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Clumping of acetylcholinesterase activity in the developing striatum of the human fetus and young infant

(basal ganglia/striatum/acetylcholine/brain development/histochemical compartments)

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ABSTRACT **The distribution of acetylcholinesterase activity (acetylcholine acetylhydrolase, EC 3.1.1.7) in the developing human striatum has been studied histochemically in autopsy material from fetal brains of estimated gestational ages 16–29 weeks (180–1000 g) and from the brains of infants 2 days to 4 months old. The findings provide evidence that striatal acetylcholinesterase activity in the human fetus and neonate is concentrated in a network of densely stained zones that appear in cross section as variably shaped 0.5- to 1.0-mm-wide dark patches distributed in a lighter background matrix. An orderly arrangement of the patches seemed well established in the putamen by the 16th–18th week of gestation (crown–rump length 14–15 cm) but in the caudate nucleus the pattern was still not fully elaborated at these ages. The lateroventral part of the caput was mainly dark and its rostromedial margin, though rich early on in pseudocholinesterase activity, was still without strong acetylcholinesterase activity as late as 20–21 weeks (crown–rump length 16–20 cm). The ganglionic eminence at these ages was sharply divided into a dorsal part with little cholinesterase activity and a ventral part with a high content of pseudocholinesterase. Little information was gained about striatal development during late stages of gestation, but in three 5- to 7-month fetal specimens not only dark patches but also patches with dark perimeters and pale centers were present. Clumping of cholinesterase activity appeared at birth and up to the third month of postnatal life. The patches in both caudate nucleus and putamen were dark and of fairly uniform tint in the striatum of the young infant and the matrix staining was darker than in the fetuses. Around the fourth postnatal month hints of the mature pattern were present, with zones of low cholinesterase activity appearing against a dark background in the caudate nucleus and (in one case) a nearly homogeneous staining pattern appearing in the putamen.**

The mature striatum is divided into distinct histochemical compartments in cat, monkey, and man (1, 2). With conventional acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) staining methods, 0.5- to 1.0-mm-wide zones of low cholinesterase activity can be detected in frontal sections through the caudate nucleus in all three species, and serial-section reconstructions suggest that the pale zones form parts of highly branched three-dimensional labyrinths embedded in a matrix of much higher cholinesterase activity. In the adult cat this histochemical organization appears to be related to a comparably complex compartmentalization of striatal afferent and efferent connections visualized by anterograde and retrograde tracer methods (3, 4).

During the fetal and neonatal period, striking changes in the disposition of striatal cholinesterase have been demonstrated in experimental animals (2, 5–9). In the fetal cat, we have found a progressive and apparently systematic development in which early nearly homogeneous dark and light fields give way to a pattern of dark patches on a light background, the adult pattern of light patches on a dark matrix appearing only at about one month after birth (1, 2, 9). We report here preliminary observations on the pattern of cholinesterase activity in the striatum of the human fetus and young infant. The main findings are: First, that cholinesterase activity is concentrated in densely stained patches in both the caudate nucleus and the putamen of the human fetus and that the arrangement of these patches appears highly ordered, especially in the putamen. Second, dense aggregations of cholinesterase activity, although rarely seen in the adult human material so far studied, are visible in the striatum of the human infant at least for 3–4 months after birth. Third, the redistribution of striatal cholinesterase appears to be part of a widespread set of changes in cholinesterase activity in the developing human forebrain, involving the so-called nonspecific as well as the specific cholinesterases. Finally, at least at the stages of embryonic development studied, cholinesterase activity appears not only in the striatal parenchyma but also in restricted parts of the germinal matrix of the striatum, the ganglionic eminence.

MATERIALS AND METHODS

Postmortem striatal tissue was obtained at autopsy between November 1978 and September 1979 from the brains of 12 human fetuses (7 male) and 6 young infants (5 male). Postnatal ages of the infants ranged from 2 days to 4 months; death was attributed to the sudden infant death syndrome in all but the youngest child (female), whose autopsy diagnosis was cardiac hypoplasia. Ten fetuses were delivered after prostaglandin-induced labor at gestational ages estimated at autopsy to be 16–21 weeks. One female and one male fetus, delivered prematurely at 22 and 27–29 weeks estimated gestational age as a result of spontaneous precipitous labor, survived for 5 hr and 4 days, respectively.

All tissue blocks but one were fixed by immersion in 10% formol/saline solutions at times beginning 1.5–4 hr after death following induced labors, 12–24 hr postmortem following spontaneous labors, and 5–36 hr following the estimated time of death for the infants. Immersion–fixation was continued for up to 2 days, and 5–10% sucrose was usually added to the solutions after a few hours. Blocks were cut at 75 μm on a freezing microtome in the frontal plane except for one (from a 20-week fetus), which was cut in near-sagittal plane roughly tangent to the lateral face of the putamen. The unfixed tissue block, from a 19-week fetus, was frozen 2 hr postmortem and cut on a cryostat.

Sections from all specimens were stained by modifications of the copper thiocholine method (10). Most frequently the Geneser-Jensen and Blackstad protocol (11) was followed for incubation; sections were then washed in 0.1 M phosphate buffer (pH 7.4), mounted, and developed on the slide either

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Abbreviations: BuSCho, butyrylthiocholine; AcSCho, acetylthiocholine.

with potassium ferricyanide (12) or with sodium sulfite and silver nitrate for silver intensification (13). Incubation times ranged from 2 hr to 2 days. Sections from some blocks were stained by the copper ferricyanide method of Karnovsky and Roots (14). Incubations were routinely carried out in the presence of the pseudocholinesterase inhibitor ethopropazine (Parsidol, Warner–Chilcott). Sections from six brains were treated as controls for the specificity of the esterase stain. Procedures included (*i*) omission of substrate, (*ii*) substitution of butyrylthiocholine iodide (BuSCho) for acetylthiocholine iodide (AcSCho), (*iii*) omission of ethopropazine from the AcSCho and BuSCho solutions, (*iv*) incubation with AcSCho and BuSCho in the presence of the cholinesterase inhibitor eserine sulfate (0.1 mM), and (*v*) incubation with AcSCho and BuSCho in the presence of the acetylcholinesterase inhibitor BW284c51 (Burroughs Wellcome) in final concentrations of 10^{-3}, 10^{-5}, and 10^{-7} M. In 13 cases sections spaced at regular intervals were stained for Nissl substance.

RESULTS AND DISCUSSION

Fetal Striatum. Densely stained patches of acetylthiocholinesterase activity appeared in the striatum at all fetal ages studied. In cross sections through the putamen (Fig. 1 *A–C*) the patches were about 200–600 μm wide, often had rather angular profiles, and tended to be elongated dorsoventrally. The borders of the patches were fairly sharp, especially ventrally, where the patches were darkest. Sometimes the background stain immediately around the patches was pale. In many transverse sections the dark patches gave the impression of streaming up from the ventral walls of the putamen, especially the lateral

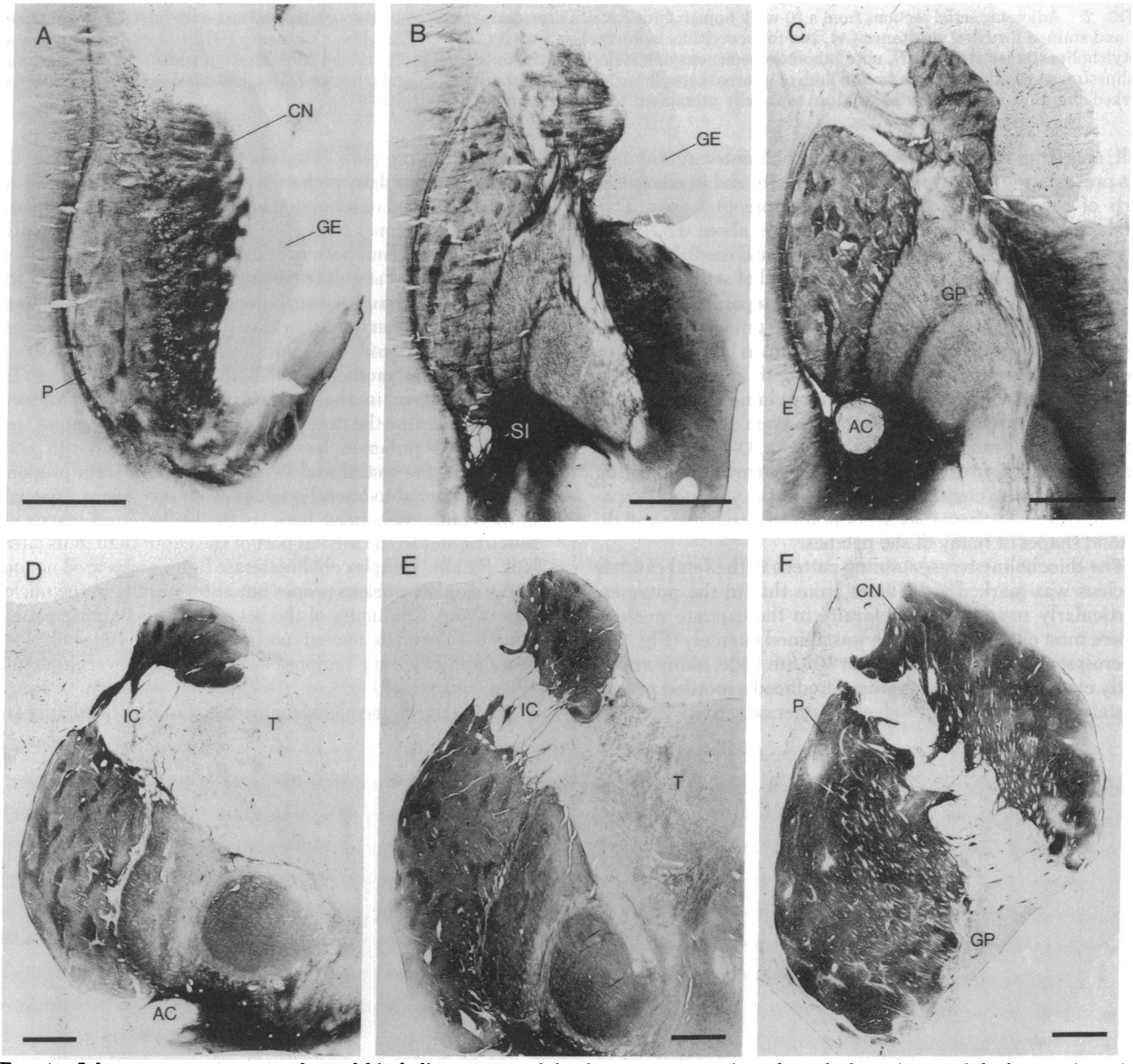

FIG. 1. Inhomogeneous patterns of acetylthiocholinesterase staining in transverse sections through the striatum of the human fetus (*A* and *B*, 17–18 weeks; *C*, 20–21 weeks), infant (*D*, 1.5 months; *E*, 3 months), and adult (*F*, 74 years). In *A*, note contrast between caudate nucleus (CN) and putamen (P); dark fibers in internal capsule (IC) between them can be traced to mediodorsal nucleus (see *B*, *C*, and Fig. 3*C*). Ganglionic eminence (GE) is pale. *B* and *C* show dense cholinesterase activity in substantia innominata (SI) and streams of cholinesterase rising from SI into external capsule (*E*), putamen, and medullary laminae of globus pallidus (GP). AC, anterior commissure; T, thalamus. For each photograph, bar marks 3 mm.

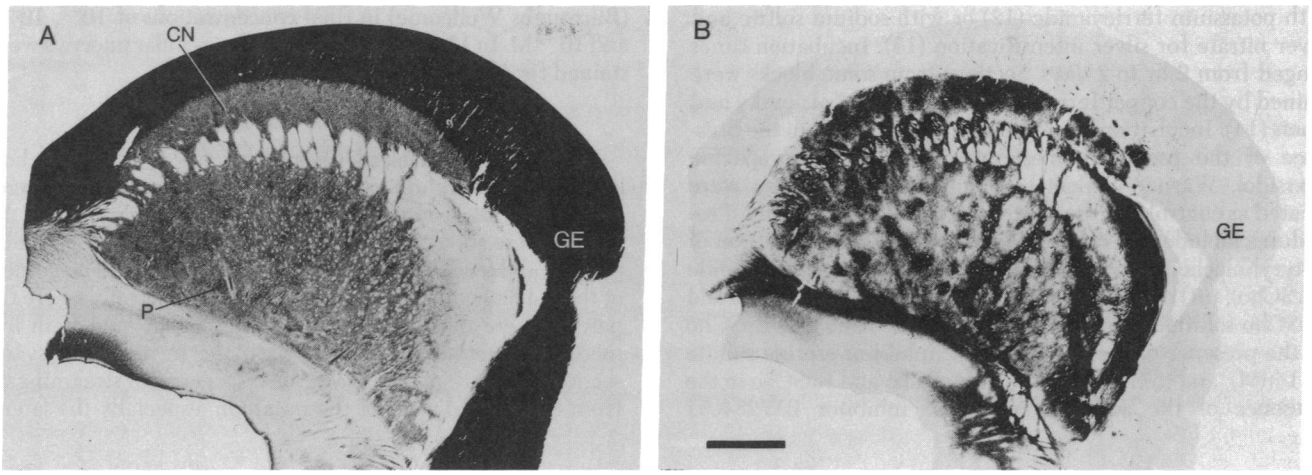

FIG. 2. Adjoining serial sections from a 20-week human fetus cut in a near-parasagittal plane through the caudate nucleus (CN) and putamen (P) and stained for Nissl substance (*A*), and for acetylthiocholinesterase activity (*B*). The ganglionic eminence (GE) is dark in Nissl, pale in acetylcholinesterase stain. In *B*, note inhomogeneous enzyme stain in striatum; also densely stained fiber-stratum ventral to putamen, and cholinesterase-positive fibers in frontal limb of internal capsule (separating caudate nucleus and putamen). Matched Nissl section (*A*) illustrates marked clumping of cells in fetal striatum especially prominent here in putamen. Bar indicates 3 mm.

wall, and from its foot, where intense cholinesterase staining was present around the anterior commissure and in adjoining parts of the substantia innominata and preoptic area. The patches were usually arranged along curves about 0.5–1 mm apart running roughly parallel to the lateral and medial margins of the putamen. An elongated broken band of stain appeared in most sections along the lateral margin, separated from the dark external capsule by a narrow (*ca.* 100 μm wide) gap. Occasionally, streamers of cholinesterase stain as long as 3–5 mm appeared in the core of the putamen, both in the transverse plane and in the plane tangent to the putamen's lateral face (Fig. 2*B*). More often, patches formed broken chains that were linked by side branches or forks where H-, U-, and Y-shaped cholinesterase figures appeared. Much narrower connecting bridges were also common, especially in the youngest preparations (Fig. 1*A*); these may account for the triangular and diamond shapes of many of the patches.

The thiocholinesterase staining pattern in the fetal caudate nucleus was markedly different from that in the putamen, particularly rostrally and ventrally in the caudate nucleus, where most of the parenchyma was stained intensely (Fig. 1*A*). In cross sections, dark zones about 300 μm wide, many apparently confluent with one another, produced a mottled pattern of staining and fingers of high cholinesterase activity stretched medially, leaving pale islands between them and giving the border of the caudate nucleus next to the pale ganglionic eminence a scalloped or lobulated appearance. Deeper in the nucleus the dark zones tended to stretch obliquely toward the internal capsule and both here and more medially they were sometimes spaced at quite regular 300- to 400-μm intervals, and sometimes were part of a turbulent mixture of light and dark. Overall, the patches tended to have more rounded profiles, were more closely spaced, and more frequently merged with one another in the caudate nucleus than in the putamen. The staining pattern in the caudate nucleus was not uniform, however, because the dorsal and caudal parts of the caput were most like the putamen, having a high ratio of matrix to patch, whereas in the rostral and ventral parts dark stain predominated. A medial-to-lateral gradient was also evident, especially in the young specimens, because the background matrix was much lighter in the medial part of the caput than in its lateral half. Finally, complex cholinesterase figures appeared not only in the caudate nucleus proper but also ventrally in the nucleus accumbens. Continuity of the heterogeneous staining patterns from both caudate nucleus and putamen into the ventral striatum and adjoining regions could be seen in every specimen (Figs. 1*A* and 3*A*).

Exceptions to the clumping pattern just described appeared

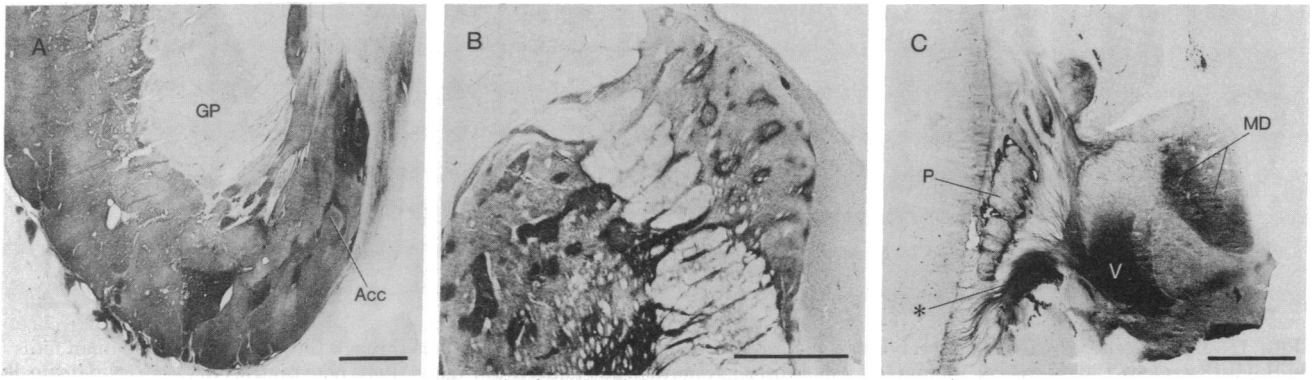

FIG. 3. (*A*) Inhomogeneous cholinesterase staining in ventral striatum of 3-month-old human infant. Acc, nucleus accumbens. (*B*) Ring-and-hollow pattern in cholinesterase patches of the caudate nucleus (but not putamen) in 26- to 29-week fetus. (*C*) Acetylthiocholinesterase section showing densely stained fiber band (asterisk) enroute to substantia innominata and dense staining in mediodorsal (MD) and ventral (V) thalamic nuclei. P, putamen. For each photograph, bar marks 3 mm.

in three fetal brains, in each of which patches in particular parts of the caudate nucleus and putamen had densely stained outer rings surrounding weakly stained centers, as though the stain in the inner parts of the patches had faded. In one brain (27–29 weeks, Fig. 3*B*) virtually all of the patches in the caudate nucleus took this form, though in the putamen the patches were uniformly dark. In the second specimen (21 weeks), light-centered ring-formations appeared in the dorsal part of the putamen, but not elsewhere. In the third brain (22 weeks), patches with dense perimeters and pale interiors were present in the pericapsular striatum of both the caudate nucleus and putamen. It is not yet clear whether these striking ring-and-hollow figures represent abnormalities, transition stages in the patterning of cholinesterase activity, or artifacts. It should be stressed, however, that they were found in the three oldest fetal specimens available for study (21, 22, and 27–29 weeks), that they appeared consistently from section to section in restricted parts of the striatum in each brain, and that in one of the specimens (21 weeks) fixation was begun within 2 hr after death.

Striatum of the Neonate and Young Infant. During the neonatal period, clumping of cholinesterase activity in the striatum was pronounced and the main patterns of distribution were similar at 2 days and 1.5, 2.5, and 3 months. The arrangement in the anterior part of the caudate nucleus is illustrated for a 3-month-old infant in Fig. 4*A*, which shows rounded 0.5- to 0.8-mm wide dark profiles spaced at fairly regular 1- to 1.5-mm intervals throughout the nucleus. In the putamen (Fig. 1*E*) the clumps had about the same dimensions and interspaces but tended to be longer and more highly branched than those in the caudate nucleus. Overall, the clumping pattern in the putamen was surprisingly like that seen in the fetal brains, with the patches arranged as though they lay in curved lamellae, but the individual patches were noticeably less angular than in the fetal brains and the background stain was darker. Often in the infant putamen, the patches were set off from the background matrix by thin (*ca.* 100 μm wide) pale sheaths that bordered them along one side. These weakly stained septa were rarely found in the caudate nucleus. More often in the infants than in the fetuses, the regular arrangement of clumps could be traced from the putamen through cell bridges into the dorsal part of the caudate nucleus. Here and elsewhere in the caput of the caudate nucleus the clumps tended to be more regular in size and shape, and the background matrix more uniform, than in the fetuses.

It was only in the two oldest infants (3.5 and 4 months) that the distribution of cholinesterase activity began to take on characteristics of the adult pattern and, at the same time, began to lose the clumping pattern typical of the fetus. In both infants, 0.5- to 1-mm-wide zones of low cholinesterase activity appeared in the caudate nucleus, especially ventrally, and formed pale bands stretching obliquely away from the ventricular face into the adjoining ventral striatum (Fig. 4*B*). In many sections, the pale zones were the main figures visible in the caudate nucleus, whereas in others both dark and pale zones appeared against an intermediate background stain. Crisply defined dark patches no longer predominated as they did in the younger specimens. Finally, though patches typical of the other young infants could be detected in the putamen of one of these two brains, there were scarcely any such patches visible in the putamen of the other case (3.5 months old). Occasionally, pale sheaths partly enclosed small almond-shaped zones of the putamen as in the younger specimens, but the tint of the zones was virtually identical to the color of the matrix so that the main impression (as in the adult putamen) was of homogeneity.

Control Experiments. Control procedures were carried out on sections from the fetal material only. All produced marked changes in the histology (Fig. 5). Staining was abolished by the addition of eserine and sections were unstained (or nearly so) when incubated with BuSCho in the presence of ethopropazine or with AcSCho in the presence of ethopropazine and 1 mM BW284c51. These findings indicate that striatal staining in the normal series was attributable to cholinesterase activity. The BuSCho controls further suggested that both pseudocholinesterase (acylcholine acylhydrolase, EC 3.1.1.8) and acetylcholinesterase were present in the striatum of the fetal brains,

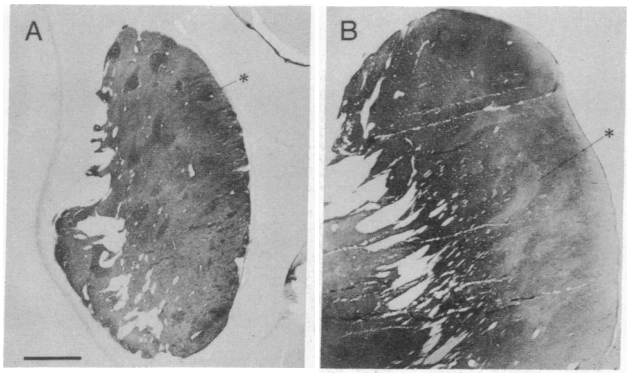

FIG. 4. Sections through anterior part of caudate nucleus stained for acetylthiocholinesterase from 3-month-old (*A*) and 3.5-month-old (*B*) human infants. Asterisks mark enzyme-rich patches in the 3-month-old and enzyme-poor zones in the 3.5-month-old. Bar indicates 3 mm.

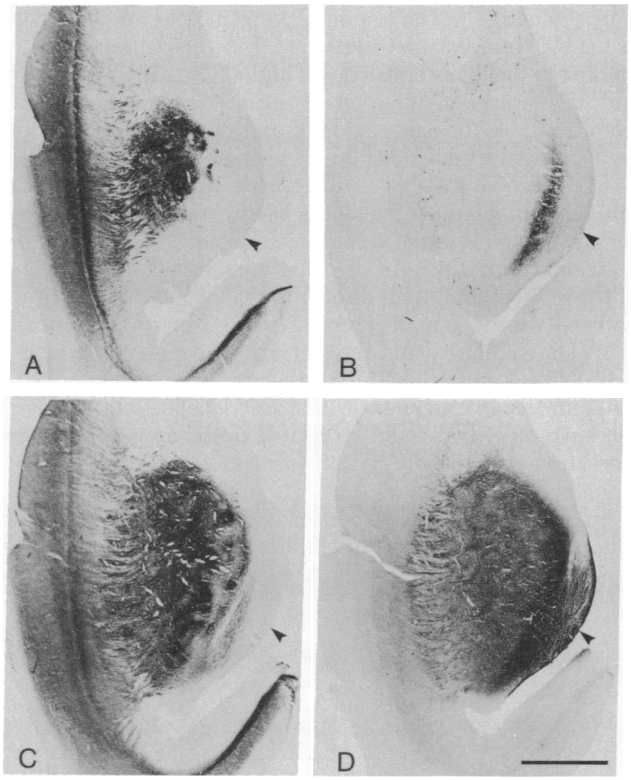

FIG. 5. Results of control procedures (see text) for testing specificity of cholinesterase stain (*B* and *C*) compared with stain by normal protocol (*A*) in closely spaced sections from 21-week human fetus. Geneser-Jensen and Blackstad incubation: (*A*) with ethopropazine; (*B*) without ethopropazine, in presence of 1 mM BW284c51; (*C*) without ethopropazine; (*D*) with BuSCho substrate, in absence of ethopropazine. In each photograph, arrow touches edge of ganglionic eminence. Bar indicates 3 mm.

but that their distributions were in part complementary. Striking findings with BuSCho incubation were (*i*) dense staining of the ventral half to two-thirds of the ganglionic eminence (unstained or very weakly stained in the normal series); (*ii*) dark staining of the medial rim of the caudate nucleus (pale in normal series from the younger specimens); (*iii*) murky but not fully homogeneous staining of much of the rest of the matrix of the caudate nucleus and putamen (weakly or moderately stained in the normal series); and (*iv*) loss of the band of intense cholinesterase activity appearing under the cortical plate in the normal series and loss of most of the staining of the external capsule. Staining with the BuSCho substrate was as intense in the presence of BW284c51 as without this acetylcholinesterase inhibitor. These observations, illustrated in part in Fig. 5, suggest that pseudocholinesterase activity was concentrated in the ventral part of the ganglionic eminence and adjoining medial margin of the caudate nucleus and that acetylcholinesterase activity was low in these regions. By contrast, pseudocholinesterase activity appeared to be more diffusely distributed in the rest of the caudate nucleus and in the putamen, regions where acetylcholinesterase appeared preferentially in patches.

Comment. The present findings suggest that during a developmental period extending into infancy a major reorganization occurs in the distribution of cholinesterase activity in the caudate nucleus and putamen of the human brain. Both specific and nonspecific cholinesterases appear to be involved, with acetylcholinesterase being concentrated in clumps and bands in the immature striatum whereas pseudocholinesterases are especially though not exclusively associated with striatal tissue closest to the germinal matrix and with the ventral part of the matrix zone itself. The origin of acetylcholinesterase activity in the striatum was not identified, but the densest focus of staining in the telencephalon was in the substantia innominata and striatal acetylcholinesterase activity seemed to emanate from this sublenticular region rather than from the ganglionic eminence. A broad band of acetylcholinesterase positivity could be traced caudally and medially from the substantia innominata into the subthalamus (Fig. 3C) whereas a much thinner band remained lateral to the cerebral peduncle. These tracts were lost caudally, but it is striking that the subthalamic trajectory of the main medial tract resembles that of the catecholamine pathway described in the human fetus by Nobin and Björklund (15). This similarity and the fact that catecholamine fluorescence, like acetylcholinesterase, is clumped in the human fetal striatum (15, 16), suggests that the acetylcholinesterase patterns we have described could be related to the dopaminergic innervation of the striatum (2, 8) or be subject to similar developmental constraints.

In considering the developmental implications of the present findings, it is important to emphasize that Kostović and his colleagues (17, 18) have recently discovered maturational shifts in the cholinesterase activity in the cerebral cortex of the human fetus, including an early columnar distribution in the frontal cortex apparently as highly ordered as the pattern here described for the cholinesterase-positive zones in the fetal putamen. Such widespread changes in the distribution of acetylcholinesterase during development support the view that this enzyme may be a signature of particular stages in the maturational history of central nervous connections (7) as well as a marker for histochemically distinct pathways in the adult human brain.

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1. Graybiel, A. M. & Ragsdale, C. W., Jr. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 5723–5726.
2. Graybiel, A. M. & Ragsdale, C. W., Jr. (1979) *Prog. Brain Res.* **51,** 239–283.
3. Graybiel, A. M., Ragsdale, C. W., Jr. & Moon Edley, S. (1979) *Exp. Brain Res.* **34,** 189–195.
4. Ragsdale, C. W., Jr. & Graybiel, A. M. (1979) *Neurosci. Abstr.* **5,** 78.
5. Krnjević, K. & Silver, A. (1965) *J. Anat.* **99,** 711–759.
6. Krnjević, K. & Silver, A. (1966) *J. Anat.* **100,** 63–89.
7. Silver, A. (1971) *Prog. Brain Res.* **34,** 345–355.
8. Butcher, L. L. & Hodge, G. K. (1976) *Brain Res.* **106,** 223–240.
9. Ragsdale, C. W., Jr. & Graybiel, A. M. (1979) *Neurosci. Lett.*, Suppl. 3, S26.
10. Koelle, G. B. & Friedenwald, J. B. (1949) *Proc. Soc. Exp. Biol. Med.* **70,** 617–622.
11. Geneser-Jensen, F. A. & Blackstad, T. W. (1971) *Z. Zellforsch. Mikrosk. Anat.* **114,** 460–481.
12. Mesulam, M.-M. & Van Hoesen, G. W. (1976) *Brain Res.* **109,** 152–157.
13. Hardy, H., Heimer, L., Switzer, R. & Watkins, D. (1976) *Neurosci. Lett.* **3,** 1–5.
14. Karnovsky, M. J. & Roots, L. (1964) *J. Histochem. Cytochem.* **12,** 219–221.
15. Nobin, A. & Björklund, A. (1973) *Acta Physiol. Scand. Suppl.* **388,** 1–40.
16. Olson, L., Boréus, L. O. & Seiger, Å. (1973) *Z. Anat. Entwickl.-Gesch.* **139,** 259–282.
17. Kostović, I., Kelović, Z., Krmpotić-Nemanić, J. & Kračun, I. (1979) *Anat. Rec.* **193,** 591–592.
18. Kostović, I. (1979) *Neurosci. Lett.*, Suppl. 3, S22.