

Organization of the GABAergic System in the Rat Hippocampal Formation: A Quantitative Immunocytochemical Study

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ABSTRACT

Specific antibodies against gamma aminobutyric acid (GABA) and glutamic acid decarboxylase (GAD) were used to study the organization of the GABAergic system in the rat hippocampal formation. Both the number of GABA-like-immunoreactive (Li) somata and neuropil density were assessed in semithin sections. Cell counts revealed that approximately 11% of the hippocampal neuronal population showed GABA-Li within the various planes of section.

Each layer in the hippocampal formation had a characteristic organization of GABA-Li elements. In Ammon's horn, 80-95% of the neuronal somata within the apical and basal dendritic regions were GABA-Li positive. Within the pyramidal cell layer 5-8% of the cells were GABA-Li in the CA1 to CA3 subfields of Ammon's horn and only 3% were GABA-Li within that portion of the pyramidal cell layer that inserts into the hilus. Only slight differences in the density of the GABA-Li neuropil were observed within the CA1-CA3 dendritic regions. Restricted to the stratum lucidum was a dense band of GABA-Li label. Counts of immunoreactive grains localized on the perimeter of pyramidal (CA1-CA3) and granule somata revealed more terminal boutons on the CA3 cells than on CA1 and granule neuronal somata.

A topographical distribution of GABA-Li somata and neuropil was found in the fascia dentata: There the label particularly concerned its suprapyramidal and rostrolateral portions. Approximately 40% of neurons in the molecular layer, 60% in the polymorph layer, and 18% within the hilar region were GABA-Li. Within the granule cell layer only 2% of the neurons were GABA-Li positive. Distinct differences in the density of the GABA-Li neuropil were present in the molecular, pericellular granular, and hilar regions of the fascia dentata.

While the morphology of GABA-Li neuronal somata varied according to their hippocampal layer, the most heterogeneous cell types were found in the regio inferior of the hippocampus. There we have identified neurons that are reminiscent of the inferior region interneuron described in Golgi material by Amaral and Woodward (*Brain Res.* 124:225-236, '77). Moreover, particularly in the sagittal plane, we have identified oval, triangular, and round cells and that have processes oriented in a parallel arrangement, appearing to be aligned along the granule cell mossy fibers.

Key words: gamma aminobutyric acid, interneurons, quantitative organization

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Whereas much of our present knowledge on the morphology of the neuronal types that make up the hippocampal interneuronal plexus is derived from the classical Golgi studies of Cajal ('11) and Lorente de Nó ('34), additional neuronal types in the hilar region have been carefully described by Amaral ('78). In the hippocampal formation, this plexus has been proposed to mediate tonic inhibition, which includes the classical recurrent inhibitory feedback circuitry (Andersen et al., '64; Andersen, '75) and direct feed-forward inhibition via a portion of the hippocampal commissural projection (McNaughton et al., '78; Buzsáki and Czéh, '81; Buzsáki and Eidelberg, '81). More recently, Frotscher et al. ('84) have provided direct anatomical evidence in support of the latter physiological studies.

The inhibitory role of gamma (γ)-aminobutyric acid (GABA) in the vertebrate central nervous system is now well established (see Roberts, '80, for a review). Biochemical methods have been used to identify GABA in the hippocampal formation by measuring the activity of its synthesizing enzyme, L-glutamate decarboxylase, GAD (Storm-Mathisen, '72, '76; Nadler et al., '74). Presently, immunocytochemical methodology is the most commonly used approach with which to identify stores of this neurotransmitter; its localization in the hippocampal formation has been demonstrated with antibodies against GAD (Barber and Saito, '76; Ribak et al., '78; Köhler and Chan-Palay, '83; Seress and Ribak, '83; Somogyi et al., '84; Kosaka et al., '84; Ottersen and Storm-Mathisen, '84; Kunkel et al., '86), against GABA-transaminase, which converts GABA and ketoglutarate to succinic semialdehyde and glutamate (Barber and Saito, '76; Nagai et al., '83), and against GABA (Ottersen and Storm-Mathisen, '84; Somogyi and Hodgson, '85; Somogyi et al., '85; Gamrani et al., '86; Sloviter and Nilaver, '87).

The above biochemical and immunocytochemical studies agree that 1) all hippocampal layers contain GABAergic markers and 2) high concentrations of GABA-Li and GAD-Li in the puncta are observed in the pyramidal cell subfields of Ammon's horn and the dentate granule cell layer and within the outer third of the dentate molecular layer. In addition, an electron microscopic analysis has shown that GABAergic axonal processes synapse upon both the initial segment of the axon of the dentate granule cells (Kosaka et al., '84) and the CA1 pyramidal cell axon (Somogyi et al., '84; Kunkel et al., '86).

However, our present state of knowledge concerning the organization of the hippocampal GABAergic system is deficient notably in respect to the topographic distribution of GABA-Li along various hippocampal axes. Furthermore, the organization has not been mapped quantitatively and the possibility of GABA-Li gradients has not been examined. This study provides data on these issues.

MATERIALS AND METHODS

Adult Wistar rats ($n = 41$) were used in the present study, in which both thick and semithin sections were prepared. As each procedure differed, a detailed description of these protocols is provided below.

Thick sections

Tissue preparation. The rats were anesthetized with phenobarbital (35 mg/Kg) and perfusion fixation was initiated by intracardial flow of 150 ml of saline (9/1,000) followed by 200 ml of 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.6). Following the removal of the brains, the tissue was postfixed for 1 hour in the same fixative, and vibratome sections of the entire hippocampal formation were cut in the frontal plane at 50- μ m thickness. The sections were then collected in cold 0.1 M cacodylate buffer (pH 7.6) and stored overnight at 4°C.

Immunocytochemical staining. In the present experiments antiserum directed against GABA (Immunotech, France, and courtesy of Dr. Geffard, CNRS, France) has been used. In parallel experiments GAD antibodies (courtesy of Dr. Tappaz, CNRS, France) have been used to evaluate the adequacy of GABA immunocytochemistry as a marker of GABAergic systems. The protocol used for the latter marker has been described by Oertel et al. ('81a,b).

For GABA immunocytochemistry the immunoperoxidase bridge technique (Sternberger, '79) has been performed as follows. The sections were washed in 0.1 M Tris buffer (pH 7.6) and exposed to the primary antibody (anti-GABA) at a dilution of 1:5,000 overnight at 4°C on a rotating apparatus. The next morning the sections were left at room temperature and then rinsed 3 \times 10 minutes in 0.1 M Tris (pH 7.6). Afterward, the sections were exposed to the secondary antibody (goat antirabbit, IgG) at a dilution of 1:200 for 3 hours at room temperature. Following the same rinsing procedures as indicated above, the sections were incubated with the peroxidase-antiperoxidase (PAP) complex at 1:600 dilution for 1 hour at room temperature. The sections were then rinsed as above and exposed to diaminobenzidine (DAB, Sigma: 25 mg/100 ml) in 0.1 M Tris buffer containing 1% H₂O₂ for approximately 20 minutes. The sections were then mounted on gelatin-coated slides and coverslipped either without counterstaining or after exposure to Nissl stain.

Semithin sections

Tissue preparation. The rats were anesthetized with phenobarbital (35 mg/kg) and perfusion fixation was initiated by intracardial flow of 100 ml of saline (9/1,000) followed by 200 ml of 5% glutaraldehyde—0.5% paraformaldehyde in 0.1 M Tris buffer (pH 7.6). Following the removal of the brains, the tissue was postfixed for 1 hour (in the same fixative). Afterward, the hippocampi of both sides were cut into 1-mm-thick blocks, serially ordered from caudal to rostral (frontal plane), medial to lateral (sagittal plane), and dorsal to ventral (horizontal plane). The specimens were dehydrated in graded alcohol and embedded in

Abbreviations

a	alveus
ar	area retrosplenialis
ael	area entorhinalis lateralis
aem	area entorhinalis medialis
CA1-	subfields of Ammon's horn
CA3	
cc	corpus collusum
fimb	fimbria
hf	hippocampal fissure
ir	interface region
pl	polymorph layer
sl	stratum lucidum
slm	stratum lacunosum moleculare
sg	stratum granulosum
sm	stratum moleculare
so	stratum oriens
sp	stratum pyramidale
sr	stratum radiatum
sub	subiculum

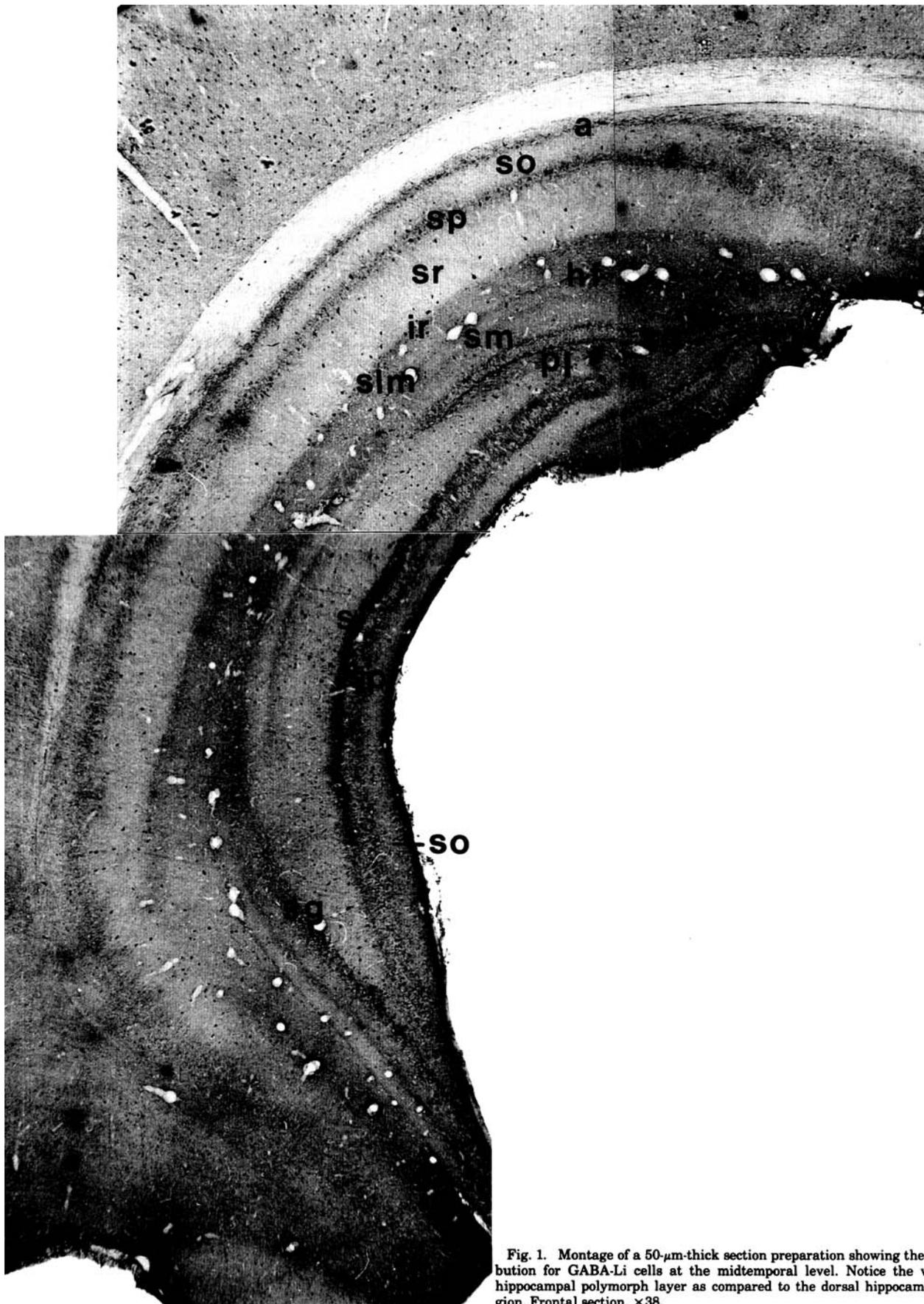
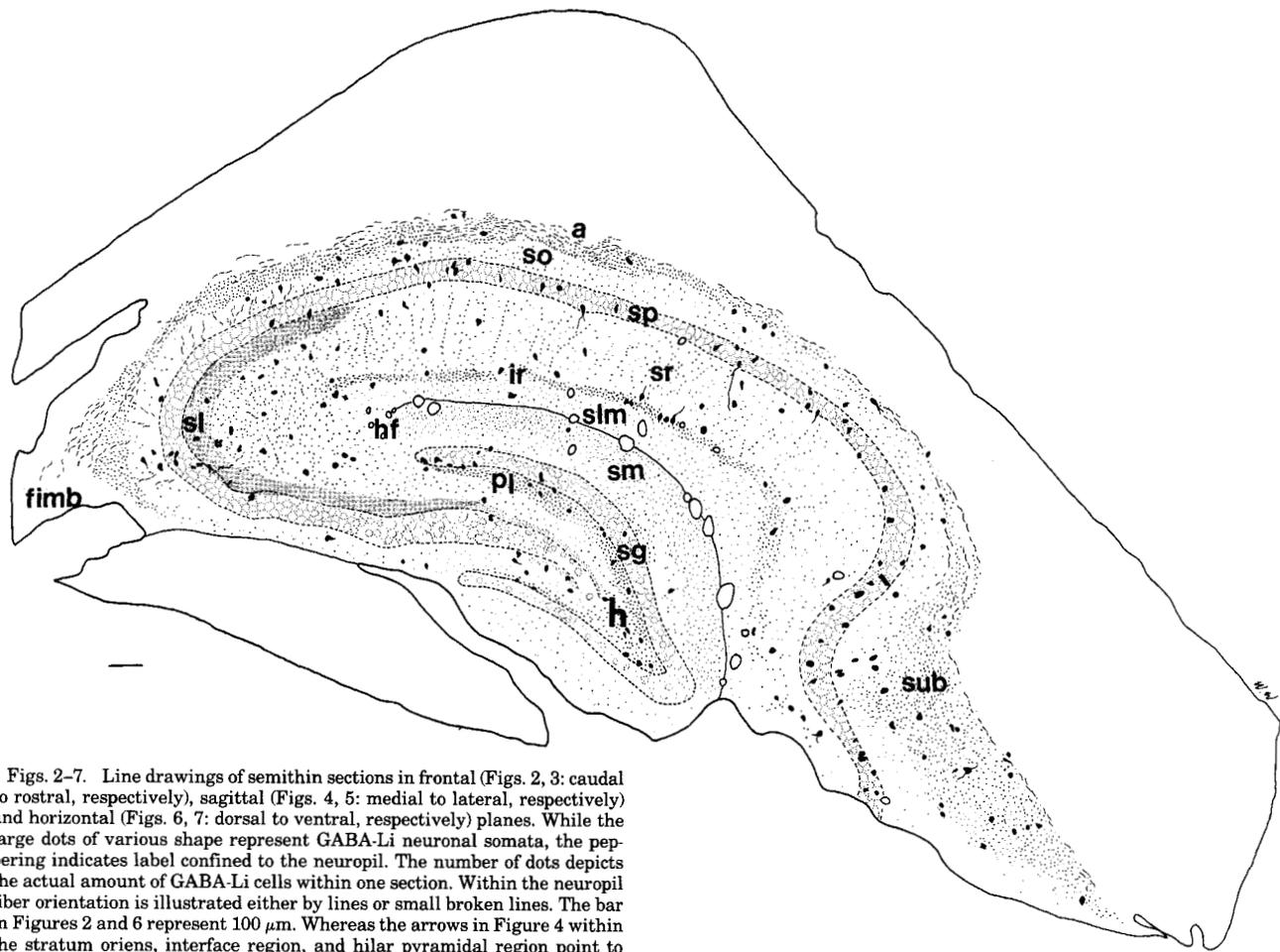


Fig. 1. Montage of a 50- μ m-thick section preparation showing the distribution for GABA-Li cells at the midtemporal level. Notice the ventral hippocampal polymorph layer as compared to the dorsal hippocampal region. Frontal section. $\times 38$.



Figs. 2-7. Line drawings of semithin sections in frontal (Figs. 2, 3: caudal to rostral, respectively), sagittal (Figs. 4, 5: medial to lateral, respectively) and horizontal (Figs. 6, 7: dorsal to ventral, respectively) planes. While the large dots of various shape represent GABA-Li neuronal somata, the peeping indicates label confined to the neuropil. The number of dots depicts the actual amount of GABA-Li cells within one section. Within the neuropil fiber orientation is illustrated either by lines or small broken lines. The bar in Figures 2 and 6 represent 100 μ m. Whereas the arrows in Figure 4 within the stratum oriens, interface region, and hilar pyramidal region point to areas also shown in photomicrographs in Figures 9C, 10D, and 13A, respectively, the arrow in Figure 5 within the regio inferior of the hippocampus points to a similar cell arrangement shown in photomicrograph in Figure 13E.

Figure 2

araldite (Fullman, 501). Following the embedding procedures, the blocks were cut 1 μ m thick on a Riegert-Jung ultramicrotome and stained with toluidine blue to obtain sections showing the full surface of the block. Afterward, the sections were mounted on gelatin-coated slides placed on a hot plate at 50°C and collected in four series. While the first slide was always stained with toluidine blue, the other three were used for control and immunocytochemical staining.

To remove the araldite, the slides were processed as follows. They were dipped in a 6% solution of the sodium methylate (15-30 minutes), then in a 1:1 methanol-benzene solution (2 minutes), in pure acetone (2 \times 2 minutes), in distilled water (2 \times 5 minutes), in 2 mM CaCl₂ + 0.02 M Tris buffer at room temperature (5 minutes), in 2 mM CaCl₂ + 0.02 M Tris + pronase (0.001 mg/ml) at 37°C (5 minutes), and in 0.1 M Tris buffer (pH 7.6) at 37°C (5 minutes). The slides were subsequently exposed to 3% H₂O₂ for 10 minutes to block nonspecific activity and then exposed to a solution of Tris buffer containing 0.1% Triton X-100 (4 \times 5 minutes).

Immunocytochemical staining. The immunoperoxidase bridge technique (Sternberger, '79) has been used to detect GABA-like immunoreactivity (GABA-Li). The sections were exposed to anti-GABA antisera at a dilution of 1:2,000 for

at least 24 hours at room temperature. After incubation the slides were washed in 0.1 M Tris buffer (pH 7.6) and then in 0.1% Triton X-100 in Tris buffer (4 \times 5 minutes) and then exposed to the secondary antibody (goat antirabbit, IgG) at a dilution of 1:100 for 45 minutes at room temperature. Afterward, the slides were again washed and exposed to Triton X-100, subsequently exposed to PAP at a dilution of 1:200 for 1 hour at room temperature, and then washed in 0.1 M Tris (pH 7.6) followed by four washes of 5 minutes each in 0.1% Triton X-100. The slides were then exposed to diaminobenzidine (30 mg/100 ml, Sigma) in 0.1 M Tris buffer containing 1% H₂O₂ for 20-30 minutes. After another wash, the slides were exposed to 0.04% osmium tetroxide for 20 seconds, dehydrated, and coverslipped with Permount.

In some experiments, instead of using the PAP complex the sections were treated with glucose oxidase-antiglucose oxidase (GAG, Jackson immunoresearch) in order to illuminate background labelling (see Clark et al., '82, for details on this technique).

Specificity of the immunoreaction

The process of the production of the antiserum anti-GABA and the biochemical controls of its specificity and cross-reactivity with other amino acids have been described (see

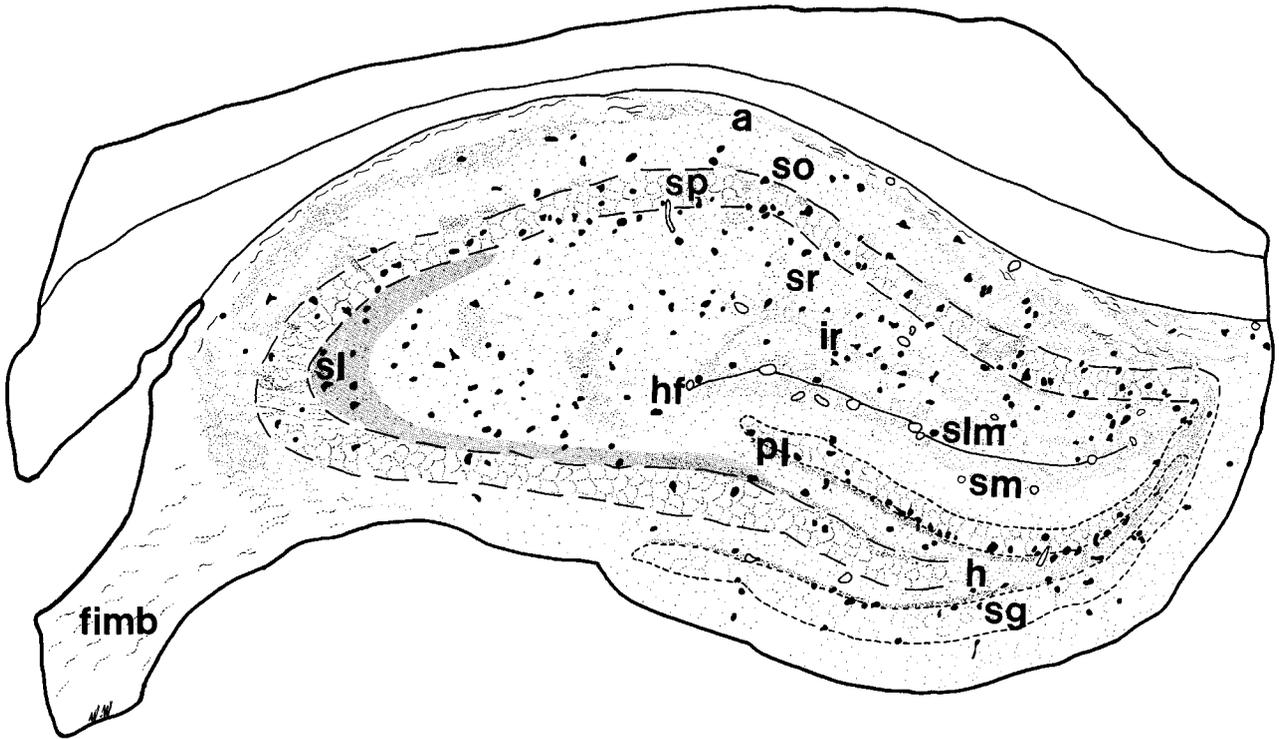


Figure 3

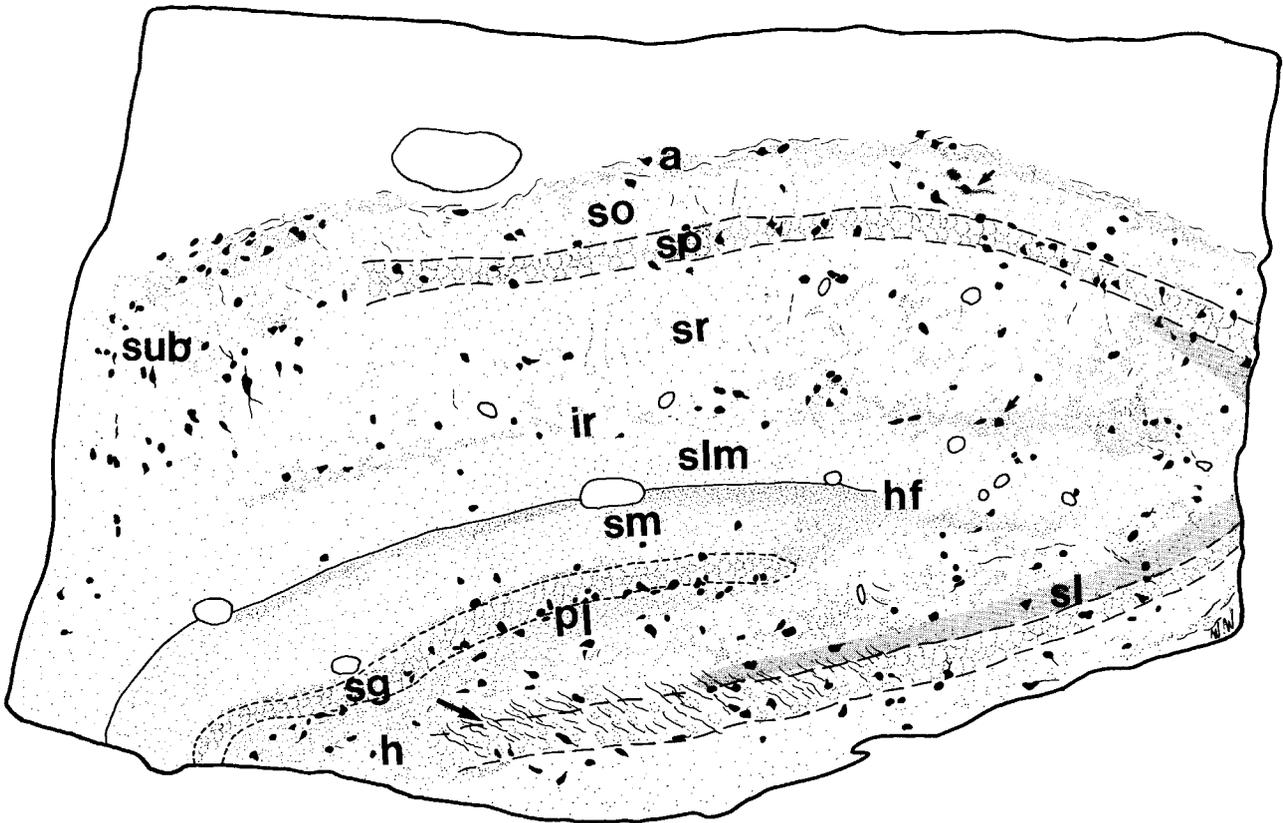


Figure 4

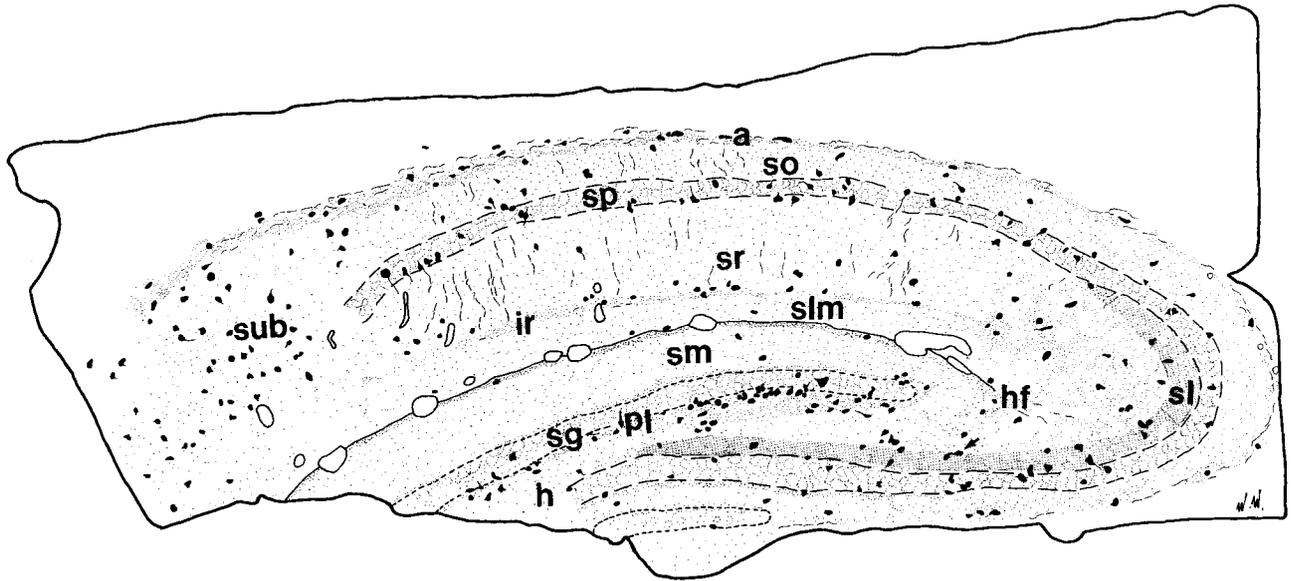


Figure 5

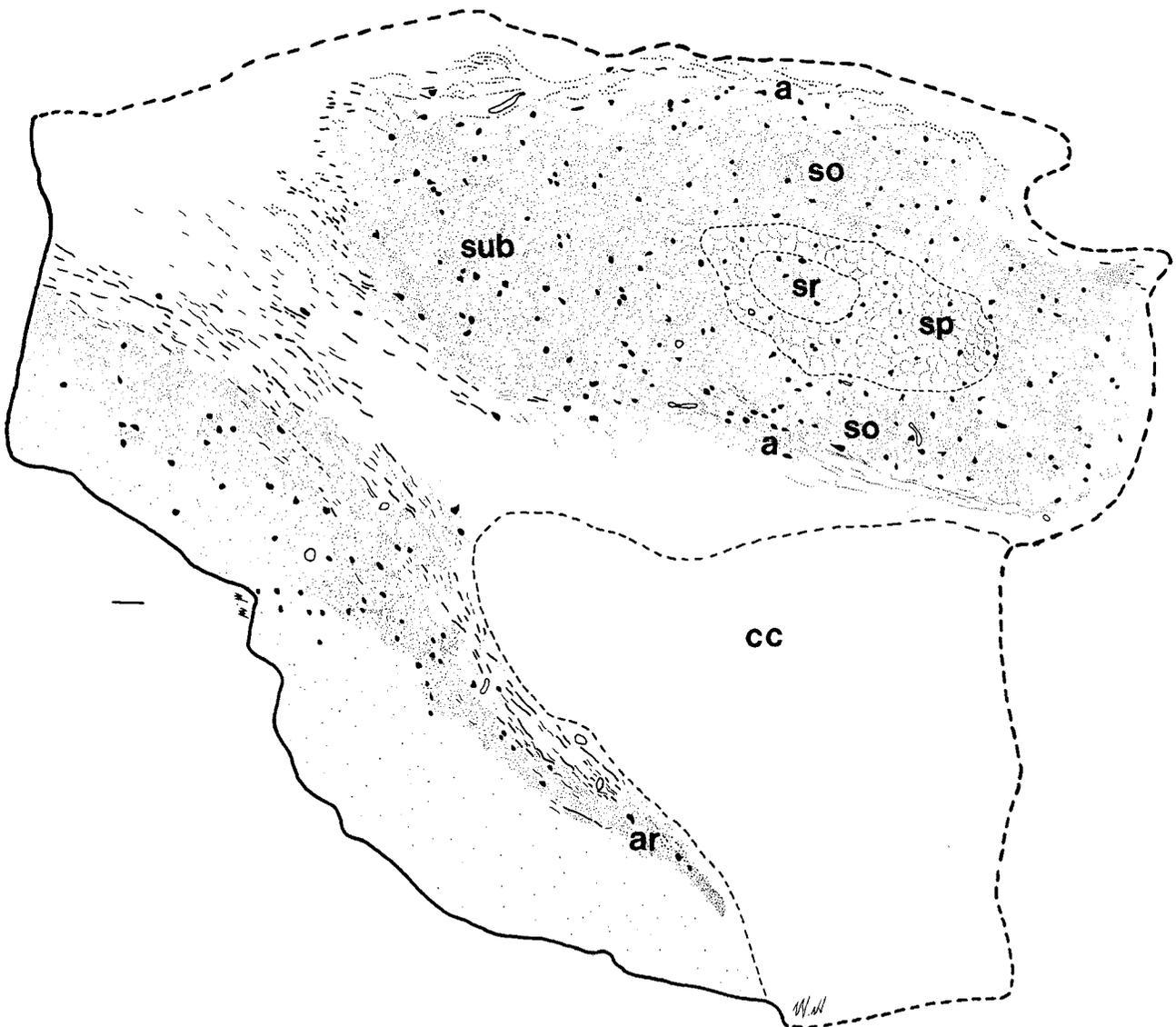


Figure 6

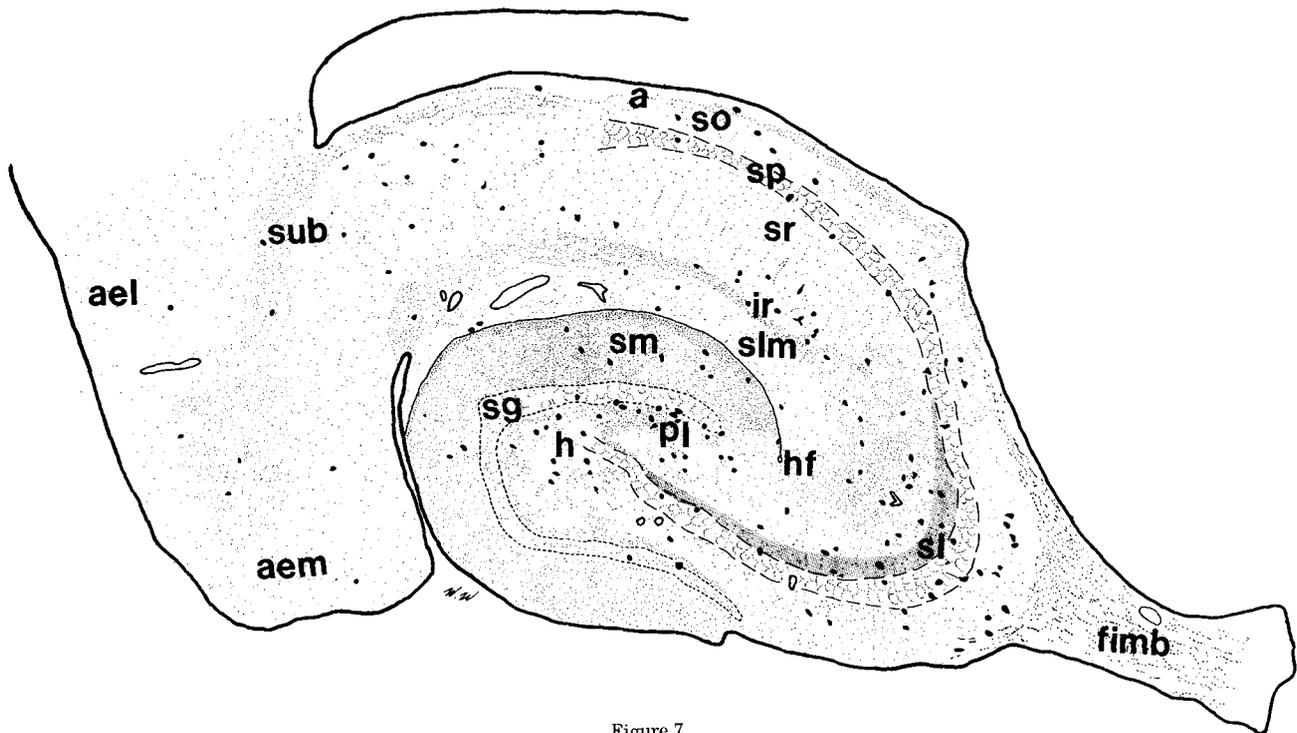


Figure 7

Sequela et al., '84). For the histochemical controls of the antibody specificity two procedures were used: 1) the omission of the primary antibody followed by all other immunocytochemical steps and 2) incubation of the slides with primary antisera absorbed with synthetic antigen (courtesy of Dr. Geffard, CNRS, France) at a dilution of 1:2,000.

Data analysis

Following examination of the slides, camera lucida drawings of semithin sections were prepared, in each plane, and both labelled neuropil and neuronal somata were plotted onto the outlined sections. As the intensity and granular size of the GABA-Li neuropil differed according to the layer within the hippocampal formation, the outlined drawings in Figures 2 to 7 only illustrate the general pattern seen in the section. Density measurements were then conducted in order to quantify the organization. For the quantitative analysis three different cases in each plane of section (i.e. 2 frontal, 2 sagittal, and 2 horizontal) were sampled. The entire quantitative analysis was performed with a microscope attached to a Nachet NS 1000 module computer equipped with graphics tablet. This allowed the visual image under the microscope to be projected onto the screen. For the calculation of GABA-Li grain density (see the following section) in the neuropil, a box representing a surface area of $100 \mu\text{m}^2$ was made on the screen under a magnification of $1,000\times$. The semithin sections were then placed onto the microscope stage and the box was micromanipulated over 5 different locations within each layer of the hippocampal formation under the same magnification. Each GABA-Li-positive grain within the neuropil (regardless of size) was then entered into the box. Only the sections which had a homogeneous dark immunoprecipitate were examined (see following section).

The calculation of the percent of GABA-Li neuronal somata was made from semi-thin sections stained with tolu-

idine blue. The total number of cells with visible nucleoli in each layer of the hippocampal formation was counted under magnifications of 160 and $250\times$. This number was then compared to the adjacent section incubated in the GABA antibody.

Comments on the GABA-Li label

In our material, the staining intensities of GABA-Li neuronal somata varied between light and dark brown: a greater immunoprecipitate was generally found within the nucleus as compared to the cytoplasm. However, these differences are usually attributed to variations in the physiological state of the neuron (see Ottersen and Storm-Mathisen, '84). As the pattern of GABA-Li confined to the neuropil had a granular appearance we shall use the term GABA-Li grains in the following description. Finally, depending on the degree to which the araldite had been removed, the immunoreactive product confined to GABA-Li elements was either light or dark. However, this did not appear to have an effect either on the number of GABA-Li neuronal somata or on the amount of GABA-Li positive grains in the neuropil.

RESULTS

The results were based on cases that underwent GAD and GABA immunocytochemistry. Whereas both antibodies revealed a similar pattern of GABA-Li labelling within the hippocampal formation, a much more extensive label was observed with the GABA antiserum, which avoids the use of colchicine to enhance labelling (see Ribak et al., '78). Therefore, the following description is primarily based on semithin sections incubated with the latter antibody.

Nomenclature

In the present study the hippocampal formation includes the pyramidal subfields of Ammon's horn, and the fascia

dentata. The subiculum was not included in our quantitative analysis. Whereas, the pyramidal cell layer of Ammon's horn is subdivided into CA1, CA2, and CA3 according to Lorente de N6 ('34), CA2 is only considered as a narrow "transitional zone" between CA1 and CA3, having slightly larger cells and a looser arrangement of neuronal somata than CA1. Within the CA1-CA2 pyramidal cell dendritic field the term "interface region" refers to the boundary between the stratum radiatum and the stratum lacunosum moleculare. The polymorph layer is the subgranular zone boarding the hilus. This region contains many GABA-Li neuronal somata. The fascia dentata hilar region is subdivided on the basis of the pattern of GABA-Li label into (see Fig. 10F) 1) the subdivision of the hilar region adjacent to the polymorph layer, which is very immunoreactive in neuropil label, and 2) the portion of the hilar region just ventral to the above zone containing less immunoreactive product within the neuropil. However, both hilar subdivisions contain very few GABA-Li neuronal somata. The hilar pyramidal cell layer creates a distinct aggregate of cells surrounded by GABA-Li pericellular networks. The term "suprapyramidal blade of the fascia dentata" refers to the portion of the granule cell layer facing the hippocampal fissure, and "infrapyramidal blade" indicates that portion immediately above the third ventricle.

In referring to the various planes of sectioning of the hippocampal formation it is necessary to view this structure as a twisted "c" in form along its rostrocaudal axes. The extreme septal and temporal poles are oriented rostradorsal and caudoventral, respectively.

Distribution of GABA-Li neuronal somata

General Organization. GABA-Li neuronal somata were observed in all layers of the hippocampal formation (see Figs. 1 to 7), and bands of cells were found in the superficial stratum oriens, stratum pyramidale, interface region, and the polymorph layer. Rostrocaudally the greatest number of GABA-Li positive cells were observed at midseptotemporal levels (Fig. 1). The most characteristic feature of the distribution of GABA-Li in the sagittal plane was the presence of many GABAergic processes in the pyramidal cell layer of the hilar region (Fig. 4). Within this area and the stratum lucidum, a unique assemblage of GABA-Li neuronal somata were observed (see below).

Within the dorsal fascia dentata topographical differences in the distribution of GABA-Li neuronal somata were observed and 3 gradients can be noted: a dorsoventral (frontal plane, Figs. 2, 3), a rostrocaudal (sagittal plane, Figs. 4, 5), and a mediolateral (horizontal plane, Fig. 7). The three planes of section showed that more GABA-Li neuronal somata were in the suprapyramidal blade (Figs. 2, 3, 5), particularly in its rostral (Figs. 4, 5) and lateral (Fig. 7) portions. While at midseptotemporal levels (frontal plane, Fig. 1) many GABA-Li neuronal somata were also observed particularly in the suprapyramidal blade, more cells were found in the ventral hippocampal formation. A slight dorsoventral topography was found in other hippocampal layers in which more GABA-Li cells were observed in the regio superior of both the alveus-stratum oriens (compare Figs. 6 and 7) and the pyramidal cell layer subfields of Ammon's horn (Figs. 2, 3).

Quantitative analysis. Cell counts performed in the hippocampal formation in frontal, sagittal, and horizontal semithin sections revealed that the total number of toluidine blue stained neuronal somata ranged between 1,300

to 2,300. As the number of GABA-Li cells in adjacent sections varied between 130 and 250, approximately 11% of the total neuronal population exhibit GABA-Li. In this connection, we have found only a small variation in the total number of granule cells to pyramidal neurons of Ammon's horn in the different planes (0.69 ± 0.2 , as compared to an estimated ratio of 0.66 suggested in Schlessinger et al., '75).

We then proceeded to estimate the percentage of GABA-Li cells within each layer of the hippocampal formation. In as much as only slight differences in this percentage were observed in the different planes, the values shown in Figure 8 represent a grouping of frontal, sagittal, and horizontal cases. As noted in Figure 8, in the pyramidal cell dendritic subfields approximately 90% of the neurons were labelled with the antibody. Only slight differences were observed within the pyramidal cell layer of subfields CA1-CA3 (5-8%), in spite of the differences between the planes of section in the total number of pyramidal neurons (900, 600, and 300 neurons in the sagittal, frontal and horizontal planes, respectively). A low percentage of nonpyramidal neurons had been suspected in a recent study (see Boss et al., '87).

In contrast, the fascia dentata revealed differences in the distribution of GABA-Li-positive cells. The dendritic regions of the fascia dentata contained only a few cells stained in toluidine blue, most of which were found in the suprapyramidal blade. Of these, approximately 42% were GABA-Li positive. In the polymorph layer of the suprapyramidal blade, an average of 50 toluidine-blue-stained cells were observed, of which approximately 60% were labelled for GABA-Li (in agreement with Seress and Ribak, '83; see the Discussion). In contrast, the polymorph layer of the infrapyramidal blade had a lower percentage of GABA-Li cells (approx. 20% out of 25-30 toluidine-blue-stained cells).

Within the two subdivisions of the hilar region we found that approximately 18% of the cells were GABA-Li positive. As for the portion of the pyramidal cell layer which inserts into the hilus, a slightly lower percentage (approx. 3%, not illustrated) was observed as compared to the CA1-CA3 subfields of Ammon's horn. However, this value could conceivably be greater if the ventral hippocampus at midtemporal levels had been counted (see Fig. 1).

The stratum granulosum had the lowest percentage of GABA-Li neuronal somata within the hippocampal formation (approx. 2%; this value includes the GABA-Li positive cells in the polymorph layer which has many labelled cell bodies). Also, a difference was observed between the suprapyramidal and infrapyramidal blades: a higher value was found in the former as compared to the latter (2.9%/586 counted cells and 0.71%/699 in the suprapyramidal and infrapyramidal blades, respectively). The above differences between the supra- and infrapyramidal blades can be observed in Figures 1-3, 5, and 7 (see discussion concerning the horizontal section).

Morphology of GABA-Li neurons. We first examined whether the GABA-Li neurons belonged to a homogeneous population in terms of size. With the exception of the superficial stratum oriens and the CA3 subfield of the pyramidal cell layer, the surface area of GABA positive neurons did not significantly differ (approximately $104 \pm 12 \mu\text{m}^2$). In the alveus-stratum oriens GABA-Li neurons had smaller somata ($70 \pm 28 \mu\text{m}^2$), whereas GABA-Li-positive neurons of the CA3 pyramidal cell region were larger ($143 \pm 15 \mu\text{m}^2$).

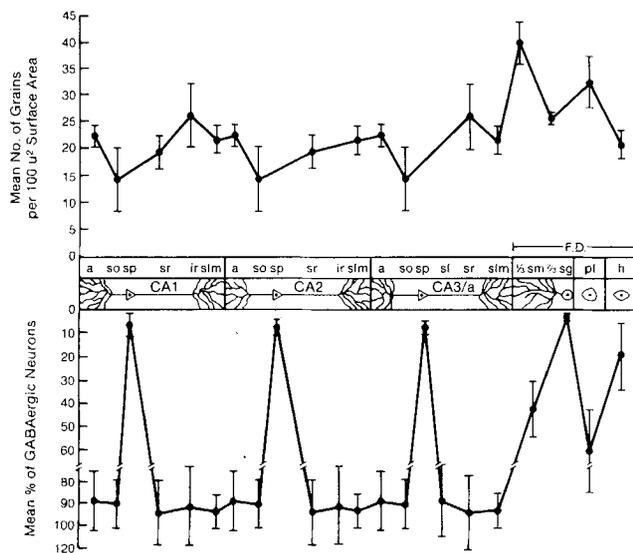


Fig. 8. Results obtained from semithin sections in frontal, sagittal, and horizontal planes illustrating both the density of GABA-Li grains and the percentage of GABA-Li neuronal somata within each layer of the hippocampal formation. Notice the distinct difference between the hippocampus and the fascia dentata with respect to the organization of GABA-Li. The grain density measurement above PL refers to the subdivision of the hilar region adjacent to the subgranular polymorph zone, and h refers to the ventral subdivision.

Morphological features of GABA-Li-positive cells differed according to the hippocampal layer. Thus, within the stratum oriens many GABA-Li neurons were horizontal cells with processes generally oriented parallel to the surface of the alveus (Fig. 9). Labelled neuronal somata within the CA1-CA2 pyramidal cell layer were often observed to have long processes descending into the stratum radiatum (Fig. 10A,B). At the CA1-CA2 interface region two types of GABA-Li-positive cells have been found. The first was a round-oval-shaped cell having a thick process directed toward the above pyramidal cell layer (Fig. 10C). The second type of neuron was a rather triangular shaped cell having a process oriented towards the interface region (Fig. 10C).

Three neuronal types were observed in stratum radiatum of CA3 in the vicinity of the stratum lucidum; these neurons most likely contribute to the intense immunoreactive label present in the mossy fiber region (see below and Discussion). The first was a round-oval-shaped cell located just at the interface between the stratum lucidum and stratum radiatum. This neuron had a thick dendritic process extending from the top of the neuron directed toward the stratum radiatum, with an axon extending from the side of the neuron directed toward the stratum lucidum (Fig. 11D,E). This cell seems to resemble the inferior region interneuron described in Golgi material by Amaral and Woodward ('77). A second cell type was observed to have a thick process leaving from the side of the soma oriented toward and penetrating into the stratum lucidum (Fig. 12A). This process becomes totally embedded within the GABA-Li label in the stratum lucidum. A third GABA-Li-positive neuron was triangular shaped, embedded within the deep portion of the stratum lucidum, having its apex facing this region and its base bordering the stratum radiatum (Fig. 12B). The thick processes of this cell type leave from both sides of its base oriented along the border between strata

radiatum and lucidum, respecting the border between the two regions. Pyramidal cell dendrites surround both sides of this neuronal type. In addition, GABA-Li neurons were present in the pyramidal portion of the hilar region; these neurons, which were particularly conspicuous in the sagittal plane (Fig. 13), had processes oriented along the many GABA-Li processes in this region. It appears that both the cells and processes may be aligned along the granule cell mossy fibers.

As mentioned above, the immunoreactive label within the stratum lucidum appeared as a uniform band of GABA-Li label (Figs. 1-5, 7). This label was present in sections incubated with either the peroxidase-antiperoxidase (which has a high background staining due to endogenous peroxidase activity) or glucose oxidase-anti-glucose-oxidase (which eliminates background staining as it is not a mammalian enzyme). Moreover, this pattern of labelling was observed in our GAD material (Fig. 11E). It seems likely that the various GABA-Li neuronal types observed in the regio inferior contribute to the label in the mossy fiber region (see discussion).

Organization of the GABA-Li neuropil

General organization. The most distinct topography was observed within the fascia dentata. GABA-Li grains were more numerous in the dorsal (frontal and sagittal planes, (Figs. 2, 3, 5) and lateral (horizontal plane, Fig. 7) portions of the molecular and granule (pericellular networks) layers and hilar region. Moreover, GABA-Li grains and fibers were more numerous in the alveus of the regio superior as compared to the inferior portion (Figs. 2-5, 7). The abundance of fibers within the regio superior is evident in the dorsal hippocampus (Fig. 6). It is also noticed that possible GABA-Li grains were present within a region that may correspond to the area retrosplenialis.

Quantitative analysis. The calculation of GABA-Li within the neuropil was performed only in our semithin sections, as the visibility of GABA-Li-positive grains was remarkably clear. In that the various cases were rather similar in density for the different hippocampal layers, the values presented in Figure 8 are a grouping of the results obtained from several cases in different planes of section. These results are in keeping with biochemical studies in which GAD activity was measured within each layer of the hippocampal formation (see Discussion).

Within the pyramidal cell dendritic subfields of Ammon's horn a more-or-less homogenous grain density was observed (Fig. 8). In this area, the interface region and the alveus had the highest grain densities (26 ± 6 and 22 ± 2 respectively, per $100 \mu\text{m}^2$). Furthermore, a slightly higher density was found in the stratum radiatum of CA3/a as compared to that of CA1-CA2 (25 ± 6 and 19 ± 3 respectively).

In contrast to the dendritic subfields of Ammon's horn, differences in grain density were observed within the dendritic regions of the fascia dentata. Here, not only was there a difference between the outer third and the inner two-thirds of the dentate molecular layer of the suprapyramidal blade (40 ± 4 and 26 ± 1 , respectively, Fig. 8), but we have also observed a slightly lower value in the infrapyramidal (approx. $22/100 \mu\text{m}^2$) and rostromedial (27 ± 3) portions of the dentate molecular layer (not illustrated, see Figs. 2, 3, 5, 7). In addition, differences in the density of GABA-Li grains were found between the two subdivisions of the hilar region (33 ± 5 and $21 \pm 3/100 \mu\text{m}^2$, respectively, Fig. 8). Moreover, the hilar region showed a slight difference be-

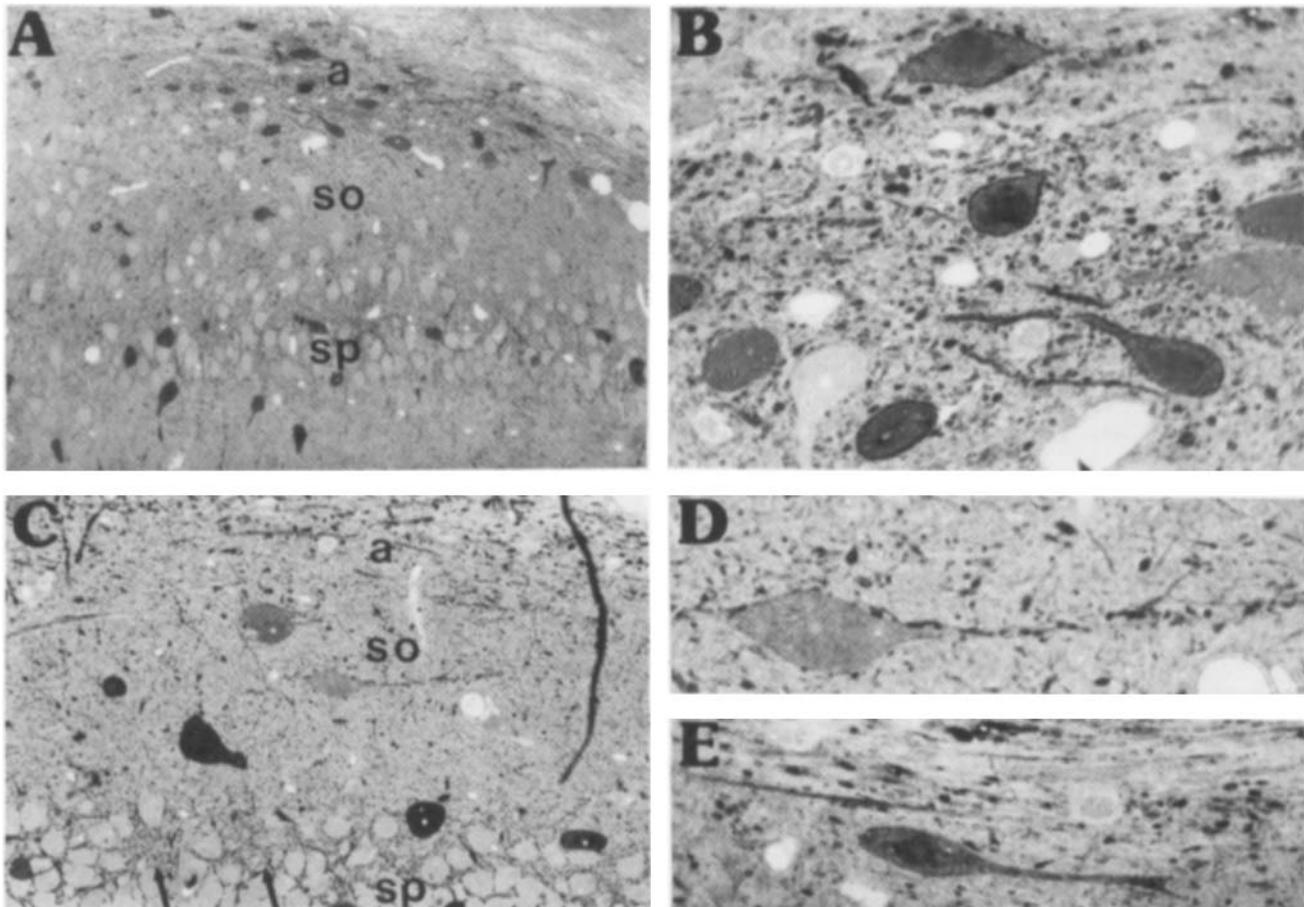


Fig. 9. Photomicrographs showing GABA-Li in the subicular and CA1 regions of Ammon's horn in semithin sections. A,B: Low and high magnifications of GABA-Li in the CA1-subicular region. Notice the distinct difference in GABA-Li grain size in B. Photomicrographs taken from the section illustrated in Figure 4. Sagittal plane. $\times 230$, and $\times 1,032$, respectively. C,D: Label of GABA-Li in the CA1 region of the stratum oriens. GABA-GABA interactions are evident on the lighter stained GABA-Li cells. A

high magnification of the labelled horizontal cell is shown in D. Within the superficial portion of the pyramidal layer in C, pockets of GABA-Li grains are observed (arrows). Sagittal plane. $\times 131$ and $\times 2,100$, respectively. E: High magnification of a similar GABA-Li-positive cell within the stratum oriens; however, no GABA-Li terminals are seen on its perimeter. Sagittal plane. $\times 944$.

tween its lateral and medial portions in the horizontal plane (34 ± 3 and $27 \pm 6/100 \mu\text{m}^2$, respectively, not illustrated, see Fig. 7).

Finally, we have counted the number of GABA-Li-positive grains attached to the perimeter of pyramidal and granule neuronal somata (Fig. 14). These data indicated a significant difference ($P < .01$) in the number of terminal grains from granule cell to CA1-CA2 pyramid to CA3 neuron (granule cell: approx. 21 grains/soma; CA1-CA2: approx. 34 and CA3 approx. 43).

In conclusion, these results (Figs. 8, 14) show that 1) whereas few variations in GABA-Li grain density are found within the dendritic regions of Ammon's horn, distinct differences are observed in the molecular layer and the hilar region of the fascia dentata; 2) the number of GABA-Li-positive grains attached to the neuron is correlated to the perimeter of the somata.

Morphology of the GABAergic neuropil. In our semithin material both coarse and fine GABA-Li grains were evident (Figs. 9B, 10D-G). The arrangement of the coarse GABA-Li grains within the interface region was very distinct from

the label within either the stratum radiatum or the stratum lacunosum moleculare (compare Fig. 10C-E). This label was often continuous and similar to the prominent GABA-Li grains within the stratum radiatum of CA3/a (see Figs. 2, 4, 5, 7). It is noticed in Figure 8 that the density within the two regions was very similar.

In Figure 10E, there is a considerable difference in the amount of immunoreactive product between the molecular layers of Ammon's horn and the fascia dentata which can be observed in our grain density measurements (see Fig. 8). Moreover, Figure 10F shows a striking distinction between the two subdivisions of the hilar region in respect to immunoreactive product. Possibly related is that many cut GABA-Li processes are located just under and aligned parallel to the granule cell layer (Fig. 10G). As already mentioned there is also a considerable difference in the grain density measurements between the two areas.

Several additional points concerning the GABAergic neuropil deserve emphasis. In agreement with Kunkel et al. ('86) we have observed GABA-Li somata surrounded by GABA-Li-positive terminals in the stratum oriens and the

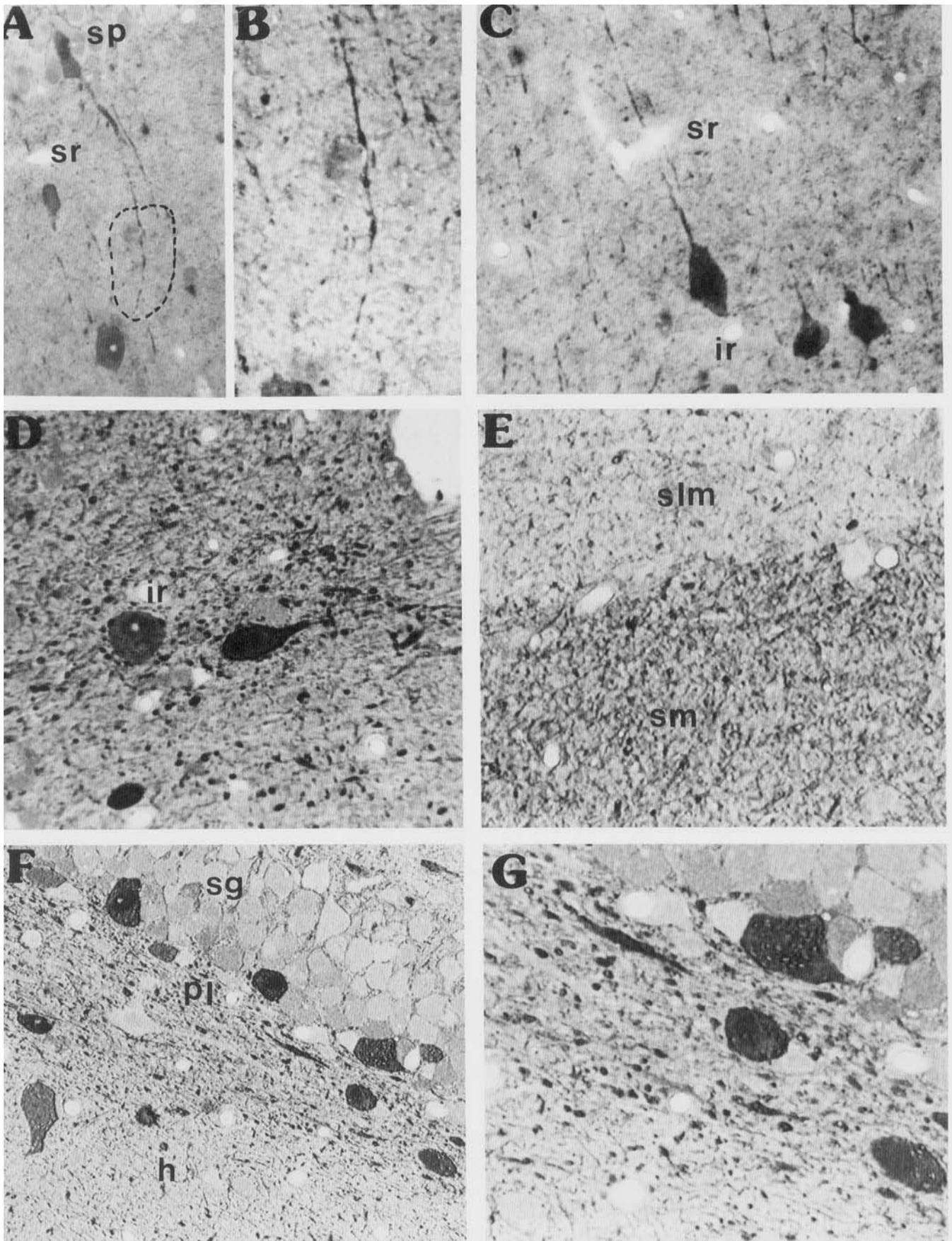


Figure 10

interface region (Figs. 9C,D, 10D). In Figure 9E a similar GABA-Li-positive neuron is located within the stratum oriens; however, no immunoreactive grains were seen on its cell body, suggesting that only certain GABA-Li cells in this layer receive GABAergic input. Furthermore, each layer had a characteristic grain pattern. Thus within the stratum oriens of CA1-CA2 the grains, primarily of the coarse type, were basically oriented parallel to the surface of the alveus (Figs. 2-7, 9C). It was noticed that this label is continuous with the fiberlike arrangements within the fimbria (Figs. 2, 3, 7). The subiculum and to a lesser extent the stratum oriens of CA3 had a scattered distribution of GABA-Li grains (Figs. 2-7). Within the stratum pyramidale, in addition to the classical pericellular terminal networks, "packets" of GABA-Li grain accumulations were in the pyramidal layer and at its border with the stratum oriens (Figs. 3, 9C). These packets were more frequent within the regio superior of the hippocampal formation.

Finally, there were two distinguishing features concerning the labelling in the stratum radiatum of CA1-CA2: 1) All planes of section showed GABA-Li grains to be oriented toward the above pyramidal cell layer (Figs. 2, 4, 5, 7, 10C). It would seem that this immunoreactive labelling is largely due to the cut processes of GABAergic neurons located within both the CA1-CA2 pyramidal cell layer and interface region (see Figs. 10A-C), and 2) Varicose swellings on GABAergic processes were frequent within this region (Fig. 10B).

DISCUSSION

General organization of GABA-Li in the hippocampal formation

The present description of GABA-Li in the hippocampal formation is confirmatory of the many studies which have used various GABAergic markers to identify this inhibitory neurotransmitter (Storm-Mathisen, '72; Nadler et al., '74; Barber and Saito, '76; Ribak et al., '78; Köhler and Chan-Palay, '83; Seress and Ribak, '83; Nagi, et al., '83; Somogyi et al., '84; Kosaka et al., '84, '85; Ottersen and Storm-Mathisen, '84; Somogyi and Hodgson, '85; Gamrani et al., '86; Kunkel et al., '86; Storm-Mathisen and Ottersen, '86; Sloviter and Nilaver, '87). However, the comparison between the various planes and the quantitative estimation of both GABA-Li-positive cells and neuropil density have provided additional information.

1) Approximately 11% of the "total population" of hippocampal neuronal somata in our sections are GABA-Li positive. If this estimate is applicable throughout the hippocampal formation, then of the approximately 1,300,000 neuronal somata in the adult Wistar rat (see Boss et al.,

'87), over 100,000 cells could exhibit GABA-Li throughout its 5.6-mm rostrocaudal extent. (See Amaral, '78, concerning the hippocampal length.) However, this point awaits clarification. The highest percentage of these neurons are found in the stratum oriens, stratum radiatum, interface region, and the polymorph layer.

Furthermore, 5-8% of the cells within the pyramidal cell layer of subfields CA1-CA3 are GABA-Li positive, thus supporting the observation of Boss et al. ('87) that the number of nonpyramidal neurons is quite small. In this connection a value of 3% is observed within the hilar pyramidal cell layer and 1-2% within the fascia dentata granule cell layer. Whereas the total percentage of GABA-Li neuronal somata within the pyramidal cell layer of Ammon's horn is quite similar to the 20% estimation of the total percentage of nonpyramidal neurons (Braitenberg and Schuz, '83), our estimate of 1-2% in the granule cell layer is just under the value (5%) given by Seress and Pokorný ('81). The percentage found in the suprapyramidal polymorph layer (60%), not the "hilar region," is in agreement with earlier studies (See Seress and Ribak, '83, concerning their percentage within the "hilar region".) These observations reinforce the classical view that most of the (somatic) GABAergic innervation originates from the extra-pyramidal (or granular) layers where most of the GABAergic neurons are located.

2) Only slight differences in GABA-Li neuronal somata percentage (and grain density, see below) are found within the pyramidal cell dendritic subfields, which is in contrast to distinct differences in these GABA-Li elements within the dendritic regions of the fascia dentata. Thus, the 80-95% estimate for the pyramidal cell dendritic subfields justifies the notion that many of the heterogeneous population of cells described with Golgi stains in Amaral's ('78) zone 2 (see his Fig. 1A) are presumably GABA-Li positive. It was his conclusion, in fact, that these cells would mainly operate as interneurons. However, it is evident that these cells do give rise to long associational projections (see Köhler and Chan-Palay, '83). Along these lines, there are clear fiberlike arrangements within the superficial stratum oriens-alveus of the CA1-CA2 region. Coarse and fine GABA-Li grains (suggestive of termination) are also found in the stratum oriens of CA3 just next to and continuous with the label in the fimbria and in the subiculum. Both of these regions are targets for the termination of associational-commissural projections (Swanson et al., '78). Our finding of GABA-Li fiber arrangements in the fimbria (in agreement with Ottersen and Storm-Mathisen, '84) might indicate that at least some of the commissural projection is GABAergic, as has already been suggested by physiological study (Buzsáki and Czéh, '81). However, 60% would be well above our estimation (see Seress and Ribak, '83).

The grain density in the pyramidal cell dendritic subfields varies between 20 and 25 grains per $100 \mu\text{m}^2$. This distribution is to be compared with the measurements of GAD activity per dry weight (Storm-Mathisen, '76) which indicate that there is a homogeneous distribution in most hippocampal layers with the exception of the distal dendritic segment in the stratum lacunosum moleculare where higher values were found. Also very similar to these GAD activity measurements is the rather large difference between the GABA-Li grain counts within the outer (40 ± 4) and inner (26 ± 1) portions of the dentate molecular layer. Interestingly, as the stratum radiatum extends into the suprapyramidal hilar region (see Amaral, '78), our grain

Fig. 10. Photomicrographs demonstrating the distribution of GABA-Li in the CA1 pyramidal and fascia dentata regions. A,B: Typical GABA-Li "basket" neuron with process extending into the stratum radiatum. The circled region in A is enlarged in B. Frontal plane. $\times 371$ and $\times 912$, respectively. C: GABA-Li neuronal somata within the interface region. The oval and semitriangular neurons extend their processes into the stratum radiatum. Frontal plane. $\times 320$. D: GABA-Li neuronal and neuropil label within the interface region. Sagittal plane. $\times 1,000$. E: Illustration showing the distinct difference of immunoreactive product between the CA1-CA2 and fascia dentata molecular layers. Sagittal plane. $\times 900$, Nomarski optics. F,G: Low and high magnifications of GABA-Li in the hilar region and polymorph layer. In F a clear difference is found between the GABA-Li in the different subdivisions of the hilar region. Also notice the thick process just under the subgranular polymorph layer. Sagittal plane. $\times 450$ and $\times 900$, respectively; Nomarski optics.

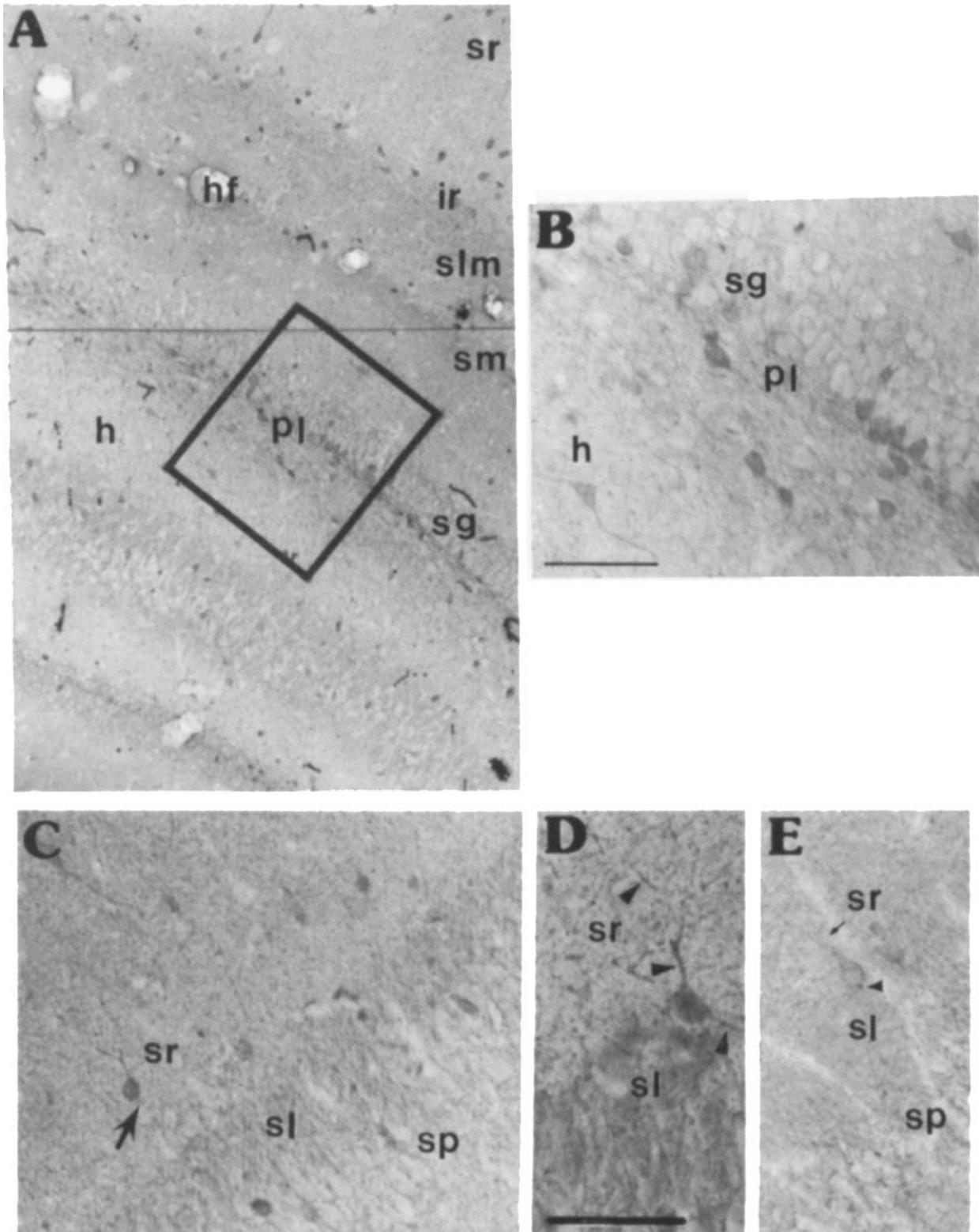


Fig. 11. Photomicrographs illustrating the general distribution of GABA-Li and GAD-Li in a thick section ($50\ \mu\text{m}$) preparation. **A:** Montage showing GABA-Li within the CA1 strata radiatum ~ lacunosum moleculare dendritic and hilar region. Notice the numerous GABA-Li-positive neuronal somata within the interface and polymorph regions. GABA-Li within the neuropil appears as a dust-like homogeneous label in contrast to the clear granular appearance in semithin sections. $\times 80$. The box in the hilar region is enlarged in **B:** Bar equals $220\ \mu\text{m}$. **C:** Photomicrograph showing the

intense GABA-Li label in the stratum lucidum. Notice the cell (arrow) resembling the Amaral and Woodward ('78) interneuron within the stratum radiatum. $\times 220$. **D:** High magnification of a inferior region interneuron stained with anti-GABA antibody. Note the thick dendrites (arrowheads) directed toward the stratum radiatum. The thin process, presumably an axon, penetrates the stratum lucidum. Bar equals $100\ \mu\text{m}$. **E:** GAD immunocytochemical demonstration of a similar interneuron in the stratum lucidum. Notice the intense GAD-Li label in the axonal hillock. $\times 240$.

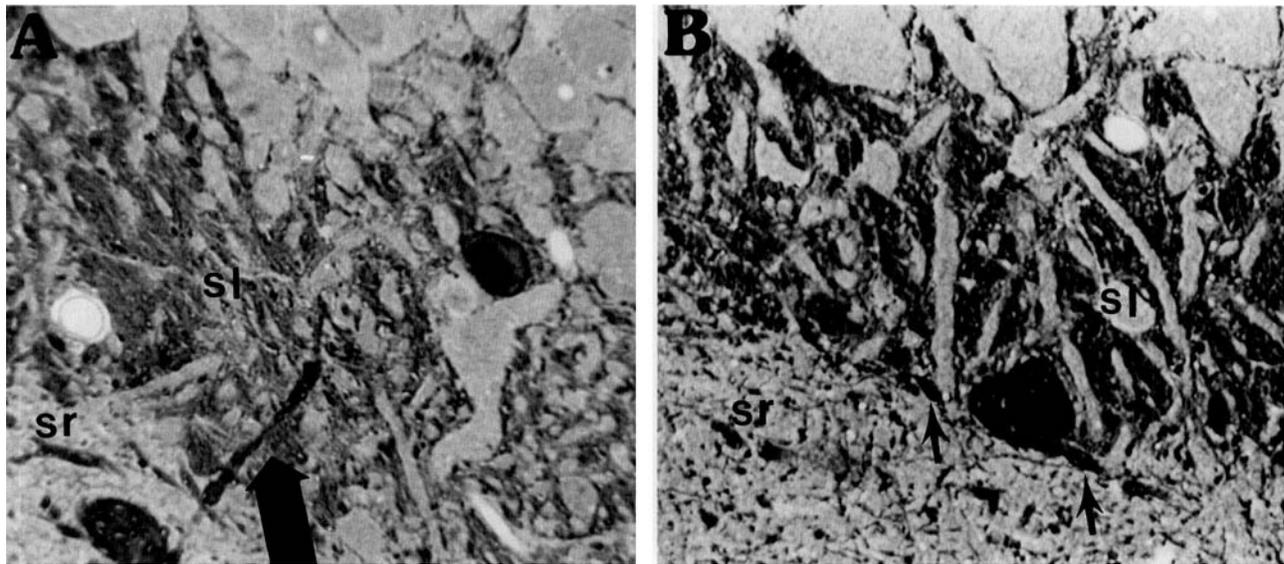


Fig. 12. Photomicrographs showing GABA-Li cell types at the stratum radiatum ~ stratum lucidum interface. A: Oval-shaped cell with thick process (arrow) extending into the stratum lucidum. Sagittal plane. $\times 400$. B: Triangular-shaped neuron with processes oriented parallel to the inter-

face between the strata radiatum and lucidum (arrows). Notice the surrounding pyramidal cell processes embedded within the GABA-Li-positive stratum lucidum. Sagittal plane. $\times 900$, Nomarski optics.

density measurements show a similarity between the two areas. Finally, when expressed in terms of the number of GABA-Li terminal grains per soma we find an increase from granule cell to CA1-CA2 pyramid to CA3 neuron, suggesting that the amount of GABAergic innervation on the somata perimeter depends largely on the available membrane surface.

Gradients of GABA-Li distribution in the fascia dentata

A most distinct topographical pattern of GABA-Li is found in the fascia dentata. Here, the different planes of section suggest several gradients in which the number of cells decrease from dorsal to ventral and from lateral to medial: 1) frontal: dorsal (suprapyramidal) to ventral (infrapyramidal); 2) sagittal: rostradorsal to rostroventral and 3) horizontal: rostrolateral to rostromedial. As the lateral and medial portions in horizontal sections are equivalent to the dorsal and ventral portions, respectively, in the frontal and sagittal planes (see Schlessinger et al., '75), the GABA-Li gradients are completely in accord with each other.

The above GABA-Li topographical arrangements bear close similarity to both the neurogenic gradients of granule cell formation and the neurogenesis and distribution of mossy fibers. A consideration of each of these points is therefore valid.

It is well known that a vast majority of the dentate granule cells develop postnatally (Schlessinger et al., '75; Bayer, '80). Schlessinger et al. and Bayer have delineated several neurogenic gradients which are observed during granule cell ontogenesis. These are 1) a dorsal (suprapyramidal) to ventral (infrapyramidal) gradient (i.e., granule cell layer proliferation begins in the dorsal blade prenatally and then within the ventral blade postnatally), 2) an ectal to endal gradient (Bayer, '80), which is equivalent to the lateral to medial definition of Schlessinger et al. ('75), and 3) a temporal to septal gradient (Schlessinger et al., '75).

According to Bayer ('80) the postnatal granule cell formation in the medial or ventral blade lags behind that of the dorsal blade by approximately a few days. Moreover, these authors state that "the earlier"-forming granule and hilar cells are influenced by these gradients.

It is therefore evident that the gradients defined in our GABA-Li material closely resemble the above neurogenic gradients. This seems to provide evidence which points to the idea that the earlier-forming regions within the fascia dentata affect the postnatal distribution of GABA-Li. However, a possible differential GABA-Li cell death within the fascia dentata certainly cannot be discounted.

Amaral and Dent ('81) have shown similar neurogenic gradients for the maturation of mossy fibers. Seemingly, the overall postnatal distribution of GABA-Li may additionally be influenced by this gradient as the chronological establishment of recurrent inhibition is most likely of importance. Furthermore, the slight rostral inclination of mossy fibers (Lynch et al., '73; Swanson et al., '78,) may also influence the rostral accumulation of GABA-Li within the polymorph layer.

The fact that the regio superior pyramidal cell dendritic subfields of the stratum oriens contain more GABA-Li neuronal somata than the regio inferior is not compatible with any specific neurogenic gradient. However, this difference may be related to a differential migration of GABA-Li-positive cells, since a recent study suggests that the highest amount of GABA-Li neurons in the hippocampal formation is found in the regio superior within the superficial stratum oriens aligned within and just beneath the germinal zone during the first postnatal days (Rosenberg, et al., in preparation).

GABA-Li labelling in the stratum lucidum

The present results indicate that stratum lucidum is enriched in GABA-Li label. Furthermore, in agreement with a brief earlier immunocytochemical description (Barber and

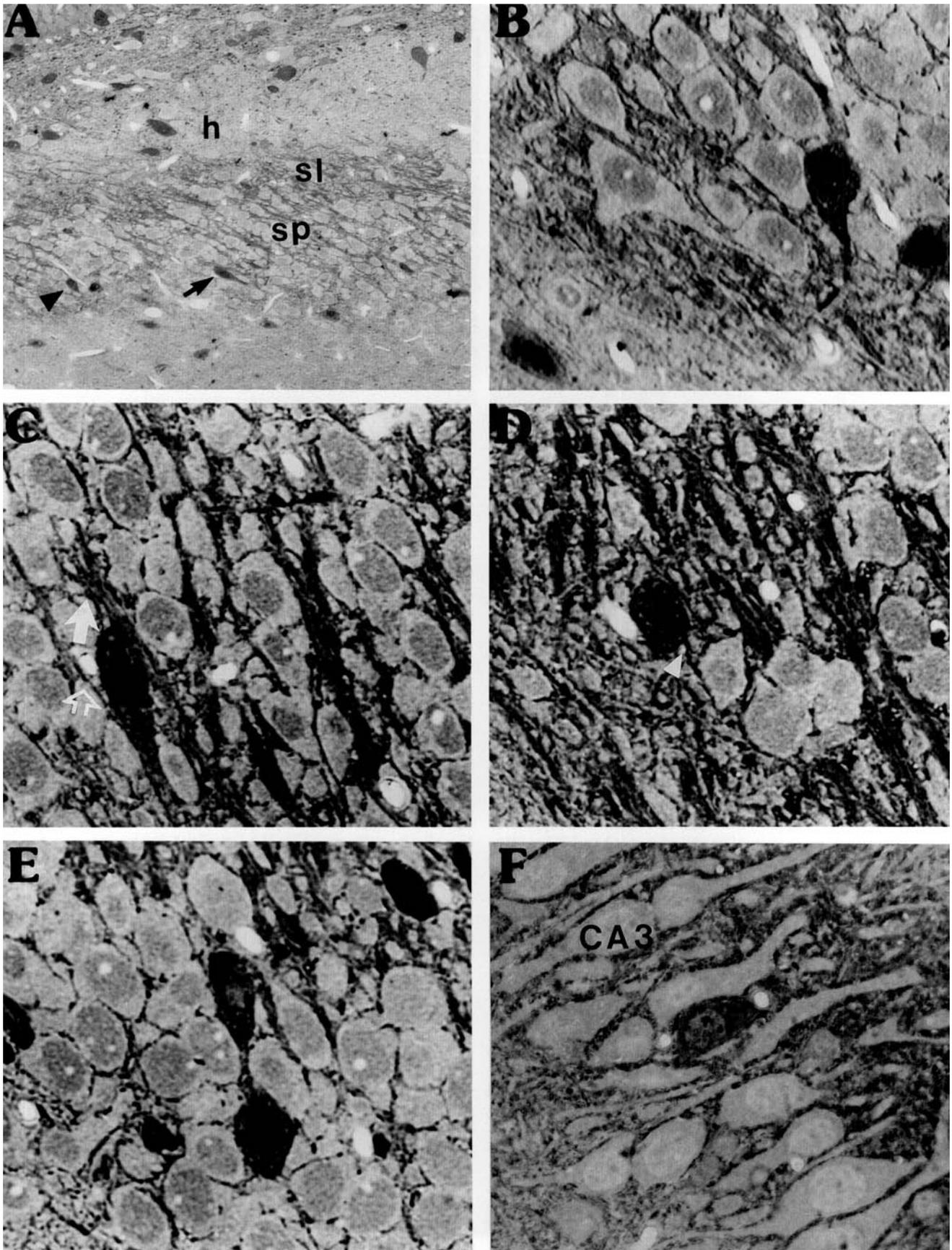


Figure 13

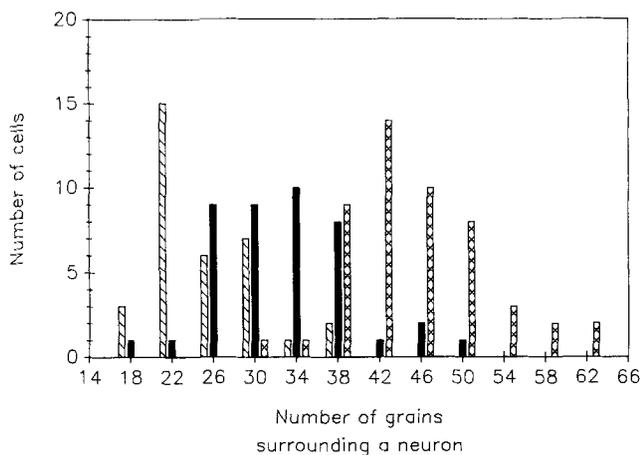


Fig. 14. Results illustrating GABA-Li grains surrounding granule (slanted lines) and pyramidal (CA1, solid lines, and CA3, hatched lines) neurons. $P < .01$. Student's *t*-test.

Saito, '76), we found that GAD is also present in this zone, suggesting that the labelling is due to a conventional dense GABAergic innervation and not GABA, which would be formed by other routes not involving GAD as suggested by Storm-Mathisen and Ottersen ('86).

To account for this labelling, the following possibilities must be considered. First, the labelling may reside in the mossy fibers themselves, as suggested by Ottersen and Storm-Mathisen ('84). This possibility can be excluded, since following a complete destruction by colchicine of the granule cells and the mossy fibers, both GABA-Li-positive neurons and the GABA-Li labelling in the stratum lucidum are unaffected (Robain, et al., in preparation). Second, there is evidence which suggests that the mossy fibers release an excitatory amino acid (Storm-Mathisen, '81). Therefore, it is probable that the labelling is due to a dense network of GABAergic elements that originate from interneurons or projecting GABAergic neurons. As seen in our material, there is in the vicinity of the stratum lucidum a heterogeneous population of GABA-Li-positive neurons, which have processes intimately related to the label found in the mossy fiber region. The possible alignment of GABA-Li processes along the mossy fibers (compare Fig. 13 A-C to Fig. 8 of Braitenberg and Schuz, '83) is of interest as this finding, to our knowledge, has not been reported. However, electron microscopic studies are necessary to resolve the nature of the GABA-Li label within the stratum lucidum.

Fig. 13. Photomicrographs demonstrating GABA-Li neuronal types within the hilar region and CA3 pyramidal cell layer. A-C: Low and high magnifications of GABA-Li neuronal somata in the hilar pyramidal layer. Notice the parallel orientation of the GABA-Li processes. The arrowhead and stemmed arrow in A indicate the triangular and bipolar neurons shown at high magnification in B and C. The arrows in C and D indicate immunoreactive processes within this region. Sagittal plane. A, $\times 256$; B and C: $\times 900$, Nomarski optics. D: A round GABA-Li neuron within the hilar pyramidal region having processes oriented within the GABA-Li-positive label. (arrowhead). Sagittal plane. $\times 900$, Nomarski optics. E: GABA-Li neuronal arrangement in the hilar pyramidal layer. Sagittal plane. $\times 900$, Nomarski optics. F: The frequently observed round GABA-Li neuron in the CA3 pyramidal cell layer. Horizontal plane. $\times 500$.

Morphology of GABA-Li neurons

Morphological details of GABA-Li neuronal somata were quite evident in our material. The most important points to be emphasized include the existence of GABA-GABA interactions and the presence of a heterogeneous population of GABA-Li neuronal somata within the hippocampal formation, particularly as concerns the regio inferior. In respect to the former point, GABA-Li terminals cover both the soma and processes of GABA-Li-positive cells in the stratum oriens (in agreement with Kunkel et al., '86). It was noted that these interactions are basically observed on the lighter-stained GABA-Li-positive neuronal somata, which most likely reflect the physiological state of the neuron (see Ottersen and Storm-Mathisen, '84). A possible source of these GABA-Li terminals is from long associational-commissural projections in CA1 and CA2 (see Kohler and Chan-Palay, '83).

As GABA-GABA connections have additionally been reported in the pyramidal cell layer and visual cortex (see Roberts, '80, for a review), this may be a general organizational arrangement within the central nervous system. The functional significance of GABA-GABA interactions would most likely be related to disinhibition.

The morphological features of many hippocampal neurons have been described in Golgi material by Ramón y Cajal ('11), Lorente de Nó ('34), Amaral and Woodward ('77), and Amaral ('78). Figures 6-8 of Lorente de Nó ('34) show neurons that resemble the immunoreactive cells in our material. According to these figures, while the horizontal GABAergic interneurons of the stratum oriens basically have dendritic processes ramifying within the layer, the basket cell neurons within the pyramidal layer of CA1 and CA2 and interneurons at the stratum radiatum/stratum lacunosum moleculare interface of the same region have dendritic processes oriented opposite to each other ramifying within the stratum radiatum.

Neither the above Golgi studies nor our material showed morphological details of GABA-Li-positive cells in the stratum radiatum of the CA3/a region. In contrast, our material did reveal excellent morphological details of labelled neuronal somata in both the CA3/b,c stratum radiatum and the hilar pyramidal cell layer. In addition to our possible inferior region interneuron (see below), we could identify at the interface between the strata radiatum and lucidum a triangular neuron. This has not been described in Golgi material. As the processes of this GABA-Li neuron are uniquely aligned parallel to the interface between the two areas, it is possible that this cell type functions specifically at the interface. Indeed, as this cell belongs to Amaral's ('78) zone 2, it would fit his description of the neuronal constituents of this area, which are specialized to function at the interface between the fascia dentata and the hippocampus.

Within the hilar pyramidal cell layer, we have observed a heterogeneous population of very distinctive GABA-Li neurons, particularly in the sagittal plane. These cells are round, oval, and triangular and are characterized by having thick processes oriented parallel to the many GABA-Li processes in this area. It is our tentative belief that these cells could represent different variations of Amaral's ('78) long-spined multipolar cell (see his Fig. 25). According to him, this cell type is highly unique in that it has a very extensive longitudinal spread of dendrites.

Finally, we could additionally identify a neuron that resembles Amaral and Woodward's ('77) inferior region inter-

neuron. This cell type is in an interesting position since it may receive input from the entorhinal cortex (i.e., the perforant path) and possibly commissural and cholinergic septal afferents (see Amaral and Woodward, '77). There is also some evidence that this neuron may receive mossy fiber termination (Amaral and Woodward, '77), and there is anatomical evidence that mossy fibers directly innervate the GABAergic pyramidal basket cells of the CA3 region (Frotscher '85). Therefore, the inferior region interneurons constitute an obvious candidate for the important feed forward inhibition described in CA3 neurons (Buzsáki and Czéh, '81; Alger and Nicoll, '82; Frotscher et al., '84).

These neurons are probably destroyed following severe limbic seizures produced by kainate, along with the destruction of the pyramidal cell layer of CA3 (see Ben-Ari, '85). This procedure spares the mossy fibers, and there is electrophysiological evidence of a decrease of hippocampal GABAergic neurons in kindled rats (Kamphuis et al., '87). This would tend to enhance excitability and is likely to contribute to the presence of epileptic discharge. Recently, however, Sloviter ('87) has proposed the loss of a basket-cell-activating system in the pathology of the epileptic state.

CONCLUSION

The organization of GABAergic system in the hippocampal formation is highly complex, in keeping with the multiplicity of effects of GABA described in electrophysiological experiments (see Andersen et al., '82). Furthermore, the hippocampal GABAergic cell population is heterogeneous and probably consists of many different specialized types, such as the axoaxonal cell (see Somogyi et al., '84), and the unique assemblage of GABA-Li cell types which appear aligned along the mossy fibers. The heterogeneity of GABA-Li cells is further reflected by their coexistence with various neuropeptides: Cholecystokinin octapeptide (CCK), vasoactive intestinal polypeptide, neuropeptide y, and somatostatin (Somogyi et al., '84; Kosaka et al., '85; Köhler et al., '86). It also appears that GABA-Li somatostatin and CCK cells are distributed differently within the hippocampal layers (Somogyi et al., '84; Sloviter and Nilaver, '87). Clearly this heterogeneity must be taken into consideration in order to comprehend better the physiological role of the GABAergic system in the hippocampal formation.

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