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Development of Cholinergic Innervation in the Hippocampal Formation of the Rat

II. Quantitative Changes in Choline Acetyltransferase and Acetylcholinesterase Activities

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The postnatal development of choline acetyltransferase (ChAc) and acetylcholinesterase (AChE) activities has been studied in the hippocampal region of the rat brain and in discrete layers of the dentate gyrus. Because the hippocampal region receives its cholinergic innervation predominantly from a single source, these data could be related to development within a defined central cholinergic projection. The specific activity of ChAc is low and uniform in all layers of the dentate gyrus at 11 days of age. It attains adult values within a brief period around 16-17 days. We interpret this finding as a correlate of cholinergic synaptogenesis. It is not accompanied by an increase of similar magnitude in AChE activity, which attains mature values later and at a more gradual rate. These findings are integrated with AChE histochemistry to formulate a developmental sequence in the cholinergic innervation of the dentate gyrus. Less-detailed data on development of ChAc and AChE activities in the hippocampal region as a whole suggest a similar sequence in divisions other than the dentate gyrus.

INTRODUCTION

The hippocampal formation of the rat brain is distinguished by a fairly rigid stratification of intrinsic neurons and synaptic input (Blackstad, 1958, 1967; Raisman *et al.*, 1965; Ramón y Cajal, 1968). Its anatomical simplicity and the occurrence of extensive postnatal developmental changes, particularly in the dentate gyrus (Altman and Das, 1965, 1966; Crain *et al.*, 1973), facilitate correlations between development of enzyme activities related to neurotransmission and development of the morphologic substrate. We exploited these advantages in our previous histochemical study of the ontogenesis of cholinergic innervation in this region of the central nervous system (CNS) (Matthews *et al.*, 1974). In the present study, we have followed the postnatal development of choline acetyltransferase (ChAc) and acetylcholinesterase (AChE), the enzymes associated with cholinergic neurotransmission.

ChAc and AChE are localized in discrete layers in the hippocampus and dentate gyrus (Fonnum, 1970; Storm-Mathisen, 1970). In adult rats these layers correspond to the regions of histochemical AChE staining (Shute and Lewis, 1963; Storm-Mathisen and Blackstad, 1964; Lewis and Shute, 1967) and match fairly closely the terminal regions of the septohippocampal projection (Mosko *et al.*, 1973). These enzymes largely disappear from the hippocampal region following transection of this projection (Storm-Mathisen, 1970, 1972), as does the histochemically demonstrable AChE activity (Shute and Lewis, 1963; Lewis *et al.*, 1967; Lynch *et al.*, 1972; Storm-Mathisen, 1972). These and other lines of evidence (summarized by Matthews *et al.*, 1974) have led to the conclusion that the cholinergic innervation of the hippocampal region is mainly derived from septohippocampal afferent fibers.

The ontogenic development of histo-

chemical AChE staining in the hippocampus and dentate gyrus of the rat has been described (Matthews *et al.*, 1974). At the light microscope level this staining characterizes the septohippocampal afferent fibers (Shute and Lewis, 1966), but cannot be used to follow the development of cholinergic synaptic terminals. For this purpose, quantitative determinations of ChAc activity were employed. A sharp increase in ChAc activity has been related to development of presynaptic cholinergic terminals (Burt, 1968; Filogamo and Marchisio, 1971; Black *et al.*, 1971) and, in homogenates of the rat hippocampal region, this enzyme has been recovered mainly in the synaptosomal fraction (Fonnum, 1970).

The ontogenic development of ChAc and AChE activities was first determined in the hippocampal region as a whole. Recognizing that analysis of an entire region of the nervous system does not permit proper appreciation of changes within each of its divisions, we extended these investigations to discrete layers of the dentate gyrus by microanalytical techniques. The results have been correlated with the development of histochemical staining for AChE activity, and possible relationships to synaptogenesis are suggested.

METHODS

Preparation of tissue. Sprague-Dawley rats of various ages were used in these experiments. The day of birth was taken as day 0, and animals were considered to be adult at 90 days of age.

In studies of enzyme activity, rats were killed by decapitation following a sharp blow on the neck, and the brains were quickly removed. For measurement of activity in the total hippocampal region, the paired regions from four animals, which included the hippocampus, dentate gyrus, subiculum, and presubiculum were isolated, weighed, and pooled. The pooled hippocampi were homogenized in 1.0–2.0 ml of H₂O with a Dounce homogenizer.

The homogenate was sonicated briefly. Enzyme and protein determinations were performed on suitable portions immediately after sonication.

Enzyme activity in layers of the dentate gyrus was determined by quantitative histochemical techniques (Lowry and Passonneau, 1972). For this purpose, the cerebellum, lower brain stem and anterior third of the cerebrum were removed from the brain, and the remainder was frozen in a solution of 8% (v/v) methylcyclohexane in 2-methylbutane cooled by liquid N₂. The frozen brain was mounted with H₂O in a cryostat maintained at -18° to -22° C. Sections of 100 μ m thickness were cut in the horizontal plane, and those which had been cut transverse to the long axis of the temporal half of the hippocampus were transferred to glass slides within the cryostat. The slides were fastened to aluminum blocks with holes of a size similar to the sections, and these assemblies were quickly transferred to a freeze-drying flask packed in solid CO₂ and freeze-dried overnight. Then the flask was allowed to reach room temperature under continued evacuation to remove any remaining traces of H₂O. Until this time, the sections were never permitted to warm above the temperature of the cryostat, and they did not adhere to the slides. After freeze-drying, the sections were either dissected immediately or stored *in vacuo* at -70° C for not longer than 2 weeks.

The four animals used in each experiment on the total hippocampal region were litter-mates, except in studies of adult rats. Rats from at least two different litters were used at each age in studies of layers of the dentate gyrus. Animals were taken from litters which varied in size. However, the size of the litter probably had little effect on the enzyme activities which were determined, since the variation among animals from the same litter was as great as the variation among animals from different litters.

Dissection. Freeze-dried sections were dissected freehand under a binocular microscope with a broken razor blade and hair loop glued to Pasteur pipettes. The morphology of the tissue was well preserved, and illumination from above revealed sufficient detail to permit accurate dissection of the dentate gyrus.

The dentate gyrus, both internal and external leaves, was divided into five layers: outer (MO), middle (MM), and inner (MI) portions of the molecular layer, granule cell layer (G) and the region of the hilus (H) immediately deep to the granule cell bodies (see Matthews *et al.*, 1974, for definition of the anatomical terms), except that the outer and middle portions of the molecular layer from 11-day-old rats were combined. To check the accuracy of the dissection, marks were placed on the boundaries between the layers of adult

dentate gyrus with a broken razor blade, and the sections were stained for AChE activity (Naik, 1963) (Fig. 1). Promethazine ($1.8 \times 10^{-4} M$) was included in the incubation medium to inhibit nonspecific cholinesterases (Todrck, 1954). The layers coincided with zones of histochemical staining. Thus the contamination of each layer with material from adjacent layers was small despite the thickness of the sections. The use of 100- μm sections was necessary to obtain enough tissue for assay from young animals without having to dissect an unreasonably large number of sections.

Each sample consisted of layers from eight to twelve transverse sections of the temporal half of the dentate gyrus from one rat brain. The pooled tissue was dispersed uniformly in 50 μl of H₂O with a Vortex mixer. No particulate matter was observa-



FIG. 1. Demarcation of layers of the dentate gyrus on a section stained for AChE activity. Note that the width of certain layers varies at different points. See Methods for explanation of abbreviations. Arrows indicate the hippocampal fissure. $\times 50$.

ble after a brief agitation. The samples were then stored overnight at -20°C before enzyme activities were determined.

Determination of enzyme activities and protein. ChAc activity was assayed by a radiometric method similar to that of Coggeshall *et al.* (1972), which is based on the conversion of $[1-^{14}\text{C}]$ acetyl-CoA to $[1-^{14}\text{C}]$ acetylcholine (ACh). The labeled ACh is quantitatively extracted and counted.

A buffer-substrate mixture was so prepared that the final composition of the incubation medium would be: 60 mM sodium phosphate, pH 7.9, 200 mM NaCl, 16.5 mM MgCl_2 , 0.12 mM physostigmine sulfate, 1 mM EDTA, 0.6 mg/ml bovine serum albumin, 20 mM choline chloride, 0.2% v/v Triton X-100 and 0.4 mM $[1-^{14}\text{C}]$ acetyl-CoA (New England Nuclear Corp; diluted to 2.14 mCi/mole for studies of the total hippocampal region and to 9.9 mCi/mole for studies of layers of the dentate gyrus). In experiments on the total hippocampal region, 10 μl of cold buffer-substrate mixture were added to 2 μl of homogenate and in studies on freeze dried material, 5 μl of cold buffer-substrate mixture were added to 5 μl of dispersed sample. The reaction mixtures were preincubated 15 min in ice so that the Triton X-100 could release occluded enzyme activity. Samples were then incubated at 37°C for 30 min, after which they were placed on ice, and 100 μl of sodium tetraphenyl boron solution (50 mg/ml in 3-heptanone) were added. The samples were mixed and centrifuged briefly to separate the phases. Then 75 μl of the organic layer were treated with 50 μl of 50 mM sodium phosphate, pH 7.3. The samples were again mixed and centrifuged, and 50 μl of organic phase were counted by the "tT 21" (toluene-PPO-POPOP-Triton X-100) liquid scintillation counting method of Patterson and Greene (1965).

AChE activity was determined by an adaptation of the radiometric procedure of Fonnum (1969), in which $[1-^{14}\text{C}]$ acetylcho-

line is hydrolyzed and the resulting $[1-^{14}\text{C}]$ acetate is counted following extraction of the residual substrate.

A buffer-substrate mixture of the following composition was prepared: 80 mM sodium phosphate, pH 7.3, 1.6 mM $[1-^{14}\text{C}]$ acetylcholine chloride (New England Nuclear Corp.; diluted to 0.7–0.9 mCi/mole) and 0.6 mM promethazine hydrochloride. To 1 μl of fresh homogenate or 2 μl of dispersed freeze-dried tissue were added 20 μl of cold buffer-substrate mixture. The samples were incubated at 37°C for 30 min. They were then placed on ice, and 100 μl of sodium tetraphenyl boron solution were added. After mixing and a brief centrifugation, the organic layer was completely removed by aspiration, and 10 μl of the aqueous phase were counted. Activities of both ChAc and AChE were proportional to protein within the range used in these studies.

Protein was determined by the method of Lowry *et al.* (1951).

Enzyme activities of freeze-dried material were compared with activities of homogenates prepared from fresh dentate gyrus dissected from 1–2 mm slabs of adult rat brain to estimate any loss of activity due to the manipulations that were employed. The specific activities were (in μmoles ACh synthesized or hydrolyzed in 30 min per gram of protein \pm SEM; $N = 6$ in each case): ChAc, 14.8 ± 1.1 and AChE, 1340 ± 160 for fresh dentate gyrus, and for freeze-dried tissue, ChAc 13.0 ± 1.7 and AChE, 1660 ± 80 . Therefore the enzyme activities of freeze-dried material, as determined by our methods, were not significantly different from activities of fresh tissue.

Dimensions of the hippocampal region. Rats which were 4, 11, 16, 25, 30, 43, and more than 90 days of age were perfused through the heart with a buffered paraformaldehyde-glutaraldehyde solution (Vaughn and Peters, 1966). The hippocampal region was exposed by removal of the

skull and overlying neocortex and was photographed at $3.7\text{--}4.7\times$ magnification. The dimensions of the dorsal hippocampal region were measured from the photographs at its greatest length and width.

RESULTS

Dimensions, Wet Weight, and Protein Content

On each side of the 4-day-old rat brain, the dorsal hippocampal region measured 0.23×0.53 cm at its greatest width and length. In rats 11 days of age or older, the dimensions were 0.33×0.70 cm on the average with a maximum deviation of 10%. Thus the mature size of the hippocampal region is probably attained by 11 days.

As one might expect, a large (about 4-fold) increase in wet weight and protein content accompanied this physical growth (Fig. 2). After 14 days, the wet weight rose more gradually toward adult values, while the protein content appeared to increase rapidly until about 20 days. The additional doubling of wet weight and the 4-fold increase in protein content subsequent to the attainment of mature size evidently reflected a structural maturation within fixed boundaries.

ChAc and AChE in the Hippocampal Region

The specific activity of ChAc in the hippocampal region increased 9-fold be-

tween the day of birth and maturity (Table 1). In absolute terms, the adult hippocampal region had 120 times the ChAc activity of the neonatal region. These increases took place mainly during two discrete periods of development, from birth to 7 days of age and from 11 to 21 days of age. During the earlier period the absolute activity of ChAc increased about 10-fold, which resulted in a 4- to 5-fold increase in specific activity. Absolute activity increased by about 4-fold and the specific activity more than doubled during the later period. By the end of this time adult values for specific activity of ChAc had been attained.

The absolute activity of AChE increased by more than 40-fold during postnatal development, which produced a 4-fold rise in specific activity. Changes in the activity of AChE seemed to be confined to discrete periods of development similar to the changes in activity of ChAc, but the temporal pattern was somewhat different for the two enzymes. The earliest postnatal increase in AChE activity took place between 4 and 7 days of age and a second sharp rise occurred between 11 and 14 days. The absolute activity of AChE increased 3-fold between 14 days of age and maturity, but at a gradual rate without additional abrupt changes. Protein content increased at a similar rate, so that there was little change in the specific activity of the enzyme.

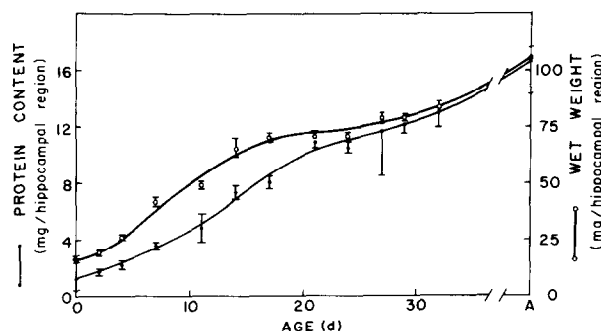


FIG. 2. Postnatal increase of wet weight and protein in the hippocampal region of the rat brain. Values are expressed as means \pm SEM for 8–20 animals (wet weight) or 2–5 groups of four animals (protein content) at each age.

TABLE 1
ACTIVITIES OF ChAc AND AChE IN RAT HIPPOCAMPAL REGION DURING DEVELOPMENT^a

Age (days)	ChAc		AChE	
	Specific activity	Absolute activity	Specific activity	Absolute activity
0	0.9, 1.0	0.002, 0.001	500	0.9
2	1.3 ± 0.3 (3)	0.002 ± 0.001	530, 350	1.0, 0.8
4	3.7 ± 1.4 (3)	0.008 ± 0.004	560	1.0
7	4.5 ± 2.4 (3)	0.015 ± 0.008	1100, 1160	4.0, 4.4
11	4.5 ± 0.5 (4)	0.021 ± 0.002	1040, 1120	4.4, 7.1
14	4.2, 6.7	0.052, 0.029	1650	12.8
17	9.0, 4.6	0.076, 0.036	1580	12.1
21	10.1, 9.9	0.114, 0.105	1400	14.7
24	9.5, 8.0	0.086, 0.097	1650	17.8
27	10.0, 6.0	0.139, 0.056	2250	21.2
29	8.0, 7.0	0.090, 0.094	1700	21.6
32	8.1 ± 1.8 (3)	0.108 ± 0.028	1990, 2150	25.2, 26.1
Adult	8.8 ± 1.2 (5)	0.147 ± 0.023	2090 ± 70 (3)	37.6 ± 3.8

^a The units of enzyme activity are: specific activity, μ moles ACh synthesized or hydrolyzed in 30 min per gram of protein, and absolute activity, mmoles in 30 min per hippocampal region. Values are expressed as means \pm SEM for the number of experiments in parentheses or as results of single experiments.

Development in the Dentate Gyrus

The specific activity of ChAc was greater in the dentate gyrus of the adult rat than in the hippocampal region as a whole, while the specific activity of AChE was slightly lower (Tables 2 and 3). Laminar analysis revealed peak activities for both enzymes in layer G and, to a lesser extent, in layer H. This distribution was similar to that reported by other investigators (Fonnum, 1970; Storm-Mathisen, 1970, 1972) and consistent with a primary localization of ChAc in septohippocampal synaptic terminals (Mosko *et al.*, 1973).

At the age of 11 days the specific activity of ChAc in the dentate gyrus as a whole was comparable to that in the entire hippocampal region, and the enzyme was distributed among the layers simply in proportion to protein content (Table 2). The activity in layers of the dentate gyrus was just great enough to be detected by our methods. Hence we did not attempt to investigate its development in younger animals. The absolute activity of ChAc (total activity in a 1.2 mm segment of temporal dentate gyrus) increased 15-fold between

11 days and maturity, and this resulted in a 3- to 4-fold increase in specific activity.

By far the largest rise in specific activity of ChAc took place between 11 and 16-17 days. By the end of this period adult values were attained. Although values from 17-day rats were generally greater than those from 16-day animals, the difference was not statistically significant and therefore the data from all animals of these ages have been combined. Specific activities at 16-17 days ranged from values typical of 11-day-old rats (about 4 μ moles ACh synthesized/30 min/gm protein) to as high as 15-20 μ moles ACh synthesized/30 min/gm protein, with the higher values derived mainly from 17-day animals. These data suggest a massive addition of ChAc activity in excess of protein within a period of only 2 or 3 days. Indeed the absolute activity of ChAc rose by about 4.5-fold on the average, while protein content increased by less than 2-fold.

Between 17 days and adulthood the absolute activity of ChAc increased at a rate only slightly higher than the rate of increase in protein content. The increase in specific activity was confined to layers

TABLE 2
SPECIFIC AND ABSOLUTE ACTIVITIES OF ChAc IN THE DENTATE GYRUS DURING DEVELOPMENT^a

Age (days)	Layer ^b					Whole dentate gyrus ^c
	MO	MM	MI	G	H	
<i>Specific activity (μmoles ACh synthesized/30 min/gm protein)</i>						
11	4.1 ± 0.6		4.7 ± 1.2	4.2 ± 0.8	4.8 ± 1.7	4.2 ± 0.7
16-17	9.0 ± 2.5	12.0 ± 2.5	10.2 ± 1.9	10.3 ± 1.7	9.1 ± 1.9	10.1 ± 1.9
30	6.8 ± 0.9	11.2 ± 1.8	7.2 ± 0.7	12.4 ± 0.8	9.8 ± 1.2	9.7 ± 0.7
43	10.0 ± 1.7	11.9 ± 1.6	10.1 ± 1.8	12.2 ± 0.7	12.4 ± 1.5	11.3 ± 1.1
Adult	13.0 ± 0.9	11.6 ± 1.0	10.6 ± 1.3	16.2 ± 1.2	13.2 ± 1.3	13.0 ± 0.7
<i>Absolute activity (nmoles ACh synthesized/30 min/12 × 100 μm sections)</i>						
11	0.109 ± 0.030		0.025 ± 0.006	0.044 ± 0.008	0.023 ± 0.006	0.21 ± 0.03
16-17	0.16 ± 0.04	0.24 ± 0.05	0.08 ± 0.01	0.30 ± 0.05	0.12 ± 0.02	0.90 ± 0.16
30	0.18 ± 0.03	0.31 ± 0.04	0.08 ± 0.01	0.33 ± 0.04	0.14 ± 0.02	1.04 ± 0.10
43	0.36 ± 0.10	0.48 ± 0.09	0.17 ± 0.04	0.43 ± 0.05	0.26 ± 0.04	1.70 ± 0.30
Adult	0.90 ± 0.11	0.70 ± 0.06	0.27 ± 0.04	0.74 ± 0.04	0.47 ± 0.05	3.08 ± 0.21

^a Values are expressed as means ± SEM for *N* as follows: 11 days, 6; 16-17 days, 9; 30 days, 6; 43 days, 8; adult, 6.

^b See Fig. 1.

^c Total activity in all layers (absolute) or total activity in all layers ÷ total protein in all layers (specific).

TABLE 3
SPECIFIC AND ABSOLUTE ACTIVITIES OF AChE IN THE DENTATE GYRUS DURING DEVELOPMENT^a

Age (days)	Layer					Whole dentate gyrus
	MO	MM	MI	G	H	
<i>Specific activity (μmoles ACh hydrolyzed/30 min/gm protein)</i>						
11	380 ± 60		300 ± 170	380 ± 140	760 ± 280	420 ± 100
16-17	400 ± 40	520 ± 60	550 ± 70	580 ± 70	720 ± 70	550 ± 50
30	720 ± 70	830 ± 140	630 ± 160	1230 ± 150	1280 ± 190	940 ± 120
43	830 ± 100	1130 ± 90	1020 ± 150	1560 ± 90	1400 ± 90	1190 ± 80
Adult	1380 ± 80	1360 ± 90	1640 ± 90	2200 ± 140	2020 ± 190	1660 ± 80
<i>Absolute activity (nmoles ACh hydrolyzed/30 min/12 × 100 μm sections)</i>						
11	10.8 ± 2.2		1.5 ± 0.9	3.9 ± 1.0	3.7 ± 0.9	19.9 ± 2.8
16-17	7.8 ± 1.4	11.3 ± 2.0	4.9 ± 1.0	18.1 ± 3.2	10.1 ± 1.2	52 ± 8
30	19 ± 3	25 ± 6	8 ± 3	32 ± 5	19 ± 4	104 ± 18
43	31 ± 8	44 ± 6	17 ± 4	56 ± 7	30 ± 6	177 ± 29
Adult	96 ± 13	83 ± 8	41 ± 3	103 ± 10	72 ± 8	395 ± 32

^a See Table 2 for details.

MO, G, and H. This selectivity produced the mature laminar distribution, which was difficult to discern at 16 or 17 days.

The specific activity of AChE was much lower in the dentate gyrus of the 11-day-old rat than in the total hippocampal region and was comparable to that in the hippocampal region of rats less than 5 days of

age (Table 3). AChE activity was distributed among the layers in proportion to protein content except in layer H, which was relatively enriched in this enzyme. This finding suggests a significant contribution from those polymorphic neurons in this layer which stain positively for AChE activity (Shute and Lewis, 1966; Lewis and

Shute, 1967; Matthews *et al.*, 1974). Between 11 days and adulthood, the absolute activity of AChE increased 20-fold and the specific activity 4-fold.

In contrast to the development of ChAc, the specific activity of AChE in the dentate gyrus increased only modestly in layers MO, MM, MI, and G between 11 and 16–17 days and not at all in layer H. Thus the abrupt increase in the specific activity of ChAc which occurred at 16 days or so was not accompanied by a comparable increase in AChE activity.

Between 17 days and adulthood the absolute activity of AChE increased at a rate which exceeded the rate of increase in protein content, and thus there was a progressive rise in specific activity. No abrupt increase in specific activity to adult values was evident. The mature laminar pattern of distribution appeared by 30 days. After this age, the specific activity of AChE increased at the same rate in all layers.

The ratio of the specific activity of AChE to that of ChAc in the dentate gyrus as a whole was about 100 at all ages except at 16–17 days, when it was reduced to half this value by the addition of ChAc activity substantially in excess of addition of AChE activity. This ratio was very similar in each layer, except that layer MO tended to be relatively enriched in ChAc activity and layer H in AChE activity.

The increase in protein content of the dentate gyrus with maturation was some-

what discontinuous (Table 4). Between 11 and 16–17 days the major part of the increase was restricted to layers G and H, whereas the small increase between 17 and 30 days was entirely confined to the molecular layer. After 30 days the percentage increase in protein content was very nearly the same in all layers of the dentate gyrus.

DISCUSSION

Most regions of the mammalian CNS contain a variety of cholinergic elements, many of unknown origin. Thus changes in levels of ChAc and AChE activities or of the transmitter, ACh, have heretofore been largely uninterpretable in morphologic terms. In this regard, the unique advantages of the hippocampal formation are immediately evident. Its relatively simple laminar organization permits detailed analysis at a much finer than regional level. Most important, the predominant cholinergic innervation is apparently derived from two well-defined groups of cell bodies within the septum (Daitz and Powell, 1954; Shute and Lewis, 1963). This septohippocampal pathway contributes more than 80% of the ChAc and AChE activities in the hippocampal formation (Storm-Mathisen, 1970, 1972). There are few, if any, intrinsic cholinergic neurons. Thus developmental studies of enzymes involved in cholinergic transmission can be related to development of a single morphological entity.

TABLE 4
PROTEIN CONTENT OF THE DENTATE GYRUS DURING DEVELOPMENT^a

Age (days)	Layer					Whole dentate gyrus
	MO	MM	MI	G	H	
11	28.7 ± 4.9		5.4 ± 0.6	12.3 ± 2.8	7.0 ± 1.9	53 ± 6
16–17	19 ± 2	21 ± 2	9 ± 1	30 ± 2	14 ± 1	92 ± 5
30	26 ± 2	28 ± 3	12 ± 2	26 ± 2	14 ± 1	107 ± 7
43	35 ± 5	39 ± 3	16 ± 2	35 ± 3	20 ± 3	144 ± 13
Adult	70 ± 12	61 ± 6	25 ± 1	47 ± 4	37 ± 5	240 ± 22

^a Units are $\mu\text{g}/12 \times 100 \mu\text{m}$ sections. For other details see Table 2.

Development of the Septohippocampal Projection

The biochemically determined AChE activity of the hippocampal region doubles between 4 and 7 days after birth and again between 11 days and maturity. ChAc activity increases rapidly during the first week of postnatal life and again between 11 and 21 days after birth. After the third week of postnatal life, the development of ChAc activity is essentially complete. Our studies on discrete layers of the dentate gyrus reveal a similar, but more clear-cut, pattern. The specific activity of ChAc is less than one-third the adult value at 11 days of age and is uniformly distributed in all layers. It reaches mature levels about 16–17 days after birth. This remarkably large and abrupt increase in ChAc activity is unaccompanied by a development of similar magnitude in AChE activity. The latter enzyme activity develops more slowly, not attaining mature values of specific activity until more than 43 days of age. In our discussion of these data, we first consider possible morphogenetic relationships. We then propose a model of growth and development for the septohippocampal projection. Particular emphasis is placed on innervation of the dentate gyrus, the division of the hippocampal region for which the most detailed morphogenetic information has been amassed.

The sudden increase in biochemically determined AChE activity during the latter half of the first postnatal week corresponds well to the increased histochemical staining of the hippocampal formation which is observed simultaneously (Matthews *et al.*, 1974). The histochemical patterns of development strongly suggest that this increased activity results mainly from the growth of septohippocampal axons into the hippocampal region. Generation of these fibers may also introduce some ChAc activity into this region. The period during which this growth of fibers probably takes place (4–11 days of age)

coincides with the time of most rapid increase in size, wet weight and protein content of the hippocampal formation. The times of invasion of the hippocampal formation by other axons has not been studied. It would be of interest to determine how much of a role ingrowth of extrinsic axons plays in development of these characteristics.

Several factors could be responsible for the dramatic increase in ChAc activity during the third week of postnatal life including synaptogenesis, increased synthesis of enzyme and transport to existing synaptic terminals and release from inhibitory regulation. However, studies on developing chick sympathetic ganglia (Marchisio and Consolo, 1968) and spinal cord (Burt, 1968) and on mouse superior cervical sympathetic ganglion (Black *et al.*, 1971) have demonstrated a close temporal correlation between increases in ChAc activity during ontogenesis and formation of cholinergic synapses. They indicate that cholinergic synaptic development is the major contributor to the ontogenic increase in ChAc activity. Thus this enzymatic change may serve as an index of such synaptic development.

By analogy with these data we interpret the development of ChAc activity in the hippocampal formation during the period between 11 and 21 days after birth as a correlate of synaptogenesis. This conclusion derives further support from the fact that, in the dentate gyrus, this phase occurs around 16–17 days of age, a period of rapid synaptic development in that region (Crain *et al.*, 1973). We conclude that this developmental change reflects attainment of high levels of ChAc activity within the septohippocampal tract, since nearly all hippocampal ChAc activity in adult rats is associated with this input, and these axons enter all parts of the hippocampal region before 11 days of age.

Our data demonstrate that the AChE activity in the temporal part of the dentate gyrus develops more gradually and some-

what later than ChAc activity. Although much of the AChE activity in the hippocampal region of adult rats may be associated with presynaptic terminals (Fonnum, 1970), the ontogenic development of this enzyme appears to be independent of synaptic development.

Other investigators have pointed out that a developmental increase in AChE activity need not accompany the change in ChAc activity coincident with alterations in cholinergic synaptic relations. For example, the development of mature levels of AChE activity in chick sympathetic ganglia follows the developmental increase in activity of ChAc by 4 days (Giacobini *et al.*, 1970) and relates less well to the period of synaptogenesis. Also the developmental increase in ChAc activity associated with synaptic development in the brachial spinal cord of the chick (Burt, 1968) and the reduction of ChAc activity, which is brought about by denervation of the ventral part of this region (Burt and Narayanan, 1970) are unaccompanied by changes in AChE activity. Thus changes in AChE activity are not generally useful indicators of cholinergic synaptogenesis.

The delay we have observed in development of the capacity to metabolize ACh may play a physiologically significant role in regulating the efficacy of synaptic transmission in developing cholinergic systems. A complete explanation for this finding must obviously await detailed elucidation of synaptic physiology during development. In particular, we need to know how much effect a three-fold increase in the specific activity of AChE really has on cholinergic transmission in the dentate gyrus. However, a possible function for AChE in regulating presynaptic stores of transmitter is suggested. Development of AChE activity may be postponed until a sufficient steady-state level of ACh has been synthesized. Alternatively, a relatively low AChE activity may facilitate cholinergic neurotransmission in the dentate gyrus. The latter interpretation im-

plies a more significant role for the septohippocampal input in the developing animal than in the adult. Unfortunately, our data are inconclusive with regard to the development of AChE activity within other divisions of the hippocampal region. It would be interesting to know whether the temporal sequence of enzymatic development we have described for the dentate gyrus is generally true for all developing central cholinergic systems.

It is of some interest that the protein content of the dentate gyrus increases in a gradual fashion during postnatal development. Most of the net change occurs not in association with neuronal differentiation or synaptogenesis, which are complete by 30 days after birth (Altman and Das, 1965, 1966; Crain *et al.*, 1973), but within a largely mature structure. In this connection, contributions from glial development, myelination, dendritic ramification and further synaptic specialization, among others, should be considered. Possibly a large part of the developmental increase in protein content may not be associated with obvious structural change, but rather with intracellular events.

Combining the histochemical data with results of our biochemical studies enables us to propose an integrated model of the development of cholinergic innervation in the temporal part of the dentate gyrus. Septohippocampal axons begin to invade this region at the age of 6 days, as indicated by the initial appearance at this age of histochemical AChE staining associated with afferent fibers. These axons become distributed in their mature laminar arrangement by 11 days of age. However, the increased intensity of staining at later ages suggests continuing elaboration of cholinergic neuropil. ChAc activity develops mainly around 16–17 days after birth, a finding which implies development of functional cholinergic synaptic terminals at this age. Finally, the major part of the AChE activity develops in the period following synaptogenesis. While comparably

detailed studies on other divisions of the hippocampal region are lacking, the information from our own investigations suggests a similar sequence of events in these areas. An evaluation of whether the developmental scheme in the temporal part of the dentate gyrus typifies cholinergic ontogeny in the CNS must await studies of other regions at a comparable level of resolution.

Intrinsic Enzyme Activity during Development

The increase of ChAc activity in the hippocampal region during the first postnatal week cannot easily be interpreted in terms of synaptogenesis. The histochemical evidence suggests the presence of very few cholinergic elements in this region before 4 days of age aside from some polymorphic neurons, and these cells may be cholinceptive rather than cholinergic (Shute and Lewis, 1966). On the basis of our studies on the dentate gyrus of 11-day-old rats, it seems unlikely that these neurons contain much ChAc activity. However, considerable evidence suggests that the cholinergic system plays a role in the growth and development of many neurons independent of its function in neurotransmission (Filogamo and Marchisio, 1971; Silver, 1971). For example, neurons in spinal ganglia of the chick, which never receive cholinergic innervation and are not themselves likely to be cholinergic, possess considerable ChAc activity during ontogenesis (Marchisio and Consolo, 1968). The times of peak ChAc activity coincide with the proliferation and maturation of specific groups of neurons. Possibly some or all of the noncholinergic intrinsic neurons of the hippocampal region also require ChAc activity at a stage in their development. Neurons in chick spinal ganglia also possess AChE activity during their development (Giacobini *et al.*, 1970), as do many other noncholinergic neurons (Silver, 1971). These findings raise the possibility that the low AChE activity which is pres-

ent in the hippocampal region at birth may also subserve a nonsynaptic function within intrinsic neurons.

Correlations with Histochemistry

Storm-Mathisen (1970) has shown that histochemical AChE staining serves as a reliable index of the actual biochemical activity of this enzyme in the hippocampal formation of adult rats. However, quantitative changes in AChE activity do not always correlate with similar changes in histochemical staining of the developing hippocampal formation (Matthews *et al.*, 1974). During the first week of postnatal life the correlation is quite good. The relatively low and constant specific activity of AChE from birth to 4 days of age corresponds well to the lack of axonal staining in the hippocampal region during this period. Between 4 and 7 days the increase in biochemically determined activity parallels the initial appearance and rapid spread of AChE-dependent staining associated with nerve fibers. However, the development of AChE activity in the temporal part of the dentate gyrus after 11 days bears little relation to the pattern of staining. Quantitative measurements show that the discrete laminar distribution of AChE activity, characteristic of the adult dentate gyrus, does not develop until later than 17 days of age, but the mature pattern of AChE staining can be detected as early as 11 days. Consider also that layer H at 11 and 16–17 days has the same specific activity as layer MO at 30 days and the specific activities of layers G and H at 30 days are similar to that of layer MO in the adult. However, the areas included in layers G and H stain heavily for AChE activity at the ages mentioned, but little intensity of staining ever develops along the superficial edge of the molecular layer. Lack of agreement between the two techniques is evidently not unique to the dentate gyrus. Eränkö (1972) has reported discrepancies between the biochemical and histochemical development of AChE activ-

ity in the superior cervical sympathetic ganglion of the rat similar to those we have observed.

While the discrepancies in laminar development may be attributable partially to the relative grossness of the microdissections (for example, layer G averages all of one and part of another band of intense activity with the nonstaining granule cell bodies), we emphasize that the copper-thiocholine histochemical technique often fails to demonstrate the total AChE activity in tissue (Eränkö *et al.*, 1964; Koelle *et al.*, 1970; Friedenbergl and Seligman, 1972). Previous investigators have suggested that several factors contribute to this phenomenon: (1) Part of the total AChE activity is generally present in a soluble form which is eluted from the tissue during fixation. (2) Fixation inactivates some isoenzymes of AChE more than others. (3) The histochemical substrate, acetylthiocholine, penetrates poorly into tissue and thus may not reach all sites of AChE activity. (4) The intensity of staining is dependent not only on the amount of deposit, but also on its distribution. In preliminary studies, we have found that the AChE activity of aldehyde-fixed hippocampal formation at the pH of the histochemical reaction (5.3) is only 15–20% of the activity under the assay conditions used in our biochemical investigations. Hence the histochemical staining is dependent on a rather small percentage of the total AChE activity. Accordingly, caution must be exercised in relating the histochemical reaction to biochemical data. In our studies the histochemical procedure probably demonstrated a form or forms of AChE associated with the ingrowing septohippocampal axons. At least in the temporal part of the dentate gyrus, the development of other forms of this enzyme occurs independently and at a later time.

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REFERENCES

- ALTMAN, J., and DAS, G. D. (1965). Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J. Comp. Neurol.* **124**, 319–336.
- ALTMAN, J., and DAS, G. D. (1966). Autoradiographic and histological studies of postnatal neurogenesis. *J. Comp. Neurol.* **126**, 337–390.
- BLACK, I. B., HENDRY, I. A., and IVERSEN, L. L. (1971). Trans-synaptic regulation of growth and development of adrenergic neurones in a mouse sympathetic ganglion. *Brain Res.* **34**, 229–240.
- BLACKSTAD, T. W. (1958). On the termination of some afferents to the hippocampus and fascia dentata. An experimental study in the rat. *Acta Anat.* **35**, 202–214.
- BLACKSTAD, T. W. (1967). Cortical grey matter: a correlation of light and electron microscopic data. In "The Neuron" (H. Hydén, ed.), pp. 49–118. Elsevier, Amsterdam.
- BURT, A. M. (1968). Acetylcholinesterase and choline acetyltransferase activity in the developing chick spinal cord. *J. Exp. Zool.* **169**, 107–112.
- BURT, A. M., and NARAYANAN, C. H. (1970). Effect of extrinsic neuronal connections on development of acetylcholinesterase and choline acetyltransferase activity in the ventral half of the chick spinal cord. *Exp. Neurol.* **29**, 201–210.
- COGGESHALL, R. E., DEWHURST, S. A., WEINREICH, D., and McCAMAN, R. E. (1972). Aromatic acid decarboxylase and choline acetylase activities in a single identified 5-HT containing cell of the leech. *J. Neurobiol.* **3**, 259–265.
- CRAIN, B., COTMAN, C. W., TAYLOR, D., and LYNCH, G. (1973). A quantitative electron microscopic study of synaptogenesis in the dentate gyrus of the rat. *Brain Res.* in press.
- DAITZ, H. M., and POWELL, T. P. S. (1954). Studies of the connexions of the fornix system. *J. Neurol. Neurosurg. Psychiat.* **17**, 75–82.
- ERÄNKÖ, L. (1972). Biochemical and histochemical observations on the postnatal development of cholinesterases in the sympathetic ganglion of the rat. *Histochem. J.* **4**, 545–559.
- ERÄNKÖ, O., HÄRKÖNEN, M., KOKKO, A., and RÄISÄNEN, L. (1964). Histochemical and starch gel electrophoretic characterization of desmo- and lyoesterases in the sympathetic and spinal ganglia of the rat. *J. Histochem. Cytochem.* **12**, 570–581.
- FLOGAMO, G., and MARCHISIO, P. C. (1971). Acetylcholine system and neural development. *Neurosci. Res.* **4**, 29–64.
- FONNUM, F. (1969). Radiochemical micro-assays for the determination of choline acetyltransferase and

- acetylcholinesterase activities. *Biochem. J.* **115**, 465-472.
- FONNUM, F. (1970). Topographical and subcellular localization of choline acetyltransferase in rat hippocampal region. *J. Neurochem.* **17**, 1029-1037.
- FRIEDENBERG, R. M., and SELIGMAN, A. M. (1972). Acetylcholinesterase at the myoneural junction: cytochemical ultrastructure and some biochemical considerations. *J. Histochem. Cytochem.* **20**, 771-792.
- GIACOBINI, G., MARCHISIO, P. C., GIACOBINI, E., and KOSLOW, S. H. (1970). Developmental changes in cholinesterases and monoamine oxidase in chick embryo spinal and sympathetic ganglia. *J. Neurochem.* **17**, 1177-1185.
- KOELLE, W. A., HOSSAINI, K. S., AKBARZADEH, P., and KOELLE, G. B. (1970). Histochemical evidence and consequences of the occurrence of isoenzymes of acetylcholinesterase. *J. Histochem. Cytochem.* **18**, 812-819.
- LEWIS, P. R., and SHUTE, C. C. D. (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**, 521-540.
- LEWIS, P. R., SHUTE, C. C. D., and SILVER, A. (1967). Confirmation from choline acetylase analyses of a massive cholinergic innervation of the rat hippocampus. *J. Physiol. (London)* **191**, 215-224.
- LOWRY, O. H., and PASSONNEAU, J. V. (1972). "A Flexible System of Enzymatic Analysis," pp. 229-235. Academic Press, New York.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.
- LYNCH, G. MATTHEWS, D. A., MOSKO, S. PARKS, T., and COTMAN, C. (1972). Induced acetylcholinesterase-rich layer in rat dentate gyrus following entorhinal lesions. *Brain Res.* **42**, 311-318.
- MARCHISIO, P. C., and CONSOLO, S. (1968). Developmental changes of choline acetyltransferase (ChAc) activity in chick embryo spinal and sympathetic ganglia. *J. Neurochem.* **15**, 759-764.
- MATTHEWS, D. A., NADLER, J. V., LYNCH, G. S., and COTMAN, C. W. (1974). Development of cholinergic innervation in the hippocampal formation of the rat. I. Histochemical demonstration of acetylcholinesterase activity. *Develop. Biol.* **36**, 130-141.
- MOSKO, S., LYNCH, G. S., and COTMAN, C. W. (1973). Distribution of the septal projection to the hippocampal formation of the rat. *J. Comp. Neurol.* in press.
- NAIK, N. T. (1963). Technical variations in Koelle's histochemical method for demonstrating cholinesterase activity. *Quart. J. Microsc. Sci.* **104**, Part I, 89-100.
- PATTERSON, M. S., and GREENE, R. C. (1965). Measurement of low energy beta-emitters in aqueous solution by liquid scintillation counting of emulsions. *Anal. Chem.* **37**, 854-857.
- RAISMAN, G., COWAN, W. M., and POWELL, T. P. S. (1965). The extrinsic afferent, commissural and association fibres of the hippocampus. *Brain* **88**, 963-996.
- RAMÓN Y CAJAL, S. (1968). "The Structure of Ammon's Horn" (L. M. Kraft, transl.). Thomas, Springfield, Illinois.
- SHUTE, C. C. D., and LEWIS, P. R. (1963). Cholinesterase-containing systems of the brain of the rat. *Nature (London)* **199**, 1160-1164.
- SHUTE, C. C. D., and LEWIS, P. R. (1966). Electron microscopy of cholinergic terminals and acetylcholinesterase-containing neurones in the hippocampal formation of the rat. *Z. Zellforsch. Mikrosk. Anat.* **69**, 334-343.
- SILVER, A. (1971). The significance of cholinesterase in the developing nervous system. *Progr. Brain Res.* **34**, 345-355.
- STORM-MATHISEN, J. (1970). Quantitative histochemistry of acetylcholinesterase in rat hippocampal region correlated to histochemical staining. *J. Neurochem.* **17**, 739-750.
- STORM-MATHISEN, J. (1972). Glutamate decarboxylase in the rat hippocampal region after lesions of the afferent fibre systems. Evidence that the enzyme is localized in intrinsic neurones. *Brain Res.* **40**, 215-235.
- STORM-MATHISEN, J., and BLACKSTAD, T. W. (1964). Cholinesterase in the hippocampal region. *Acta Anat.* **56**, 216-253.
- TODRICK, A. (1954). The inhibition of cholinesterases by antagonists of acetylcholine and histamine. *Brit. J. Pharmacol.* **9**, 76-83.
- VAUGHN, J. E., and PETERS, A. (1966). Aldehyde fixation of nerve fibres. *J. Anat. (London)* **100**, 687.