 10 and then decline must be taken into account. In addition, a protein found both in the blood and cerebrospinal fluid during the neonatal period, α-fetoprotein (AFP), competes with estrogen receptors for circulating estradiol (16, 25). It is possible that the concentration of plasma estrogen rose sufficiently by day 10 to overcome the interference by AFP. Maclusky *et al.* (16) concluded that this was unlikely. They maintain that "even as late as the second week after birth, AFP presents an almost insurmountable barrier to plasma estrogen." If this is indeed the case, why does ovariectomy affect cerebral cortical thickness and the frequency of split synapses together with synaptic curvature? How does one explain the evidence that early exposure to estrogen can affect both myelinization (4) and amino acid concentrations in the cortex (14), and that a spurt of both cortical differentiation (1) and myelinization (15) occurs about the same time that receptor concentrations reach their peak? (The possibility does exist that some of these observations may be accounted for by other hormones which estrogen affects: prolactin, luteinizing hormone, thyroxine, and the corticoids (24, 27).) What purpose do the receptors for estrogen serve that are present in the cortex to day 10? What is the significance of the difference in distribution of estrogen receptors between adults and neonates? These are questions which further research must seek to answer. One possible experiment which would help to determine if the results observed are indeed due to differences in concentrations of estrogen is administration of estrogen to ovariectomized animals.

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