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Local circuit neurons in both the dentate gyrus and Ammon's horn establish synaptic connections with principal neurons in five day old rats: a morphological basis for inhibition in early development

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Summary. Glutamate decarboxylase (GAD)-positive and Golgi impregnated local circuit neurons of the hippocampal formation of five day old rats were examined in light and electron microscopic preparations. The ultrastructural features of these neurons were similar in both the dentate gyrus and CA1 area of Ammon's horn. Somata displayed a perikaryal cytoplasm rich in organelles but lacked organized Nissl bodies. Most nuclei showed intranuclear infoldings of varying degrees but no intranuclear sheets or rods were found. Somata and dendrites were contacted by relatively immature axon terminals that formed mainly symmetric synapses. The axons of local circuit neurons in both the dentate gyrus and Ammon's horn formed symmetric synapses with somata and dendrites of the principal neurons in these regions. Thus, both GAD-positive and Golgi-impregnated terminals of local circuit neurons were observed to form synapses with pyramidal and granule cells. These terminals were usually small and contained relatively few pleomorphic synaptic vesicles. The results show that a circuitry for inhibition is established in the 5 day old dentate gyrus and Ammon's horn, even though the local circuit neurons lack some of the typical adult ultrastructural features at this age.

Key words: Inhibition – Hippocampal formation – Development – GABAergic neurons – Rat

Introduction

Previous studies have shown that GABAergic inhibitory neurons of the dentate gyrus are generated

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prenatally whereas the majority of granule cells are formed postnatally (Amaral and Kurz 1985; Lübbers et al. 1985; Bayer 1980). In contrast, both pyramidal cells and GABAergic neurons are generated during the prenatal period in Ammon's horn. Recent physiological studies indicate that inhibition matures at different rates in the CA 1 and CA 3 regions (Swann et al. 1989; Janigro and Schwartzkroin 1988). For example, the first appearance of recurrent inhibition of pyramidal cells in CA 3 was shown to occur in 5 day old rats whereas a similar inhibition of pyramidal cells in CA 1 appeared several days later (Swann et al. 1989).

These physiological findings have not been supported by recent anatomical studies of the development of GABAergic neurons in these regions. For example, a parallel appearance of glutamate decarboxylase (GAD)- and GABA-positive neurons occurs in the dentate gyrus and in the CA 1 and CA 3 subregions of the Ammon's horn during postnatal development (Seress and Ribak 1988a). In addition, the number and light microscopic distribution of GAD-positive puncta among granule and pyramidal cells appeared to be similar in 5 day old rats (Lübbers and Frotscher 1988). Also, basket cells in the 5 day old dentate gyrus display abundant cisternae of the granular endoplasmic reticulum, the anatomical substrate for GAD synthesis (Seress and Ribak 1988b) and GAD-positive terminals establish synaptic connections with granule cells (Lübbers and Frotscher 1988). However, it was not shown what type of cell gives rise to these GAD-positive terminals and whether such cells receive afferent synaptic connections at this age. In addition, it is also not known whether the GAD-positive axon plexus is organized the same way as

that found in the adult. The present study was undertaken to determine whether:

1. Golgi-impregnated basket cells of the dentate gyrus and local circuit neurons of the Ammon's horn establish synaptic connections with principal neurons in five day old rats, 2. the ultrastructure of these cells is similar in the two regions of the hippocampal formation of young rats, 3. the ultrastructural characteristics of these cells are the same as that found in adult animals, 4. these neurons are contacted by axon terminals, and 5. GAD-positive terminals form synapses with granule and pyramidal neurons at this age.

Material and methods

Combined Golgi-electron microscopic procedure

Ten Sprague-Dawley rats (five day old, day 0 is the day of birth) were fixed under ether anesthesia by intracardiac perfusions with a single solution containing 4.0% paraformaldehyde, 1.25% glutaraldehyde and 0.002% calcium chloride in a 0.12 M phosphate buffer at pH 7.2. The perfused animals were stored overnight at 4° C, and the brains dissected out and processed for the combined Golgi-electron microscopic method according to Fairén et al. (1977).

The entire brain was rinsed and placed into a solution containing 2.0% OsO_4 and 2.4% $K_2Cr_2O_7$. Each specimen was immersed in 50 ml of this solution and kept in the dark at room temperature for 4 days. The tissue was then washed briefly in 0.75% $AgNO_3$ and stored in this solution for 3 days. Following impregnation, the brains were passed through 20, 40, 60, 80 and 100% solutions of glycerol, embedded in agar and sectioned with the Sorvall tissue chopper. Sections were cut at 80–100 μm, collected on slides, coverslipped with 100% glycerol and examined with the light microscope.

Golgi-impregnated basket cells and local circuit neurons that displayed an axonal plexus were drawn with a Zeiss microscope equipped with a drawing tube, and photographed. The sections containing these cells were hydrated through a series of glycerol solutions and placed into a chilled (4° C) 0.05% gold chloride solution for about 60 min with agitation. After three rinses in cold distilled water they were placed into cold 0.05% oxalic acid for 2 min, brought to room temperature and placed into a 1% solution of sodium thiosulphate for 1–1.5 h. The sections were then rinsed in distilled water and examined with the microscope to confirm the presence of the de-impregnated somata, dendrites and axons. The sections were then processed for electron microscopy using a routine schedule that included poststaining with OsO_4, rapid dehydration with acetone, and embedding in Epon. The use of sections that were about 100 μm in thickness allowed us to visualize the de-impregnated cells in the polymerized blocks of resin. Serial thin sections were taken of critical structures. All sections were stained with uranyl acetate and lead citrate before examination with an electron microscope.

Two basket cells at the hilar border of the granular layer and two local circuit neurons in the CA 1 area were examined.

Immunocytochemistry for glutamate decarboxylase (GAD)-containing neurons

Five Sprague-Dawley rats (age 5 days) were used. The animals were perfused transcardially under ether anaesthesia with a fixative containing 4.0% paraformaldehyde, 0.08% glutaraldehyde and 150 ml saturated picric acid in 1000 ml 0.1 M phosphate buffer at pH 7.3. After dissection of the hippocampus, small tissue blocks were stored for two hours in glutaraldehyde-free fixative. Then transverse Vibratome sections of the hippocampus were prepared at a thickness of 40 μm, thoroughly rinsed in phosphate buffer, and stored overnight at 4° C.

Part of the sections destined for electron microscopy underwent cryoprotection in 10% sucrose followed by shock freezing in liquid nitrogen, thawing to room temperature, and careful washing in phosphate buffer. All specimens were incubated in 10% normal rabbit serum for 30 min and in sheep antiserum S3 against GAD (Oertel et al. 1982, dilution 1:2000 in 0.1 M phosphate buffer containing 1% normal rabbit serum and 0.1% NaN_3) for 48 h at 4° C. The antigen-antibody complex was visualized with the peroxidase-antiperoxidase (PAP) technique of Sternberger et al. (1970), which included incubation in rabbit antisheep IgG (1:40, 1.5 h) and goat PAP complex (1:40, 2 h). Between each incubation step the sections were washed in several changes of phosphate buffer. The tissue-bound peroxidase was then visualized by incubating the sections with 3,3'diaminobenzidine (0.07%) and H_2O_2 (0.002%) in Tris buffer (0.1 M, pH 7.6) for 5 to 10 min. After osmication, the sections were processed for routine embedding for electron microscopy. The sections were dehydrated and block-stained with uranyl acetate in 70% ethanol. Then, the sections were flat-embedded in Araldite between aluminum and transparent plastic foils. After removal of the aluminum foil the preparations were reexamined with the light microscope for GAD-positive cells. The sections were reembedded in plastic capsules and cut in serial sections. The sections were not stained with lead citrate so that the immunolabelling could be observed better. Five GAD-positive neurons at the hilar border of the granular layer, two GAD-positive neurons in the molecular layer of the dentate gyrus and five GAD-positive neurons in the CA 1 area were examined.

Results

Dentate gyrus

The light microscopic features of Golgi-impregnated basket cells in the dentate gyrus of 5 day old animals were similar to those described in adults (Ribak and Seress 1983; Amaral 1978), with the exception that the dendrites were shorter and the axonal plexus was smaller (Fig. 1a). These cells had a larger cell body than the granule cells and they displayed both apical and basal dendrites. The dendrites were smooth, although growth cones and varicosities were observed (Fig. 1a). Branches of the axons of basket cells were distributed mainly in the granular layer but some branches were also detected in the molecular layer.

In the electron microscope the basket cells displayed axons that formed symmetric synapses with somata and dendrites of granule cells (Fig. 1d). These terminals were small and contained few pleomorphic synaptic vesicles. The somata of basket cells were larger than those of the granule cells and they also contained more cytoplasmic organelles, such as the Golgi complex, granular endoplasmic
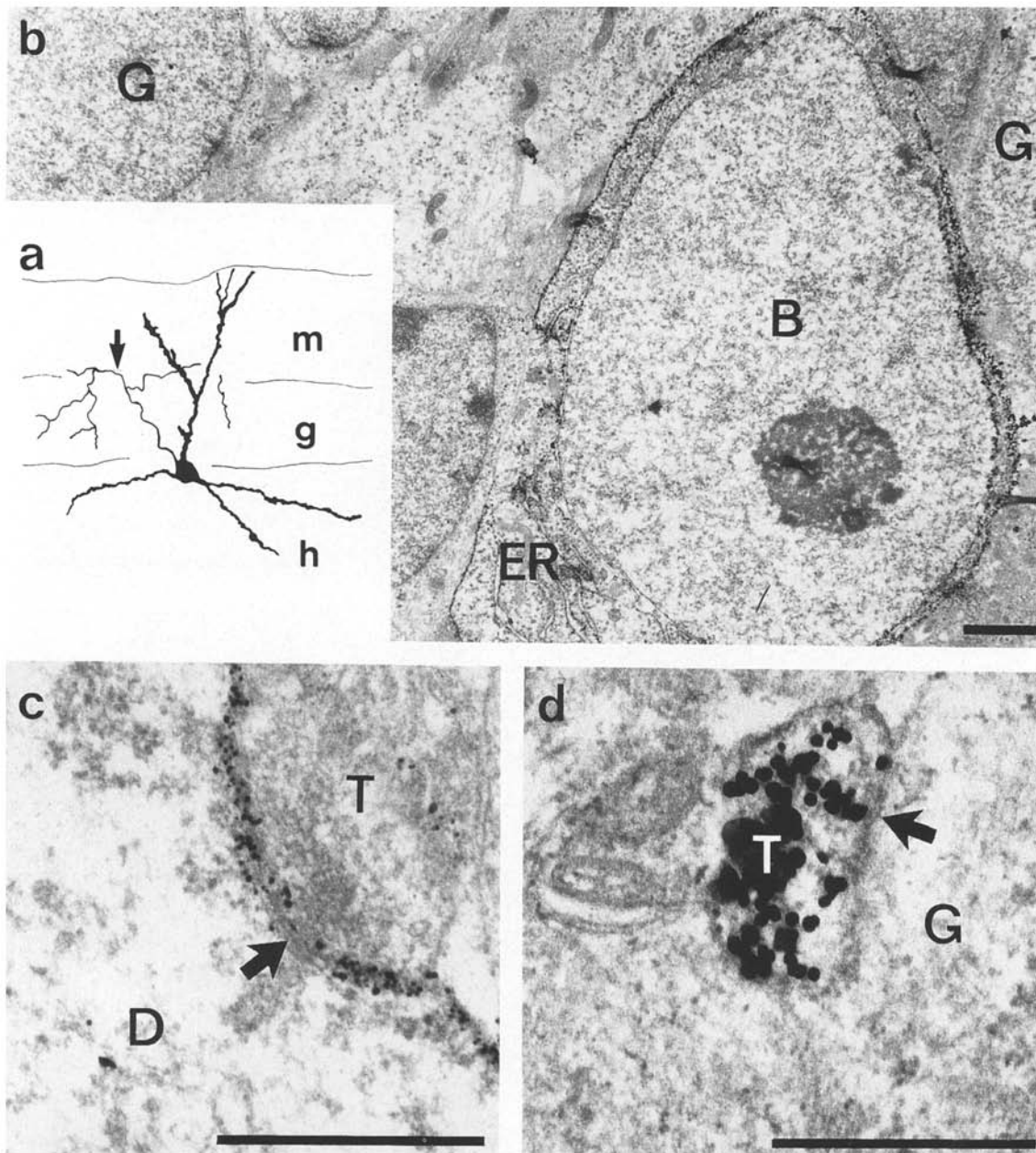
Fig. 1. a Camera lucida drawing of a Golgi impregnated basket cell at the hilar border of the granular layer (g) from a 5 day old rat. The neuron has dendrites both in the hilus (h) and in the molecular layer (m). The axon (arrow) ramifies in the granular layer. The dendrites are thick, varicose and have few side branches. **b** Electron micrograph of the basket cell (B) shown in a. The cell body is located directly underneath the layer of granule cells (G) and is mainly occupied by an ovoid nucleus which does not show infoldings or intranuclear rods. The cytoplasm is sparse but cisternae of endoplasmic reticulum (ER) as well as mitochondria and free ribosomes are present. Scale: 1 μm. **c** Electron micrograph of an axon terminal (T) that forms a synapse (arrow) with the apical dendrite (D) of the basket cell shown in a. Scale: 1 μm. **d** Electron micrograph of an impregnated terminal (T) of the basket cell shown in a. The terminal forms what appears to be a symmetric synaptic contact with the cell body of a granule cell (G). Scale: 1 μm

reticulum and free ribosomes (Fig. 1b). In addition, somata and both apical and basal dendrites of basket cells were contacted by relatively immature terminals that formed mainly symmetric synapses (Fig. 1c). Some of the axon terminals that contacted basal dendrites showed a similarity to immature mossy fiber collaterals. Some adult features of the basket cells, such as the intranuclear rods and sheets were never found at this age and the intranuclear infoldings, when present, were smaller and less complex than that in the adult animals (Fig. 1b).
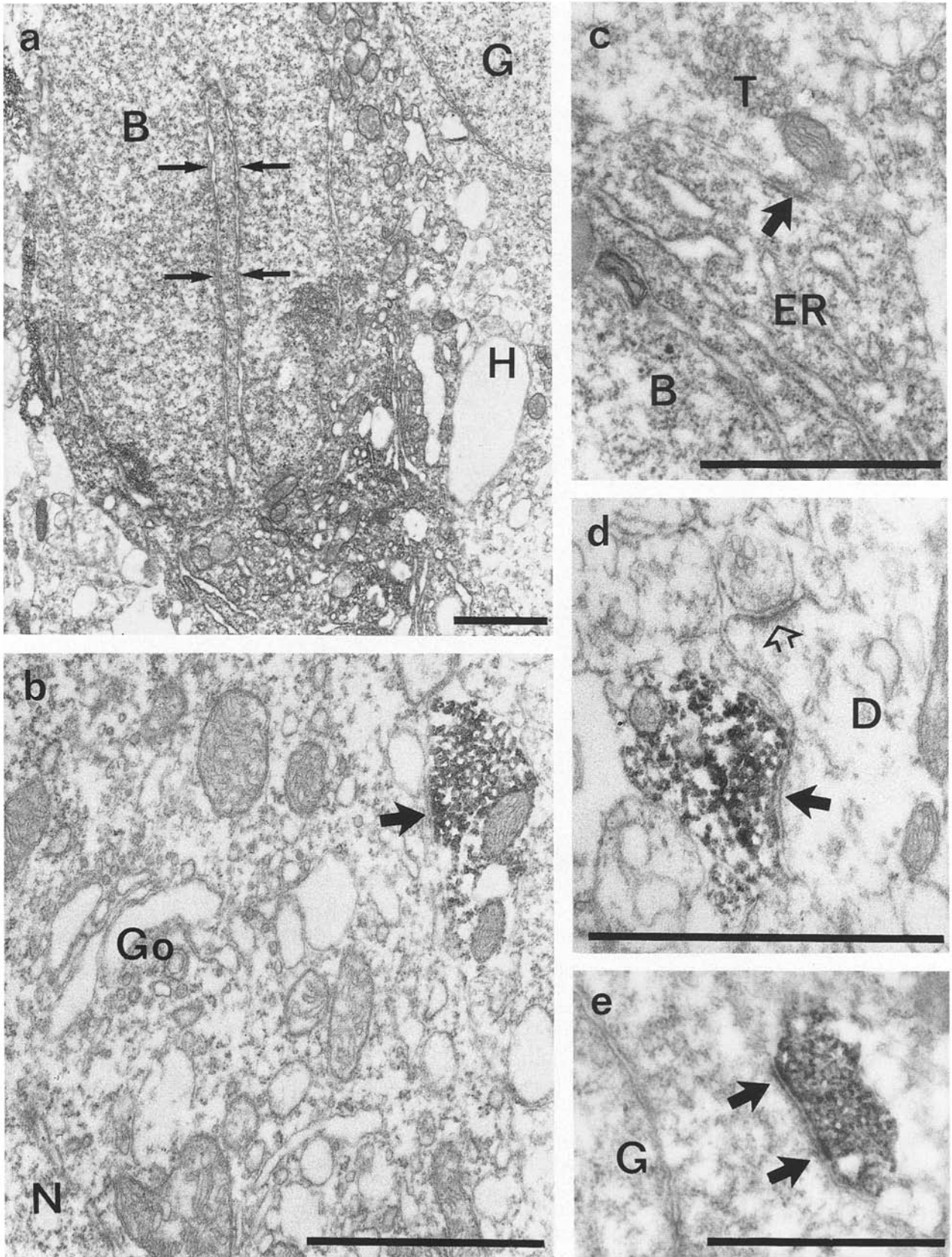
4

Fig. 2

GAD-positive neurons were present in the hilar region, at the hilar border of the granular layer and in the molecular layer. However, the number of these neurons per section was low as compared to the adult. In 40 µm thick sections, 4–6 GAD-positive neurons per section could be counted in the hilar region including the border area and only 1–2 neurons were seen in the molecular layer.

In electron microscopic preparations the pyramidal type of basket cell was found to contain GAD-positive reaction product (Fig. 2a). The cell shown in this Figure was located at the hilar border and displayed a typical pyramidal shape with a relatively large cell body. The cytoplasm to nuclear ratio was large as compared to that of the granule cells in these preparations. The cytoplasm contained many cisternae of granular endoplasmic reticulum but no grouping of cisternae that could be considered a Nissl body (Fig. 2c). The cell nucleus displayed some nuclear infoldings but intranuclear rods were never found. The GAD-positive neurons in the molecular layer showed similar characteristics although their cell bodies were smaller than those of the pyramidal basket cells. The GAD-positive cell bodies were contacted by terminals which formed both symmetric and asymmetric synapses (Fig. 2c), although distinguishing between these two types was difficult due to the young age and the deposition of reaction product adjacent to the internal face of the plasma membrane. Junctions not displaying accumulations of synaptic vesicles and a clear synaptic cleft were considered to be nonsynaptic *puncta adhaerentia* and were not included in the present study.

GAD-positive terminals were very frequent in the molecular layer as well as within the granular layer at this age. They formed symmetric synapses with cell bodies of granule cells (Fig. 2e) and with dendritic profiles in the molecular layer (Fig. 2d). Some of the terminals also formed axo-somatic synapses with the cell bodies of nongranule cells (Fig. 2b.).

Ammon's horn (CA 1 area)

In light microscopy, a wide variety of cells could be identified as local circuit neurons in Golgi prepara-

tions. These neurons were located in all layers as found in adult animals. The most frequent cell type had its cell body adjacent to the pyramidal layer or in the stratum radiatum. The cell body was multipolar and the dendrites were varicose and smooth. They were significantly shorter than in adult animals (Fig. 3a). Already at this age the features of the local circuit neurons were different from the characteristics displayed by pyramidal cells of the CA 1 area. In electron microscopic preparations, the nonpyramidal neurons showed a large cell body with a rich perikaryal cytoplasm (Fig. 3b). The cytoplasm contained a large number of organelles, including Golgi complex, mitochondria, cisternae of granular endoplasmic reticulum and free ribosomes. Although some cisternae of endoplasmic reticulum formed parallel stacks, they did not appear as a well-formed Nissl body (Fig. 3b). The cell nucleus was large, ovoid or round and showed intranuclear infoldings of variable sizes, but there were no intranuclear rods in these nuclei (Fig. 3b). The axons formed symmetric synapses with dendrites of unknown origin (Fig. 3e). The axon terminals were immature, because they contained few mitochondria and vesicles (Fig. 3e). However, they arose from an axon initial segment that displayed adult features (Fig. 3c). Terminals of unknown origin formed synapses with the cell body and dendrites of the identified nonpyramidal cells (Fig. 3f). Long, thin filopodia arose from the dendrites but they were not contacted by terminals (Fig. 3d). These filopodia may represent the growing side branches of the dendrites.

A small number of GAD-positive neurons were present in all layers of the CA 1 area of the Ammon's horn at 5 days of age. On average, 6–8 GAD-positive neurons could be identified in the CA 1 area in a single 40 µm thick section. The GAD-positive neurons were well recognized both in light and electron microscopic preparations, because dense immunoreaction product filled their perikaryal cytoplasm (Fig. 4a,b). They were large neurons with bipolar or multipolar shapes. In some cases the main proximal dendrites could also be recognized in the electron microscope (Fig. 4a,f). The cytoplasm was rich in organelles, similar to that of the pyramidal neurons which were GAD-

Fig. 2 a–e. Electron micrographs of GAD-positive neurons and synaptic terminals from the dentate gyrus of 5 day old rats. **a** GAD-positive cell body of a basket cell (B) at the hilar border (H) of the granule cell layer (G). The nucleus exhibits deep infoldings (arrows). Scale 1 µm. **b** GAD-negative cell body in the molecular layer contacted by a GAD-positive terminal (arrow). Large amounts of cytoplasm suggest that this neuron is a nongranule cell. Go, Golgi apparatus; N, nucleus. Scale: 1 µm. **c** GAD-positive cell body of a basket cell (B) in contact (arrow) with a presynaptic terminal (T). ER, endoplasmic reticulum. Scale: 1 µm. **d** A GAD-positive terminal forms a symmetric synapse (arrow) with a dendrite (D) in the inner molecular layer. An immunonegative terminal establishes an asymmetric synapse with a stubby spine of this dendrite (open arrow). Scale: 1 µm. **e** A GAD-positive terminal forms a symmetric synapse (arrows) with the cell body of a granule cell (G). Scale: 1 µm.
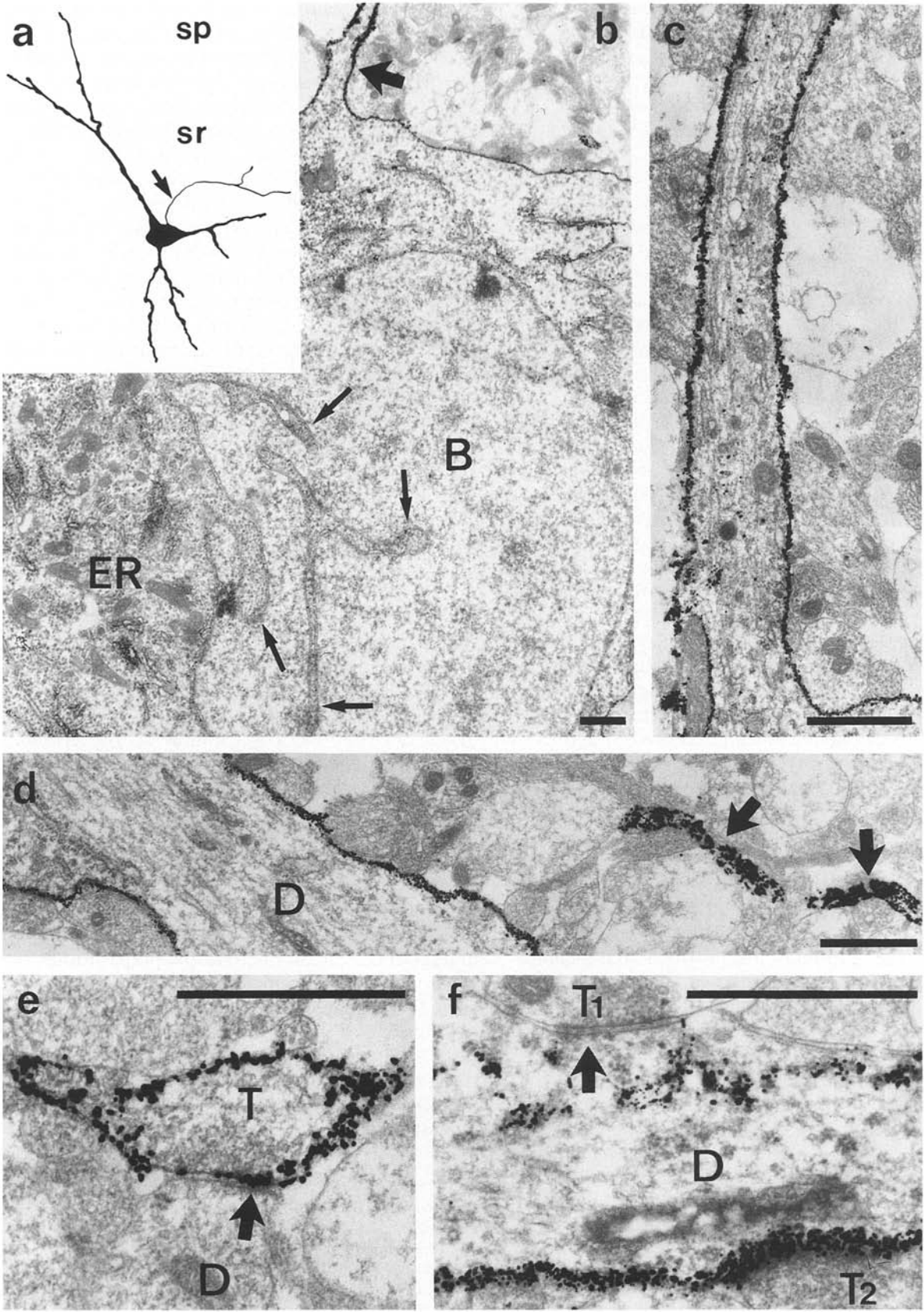
Fig. 3

negative. The cell nuclei often exhibited infoldings (Fig. 4b). The cell bodies and dendrites of GAD-positive neurons were apposed by axon terminals which frequently formed synapses (Fig. 4f). Some of the terminals that formed symmetric synapses were GAD-positive. A large number of GAD-positive terminals were found in all layers of CA 1. They formed symmetric synapses with cell bodies of neurons inside the pyramidal layer (Fig. 4c) as well as with different dendritic profiles (Fig. 4d,e) of unknown origin.

Discussion

The major finding of this study is that the GABAergic local circuit neurons of both the dentate gyrus and CA 1 region display synaptic connections in five day old rats that are similar to those observed in adult rats. The results show that the axons of these local circuit neurons form symmetric synapses with principal neurons (pyramidal neurons, granule cells) but also with nonpyramidal neurons at this age. These results were demonstrated independently with two different methods, combined Golgi-electron microscopy and GAD-immunocytochemistry. In addition, numerous axon terminals of unknown origin were shown to form synapses with the somata and dendrites of these neurons. Although the appearance of many synapses at this age is immature, it is likely that those synapses which appear mature are functional.

The ultrastructure of the local circuit neurons in both the dentate gyrus and CA 1 was similar. These neurons displayed infolded nuclei of varying degrees. However, the CA 1 cells showed deeper infoldings than those in the dentate gyrus. The cytoplasmic organelles found in neurons in both location were similar. Although many cisternae of granular endoplasmic reticulum were observed within these neurons they were not found to form an organized stack that would be considered a Nissl body. In addition, nuclear rods and sheets were not observed. These findings show that the local circuit

neurons in five day old rats display ultrastructural features that are different from those in adult preparations, because such neurons in adults always display deep nuclear infoldings, intranuclear rods and Nissl bodies. The results of this study are consistent with previous data that also showed a similar rate of development for local circuit neurons in both CA 1 and the dentate gyrus (Seress and Ribak 1988a). This finding is interesting, because the projection neurons in these regions have different developmental rates with all pyramidal cells generated prenatally whereas granule cells are mainly generated postnatally. Therefore, the GABAergic local circuit neurons appear to develop independently from their target neurons. Also, the appearance of GABAergic neurons in intracerebral transplants of hippocampus suggests an independent development of their afferentation (Frotscher and Zimmer 1987). Thus, the GABAergic hippocampal neurons appear to develop independently of both their efferent targets and afferents. What sets the time-table for the development of GABAergic neurons is unknown at present, but the most likely possibility is that it is a genetic factor.

If the different areas of the hippocampal formation develop GABAergic local circuit neurons simultaneously, then why does physiologically recorded inhibition appear earlier in one region that in another? Several possibilities could explain this phenomenon. Pyramidal neurons in the CA 3 area are generated earlier in embryonic life than the same neurons in CA 1 (Bayer 1980). Therefore, they may develop their dendritic trees and their axon collaterals earlier. In the CA 3 area, the local circuit neurons show signs of advanced development in rapid Golgi preparations compared to similar neurons of the CA 1 region (Frotscher and Lang unpublished observations). For example, they have longer dendrites with more branches than similar neurons in the CA1 area. Another possibility involves the arrival of long projection systems. The commissural and associational pathways are known to arrive and establish synaptic

Fig. 3. a Camera lucida drawing of a Golgi impregnated nonpyramidal neuron, probably a basket cell, in the stratum radiatum (sr) of CA 1 from a 5 day old rat. The dendrites are relatively short, varicose and do not have side branches. The axon (arrow) branches close to the cell body. sp, stratum pyramidale. **b** Electron micrograph of the basket cell (B) shown in a. Note large amounts of endoplasmic reticulum (ER) in the perinuclear cytoplasm. The nucleus shows deep infoldings (arrows) but no intranuclear rods or sheets. The axon (large arrow) originates directly from the cell body. Scale: 1 μm. **c** Electron micrograph of the axon initial segment of the basket cell at higher magnification. The characteristic membrane undercoating is obscured by gold particles but fascicles of microtubules are seen. Scale: 1 μm. **d** Dendrite (D) of the basket cell shown in a. It gives rise to thin filopodia (arrows), which are thinner than the axon and are, therefore, hardly seen in light micrographs. They may represent outgrowing dendritic side branches. Scale: 1 μm. **e** An axon terminal (T) of the basket cell in a forms a symmetric synapse (arrow) with a dendritic profile (D). Note the low number of vesicles and the absence of mitochondria in the terminal. Scale: 1 μm. **f** Two terminals (T 1, T 2) impinging on the dendrite of the basket cell. A synapse (arrow) is visible for only one of the terminals, T 1. Scale: 1 μm
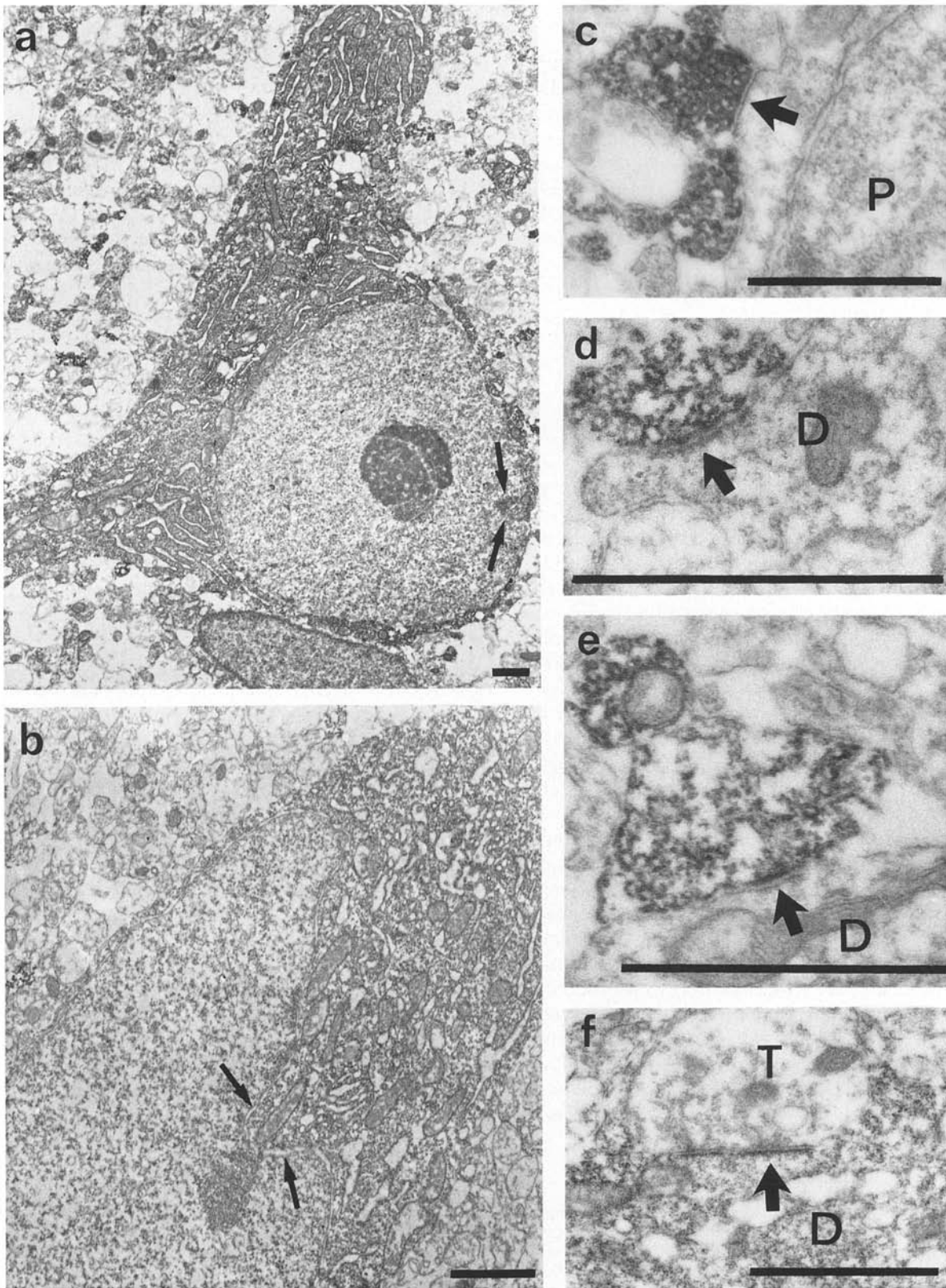
Fig. 4 a–f. GAD-positive cells, in Ammon's horn of 5 day old rats. **a** GAD-positive neuron in the stratum radiatum with the main dendrites stained. The nucleus is round with only small infoldings (arrows). Scale: 1 μm. **b** GAD-positive neuron with a large cell body in stratum radiatum of CA 1. The nucleus contains large infoldings (arrows), but no rods. Scale: 1 μm. **c** A GAD-positive terminal forms a symmetric synapse (arrow) with the cell body of a pyramidal neuron (P). Scale: 1 μm. **d** A GAD-positive terminal forms a symmetric synapse (arrow) with a dendritic profile (D) in the stratum radiatum of CA 1. Scale: 1 μm. **e** A GAD-positive terminal makes a symmetric synapse (arrow) with a dendritic shaft (D) in the outer stratum radiatum. Scale: 1 μm. **f** An immuno-negative terminal (T) establishes an asymmetric synapse (arrow) with a GAD-positive dendrite (D) of the neuron shown in Fig. 4a. Scale: 1 μm

connections 2–3 days earlier in the CA 3 region than in the CA 1 area (Loy 1980). A third possibility involves data that show different developmental patterns of GABA receptors (Janigro and Schwartzkroin 1988) in these same areas. This finding is very important in that the presence of synaptic connections between neurons, such as those shown in the five day old rats of the present study, do not necessarily mean that this circuitry is physiologically active. Therefore, physiologically detectable inhibitory events may not appear until a sufficient number of mature synaptic contacts are formed.

The development of individual synapses should also be considered. It is known that newly formed synapses contain less vesicles and mitochondria than adult synapses. Also, the active zone of synapses, including pre- and postsynaptic densities, undergoes significant postnatal changes (Cragg 1972; Dyson and Jones 1976; Jones and Revell 1970). Taken together with data on receptor development (described above) this may indicate that newly formed synapses behave differently than the adult ones.

In the young hippocampus, the number of symmetric synapses is greater than the number of asymmetric ones whereas the opposite is found in the adult hippocampus (Crain et al. 1973). For example, in 4 day old animals, the density of synapses in the molecular layer of the dentate gyrus suggests that only 1% of the adult value has formed and most are symmetric. Most of the remaining synapses (99%) which are formed between postnatal days 4 and 25 are terminals from the developing perforant and commissural pathways and they seem to form mainly asymmetric terminals. The relatively high density of GAD-positive terminals forming symmetric synaptic contacts in all layers of the 5 day old hippocampus supports these earlier findings. Therefore, it is likely that the growing number of synapses made by the afferent pathways plays a key role in the activation and maturation of neuronal circuits between principal and local circuit neurons.

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