EXPERIMENTAL NEUROLOGY 21, 502–511 (1968)

Acetylcholinesterase and Cholinesterase Activities, Protein Content and Wet Weight Measures in the Rat Brain after Early Hypophysectomy

KENNETH M. GREGORY AND MARIAN C. DIAMOND [1]

Department of Physiology-Anatomy, University of California, Berkeley, California 94720

Received April 19, 1968

The development of acetylcholinesterase (AChE) and cholinesterase (ChE) activities, weight measures and protein content were determined for specific brain samples from early hypophysectomized and control female rats at 33–35 days of age. Of the eight brain samples, only two, the somatosensory and ventral cortex, showed significantly less AChE activity in the experimental compared to the control rats. The decrease in the somatosensory AChE activity was postulated to reflect a developmental deficit of sensory-motor function due to the dwarfing of the body mass and surface. The decrease in the ventral cortical sample was interpreted as due to alterations in the development of behavioral and hormonal limbic mechanisms. Early hypophysectomy reduced the level of ChE activity in only one brain region, the hypothalamus. This decrease was thought to be due primarily to alterations in the function of the supraoptic and paraventricular nuclei whose neurosecretory products are normally released from the neurohypophysis. The protein content was not significantly different between the cortical samples from the hypophysectomized and control rats.

Introduction

Numerous experiments have indicated that the hormonal environment plays an essential role in the early development and maturation of the nervous system. The question arises, however, as to which hormones are required to ensure normal growth and development of the brain. The study of the effects of hormonal deficiencies on brain development is one avenue of approach to the solution of the problem. Investigations of selective hormonal deficiencies have shown that thyroid (5) and gonadal (20) hormones are essential for proper neurological development. Although growth hormone has been shown to have an effect on nervous system

[1] This work was supported by USPHS grants HD 101 and NB 062 93–02. The authors acknowledge the technical assistance of Mrs. J. Ehlert, Marie Hebert, Hiromi Morimoto, Anne Orme, and Edward L. Bennett. Dr. Gregory's present address is: Department of Anatomy, State University of New York, Downstate Medical Center, Brooklyn, New York, 11203.

development (2, 28), studies on early hypophysectomized rats seem to indicate that growth hormone probably does not exert a direct and essential effect on the normal growth and development of the brain (12, 25, 27).

Although hypophysectomy at four to six days of age in the rat results in major alterations in the growth and maturation of somatic and visceral structures, no changes have been observed in the growth (weight) of the brain (12, 26, 27). In order to determine more fully the effects of early hypophysectomy on brain development, and as a correlate to previously reported morphological investigations (3, 12), the present study was designed to determine whether brain biochemistry was also significantly affected after early hypophysectomy. Comparisons are made between early hypophysectomized and control rats in the development and regional concentration of two brain enzymes, acetylcholinesterase and cholinesterase, in the protein content of two specific cortical samples, and in the weights of the different brain regions.

Methods

Animals. Female Long-Evans rats, 6 days of age, were hypophysectomized via the parapharyngeal approach while under hypothermic anesthesia. The operative technique and postoperative care have been described (12). Control animals were subjected to all the same pre- and postoperative conditions as were the experimental animals. Litter size was maintained at four to six animals to maximize lactation. Body weights were recorded at 5-day intervals. Food and water were available *ad libitum*. The experimental animals and their littermate controls were weaned at 30 days of age. The animals were killed in chloroform vapor when 33–35 days of age.

Organ Weights. The thyroid gland, the adrenal glands and the ovaries were removed and weighed. In some cases the uterus, thymus, and eyes were also removed and weighed.[2] All organs were weighed to the nearest 0.1 mg.

Brain Samples. The detailed procedures for removal of the brain samples and a description of their boundaries have been published (23). Bilateral samples from the dorsal surface of the cortex include the following: (a) anterior or frontal; (b) somatosensory; (c) visual; and (d) the remaining dorsal cortex. The remaining cortex on the ventral surface includes the corpus callosum (12% of sample), hippocampus (70% of sample), amygdala, and small contributions from the posterior caudate nucleus and putamen. The remaining portion of the brain, termed the "rest of brain"

[2] Although the N is small for the number of animals from which these means were obtained, unpublished data from a larger sample indicated that the given means (Table 1) are approximately the same as those of the much larger sample.

was separated into (a) the hypothalamus, (b) the cerebellum plus medulla, and (c) the remainder of brain including the olfactory bulbs. After removal, each sample was weighed to the nearest 0.1 mg, frozen on dry ice and stored in the deep freeze until biochemically analyzed.

Enzyme Determinations. The eight brain samples were analyzed for Acetylcholinesterase (AChE) and Cholinesterase (ChE) activities using a modification of the colorimetric method of Ellman *et al.* (6).

Protein Determination. The protein content of the somatosensory and visual cortices was determined with the Folin phenol reagent as described by Lowry *et al.* (21).

Results

Physical Appearance and Behavior. A comparison of the appearance and behavior of the hypophysectomized with the control rats has been published previously (12, 26, 27). Hypophysectomized rats differed markedly from the controls in their stunted growth and retention of many infantile characteristics. The hypophysectomized rats were very lethargic and showed signs of poor thermoregulation.

Body and Organ Weights. Table 1 presents the body and organ weight data for the experimental and control rats and indicates the effect that early hypophysectomy has upon the growth of somatic, visceral, and pituitary dependent organs. Eye weights of the early hypophysectomized rats were significantly less by 19% ($p < .001$; t test) than those of the controls.

TABLE 1

MEAN BODY AND ORGAN WEIGHTS [a] OF EARLY HYPOPHYSECTOMIZED AND CONTROL FEMALE RATS AT 33–35 DAYS OF AGE

Group	Hypoph.	Contr.	Hypoph. / Contr.	6 Day [b] Onset / Contr.
Final body weight (g)	44 ± 1 (21)	114 ± 3 (17)	.39	17
Total brain weight (mg)	1520 ± 18 (21)	1567 ± 25 (17)	.97	669
Thyroid (mg)	6 ± 0.3 (21)	11 ± 0.5 (17)	.55	3
Adrenals (mg)	8 ± 0.6 (21)	29 ± 1 (17)	.28	4
Ovaries (mg)	4 ± 0.3 (8)	29 ± 2 (16)	.15	1
Uterus (mg)	18 ± 1 (8)	87 ± 13 (7)	.21	8
Thymus (mg)	85 ± 10 (8)	340 ± 16 (7)	.25	31
Eyes (mg)	145 ± 3 (6)	172 ± 3 (4)	.84	---

[a] Weights ± SE. Number of rats in parentheses.

[b] Weights taken at 6 days of age to compare changes induced by hypophysectomy or by normal growth.

TABLE 2

MEAN WET WEIGHTS [a] OF SPECIFIC BRAIN AREAS FROM HYPOPHYSECTOMIZED AND CONTROL FEMALE RATS AT 33–35 DAYS OF AGE

Group	Contr. ($N = 17$) $\bar{X}$	SD	Hypoph. ($N = 21$) $\bar{X}$	SD	% Diff.[b]	p
Total brain	1564.8	108.0	1519.8	82.0	3.0	NS
Cortex						
Total	740.4	61.8	723.4	45.4	2.3	NS
Ventral	298.1	48.2	289.0	23.2	3.1	NS
Remaining dorsal	295.7	29.8	286.3	24.4	3.3	NS
Visual	60.7	4.2	62.1	4.4	−2.3	NS
Somatosensory	53.7	3.4	57.1	4.4	−6.1	NS
Frontal	28.4	2.5	29.0	3.4	−2.1	NS
Rest of brain						
Remainder of brain	409.0	45.7	398.0	26.1	2.8	NS
Cerebellum + medulla	361.4	22.9	344.6	26.8	4.9	NS
Hypothalamus	57.7	7.1	53.8	8.7	7.2	NS

[a] Values in milligrams.

[b] Contr. − Hypoph./Hypoph. × 100 = % Diff.

Wet Weights of Specific Brain Areas. There were no significant [3] differences between the experimental and control rats in the weights of the specific brain samples or in the total cortical or total brain weights, as is indicated in Table 2. The three dorsal cortical samples all weighed slightly more in the experimental than in the control rats, although the differences were not significant.

Acetylcholinesterase Activity. The data on AChE activity for the eight specific brain samples and for the total cortex and total brain are presented in Table 3. The only dorsal cortical area showing a significant difference between the experimental and control rats was the somatosensory cortex sample, the decrease in the experimental rats being about 13% compared to the control rats ($p < .05$; $F = 5.71$, df 1,288). The ventral cortex sample showed a greater and even more significant decrease in the hypophysectomized rats, 23% ($p < .001$; $F = 45.07$, $df = 1,288$). The

[3] Statistical treatment of the data from the AChE and ChE activities, and for the wet weight measures of the eight specific brain samples consisted of a Two-Way analysis of variance. Tests made included those for simple main effects of the appropriate variables. Total cortex, total brain, and protein content were analyzed by the t test. Data analyses were performed at the S.U.N.Y., Downstate Medical Center, Computing Center, on a program prepared by Dr. S. Kahan, Dept. of Biomedical Computer Sciences.

TABLE 3
COMPARISON OF AChE ACTIVITY PER UNIT WEIGHT [a] BETWEEN HYPOPHYSECTOMIZED AND CONTROL FEMALE RATS AT 33–35 DAYS OF AGE

Group	Contr. ($N = 17$)		Hypoph. ($N = 21$)			
	X	SD	X	SD	% Diff.[b]	p
Total brain	13.1	0.6	12.5	0.6	4.8	$< .01$
Cortex						
Total	7.5	1.0	6.4	0.4	17.2	$< .001$
Ventral	10.0	1.7	8.1	0.8	23.4	$< .001$
Remaining dorsal	5.9	0.4	5.4	0.4	9.3	NS
Visual	4.8	0.4	4.3	0.3	11.6	NS
Somatosensory	5.9	0.5	5.2	0.4	13.5	$< .05$
Frontal	5.9	0.4	5.4	0.4	9.3	NS
Rest of brain						
Remainder of brain	26.3	1.8	26.2	1.7	0.4	NS
Cerebellum + medulla	10.0	0.6	9.7	0.5	3.1	NS
Hypothalamus	11.7	0.6	11.5	0.7	1.7	NS

[a] μmoles acetylcholine hydrolyzed/min/mg wet wt.
[b] Contr. − Hypoph./Hypoph. × 100 = % Diff.

total cortex and total brain differences in AChE activity are also significant ($p < .001$ and $< .01$, respectively), the differences due mainly to the somatosensory and ventral cortical samples.

Cholinesterase Activity. Table 4 indicates that the only brain sample showing a significant change in ChE activity was the hypothalamus, with a decrease in the hypophysectomized rats of a little over 5% ($p < .05$; $F = 4.91$, $df = 1,288$), compared to the control rats. Total brain and total cortex values were not different between the two groups in ChE activity.

Protein Content. Protein content determinations are indicated in Table 5 and show no differences between the experimental and control rats in the somatosensory or visual cortex samples, the only two regions analyzed.

Discussion

Brain, Body, and Organ Weights. The comparison of the mean body and organ weights between the early hypophysectomized rats with the control rats indicates the degree to which the general growth and development are impaired following early hypophysectomy. The single exception to this over-all effect is the weight of the brain which was not affected by pituitary removal. Although Walker *et al.* (26) indicated that eyeball weights did not change following hypophysectomy, the data of the present experiment and of unpublished observations show that without exception the eyeball

TABLE 4
COMPARISON OF ChE ACTIVITY PER UNIT WEIGHT [a] BETWEEN HYPOPHYSECTOMIZED AND CONTROL FEMALE RATS AT 33–35 DAYS OF AGE

Group	Contr. ($N = 17$)		Hypoph. ($N = 21$)		% Diff.[b]	p
	$\bar{X}$	SD	$\bar{X}$	SD		
Total brain	.42	.03	.42	.03	0.0	NS
Cortex						
Total	.28	.03	.28	.03	0.0	NS
Ventral	.28	.03	.28	.03	0.0	NS
Remaining dorsal	.26	.04	.26	.03	0.0	NS
Visual	.33	.05	.32	.04	3.1	NS
Somatosensory	.35	.04	.35	.04	0.0	NS
Frontal	.30	.04	.30	.04	0.0	NS
Rest of brain						
Remainder of brain	.62	.06	.62	.04	0.0	NS
Cerebellum + medulla	.44	.05	.43	.05	2.3	NS
Hypothalamus	.61	.03	.58	.05	5.2	$< .05$

[a] μmoles butyrylthiocholine hydrolyzed/min/mg wet wt.
[b] Contr. − Hypoph./Hypoph. × 100 = % Diff.

weights of the hypophysectomized rats are consistently less than those of control rats.

The lack of any significant differences between the experimental and control rats in the wet weights of the specific brain samples substantiates the gross brain weight observations reported previously (12). The slightly heavier frontal, somatosensory, and visual samples of the hypophysectomized rats are probably due to the development of shorter and broader skulls, and hence brains, of the hypophysectomized rats.

AChE Activity. An extensive literature exists which indicates that central and peripheral cholinergic systems contain an important hydrolytic enzyme,

TABLE 5
PERCENTAGE PROTEIN CONTENT OF TWO SPECIFIC BRAIN AREAS FROM HYPOPHYSECTOMIZED AND CONTROL FEMALE RATS AT 33–35 DAYS OF AGE

Group	(% Protein) Contr. ($N = 17$)		Hypoph. ($N = 21$)		% Diff.[a]	p
	$\bar{X}$	SD	$\bar{X}$	SD		
Cortex						
Visual	10.6	0.6	10.3	0.2	2.9	NS
Somato-sensory	10.6	0.6	10.4	0.4	1.9	NS

[a] Contr. − Hypoph./Hypoph. × 100 = % Diff.

AChE. AChE has been shown to play an important part of synaptic and neuroeffector cholinergic transmission. Histochemical methods have also indicated that the presence of AChE in nerve cell bodies and along the axons is a valuable method in identifying cholinergic neurons and pathways (18, 19, 24).

Of the eight specific brain regions studied, early hypophysectomy affects the development of cholinergic mechanisms in only two regions, the somatosensory and the ventral cortex. Considering first the somatosensory sample, histochemical studies (17) have shown that cortical AChE containing fibers are localized in a horizontally oriented plexus in layers V and VI and in the deeper part of layer I. The AChE stained plexus was suggested to consist of the terminal axons of neurons of the ascending reticular activating system (ARAS) and of the intracortical arcuate fibers. Additional evidence indicates that the ARAS contains prominent cholinergic components (4, 24).

Since the primary sensory pathways appear to be noncholinergic (14, 18), the observed decrease in AChE activity in the somatosensory area may be due to a decrease in sensory and motor projection to and hence involvement of cholinergic reticular mechanisms from which some of the cortical cholinergic fibers are derived.

The intracortical arcuate neurons and their axons projecting to and from the somatosensory area could contribute to a decrease in AChE activity. The enzyme change may reflect a failure on the part of the somatosensory system to develop proportionately to brain growth due to the dwarfing of the body mass and surface.

In contrast to the somatosensory area, the visual sample did not show any change in AChE activity after early hypophysectomy. Unlike the somatosensory system, the visual system was possibly not subjected to any appreciable sensory deficiency, even though the eyeballs of the hypophysectomized rats were smaller than those of the control rats. The retinas, however, were not measured separately. Reportedly, light deprivation from birth to 21 days in the rat does not affect AChE activity in the visual cortex (22).

Attempts to correlate biochemical changes with morphological measures of growth have yielded negative results. A previous study has indicated that no changes occurred in the development of the basal dendritic branching patterns of pyramidal cells from layers II and III of the somatosensory cortex (12). This failure to reveal any changes in the dendritic arbor does not mean that changes may not have occurred at a level finer than the one measured. Lateral geniculate lesions and visual deprivation in rabbits has been found to lead to a loss of or a deformity of the spines along localized regions of the apical dendrites of pyramidal cells of the visual cortex, yet

no significant variation in the overall dendritic pattern was observed (10, 11).

The other brain region showing a significantly lower AChE activity in the experimental rats compared to the control rats was the ventral cortex sample. As was mentioned earlier, the major portion of this sample consists of limbic system structures which have been shown to contain prominent cholinergic components (4, 19). It is not surprising that early hypophysectomy probably adversely affects the development and maturation of limbic mechanisms involved in those functions influenced and regulated by pituitary hormones. The lower AChE activity of the ventral cortical sample in the hypophysectomized rats may have resulted from deficiencies of the limbic mechanisms concerned with the control of gonadal hormones as a result of the absence of these hormones. Kawakami *et al.* (15), have shown that the positive and negative feedback control of progestin output from the ovary originates in limbic structures.

Numerous reports have accumulated which indicate that digestive and metabolic processes are altered by hypophysectomy (8). Such alterations could exert a marked influence on the development and function of limbic mechanisms involved in appetitive behavior. Hypophysectomy may also interfere with limbic mechanisms associated with the maintenance of body temperature and of water balance.

ChE Activity. The hypothalamus was the only brain sample showing a significantly lower ChE activity in the experimental rats compared to the control rats. With the exception of the supraoptic and paraventricular nuclei which show a marked staining, very little ChE is found in the hypothalamus (7). ChE has been localized in nerve cells (7, 18), in the endothelial cells of capillaries and blood vessels in regions protected by the blood brain barrier (13), and in neuroglia cells (16). It is also known that the supraoptic and paraventricular regions have a very rich vascular supply.

The lower ChE activity in the hypothalamus of the hypophysectomized rats may be due to the disruption of the hypothalamic control of water diuresis, with concommitant changes in the neurons and vascular system of the supraoptic and paraventricular nuclei, or both. That the developmental effect may be more vascular than neural is perhaps indicated by the fact that there were no significant decreases in AChE activity in the hypothalamus of the hypophysectomized rats compared to the control rats, even though almost all of the AChE in the hypothalamus is also localized in the supraoptic and paraventricular nuclei (1, 14).

The absence of any changes in ChE activity in the cortical samples after early hypophysectomy contrasts with the reported 15% decrease in cortical ChE activity after neonatal thyroidectomy (9).

Protein Content. The protein content of the visual and somatosensory areas was slightly but not significantly reduced in the experimental rats compared to the control rats. These data support the other evidence presented here that early hypophysectomy has very little effect on brain development, especially when compared to the developmental conditions imposed by neonatal thyroidectomy.

Conclusion. The rather specific effects of early hypophysectomy on brain AChE and ChE activities tends to support the hypothesis developed elsewhere (3, 12) that there is a generalized maintenance of brain growth due to residual thyroid hormone activity. The biochemical data presented here indicate that there may be localized deficiencies in the development of the nervous system due to somatosensory deprivation concommitant with the overall reduction in body mass and surface, and to deficiencies in the internal hormonal environment, particularly with regard to gonadal steroids.

References

1. ADAMS, C. W. M. 1965. Histochemistry of the cells in the nervous system, pp. 253–331. *In* "Neurohistochemistry," C. W. M. Adams [ed.], Elsevier, New York.
2. CLENDINNEN, B. G., and J. T. EAYRS. 1961. The anatomical and physiological effects of prenatally administered somatotrophin on cerebral development in rats. *J. Endocrinol.* **22**: 183–193.
3. DIAMOND, M. C. 1967. The effects of early hypophysectomy and hormone therapy on brain development. *Brain Res.* **7**: 407–418.
4. DOMINO, E. F., A. T. DREN, and K. YAMAMOTO. 1967. Pharmacologic evidence for cholinergic mechanisms in neocortical and limbic activating systems, *Progr. Brain Res.* **27**: 337–364.
5. EAYRS, J. T. 1966. Thyroid and central nervous development, pp. 317–402. *In* "The Scientific Bases of Medicine, Annual Reviews," Athlone Press, London.
6. ELLMAN, G. L., K. D. COURTNEY, V. ANDRES, and R. M. FEATHERSTONE. 1961. A new and rapid colorimetric determination of AChE activity. *Biochem. Pharmacol.* **7**: 88–95.
7. FRIEDE, R. L. 1967. A comparative histochemical mapping of the distribution of butyryl cholinesterase in the brains of four species of mammals, including man. *Acta Anat.* **66**: 161–177.
8. GAEBLER, O. H. 1965. Growth and pituitary hormones, pp. 85–121. *In* "Newer Methods of Nutritional Biochemistry," A. A. Albanese [ed.], vol. 2, Academic Press, New York.
9. GEEL, S., and P. S. TIMIRAS. 1967. Influence of neonatal hypothyroidism and of thyroxine on the acetylcholinesterase and cholinesterase activities in the developing central nervous system of the rat. *Endocrinology* **80** (6): 1069–1074.
10. GLOBUS, A., and A. B. SCHEIBEL. 1967. Synaptic loci on visual cortical neurons of the rabbit: the specific afferent radiation. *Exptl. Neurol.* **18**: 116–131.
11. GLOBUS, A., and A. B. SCHEIBEL. 1967. The effect of visual deprivation on cortical neurons: a Golgi study. *Exptl. Neurol.* **19**: 331–345.
12. GREGORY, K. M., and M. C. DIAMOND. 1968. The effects of early hypophysectomy on brain morphogenesis in the rat. *Exptl. Neurol.* **20**: 394–414.

13. Joó, F., and Csillik, B. 1966. Topographic correlation between the hemato-encephalic barrier and the cholinesterase activity of brain capillaries. *Exptl. Brain Res.* **1**: 147–151.
14. Karczmar, A. G. 1967. C. Pharmacologic, toxicologic, and therapeutic properties of anticholinesterase agents, pp. 163–322. *In* "Physiological Pharmacology," vol. 3, W. Root and F. Hofmann [eds.], Academic Press, New York.
15. Kawakami, M., K. Seto, E. Terasawa, and K. Yoshida, 1967. Mechanisms in the limbic system controlling reproductive functions of the ovary with special reference to the positive feedback of progestin to the hippocampus. *Progr. Brain Res.* **27**: 69–102.
16. Koelle, G. B. 1954. The histochemical localization of cholinesterase in the central nervous system of the rat. *J. Comp. Neurol.* **100**: 211–227.
17. Krnjèvic, K. 1965. Actions of drugs on single neurons in the cerebral cortex. *Brit. Med. Bull.* **21**: 10–14.
18. Krnjèvic, K., and A. Silver. 1965. A histochemical study of cholinergic fibers in the cerebral cortex. *J. Anat.* **99**: 711–759.
19. Lewis, P. R., and C. C. D. Shute. 1967. The cholinergic limbic system: projections to hippocampal formation, medial cortex, nuclei of the ascending cholinergic reticular system, and the subfornical organ and supra-optic crest. *Brain* **90**: 521–539.
20. Levine, S., and R. F. Mullins, 1966. Hormonal influences on brain organization in infant rats. *Science* **152**: 1585–1592.
21. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**: 265–275.
22. Maletta, G. J., and P. S. Timiras. 1967. Acetylcholinesterase activity in optic structures after complete light deprivation from birth. *Exptl. Neurol.* **19**: 513–518.
23. Rosenzweig, M. R., Krech, D., Bennett, E. L., and Diamond, M. C. 1962. Effects of environmental complexity and training on brain chemistry and anatomy. *J. Comp. Physiol. Psychol.* **55**: 429–437.
24. Shute, C. C. D., and Lewis, P. 1967. The ascending cholinergic reticular system: neocortical, olfactory and subcortical projections. *Brain* **90**: 497–519.
25. Stone, B., K. M. Gregory, and J. Ehlert. 1966. Regional failure of rat cortical development after early hypophysectomy. *Anat. Record* **154**: 428–429.
26. Walker, D. G., C. W. Asling, M. E. Simpson, C. H. Li, and H. M. Evans. 1952. Structural alterations in rats hypophysectomized at 6 days of age and their correction with growth hormone. *Anat. Record* **114**: 19–48.
27. Walker, D. G., M. E. Simpson, C. W. Asling, and H. M. Evans. 1950. Growth and differentiation in the rat following hypophysectomy at 6 days of age. *Anat. Record* **106**: 539–554.
28. Zamenhof, S., J. Mosley, and E. Schuller. 1966. Stimulation of the proliferation of cortical neurons by prenatal treatment with growth hormone. *Science* **152**: 1396–1397.