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Short Communications

Electron microscopic localization of acetylcholinesterase in the dentate gyrus of young and adult rats

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Acetylcholinesterase (AChE) histochemical staining occurred in neurons of the dentate gyrus at the day of birth and steadily increased in intensity and distribution during the first 3 postnatal weeks until the adult pattern was reached. Granule cells failed to display AChE staining; however, the somata of most non-principal cells in these regions showed AChE activity. It is interesting that most hilar neurons in the dentate gyrus were AChE-positive, but molecular layer local circuit neurons and pyramidal basket cells associated with the granule cell layer did not display AChE staining. AChE reaction product was localized to the nuclear envelope and cisternae of the granular endoplasmic reticulum in the labeled neuronal somata. In addition, the neuropil in the dentate gyrus displayed AChE staining associated with membranes. The possible cholinoceptive role of the AChE somata in the hilus is discussed.

The hippocampal formation contains intrinsic acetylcholinesterase (AChE) activity as well as that found within the fibers of the septohippocampal pathway. For example, Lewis and Shute[9] have shown that AChE-containing somata in the hippocampus and dentate gyrus retain their staining after transection of the cholinergic afferents in the fimbria. Also, Srebro and Mellgren[19] have shown that large numbers of hilar neurons of the dentate gyrus contain AChE reaction product 8 h after lesions of the septohippocampal pathway. In corroboration of the histochemical findings, Storm-Mathisen[20] has shown that about 20% of the total AChE activity in the hippocampus remains after destruction of this well-characterized cholinergic septohippocampal pathway. Thus, anatomical and biochemical data indicate a significant intrinsic population of AChE-containing cells that are known to be non-principal neurons of the hippocampal formation[19,21]. It is surprising that 20% of the AChE activity appears to originate from a group of neurons that comprises less than 1% of the total neuronal population in both the hippocampus[1] and the dentate gyrus[16]. Although these non-principal neurons have been considered to be local circuit neurons, a number of studies have shown that many of these neurons in both regions have projections to the septum or the contralateral hippocampus[3,21].

The development of AChE activity in the hippocampus has been addressed in several recent studies. Most of the attention has focused on AChE activity related to the septohippocampal projection[10,12,19]. It is known however, that AChE activity is present at birth in some hippocampal neurons[12]. Milner et al.[12] analyzed the development of AChE staining and found that it reaches the adult level by 14 days postnatally.

Since the time of these reports, we have studied the ultrastructural features of the non-granule cells of the dentate gyrus. Initially, we analyzed the well-known local circuit neurons associated with the granule cell layer, the basket cells[13]. Subsequently, mossy cells of the hilus were analyzed and they were shown

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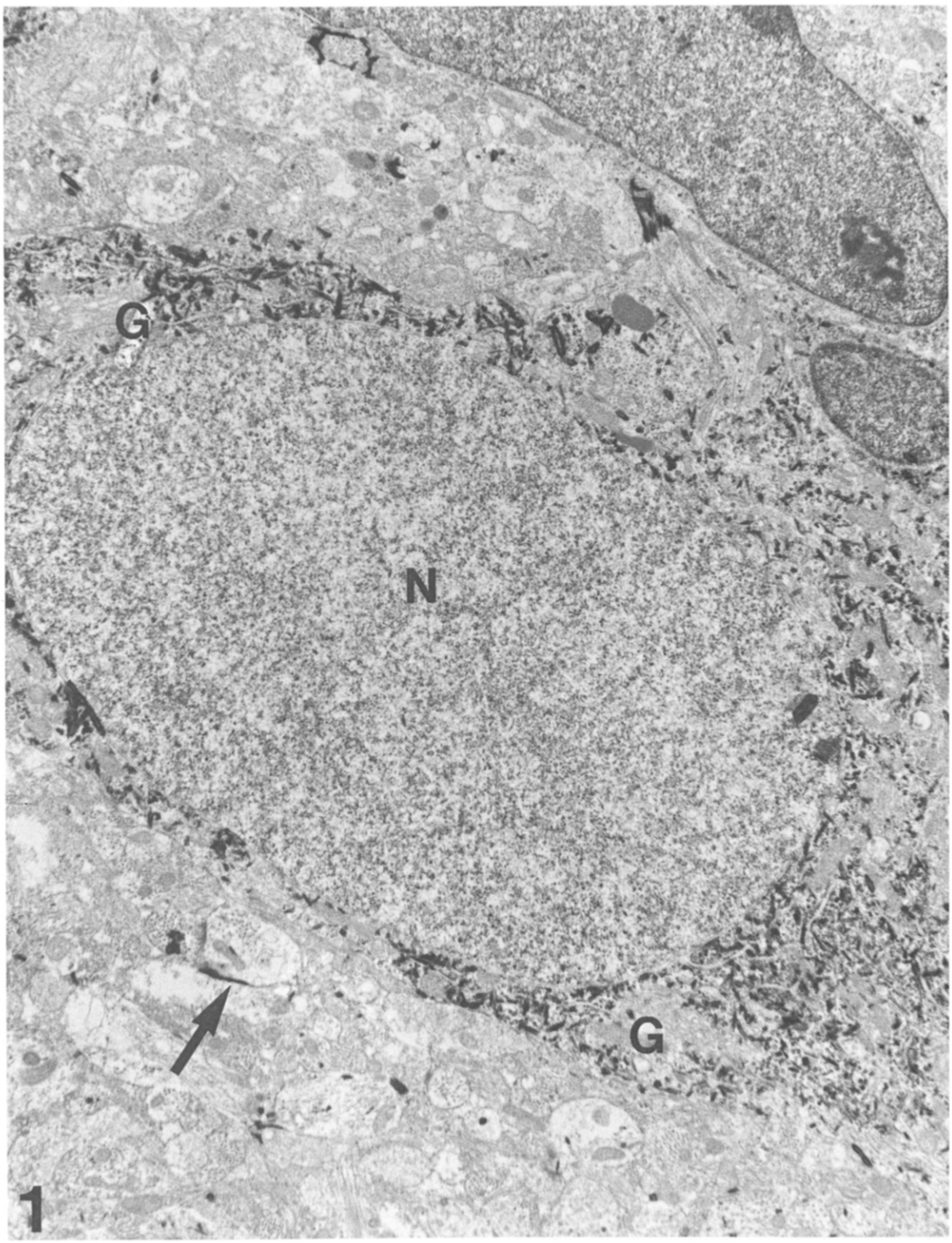


Fig. 1. Electron micrograph of a hilar neuron from an 8-day-old rat reacted for AChE. This soma shows very heavy labeling in the perikaryal cytoplasm, but the nucleus (N) is unstained. The Golgi apparatus (G) also is free of reaction product. In the neuropil, reaction product is associated with membranes of dendrites and axons (arrow). ×9000.

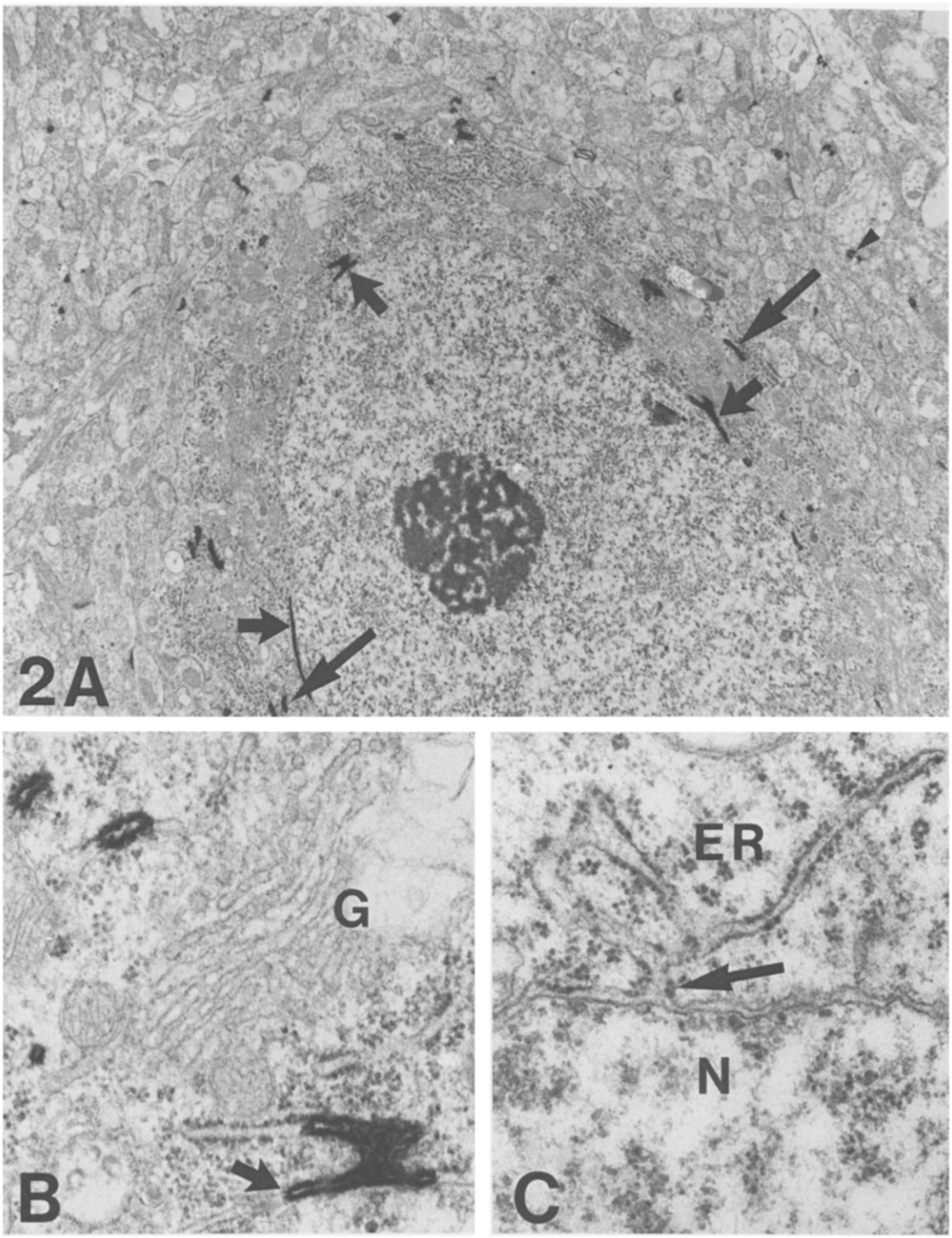
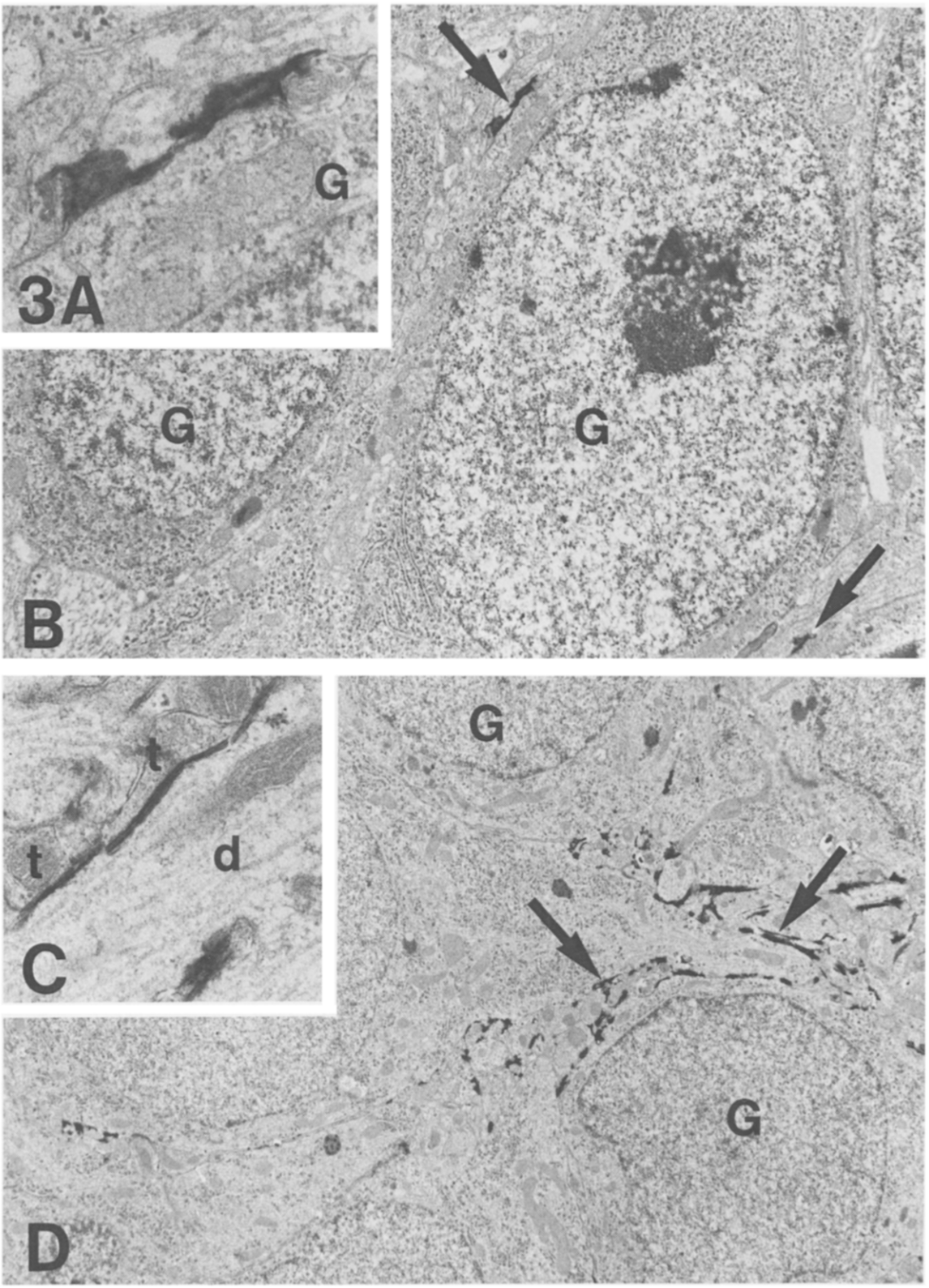


Fig. 2. A: electron micrograph of a hilar neuron from a 12-day-old rat reacted for AChE. Reaction product appears in the endoplasmic reticulum (large arrows) and the adjacent nuclear envelope (small arrows). Sparse reaction product is also present in the neuropil (arrowhead). ×8000. B: enlargement from Fig. 2A to show the AChE-staining in the endoplasmic reticulum that is probably connected with the nuclear envelope (arrow). The neighboring Golgi apparatus (G) is free of reaction product. ×50,000. C: electron micrograph of a hilar neuron from a 5-day-old rat. In this unstained neuron, the continuity (arrow) between the nuclear membrane of the cell nucleus (N) and the endoplasmic reticulum (ER) is clearly shown. ×50,000.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to have extensive mossy fiber afferents and axonal projections to the contralateral hippocampus[14]. Features of commissural projecting hilar neurons were also described[18]. With our recent advances in knowledge of hilar neurons, we investigated the AChE neurons of the dentate gyrus at the ultrastructural level to determine which class of neuron contains this enzyme and where it is located within these neurons. Also, we have determined the patterns of development of these AChE neurons.

A total of 15 animals, aged 0, 2, 4, 8, 12, 14, 25, 60 and 90 postnatal days, were used for the light and electron microscopic analyses of AChE. In addition, an extensive library of over 150 AChE-stained brains, aged 0–40 postnatal days was available for light microscopic study[15]. The method of Lewis[8] with some modifications was used[4]. Rats of 2 days and older were injected intraperitoneally with diisopropylfluorophosphate (DFP) at a dose of 2 mg/kg body weight. The rats were perfused with 2% paraformaldehyde and 0.5% glutaraldehyde in 0.12 M sodium-phosphate buffer at pH 7.3, at intervals of 3–6 h after the DFP injection. The perfusion and vibratome sectioning were carried out as described previously[4]. Tissue sections were rinsed 3 times, 15 min each, in a succinate buffer at pH 5.3 consisting of 50.0 mM succinic acid, 62.0 mM sodium sulfate, 0.1 mM calcium chloride, and 70 mM sodium hydroxide. Sections were placed at 4 °C for 30 min in a preincubation medium consisting of (in mM): cupric sulfate 6.5, glycine 32.5, succinic acid 25.0, sodium sulfate 36.6, and adjusted to pH 5.3 by addition of NaOH to a concentration of approximately 50 mM. The sections were next incubated for 4 h at 4 °C in a medium consisting of the preincubation medium and acetylthiocholine, added to reach a final concentration of 13 mM. The pseudocholinesterase inhibitor, iso-OMPA, was added to both incubation and preincubation media at a concentration of 10^{-5} M. After incubation, sections were rinsed 3 times, 15 min each in succinate buffer. The reaction product was developed and stabilized for electron microscopy by transferring sections to 3% potassium ferricyanide in succinate buffer for 15 min[4].

The AChE-reacted tissue was postfixed in 1% osmium tetroxide in 0.12 M phosphate buffer and rinsed in distilled water. Dehydration was begun in ethanol and en bloc staining was done with 1% uranyl acetate in 70% ethanol. Acetone was used to complete dehydration prior to embedding in Epon. Thin sections for electron microscopy were mounted on formvar-coated slot grids and stained for 6 min in 20% uranyl acetate in methanol.

For light microscopic studies, transverse sections stained for AChE were examined. The numbers of AChE-positive neurons were counted in these sections for each age and compared to the number from a comparable section in the adult. These counts for each age were expressed as percentages of the adult value. Similar counts of AChE-positive somata were made from thin sections with the electron microscope. These latter counts were consistent with the light microscopic results.

In agreement with Milner et al.[12], the number of AChE-containing neurons in the dentate gyrus at birth was about 30% of the adult number, whereas the hippocampus had about 50% of the adult at this time. The distribution of these labeled cells in the newborn was identical to that in the adult. By the 14th postnatal day, about 70% of the adult number of AChE neurons was found throughout the hippocampal formation. At the later ages, the number of labeled neurons increased until adult levels were reached by postnatal day 19, but the regional distribution remained the same. For the dentate gyrus, no AChE-containing neurons were ever found in the molecular or granule cell layers.

AChE reaction product could be detected in hilar neurons at all examined ages in electron microscopic preparations. At 8 days, rats displayed many densely

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Fig. 3. A: enlargement from the electron micrograph shown in Fig. 3B. The cytoplasm of a granule cell (G) is free from reaction product. Adjacent to the cell body is intense staining associated with the extracellular space and some unidentified membranes. ×48,000. B: electron micrograph of the dentate gyrus of a 12-day-old rat (reacted for AChE). The granule cells (G) are free from reaction product, but some staining is observed in association with membranes (arrows). ×9000. C: electron micrograph of a dendrite (d) in the molecular layer of the dentate gyrus in an adult rat. Reaction product is found between the dendritic surface and a few axon terminals (t) that may form synapses. ×33,000. D: electron micrograph of the granule cell layer of an adult rat stained for AChE. The granule cells (G) do not contain AChE in their cytoplasm, but reaction product is obvious in the neuropil around the granule cells (arrows). ×6000.

stained somata (Fig. 1). Reaction product was abundant in the perikaryal cytoplasm of such neurons with no staining within the nucleus. Labeled structures included portions of the nuclear envelope and many cisternae of the granular and agranular endoplasmic reticulum. Some label was also associated with polysomes. In contrast, the Golgi complex and mitochondria displayed no labeling. Some labeled hilar somata contained light staining (Fig. 2A,B). In such cases with less intense staining, AChE reaction product was found within the same structures (i.e. nuclear envelope and cisternae of granular endoplasmic reticulum) that were labeled in the densely stained somata. However, reaction product was not as widespread in the perikaryal cytoplasm in the lightly labeled somata. It is interesting to note that staining can often be found around a Golgi complex, but the cisternae and vesicles of the Golgi complex were not labeled. This finding of densely and lightly stained somata in young preparations was also observed in the adult preparations. Variation in staining intensity following DFP treatment indicates variation in the individual cells' ability to synthesize AChE. Variation in the amount or activity of the AChE message may indicate differences in the role of AChE in these cells.

In many instances, the stained regions of the nuclear envelope were associated with cisternae of the granular endoplasmic reticulum (Fig. 2B). The structure at these sites resembled sites where the granular endoplasmic reticulum was in continuity with the nuclear envelope (Fig. 2C). This localization is consistent with results from previous ultrastructural studies[4].

Neuronal somata in the granule cell layer were examined at all ages. Both basket and granule cells (Fig. 3) were free of reaction product. However, AChE reaction product was associated with processes of neurons and glia and in the extracellular space adjacent to granule cells (Fig. 3A,D). The amount of neuropil labeling was greater in the adult than in the young rat (cf. Figs. 3B and 3D). In addition, many presumed pre- and postsynaptic membranes were labeled with AChE reaction product in the adult (Fig. 3C).

A major finding of this study is the presence of AChE-positive non-principal neurons in the hippocampus and dentate gyrus from the day of birth. The adult pattern of AChE staining in fibers and neurons was reached at the 19th postnatal day. This steady development of the adult pattern is different from that observed in sensory neocortex, where AChE is expressed transiently in layer IV, the site of termination of thalamocortical afferents[15].

The AChE-stained neurons in the hippocampus are non-pyramidal and non-granule neurons. However, not all cells of these types were stained. In preparations of the dentate gyrus from all ages, the pyramidal basket cells and molecular layer local circuit neurons were unstained. The basket cells are an important GABAergic cell type that have somata beneath the granule cell layer and an axon that arborizes extensively in the inner molecular and granule cell layers to contact the somata and dendrites of granule cells[13,17]. Similarly, many of the molecular layer local circuit neurons are also GABAergic[17]. However, GABAergic and AChE-positive neurons do not comprise mutually exclusive populations of cells in the hippocampus, because Hallanger et al.[5] recently showed that GABA and AChE were co-localized in some neurons throughout the hippocampus and neocortex.

Why do some hippocampal GABAergic neurons contain AChE, whereas others do not? One possibility is that some GABAergic neurons are cholinoceptive and synthesize large amounts of AChE to hydrolyze acetylcholine secreted at afferent impinging synapses. Recent physiological data[2] suggest that some GABAergic hilar neurons are contacted by cholinergic axons. Preliminary data by Léránth and Frotscher[6] support this contention. These investigators demonstrated that cholinergic axons form synapses with both somatostatin and GABA-containing hilar neurons, which have extensive associational and commissural projections. This finding does not imply that all cholinergic synapses are formed with these non-principal neurons, because granule and pyramidal cells are also contacted by cholinergic axons. Other GABA neurons may also be contacted by cholinergic axons as well.

The problem remains as to why only certain hippocampal cells contain AChE even though many other cell types are contacted by cholinergic axons. One possibility is that the AChE-stained cells may direct the ingrowth of the cholinergic axons. For example, the AChE hilar neurons in the dentate gyrus have associational and commissural projections[21] and these

axons terminate in the same region where cholinergic axons are concentrated in the dentate gyrus[7,11]. The overlap in the distribution of these intrinsic AChE-containing fibers and the extrinsic cholinergic axons suggests some type of functional interaction, either in development or normal adult metabolism.

A final issue concerns whether the AChE cells in the hippocampus are cholinergic. Although some hippocampal intrinsic neurons stain positively for choline acetyltransferase (ChAT) and are thus presumed to be cholinergic[7,11] the two populations of ChAT- and AChE-containing cell types appear to be distinct[7,11].

The data from this correlative light and electron microscopic study are consistent with previous studies of the AChE-containing neurons[12,19]. The presence of AChE in the neuropil around the granule cells and in the protein synthetic machinery of hilar neurons indicates that AChE is probably synthesized by many hilar neurons, which have extensive associational and commissural projections to granule cells[21]. Therefore, it is likely that these AChE hilar cells provide a substantial contribution to the AChE staining found in the neuropil around the granule cells.

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