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Development of Cholinergic Innervation in the Hippocampal Formation of the Rat

I. Histochemical Demonstration of Acetylcholinesterase Activity

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The postnatal development of acetylcholinesterase (AChE) activity in the hippocampal formation of the developing rat brain, as demonstrated histochemically by the copper-thiocholine technique, serves as a marker for the ingrowing cholinergic afferent fibers. The discrete laminar pattern of staining characteristic of the adult hippocampal formation develops entirely after birth. Stain deposit is observable earliest (about 4 days after birth) at the septal end of the hippocampus. During the following week, AChE activity can be demonstrated in successively temporal segments until, about 11 days after birth, all parts of the hippocampal formation exhibit activity. Within each segment, the pattern of developing activity suggests association with three distinct fiber projections emanating from the fimbria, each with its own characteristic time of appearance and rate of growth: (1) a projection through stratum oriens of hippocampus regio inferior to stratum oriens of regio superior; (2) fibers which cross stratum pyramidale of regio inferior, run in the suprapyramidal zone of that region and continue into the supra- and infragranular zones in the external leaf of the dentate gyrus; (3) a projection through stratum oriens of regio inferior which continues into the supra- and infragranular zones in the internal leaf of the dentate gyrus.

INTRODUCTION

Few regions of the mammalian central nervous system (CNS) are suitable for detailed developmental investigations of cholinergic innervation because the various cholinergic elements cannot easily be segregated. As a result, development of enzyme activities associated with cholinergic transmission has not been related to morphologic ontogeny. Many of the difficulties inherent in such investigations can be obviated by employing the hippocampal formation of the rat brain as a model system. This region offers several important advantages. The hippocampal formation undergoes considerable postnatal development, and the granule cells of the dentate gyrus differentiate mainly after birth (Altman and Das, 1965, 1966; Das and Kreutzberg, 1967; Crain *et al.*, 1973). Its structure is relatively simple and well defined (Blackstad, 1967). Both electrophysiological and anatomical techniques define a laminar arrangement of intrinsic neurons and synaptic input (Raisman *et al.*, 1965; Andersen *et al.*, 1966; Ramón y Cajal, 1968). Finally, only one extrinsic cholinergic afferent, the septohippocampal tract, has been identified. These favorable characteristics have permitted the use of acetylcholinesterase (AChE) histochemistry to visualize a single developing central cholinergic pathway. They also facilitate biochemical investigation of the associated enzyme activities at a fine level of resolution, i.e., in discrete, morphologically defined layers.

The septohippocampal fibers arise from cell bodies in the ipsilateral medial septal nucleus and nucleus of the diagonal band (Daitz and Powell, 1954), then pass via the precommissural fornix and fimbria to terminate in discrete layers of the hippocampus and the dentate gyrus (Raisman *et al.*, 1965; Raisman, 1966; Lewis and Shute, 1967; Mosko *et al.*, 1973). Several lines of evidence support the view that this affer-

ent represents the major cholinergic synaptic input to the hippocampal formation. (1) Neuronal cell bodies in the medial septal nucleus and nucleus of the diagonal band stain histochemically for AChE activity (Lewis and Shute, 1967). Stained axons can be traced into the precommissural fornix, the fimbria, and on into the hippocampus. (2) Light (Shute and Lewis, 1963; Storm-Mathisen and Blackstad, 1964; Lewis and Shute, 1967; Mosko *et al.*, 1973) and electron (Shute and Lewis, 1966) microscopy reveal a close correspondence between the distribution of histochemical AChE staining and synaptic terminals or neuropil associated with the septohippocampal afferents. The AChE staining is abolished by transection of the fimbria or a lesion of the medial septal nucleus (Shute and Lewis, 1963; Lewis *et al.*, 1967; Storm-Mathisen, 1970, 1972; Lynch *et al.*, 1972), but not by lesions of other afferent tracts (Storm-Mathisen, 1972). (3) The distribution of AChE (Storm-Mathisen, 1970) and choline acetyltransferase (ChAc) (Fonnum, 1970) activities coincides with the regions of septohippocampal termination and histochemical staining. These enzyme activities are also severely diminished by transection of the fimbria (Lewis *et al.*, 1967, Storm-Mathisen, 1970, 1972). (4) A lesion of the medial septal nucleus reduces the acetylcholine content of the hippocampal formation and abolishes the high-affinity uptake of choline (Kuhar *et al.*, 1973). The high-affinity uptake system is probably localized in presynaptic terminals (Yamamura and Snyder, 1972). (5) Stimulation of the medial septal nucleus releases acetylcholine from the hippocampal region (Smith, 1972). (6) Neurons of the medial septal nucleus act as pacemakers for the theta rhythm of the hippocampus (Stumpf, 1965; Gogolák *et al.*, 1968). The theta rhythm can be initiated by cholinomimetic drugs and abolished by antimuscarinic agents. Lesions of the medial septal nucleus prevent its initiation by either environmental stimuli or cholinomimetic agents (Stumpf, 1965; Torii and Wikler, 1966).

Since parameters of cholinergic function in the hippocampal formation are predominantly associated with the septohippocampal afferents, developmental studies of these parameters can be related to maturation of this single element. In the present study, we follow the pattern of histochemical AChE staining throughout the postnatal period of development and formulate from our observations a spatial and temporal model of cholinergic axonal growth into the hippocampal formation. The results are integrated with relevant quantitative biochemical data in the following paper (Nadler *et al.*, 1974). Also, these basic studies have aided our understanding of the effects of experimental manipulations on connectivity in the developing CNS (Cotman *et al.*, 1973; Nadler *et al.*, 1973).

METHODS

A total of 65 Sprague-Dawley rats of various ages were used in these investigations. At ages ranging from 2 days to more than 90 days after birth, animals were anesthetized with sodium pentobarbital and perfused through the heart with a fixative that consisted of 4% (w/v) paraformaldehyde, 0.5% (w/v) glutaraldehyde, 0.54% (w/v) D-glucose and 0.1 *M* sodium phosphate, pH 7.4 (Vaughn and Peters, 1966). From two to eight animals were studied at each of 18 ages. The brains were removed and postfixed for 2 hr at 4°C in the perfusion medium. Sections of 50 μm thickness were cut in the coronal or horizontal plane with a freezing microtome. The sections were stained for AChE activity by a modification (Naik, 1963) of the Koelle (1950) copper–thiocholine method. The incubation medium consisted of 52 m*M* sodium acetate buffer, pH 5.3, 20 m*M* glycine, 6.5 m*M* cupric sulfate, 5 m*M* acetylthiocholine iodide, and 0.18 m*M* promethazine hydrochloride, an inhibitor of nonspecific cholinesterases (Todrick, 1954). After incubation at room tempera-

ture for 3, 6, or 24 hr, the sections were developed in 1% (w/v) ammonium sulfide and mounted on glass slides. Some sections were counterstained with cresyl violet. Finally, they were dehydrated with passage through butanol and xylene, and cover slips were applied with the use of Permount (Fisher Scientific Co., Fair Lawn, New Jersey).

In a few cases, BW284c51 (5×10^{-6} *M*), a specific inhibitor of AChE (Bayliss and Todrick, 1956), was included in the incubation medium. The laminar pattern of staining was abolished, indicating that the stain deposit represented AChE activity.

RESULTS

Hippocampal Morphology

Before presenting our histochemical data, we briefly review the relevant morphologic features of the hippocampal formation.

The paired cylindrical hippocampal formation (hippocampus and dentate gyrus) originates just posterior to the septum (septal or anterior end) and curves posterolaterally along the lateral ventricle to the base of the brain (temporal or posterior end). This pronounced curvature necessitates the use of horizontal and coronal planes of sectioning to examine the entire length in cross section. Coronal sections of the septal portion of the hippocampal formation of the rat brain are comparable to horizontal sections of the temporal portion.

The architectonic division of the hippocampal region into layers and fields (Fig. 1) originates from the classic descriptions of Ramón y Cajal (1968) and Lorente de Nó (1934). The hippocampus is distinguished by a discrete layer of pyramidal cells, whose axons represent the sole output of the hippocampal formation. Primarily on the basis of pyramidal cell structure and organization, the hippocampus has been divided into two fields, regio superior and regio inferior (Blackstad, 1956; Ramón y Cajal, 1968). The morphology of the pyramidal cells can also be used to distinguish seven cytoarchitectonic layers. The most superficial layer, the alveus, covers the surface of the hippocampus. It consists mainly of myelinated afferent fibers and efferent axons of the pyramidal cells. It is continuous with the fimbria. The adjoining stratum oriens contains the basal dendrites, axons and axon collaterals of the pyramidal cells, as well as cell bodies of polymorphic neurons (mainly basket-type). The closely packed pyramidal cell bodies and associated basket plexuses form the stratum pyramidale. The apical dendritic shafts of the pyramidal cells distinguish stratum radiatum. Present only in regio superior, stratum lacunosum is distinguished by bundles of parallel association fibers, the Schaffer collaterals, which originate from pyramidal cell bodies in regio inferior. Stratum moleculare contains the terminal dendritic plexus of the pyramidal cells. The latter three layers also contain several types of scattered short-axon neurons. Stratum lucidum, is a spe-

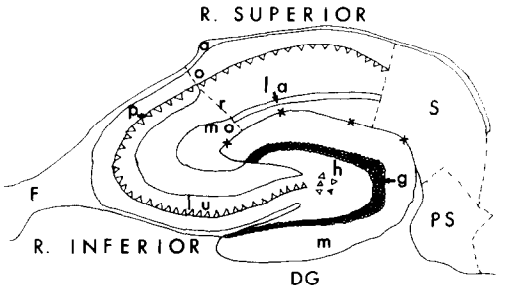

FIG. 1. Camera lucida drawing of the hippocampal region of the rat brain as seen in a horizontal section. Hippocampus consists of regio superior and regio inferior, the boundaries of which are denoted by broken lines. The hippocampal fissure (×) separates the hippocampus from the external leaf of the dentate gyrus. Abbreviations: *PS*, presubiculum; *S*, subiculum; *R. SUPERIOR*, hippocampus regio superior; *R. INFERIOR*, hippocampus regio inferior; *DG*, dentate gyrus; *F*, fimbria. Layers of the hippocampus: *a*, alveus; *o*, stratum oriens; *p*, stratum pyramidale; *r*, stratum radiatum; *la*, stratum lacunosum (regio superior only); *mo*, stratum moleculare; *lu*, stratum lucidum (regio inferior only). Layers of the dentate gyrus: *h*, hilus; *g*, granule cell layer; *m*, molecular layer.

cialized region containing the initial part of the apical dendritic shafts in regio inferior. It is the locus of the mossy fibers, the axons of the granule cells of the dentate gyrus.

The dentate gyrus may be viewed as a simplified hippocampus, with the granule cells as the major cell type. The granule cell layer is formed by an arch of tightly packed cell bodies. The dendrites of the granule cells ramify in the molecular layer, which also contains a small number of short-axon neurons. The region enclosed by the granule cells, the hilus, contains the mossy fibers, numerous polymorphic cells (mainly basket-type) and scattered pyramidal cells of hippocampus regio inferior. For descriptive purposes, the dentate gyrus can be divided into an internal and external leaf. The internal leaf adjoins the brain stem. It assumes a ventral position at septal levels and a medial position at temporal levels. The external leaf is that portion of the dentate gyrus separated from stratum moleculare of regio superior by the hippocampal fissure.

The architectonic lamination of the hippocampus and dentate gyrus is reflected in the stratification of their intrinsic and extrinsic synaptic connections (Blackstad, 1958; Raisman *et al.*, 1965). Evidence for a distinct laminar arrangement of cholinergic projections from the septum has been derived from biochemical, anatomical, and histochemical studies. Consequently, histochemical staining for AChE activity accents the cytoarchitectonic features and laminar organization of the hippocampal formation.

AChE Histochemistry in Adult Hippocampal Formation

The histochemical distribution of AChE activity in the hippocampal formation of adult rats in our study (Fig. 2E,F) agrees with the previous descriptions of Gerebtzoff (1959), Shute and Lewis (1961, 1963), Storm-Mathisen and Blackstad (1964) and Lewis and Shute (1967). A similar distribution of staining is seen in both septal and temporal portions of the hippocampus. AChE-staining fibers are scattered throughout the alveus and the external edge of the fimbria. The hippocampal pyramidal cells are bordered on both sides by zones of intense AChE activity. The heavier infrapyramidal band occupies the inner half of stratum oriens. This stain deposit is particularly heavy in regio inferior, where some can also be found between the pyramidal cell bodies. The suprapyramidal band occupies the portion of stratum radiatum (regio superior) or stratum lucidum (regio inferior) nearest the pyramidal cell bodies. The remainder of these layers is notable for relative paucity of staining, especially in regio superior. In horizontal sections through the temporal hippocampus (Fig. 2F), the entire width of stratum moleculare in the area adjacent to the subiculum (*S*; Fig. 1) appears moderately stained. This zone of staining narrows to a thin band in stratum lacunosum. At the junction of regio superior and regio inferior, the zone of staining again enlarges to cover the entire stratum moleculare. Cross sections through the septal hippocampus differ only in that AChE activity in stratum moleculare is present only at the transition of regio superior to regio inferior (Fig. 2E). At all septotemporal levels, the staining in stratum moleculare at the junction of regio superior and regio inferior is continuous with a lightly stained, triangular zone which extends across stratum radiatum from stratum pyramidale. Finally, many cell bodies of the polymorphic neurons stain positively for AChE activity, particularly in stratum oriens.

In the dentate gyrus, the stain deposit forms infra- and supragranular bands. The densely stained supragranular band is clearly evident, because of its position between the unstained layer of granule cell bodies and a clear zone (commissural zone) which contains neuropil and synaptic ter-

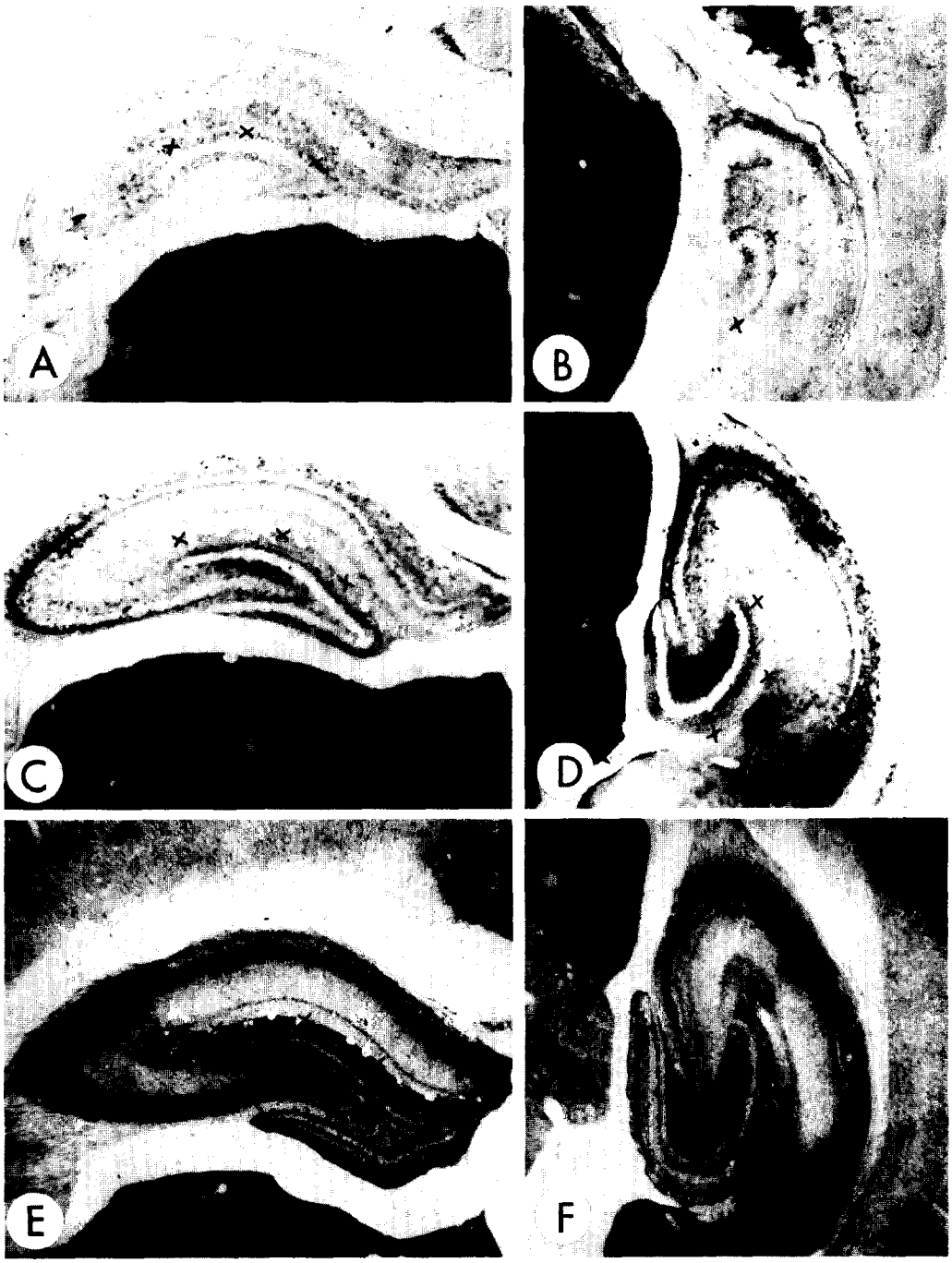

FIG. 2. Histochemical staining of AChE activity in hippocampal formation of rats at 4 days of age (A,B), 11 days of age (C,D), and maturity (E,F). Coronal sections of septal hippocampal formation on the left; horizontal sections of temporal hippocampal formation on the right. ×, Hippocampal fissure. See Fig. 1 and text for anatomical correlates. A,B, × 25; C,D, × 22; E,F, × 17.

minals associated with commissural and associational input (Blackstad, 1956; Zimmer, 1972). The somewhat more lightly stained infragranular zone merges with the well stained hilus. Many of the polymorphic cell bodies within the hilus display AChE activity. These intrinsic neurons, along with cholinergic neuropil adjacent to the scattered pyramidal cells, account for the staining of this region (Shute and Lewis, 1966). Light to moderate staining is present in the three-fourths of the molecular layer superficial to the commissural zone, especially in the septal half of the dentate gyrus. In cross sections of the temporal dentate gyrus, deposit in the middle portion of the molecular layer is noticeably heavier than in the most superficial portion. This stratification is not evident at the more septal levels.

AChE Histochemistry in Developing Hippocampal Formation

An indication of AChE activity in developing rats is present 2 days after birth in the most anterior sections of the septal end of the hippocampal formation, but activity does not become pronounced until 4 days (Fig. 2A,B). In sections through the septal end that are incubated for 24 hr (Fig. 2A), the entire area of the hippocampus and dentate gyrus not occupied by cell bodies is covered by a light diffuse deposit at 4 days. Cross sections of the septal hippocampal formation posterior to that shown in Fig. 2A stain similarly to the most dorsal sections of the temporal hippocampal formation (Fig. 2B). More intense staining in the hippocampus is largely confined to regio inferior. A moderately heavy deposit is found in stratum oriens extending from the entrance of the fimbria toward regio superior. Lighter staining can be seen in the portion of stratum lucidum which borders the pyramidal cell bodies (suprapyramidal zone). AChE-staining cell bodies are clearly evident in stratum radiatum directly opposite the fimbria.

In the external leaf of the dentate gyrus, there is an intensification of staining in the infragranular zone at its border with stratum moleculare of regio inferior. A lighter parallel intensification is found in the molecular layer just superficial to the granule cells. These areas of staining in the dentate gyrus are continuous with the light, but distinct, suprapyramidal staining in regio inferior. The molecular layer is rudimentary at 4 days and, in the internal leaf, is discernible only at the apex of the granule cell arch. In fact, at this age the dentate gyrus consists primarily of undifferentiated granule cells (Altman and Das, 1965, 1966).

The stain deposit at 4 days of age is most prominent in the more rostral sections and diminishes in the temporal direction. It is lacking in cross sections of the temporal third of the hippocampal formation. Sections from all septotemporal levels which are incubated for 3 hr show no activity.

By 6 days the pattern of AChE staining in the hippocampal formation appears more distinct, and new zones of distribution are evident (Fig. 3). The new features include a more pronounced staining of the infrapyramidal zone throughout stratum oriens in all coronal sections and to some extent in the more dorsal horizontal sections. This band extends to the subicular limit of regio superior and appears similar in all but intensity to what one sees in adult animals. In regio superior, a diffuse deposit extends from stratum pyramidale to the hippocampal fissure. Staining is most intense in the middle of this band (developing stratum lacunosum). The supragranular band is the most distinct zone of staining in the dentate gyrus at 6 days and displays the most intense precipitation of any area of the hippocampal formation. It is present only in the external leaf, where it has advanced toward the apex of the granule cell layer. Finally, AChE-staining intrinsic cell bodies become clearly visible throughout stratum oriens and the hilus of the dentate gyrus.

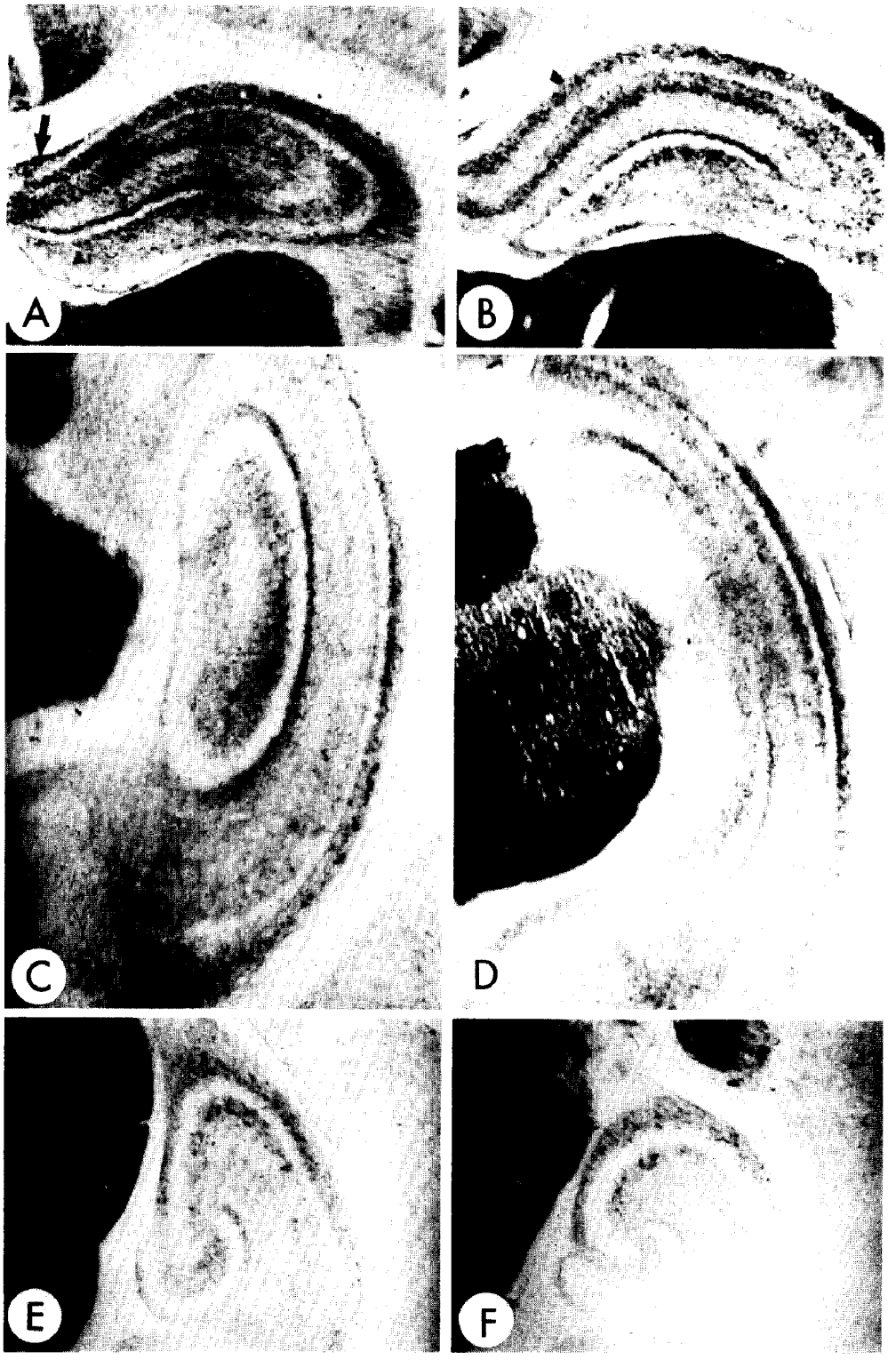

FIG. 3. Septotemporal gradient of histochemically demonstrable AChE activity illustrated by sections from a 6-day-old rat brain cut at various levels of the hippocampal formation. (A,B) Coronal sections through the septal hippocampal formation (A anterior to B). (C–F) Horizontal sections through the hippocampal formation from dorsal (C) to ventral (F). Note relative paucity of staining in the more ventral sections. Arrow denotes possible cholinergic projection via the dorsal fornix (see Discussion). × 30.

Sections cut through the hippocampal formation at 6 days illustrate most clearly the gradients of developing AChE staining. Intensity of staining is greatest in sections cut through the septal end, and it diminishes in the temporal direction. The distribution of staining within coronal sections can also be discerned in the more dorsal horizontal sections, but the most temporal horizontal sections still do not stain.

Histochemically demonstrable AChE activity increases between 6 and 11 days of age, but the laminar pattern is relatively unaltered. By 8 days the hilus of the dentate gyrus becomes heavily stained. This intense neuropil staining tends to obscure the cell bodies of polymorphic neurons which display AChE activity.

The hippocampus and dentate gyrus of 11-day-old rats stain similarly to these regions in the adult, when sections incubated for 24 hr are viewed (Fig. 2C,D). All the layers of staining are identifiable throughout the septotemporal length of the hippocampal formation. The suprapyramidal band first becomes darkly stained at this age. In the temporal part of the hippocampal formation, the deposit in stratum moleculare adjacent to the subiculum is distinguishable, as is the thin band in stratum lacunosum. The infrapyramidal band of staining in regio inferior extends toward the internal leaf of the dentate gyrus, where it appears continuous with the developing supragranular band. This latter zone of staining first becomes distinctly visible in the internal leaf at 11 days. In cross sections through the middle third of the hippocampal formation, the completion of the arch of the supragranular band can be seen to take place by the joining of its external and internal divisions in the internal leaf. In both leaves, a much less dense deposit in the more superficial part of the developing molecular layer is continuous with the supragranular band.

At later ages the intensity of AChE staining increases and the zones of staining become more sharply delineated. At 16 days of age the unstained commissural zone of the molecular layer of the dentate gyrus first becomes detectable, although it is not clearly visible until 25 days. Development of the commissural zone separates the intense supragranular band from the more lightly stained superficial three-fourths of the molecular layer.

DISCUSSION

Histochemical localization of AChE activity in the developing CNS may define the pattern of growth of cholinergic axons, when these fibers can be segregated from other sources of enzyme activity. The hippocampal formation of the rat brain meets this criterion. The development of cholinergic innervation in this region, as detected by AChE histochemistry, occurs almost entirely after birth. The ordered developmental pattern is differentiated both spatially and temporally. Within each septotemporal segment of the hippocampal formation, a characteristic sequence of events is observed, which suggests development of four distinct fiber projections. Histochemically demonstrable AChE activity first appears in hippocampus regio inferior (*A;* Fig. 4). Stained fibers enter stratum oriens from the fimbria, cross the layer of pyramidal cell bodies and project in the suprapyramidal zone toward the dentate gyrus. Upon reaching the external

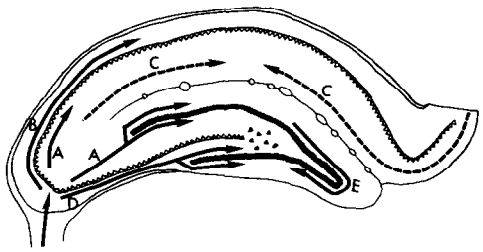

FIG. 4. Schematic presentation of pathways followed by developing cholinergic fibers in the hippocampal formation. Letters denote the order in which each pathway becomes histochemically demonstrable. Broken lines indicate pathways that may account for the staining in hippocampus regio superior. △, Pyramidal cells. ●, Granule cells.

leaf of the dentate gyrus, the projection bifurcates to form infra- and supragranular bands of staining. The infrapyramidal band in hippocampus regio superior (*B*) is the second zone of staining to become evident and the first to appear completely developed. Within 2 days of their emergence from the fimbria, the stained fibers in stratum oriens can be visualized throughout regio superior. Next, a suprapyramidal region of diffuse staining appears in regio superior (*C*), filling the area from stratum pyramidale to the hippocampal fissure. The course of the fibers responsible for this staining is not clear. Again within a 2-day period the suprapyramidal band and the staining of stratum lacunosum and stratum moleculare become separate and distinct. During the same period the infrapyramidal band in regio inferior appears (*D*). At mid-septotemporal levels, this latter projection can be seen to split as it reaches the dentate gyrus, forming infra- and supragranular bands of staining in the internal leaf. Finally, the two parts of the developing supragranular band join in the internal leaf (*E*).

Examination of cross sections from rats less than 11 days of age through all levels of the hippocampal formation reveals a septotemporal gradient in the development of AChE staining. The relative intensity of stain deposit and the degree of maturation in the laminar pattern decrease in the temporal direction. Cell bodies in the medial septal nucleus and nucleus of the diagonal band stain heavily by 2 days after birth, but the hippocampal formation is essentially unstained at this age. The development of AChE staining in the hippocampal formation commences about 4 days after birth at its septal end. In the following week progressively more temporal segments demonstrate activity. This septotemporal gradient is most clearly illustrated in stained sections from the 6-day-old animal (Fig. 3). By 11 days AChE-dependent histochemical staining can be demonstrated in all zones of the rat hippocampal formation which display this staining in adult animals. Each of the developmental gradients we have described can also be detected at somewhat later developmental stages by the use of incubation times much shorter than 24 hr.

Since the histochemically demonstrable AChE activity of the rat hippocampal formation is predominantly associated with input from the septum, our data probably illustrate the growth of this fiber tract into its target areas. Thus the gradients in staining we have observed most likely reflect the pattern of developing septohippocampal innervation. Indeed the temporal sequence we have described—staining first of cell bodies in the septum, then axons which pass through the fimbria and finally discrete zones of increasingly temporal segments of the hippocampal formation—is entirely consistent with this view. Nevertheless alternative explanations cannot be excluded. For example, we may have visualized development of histochemically demonstrable activity along fibers already in place. The view that we have demonstrated connections in the process of development depends on the assumption that septohippocampal fibers display AChE activity at all stages of their growth and development. Tennyson and Brzin (1970) have demonstrated AChE activity in the growing central process of embryonic dorsal root ganglion neurons from the time these fibers enter the spinal cord. However, the dorsal root axons are noncholinergic. Since no anatomical data directly relevant to this hypothesis are presently available, the inferences we have drawn concerning development of septohippocampal axons must be viewed as tentative.

Assuming that we have simply visualized the development of AChE activity within preformed connections rather than that within growing septohippocampal fibers, then these fibers may enter the hippocampal formation earlier than 4 days

after birth. Even if we have demonstrated the growing axons, our histochemical technique may have been insufficiently sensitive to detect the earliest fiber growth. Our data therefore provide more reliable information about the pattern of development than about the precise ages when developmental events occur. However, our histochemical data strongly suggest that at least some septohippocampal axons have invaded the hippocampal formation by 4 days after birth and have reached all zones which they eventually will innervate by 11 days.

Although the developmental pattern of staining reported here is essentially consistent with what one might expect on the basis of previously described septohippocampal projections (Raisman, 1966; Mosko *et al.*, 1973), anatomical techniques have not yet related AChE activity in stratum radiatum, stratum lacunosum, and stratum moleculare of hippocampus regio superior to an identifiable pathway. Lewis and Shute (1967) have described a projection from the septum which passes to these areas through the dorsal fornix. There was a suggestion of such a projection to the septal end of the hippocampus in the present study (*C*; Fig. 4). AChE activity was noted in the dorsal fornix, and the staining appeared to be continuous with that in regio superior (see, for example, Fig. 3A). In addition, much of the AChE staining of stratum moleculare adjacent to the subiculum in the temporal part of the hippocampus appears not to be associated with septohippocampal connections, since this staining is not diminished by transection of the fimbria (Storm-Mathisen, 1972). The present study provides no information on the origin of this enzyme activity, although we have found it to be demonstrable only rather late in the developmental sequence. Further research is necessary to resolve these issues.

Some correlations can be drawn between our results and neurogenesis in the hippocampal formation. Autoradiographic and various histochemical techniques indicate neuronal differentiation is complete in the hippocampus at birth, but the granule cells of the dentate gyrus continue to differentiate past the third postnatal week (Altman and Das, 1965, 1966). The attainment of the mature histochemical pattern in the hippocampus somewhat earlier than in the dentate gyrus is consistent with the earlier development of the pyramidal cells. Histochemical staining for AChE activity in the dentate gyrus develops initially in the external leaf, where the earliest differentiated granule cells appear and where synaptogenesis is most advanced during the first 11 days after birth (Crain *et al.*, 1973). Thus cholinergic innervation apparently develops in relation to the neurons of the hippocampal formation in the order of developmental age.

AChE histochemistry provides a simple method by which the ontogenic development of cholinergic axons can be traced, but this method alone provides little additional information. The limitations and sources of error involved in histochemical techniques have been pointed out (Gerebtzoff, 1959; Glenner, 1965), and specifically those encountered in staining for AChE activity (Malmgren and Sylvén, 1955; Lewis, 1961; Brzin *et al.*, 1966; Karczmar, 1969; Friedenberg and Seligman, 1972). Owing to the differential sensitivity of various isoenzymes to fixation and other variables which affect the copper–thiocholine reaction, the intensity of histochemical deposit bears no straightforward quantitative relationship to the total activity of AChE in the tissue (Eränkö *et al.*, 1964; Koelle *et al.*, 1970). Therefore biochemical studies are necessary to define the development of enzyme activity. In addition, AChE histochemistry at the light microscope level provides no information on cholinergic synaptogenesis. To obtain information on these aspects of development in central cholinergic systems, we have studied the ontogenic development of ChAc and AChE activities in the hip-

pocampal region. In the following paper, we coordinate our biochemical findings with these histochemical data to formulate a model of septohippocampal development (Nadler *et al.*, 1974).

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