Developmental Brain Research, 44 (1988) 197–209
Elsevier

BRD 50832

The development of GABAergic neurons in the rat hippocampal formation. An immunocytochemical study

László Seress* and Charles E. Ribak

Department of Anatomy and Neurobiology, University of California, Irvine, CA 92717 (U.S.A.)

(Accepted 26 July 1988)

Key words: γ-Aminobutyric acid; Glutamate decarboxylase; Rat; Immunocytochemistry; Light microscopy

Recent studies have indicated that hippocampal GABAergic neurons in both the dentate gyrus and Ammon's horn are generated prenatally. Although the adult distribution of GABAergic neurons has been previously described by numerous investigators, the early postnatal appearance of these neurons has not been described. In the present study, immunocytochemical methods were used to localize GABAergic neurons with antisera to both GABA and its synthesizing enzyme, glutamate decarboxylase (GAD). The GABA-positive neurons appeared at the earliest postnatal day (PND) examined, 4 PND. In contrast, GAD-positive cells were not observed until 6 PND, and the number of these neurons remained less than that of the GABA-positive neurons until 14 PND. These findings indicated that immunocytochemically detectable amounts of GAD were not present in many young GABAergic neurons. Both GABA- and GAD-positive hippocampal neurons showed two large increases in number during the 4–8 PND and 12–16 PND time periods, and they reached about 90% of adult levels before 18 PND. The regional distribution of GABA- and GAD-positive neurons throughout the hippocampal formation was homogeneous for all ages examined except 4 PND. At this age, the GABA-positive cells appeared in clusters in the proximal CA3 and the distal CA1 relative to the dentate gyrus. In addition, the number of hippocampal neurons immunostained in adult preparations for both antisera to GABA and GAD showed a similar number and distribution. The data on the developmental appearance of GABA and GAD immunoreactivities are consistent with biochemical data for the development of GABA concentration and GAD activity in the hippocampal formation. Together, these data provide important information about the functional maturation of the hippocampal GABAergic system in the first 3 weeks of rat brain development.

INTRODUCTION

γ-Aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the mammalian brain. The distribution of GABAergic neurons in the hippocampal formation of adult rats has been described using antiserum against GABA[5,11,41] and glutamate decarboxylase (GAD), its synthesizing enzyme[23,34,36]. These studies showed that the classically described local circuit neurons of the hippocampal formation[8,20] are immunoreactive for GABA and GAD whereas the major projection neurons of the hippocampus and dentate gyrus, the pyramidal and granule cells, respectively, are not labeled. These findings do not exclude the possibility that GABAergic neurons in this brain region have long projections because it has recently been demonstrated that a small proportion of GABAergic neurons display projections to the contralateral hippocampus[19,33]. In contrast to the numerous studies of GABA neuronal function in adult rats, only a few studies have analyzed the development of GABA neurons in the hippocampal formation.

The time that GABAergic neurons are generated in the rat hippocampal formation has been determined. Since all neuronal generation in the Ammon's horn occurs prenatally[7], the GABAergic neurons in this region are probably generated before birth. The situation is different in the dentate gyrus where granule cells are generated both pre- and postnatally[1]. However, the GABAergic neurons in this region have been shown to be generated prenatally[4,22,43].

* On leave from: Department of Physiology, University Medical School, Pécs, Hungary.
Correspondence: C.E. Ribak, Department of Anatomy and Neurobiology, University of California, Irvine, CA 92717, U.S.A.

Therefore, the data on the generation of GABAergic neurons indicate that they are formed prenatally throughout the hippocampal formation. Consistent with this conclusion is the finding that hippocampal neurons obtained from 17–21-day-old fetuses synthesize GABA in culture[14].

Two approaches have been used to obtain data on the functional development of GABAergic neurons. The first has used biochemical methods to measure GAD activity, GABA content and GABA receptor binding in the developing brain. At birth, only 50% of the adult level of GABA and 10% of the adult level of GAD activity are detectable[9,45]. During the first 4 weeks of postnatal development, some regions such as the cerebral cortex exhibit a 30-fold increase in GAD activity. Thus much greater levels of GABA are present at birth relative to its biosynthetic enzyme, GAD. In contrast, the development of GAD activity follows closely the development of GABA receptor binding, and therefore GAD activity may provide a more accurate index of the differentiation of the GABAergic synapses than GABA levels[9]. Recently, Swann et al.[44] have shown a similar finding for the hippocampal formation in that GABA content was relatively larger at birth than GAD activity. In addition, they showed that GAD activity in the CA3 and CA1 subregions developed at similar rates.

A second method that can be used to analyze the functional development of GABAergic neurons is electrophysiology. Swann et al.[44] have shown that inhibitory postsynaptic potentials (IPSPs) that can be blocked by GABA antagonists are found as early as 5 or 6 postnatal days (PND) for CA3 pyramidal cells in rats. However, such IPSPs develop 1–2 days later in CA1[13,44]. This functional difference in the development of inhibition in the hippocampus does not correlate with the developmental changes in GAD activity (see above). Therefore, a number of factors may be involved in the functional development of GABAergic neurons; i.e. presynaptic maturation, receptor maturation, membrane characteristics, etc. Together, these data indicate that important developmental changes of the GABAergic system may take place during the first 3 postnatal weeks.

Consistent with this notion of a functional postnatal development of GABAergic inhibition are the data for the axonal and dendritic development of hippocampal neurons. The axon arborization of Golgi-impregnated local circuit neurons in 1- and 4-day-old rats is considerably shorter than in young adult rats[39]. Only a few studies have described the development of identified cell types in the rat hippocampal formation, and the results are consistent with the notion that a significant amount of dendritic and axonal growth occurs postnatally[1,21,26,35].

The present study was undertaken to determine the postnatal appearance of GABA- and GAD-immunoreactive neurons of the hippocampus and dentate gyrus. This immunocytochemical approach was used to provide additional information about the functional development of GABAergic inhibition in the hippocampal formation. With this method, we wanted to determine: (1) whether some parts of the hippocampal formation display GABA- and GAD-immunoreactive neurons earlier than other regions, (2) when the first GABA- and GAD-immunoreactive neurons appear and (3) the age when the number of immunoreactive neurons reaches adult numbers.

MATERIALS AND METHODS

Animals

Sprague–Dawley rats that were obtained from Simonsen Laboratories (Gilroy, CA) were used in this study. Litters were generated from these rats, and the young pups were used at various postnatal ages. The day that the rats were born was denoted as PND 0. The rats that were used in the immunocytochemical study for the localization of GAD were 4, 6, 8, 10, 12, 14, 16, 18, 20, 24 and 90 PND of age. Two animals were used at each of these ages. The rats used to localize GABA were 4, 6, 8, 14, 16 and 90 PND. Either 2 or 3 rats were studied at each of these ages.

Immunocytochemistry

All animals were anesthetized with Nembutal and transcardially perfused with a fixative that contained either 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for GAD immunocytochemistry or 4% paraformaldehyde and 1.0% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for GABA immunocytochemistry. The day following the perfusion, brains were dissected from the crania and placed in phosphate-buffered saline (PBS) and 15% sucrose until they sunk. Frozen sections (40 μm) in the coronal plane through the hippocampal formation were

placed in PBS before they were processed for immunocytochemistry.

The immunocytochemical methods used for the localization of GAD and GABA were similar. Sections that were processed for GAD immunocytochemistry used a sheep anti-GAD serum characterized by Oertel et al.[24], and they were processed according to a modification of their protocol that employed an avidin-biotin-horseradish peroxidase complex[28]. Sections that were processed for GABA localization used a rabbit anti-GABA serum (Immunonuclear Co.), and they were subsequently incubated in reagents from a similar type of avidin-biotin-horseradish peroxidase complex kit (Vectastain, Vector Laboratories) as used for GAD immunocytochemistry. Briefly, free-floating sections were incubated in 0.1 M D,L-lysine in either 10% normal rabbit serum (for GAD) or 10% normal goat serum (for GABA) in 0.1 M PBS for 2–4 h followed by incubation in the primary antiserum for 24–48 h. Both GAD and GABA antisera were diluted to 1:2000 in PBS. Then, the sections were washed (3 10-min rinses in PBS) and incubated for 45 min in biotinylated secondary antibody. After another wash, the sections were incubated for 45 min in the avidin–biotin peroxidase complex. Then, the sections were reacted for 15–30 min in a diaminobenzidine (DAB) solution (6 mg DAB/10 ml PBS + 0.002% hydrogen peroxide) and washed again. Following the immunocytochemical processing, the sections were mounted on slides, dried, defatted and coverslipped.

Adjacent sections were stained with a 1% Cresyl violet solution to provide cytoarchitectonic information about the various regions of the hippocampal formation. In addition, one section from each rat was processed as described above except that the primary antiserum was omitted. These latter sections displayed no specific staining.

Quantitative analysis

Neurons that displayed GAD-immunoreactivity contained a dark brown reaction product in their perikaryal cytoplasm. The nuclei and most dendritic processes were unstained. In contrast, neurons that had GABA immunoreactivity contained reaction product in both the nuclei and cytoplasm (Figs. 1–4).

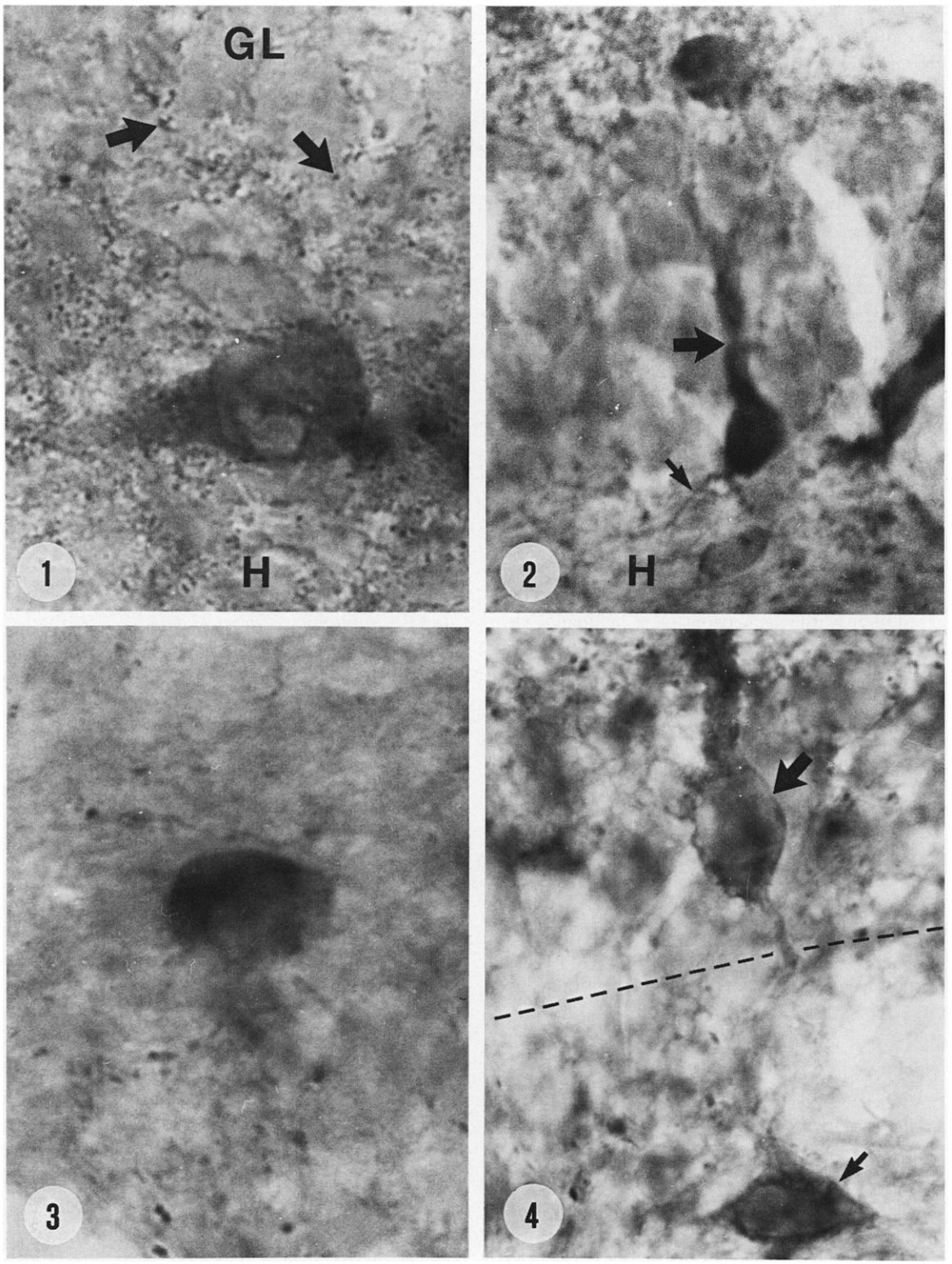
Sections from the middle of the dorsal hippocampus were used for the quantitative analysis of immunoreactive neurons in the hippocampal formation. This level is found between 4.5 and 4.9 mm rostral to the interaural line[25]. Five to 10 sections from this level were counted, and only sections that displayed good immunostaining were used. Immunoreactive neurons were counted in all parts of the hippocampus and dentate gyrus. The variation between sections at the same age was large, e.g., some sections showed 25–30% more immunostained neurons than a section that was 150–200 μm away from it. At the earlier ages, the more rostral sections consistently displayed more immunoreactive neurons than the caudal sections, and this variation could arise from the delayed development of the hippocampus in the rostral–caudal plane[12]. This variation caused the standard deviations to be more than 20% of the mean values. In addition to this intra-animal variation, animals in the same age group showed variations. These variations may be real or they could reflect differences in the immunocytochemical procedure.

RESULTS

GABA immunocytochemical data

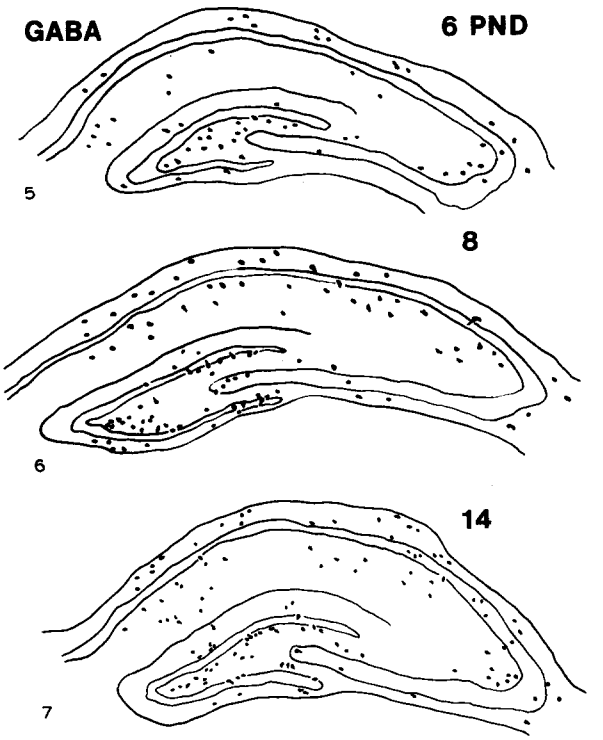
The youngest brains examined in this study displayed GABA-positive neurons in the hippocampus and dentate gyrus. Thus, at 4 PND GABA-positive neurons in the dentate gyrus were found in the hilus and molecular layer. Virtually all of these neurons were found in the hilus where they were observed in both superficial (close to the granule cell layer) and deep portions (Fig. 1). In contrast, the molecular layer rarely displayed GABA-immunoreactive neurons. At the same age, a few GABA-positive neurons were found in the CA1–3 areas where they were located either in stratum pyramidale or within 150 μm of this stratum in the adjacent strata oriens and radiatum. For CA3, most of the neurons were found in the subfield bordered by the two blades of the dentate gyrus, the CA3c. The CA1 region also showed a clustering of GABA-immunoreactive neurons, and this occurred in the part of CA1 that was most distal from CA3. The mean number of GABA-positive neurons per section was 9 for the dentate gyrus, 12 for CA3/2 and 9 for CA1.

The next examined age was 6 PND. The GABA-positive cells displayed similar staining characteristics as those studied at 4 PND, except that some


GL
1
H
2
H
3
4

showed immunoreaction product within the proximal dendrites. The extent of this staining was inadequate for the identification of a particular cell type. More GABA-positive neurons were observed in the dentate gyrus at this age but the ratio of neurons in the hilus as compared to the molecular layer appeared to be similar to that found at 4 PND. Thus, most GABA-positive cells in the dentate gyrus at 6 PND were present in the hilus as indicated in the diagram (Fig. 5).The CA1–3 regions also displayed more GABA-positive neurons at this age. In addition, their distribution was more homogeneous throughout these regions as compared to 4 PND. The same strata displayed GABA-positive neurons as that observed at 4 PND but no signs of neuronal clustering appeared in specific parts of the CA subregions (Fig. 5). The mean number of GABA-positive neurons was 19 in the dentate gyrus, 10 in the CA3/2 and 19 in CA1.

The number of GABA-positive neurons at 8 PND was much greater than that observed at 6 and 4 PND (Table I). In addition, dendritic staining was more frequently observed in the 8 PND preparations. A larger number of immunoreactive neurons was found in the molecular layer at this age as well but the large majority of GABA-positive neurons was located in the hilus, a finding consistent with the earlier ages analyzed. Many of the hilar neurons were found at the hilar/granule cell layer border. Some of these neurons had a major dendrite oriented perpendicular to the axis of the granule cell layer and frequently extended such dendrites into this layer (Fig. 2). Neurons with these features were tentatively identified as basket cells, a common GABAergic cell type in the adult dentate gyrus[34,36]. Other GABA-positive neurons were located deeper in the hilus, and some of them displayed dendrites oriented parallel to the granule cell layer. These cells were probably a type of fusiform cell[2,31]. The distribution of GABA-positive neurons in CA1–3 was homogeneous throughout the strata as indicated in the diagram (Fig. 6). However, the CA1 region displayed more neurons than the CA3/2 region. A few cells in the stratum pyramidale displayed a major dendrite oriented parallel to the plane of orientation of pyramidal cell dendrites. These cells were probably basket cells associated with the stratum pyramidale[37]. The mean number of GABA-positive neurons was 34 in the dentate gyrus, 20 in CA3/2 and 33 in CA1.



Figs. 5–7. Diagrams that show the distribution of GABA-positive cells in the hippocampal formation at 3 postnatal ages from representative, individual 40 μm sections. The orientation on the left side of these figures is similar to that shown in Fig. 14 with regard to the layers and subregions of the hippocampal formation. In addition, these figs. show CA3/2. Fig. 5 is from a 6 PND preparation. Fig. 6 is an 8 PND preparation. Fig. 7 shows a 14 PND preparation.

←

Figs. 1–4. Light photomicrographs of GABAergic neurons in the developing dentate gyrus. Fig. 1 shows a GABA-positive soma from a 4 PND preparation in a part of the hilus (H) found subjacent to the granule cell layer (GL). The presence of a dendrite oriented parallel to the GL indicates that this neuron may be a fusiform cell[31]. Note the GABA-positive puncta (arrows) found apposed to the somata of unlabeled granule cells. ×1500. Fig. 2 shows a GABA-positive pyramidal basket cell from an 8 PND preparation. It displays a thick apical dendrite (large arrow) that ascends through the granule cell layer and a thin basal dendrite (small arrow) in the hilus (H). ×1100. Fig. 3 shows a GAD-positive cell in the molecular layer of the dentate gyrus from a 6 PND preparation. Only a few GAD-positive puncta are found in the adjacent neuropil. ×1600. Fig. 4 shows two GAD-positive cells from an 8 PND preparation. One of these cells is a basket cell (large arrow) that lies within the granule cell layer whereas the other appears to be a fusiform cell (small arrow) in the hilus. ×1300.

TABLE I

Mean number of GABAergic neurons during postnatal development as demonstrated with antisera to GABA and GAD

Brain region	*4 PND*	*6 PND*	*8 PND*	*14 PND*	*16 PND*
GABA-positive neurons					
Dentate gyrus	9	19	20	38	59
CA3/2	12	10	20	20	45
CA1	9	19	33	50	73
GAD-positive neurons					
Dentate gyrus	15	16	15	34	52
CA3/2	20	14	19	36	45
CA1	30	33	27	64	70

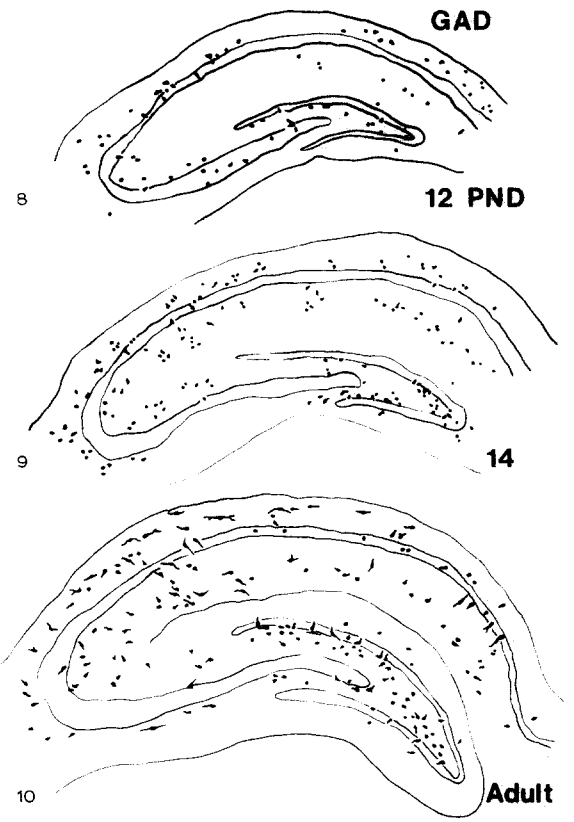
The 14 PND hippocampi showed most of the same features as that observed in preparations from 8 PND. A small increase in the number of GABA-positive neurons occurred in the dentate gyrus and CA3/2. In contrast, the number of neurons in CA1 increased much more in comparison with 8 PND (cf. Figs. 6 and 7). The extent of dendritic staining was similar in both the 8 and 14 PND preparations. The mean number of GABA-positive neurons at 14 PND was 38 in the dentate gyrus, 20 in CA3/2 and 50 in CA1.

The 16 PND preparations showed a similar number and distribution pattern of GABA-positive neurons as that observed in adult preparations. The number of GABA-positive neurons was about 90% of the adult values. These findings at 16 PND were so similar to the adult that older rats were not examined. The adult distribution of GABA-positive neurons was similar to the results previously described for the distribution of GAD-positive neurons[34,36]. The mean number of GABA-positive neurons per section in the 16 PND preparations was 59 in the dentate gyrus, 45 in CA3/2 and 73 in CA1.

The development of GABA-positive puncta or punctate structures was more difficult to describe. These puncta in the adult have been correlated with the distribution of axon terminals[34]. However, the early PND preparations displayed GABA-positive puncta that may represent other structures, such as dendrites or axonal growth cones. The 4 PND preparations displayed some puncta that were associated with somata of GABA-negative neurons (Fig. 1). However, the 6 PND preparations showed more well-developed GABA-positive puncta in the granule cell layer and the stratum pyramidale. Preparations from older ages examined in this study also displayed GABA-positive puncta and their number increased with age.

GAD immunocytochemical data

The number of GAD-positive neurons at any age up to 14 PND was less than the number of GABA-positive neurons. The youngest preparation that was used for GAD immunocytochemistry, 4 PND, lacked immunostaining. This result was unexpected because GABA-positive cells were present in preparations at this age. The next examined age, 6 PND, displayed a small number of GAD-positive neurons in both the dentate gyrus and hippocampus (Fig. 3). Their number was only about 2–4 GAD-positive neurons per section. This finding was remarkable because the number of GABA-positive cells at this age was 48 per section. Thus, the presence of GAD-immunoreactiv-



Figs. 8–10. Diagrams similar in orientation as those in Figs. 5–7 but showing the distribution of GAD-positive cells in the hippocampal formation from three ages. Fig. 8 shows GAD-positive cells from a 12 PND preparation. Fig. 9 is from a 14 PND preparation. Fig. 10 is from an adult preparation.

ity in the rat hippocampal formation occurs at a later age than that for GABA-immunoreactivity.

The number of GAD-positive neurons and their distribution at ages 8–12 PND were similar (Table I). At 8 PND, the number of GAD-positive cells was 15 in the dentate gyrus, 20 in CA3/2 and 30 in CA1. The distribution of these neurons was similar to that found for the distribution of GABA-positive neurons in preparations from 6 PND. The GAD-positive neurons in the dentate gyrus were mainly located in the hilus with only a few found in the molecular layer. In addition, the amount of dendritic staining was significant so that some cell types in the dentate gyrus could be identified, such as the basket cells at the granule cell layer/hilar border and the fusiform cells in the deep hilus (Fig. 4). The hippocampus showed many GAD-positive cells in the stratum pyramidale with others located in the strata oriens and radiatum as shown in the diagram (Fig. 8). The data for 10 and 12 PND were similar in that the mean number of GAD-positive cells in the dentate gyrus at these two ages were 16 and 15, respectively. At each of these ages, CA1 displayed larger numbers of neurons than CA3/2; CA1 had 33 and 27 cells per section at these two ages, respectively whereas CA3/2 displayed 14 and 19, respectively.

A large increase in the number of GAD-positive neurons was found in the 14 PND preparations as compared to the 12 PND ones (cf. Figs. 8 and 9). In fact, the number of GAD-positive neurons doubled in both the dentate gyrus and hippocampus during this two day period (Fig. 11). The dentate gyrus showed a significant increase in the hilus (Fig. 12). However, the increase in neuron number was the greatest in CA1 (Fig. 13). The mean number of GAD-positive neurons per section at 14 PND was 34 in the dentate gyrus, 64 in CA1 and 36 in CA3/2.

Between 16 and 20 PND, the number of GAD-positive neurons slowly grew in number to approximate 90% of the number found in adult preparations

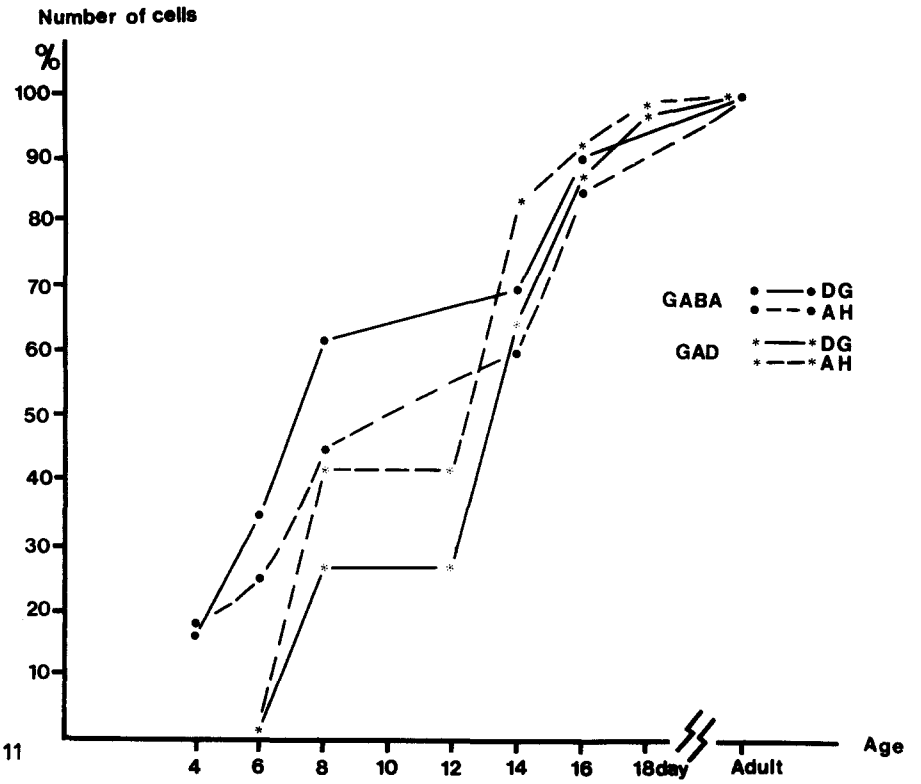
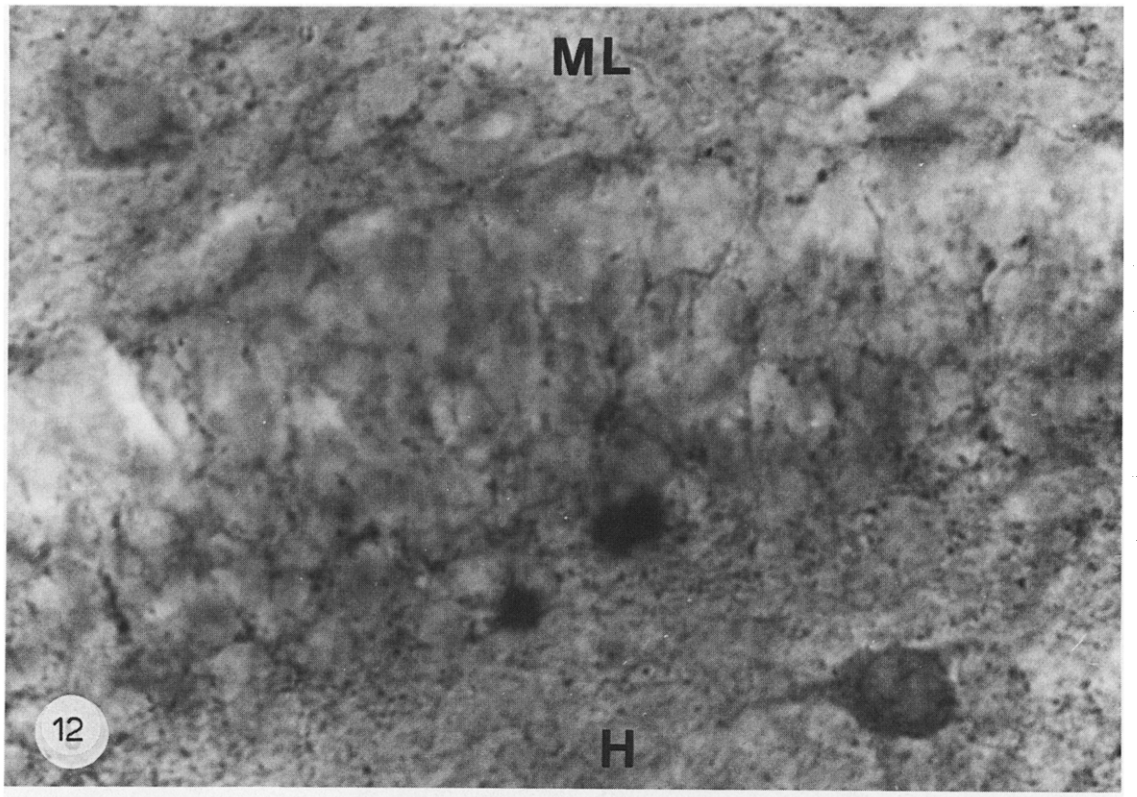
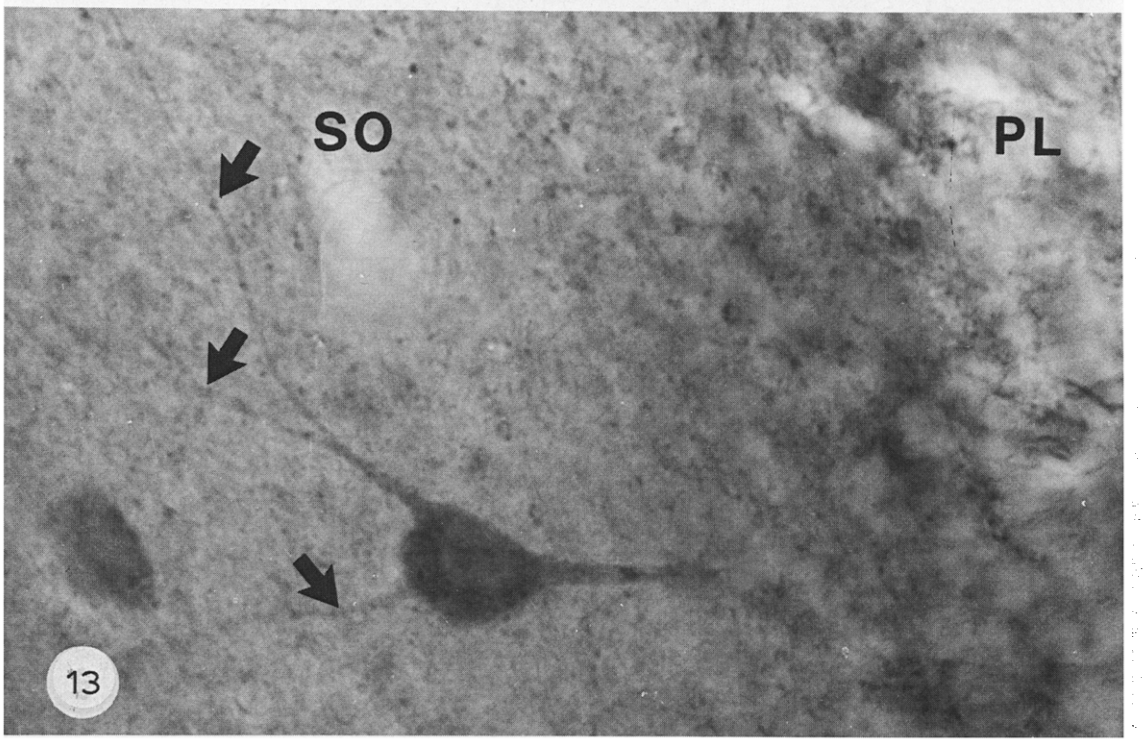


Fig. 11. Graph of the number of GABA- and GAD-positive cells in the dentate gyrus (DG) and Ammon's horn (AH) at various postnatal ages expressed as a percentage of adult values. GABA-positive cells appear earlier than GAD-positive cells. Both show rapid increases in number between 4–8 PND and 12–16 PND.





Figs. 12 and 13. Light photomicrographs of a GAD immunocytochemical preparation of the hippocampus obtained from a 14 PND rat. Fig. 12 shows two GAD-positive cells in the dentate gyrus. One is in the hilus (H) and the other is in the molecular layer (ML). ×1200. Fig. 6 shows a GAD-positive cell from CA1. It displays numerous dendrites (arrows) in stratum oriens (SO) as well as a dendrite oriented toward stratum pyramidale (PL). ×1200.

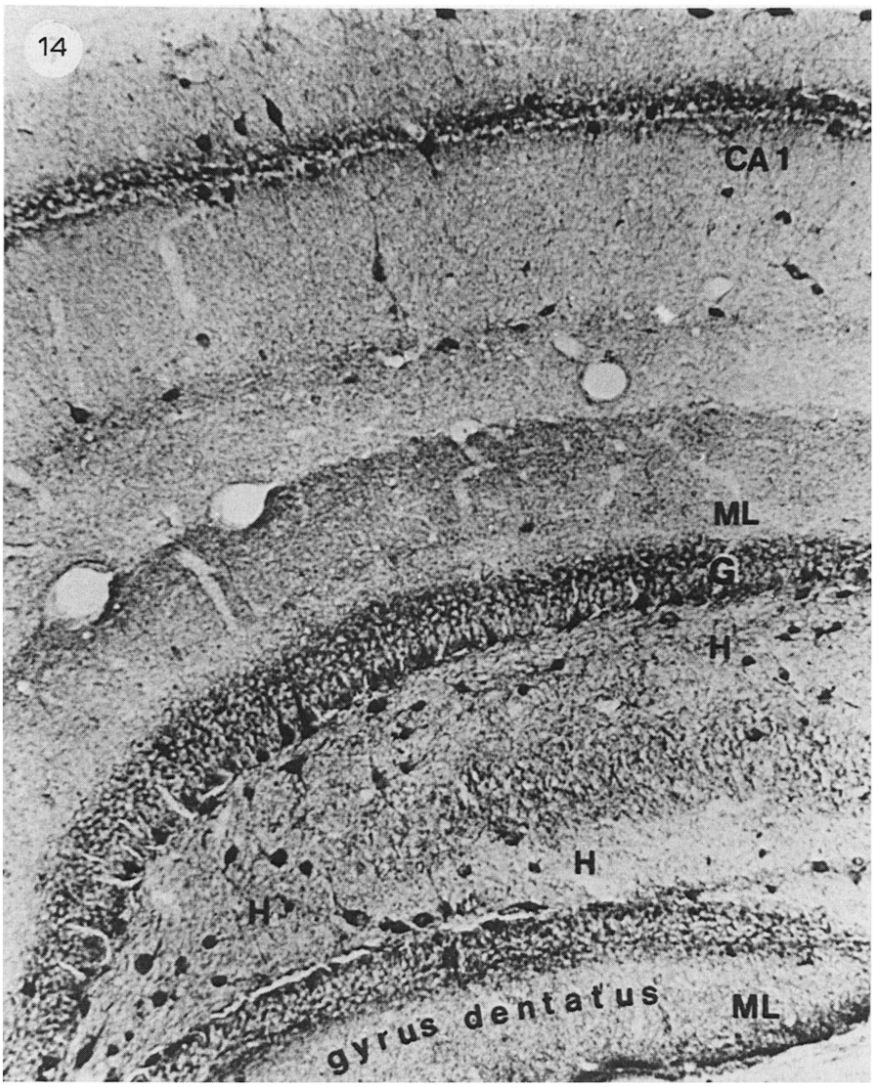


Fig. 14. Light photomicrograph of GAD-positive neurons in a hippocampal section from a 24 PND rat. The distribution of GAD-positive cells is similar to that found in the adult. The dentate gyrus contains most of these cells in the hilus (H) and the adjacent granule cell layer (G). The molecular layer (ML) of the dentate gyrus contains few GAD-positive cells. The CA1 shows GAD-positive cells in all layers. ×80.

(cf. Figs. 10 and 14). The distribution of GAD-positive neurons in the adult was similar to the results previously described[34,36].

DISCUSSION

The present study was conducted to determine the number and distribution of GABAergic neurons at various PND. Antisera to GABA and GAD were used for these studies because both antibodies have been used to detect the same population of GABAergic neurons, the only difference being that one recognizes the neurotransmitter whereas the other recognizes its synthesizing enzyme[29]. In fact, the coexistence of these two substances within the same hippocampal neuron was directly demonstrated by Somogyi et al.[42]. The immunocytochemical results of the present study indicated that GABA-positive neurons are detected earlier in development than GAD-positive neurons. Although GABA-positive neurons exceed the number of GAD-positive neurons at the earlier ages analyzed, the numbers of labeled neu-

rons immunostained with either antisera are similar at 16–18 PND when they reach adult levels. Before these data can be compared to biochemical and physiological results of the development of GABAergic inhibitory function in the hippocampal formation, potential problems with the immunocytochemical method should be discussed.

GABA and GAD immunocytochemistry

Immunocytochemically detectable levels of GABA are found at 4 PND in the present study and have been reported to be present before birth (ref. 17 and Van Eden, personal communication). In contrast, detectable levels of GAD are not found before 6 PND in the present study. However, a recent study has demonstrated GAD-positive axon terminals as early as 5 PND[21]. Thus, it is apparent that GABA-positive neurons appear in younger preparations than GAD-positive neurons even though GAD, the synthesizing enzyme for GABA, should be present within a neuron before GABA is formed. A few possibilities could explain this phenomenon. First, the most plausible explanation is that a young developing GABAergic neuron may only require a small number of GAD molecules for its proper function, and such a small number may go undetected with immunocytochemical methods. In contrast, the same neuron may synthesize a large number of GABA molecules that could be detected with immunocytochemical methods. As the neuron grows, the number of GAD molecules may increase significantly to produce a larger number of GABA molecules that would be required for the maturing neuron with its numerous axonal and dendritic processes. A second possibility, and a corollary to the first one, is that the young neurons at 1–6 PND are too immature to concentrate GAD in the perikaryal cytoplasm to detectable levels because the GAD may be widely dispersed in the cytoplasm in very low amounts. A third possibility is that the GAD molecule in very young rats is not antigenic because it may have a different conformation or may be packaged in an inaccessible cellular compartment in immature neurons. In any event, the inability to detect GAD at the same early developmental ages when GABA is detected appears to be a major finding of this immunocytochemical study. The cellular basis or cause for this finding remains to be determined.

Rate of development of GABA- and GAD-positive neurons

The percentage values of GABA- and GAD-positive neurons as compared to adult values show that the numbers of these neurons undergo a large increase in the 4–8 PND period and a second large increase in the 12–16 PND period. These two periods of rapid development are separated by a middle plateau period where only a small increase in the number of immunostained neurons occurs. Even though the development of GAD-positive neurons lags behind that of the GABA-positive neurons, both populations of neurons show a similar rate of development during these two periods that are characterized by large increases in the number of immunostained neurons.

The rate of development of GABA- and GAD-positive neurons can be compared with biochemical data on GABA and GAD activity in the developing cerebral cortex and hippocampus. Although the number of immunostained neurons fails to account for all of the GABA and GAD in the tissue because neuronal processes such as dendrites and axon terminals also contain these substances, it does provide a reasonably good indicator since cell bodies are easily quantifiable structures that can be used to generate a percentage value of adult levels. When the numerical values of cell bodies are used, it is remarkable how similar the rate of development of GABA- and GAD-immunostained neurons is with the biochemical development of GABA content and GAD activity that was recently shown by Swann et al.[44]. A comparison of the immunocytochemical data with the biochemical data indicates that the data for GABA development are very similar. In contrast, the biochemical levels for GAD activity expressed as a percentage of adult values are consistently lower than the values for the number of GAD-positive cells after 10 PND. Nevertheless, it is interesting that both sets of data show that GABA levels are higher at earlier ages than GAD levels. Therefore, a developmental lag occurs for GAD and this lag may have anatomical and physiological correlates.

It is interesting to note that the greatest increase in the number of GAD-positive neurons occurred between 12 and 14 PND. A significant increase of GAD activity was also detected during this same period[44]. At this time, most of the granule cells in the dentate

gyrus are formed, and they have begun to develop their dendritic arbor as well as their axonal plexus[1]. This growth probably increases the synaptic surface for inhibitory connections, and it also increases the number of excitatory synapses from granule cell axons in the hilar region. It is known that GABAergic basket cells as well as other types of hilar neurons are contacted by axon terminals of granule cells[30–32]. Together, these data suggest that the rapid increase in GAD activity and GAD-containing neurons is triggered by a sudden increase of synaptic (afferent and efferent) connections of GAD-positive hilar neurons which probably results in a higher rate of production of GABA as well as its synthesizing enzyme, GAD. Concomitant anatomical changes have been observed during this period in that significant ultrastructural changes occur in both the cytoplasm and nuclear structure of hilar GAD-positive neurons during the period between 10 and 16 PND[38]. It is interesting to note that a study of the development of GABAergic neurons in the cat neostriatum has demonstrated results which are consistent with this conclusion. Fisher et al.[10] showed that more neurons contain GAD and each neuron contains more of the enzyme with increasing age. In addition, they showed that the development of GAD-positive neurons and axon terminals paralleled the known biochemical increases in GAD activity. Therefore, the use of immunocytochemical methods for the description of the development of GABAergic neurons appears to provide a good indicator of their postnatal maturation.

A functional indication of the development of inhibitory circuits during this period is the appearance of theta activity at 16 PND[18]. This finding indicates that the circuit has matured enough at this age to produce a synchronized theta activity which is a characteristic of the adult hippocampal formation.

Distribution of GABAergic hippocampal neurons in development

Both GABA-positive and GAD-positive neurons were found throughout the dentate gyrus and hippocampus at each PND examined. The distribution of GABAergic neurons appeared to be somewhat homogeneous except for the GABA-positive neurons at 4 PND. At this early age, the GABA neurons were mainly found in the hilus and adjoining CA3c. The distal part of CA1 showed another group of GABA-positive neurons. This distribution may relate to the development of the trisynaptic excitatory pathways in the hippocampus[3]. At 4 PND, many granule cells have axon terminals that form synapses, but most of these are in the hilus and in the most proximal part of CA3 (CA3c). Based on the recently demonstrated topography of projections from CA3 to CA1 in the adult[15], it is known that the distal portion of CA1 receives input from CA3c pyramidal neurons. These anatomical data support the possibility that the distribution of GABA-positive neurons at 4 PND may reflect the location of active synaptic pathways at this age. Future physiological studies will be required to test this notion.

In regard to the physiological results of Swann et al.[44] that show the development of inhibition in CA3 prior to that in CA1, the results from the present study are unable to provide any anatomical basis for their findings. The data from our study show that the distribution of GABA-positive neurons at 6 PND is homogeneous throughout the hippocampus. Since the number of GABA-positive neurons at this age is much less than that found in adults, it is possible that some of their recordings from CA1 were taken from a region that lacked GABA-positive neurons. To determine if this was the case, future physiological studies of the development of inhibition in the hippocampus should be accompanied by an immunocytochemical analysis to determine the location of recording sites and GABAergic neurons in the same preparation.

The quantitative data for the development of GABAergic neurons are important because they show that a similar number of neurons are labeled with antibodies to GAD and GABA at the latest postnatal ages studied. Similar findings were observed in adults. Together, these results provide further support for the earlier studies of the number and distribution of GABAergic neurons in the hippocampal formation[34,36]. Specifically, both GABA and GAD immunocytochemical preparations show a small number of GABAergic neurons in the molecular layer of the dentate gyrus and a more substantial population in the hilus, including the GABAergic neurons located at the granule cell layer–hilus border as well as numerous types found deeper in the hilus. This finding is pertinent because reports from

Sloviter[40] and Sloviter and Nilaver[41] indicate that very few GABAergic neurons exist in the deep hilus whereas many somatostatin cells are found in this region. It was suggested that the discrepancy in the number of GABAergic neurons in the deep hilus may have resulted from different fixation protocols[27]. Further support for this suggestion was provided in the present study where GABA immunocytochemical preparations that were prepared with high concentrations of glutaraldehyde in the fixative showed large numbers of GABA-positive neurons in the deep hilus. In addition, Kosaka et al.[16] have recently shown that about 90% of the somatostatin-containing cells in the hilus are GAD-positive. Therefore, it is now apparent that the same population of GABAergic hilar neurons are stained with antibodies to both GABA and GAD.

It is generally believed that the essential timetable of brain development does not vary fundamentally among mammals. The only substantial differences with respect to brain development are represented by the timing of birth in relation to the stage of brain maturation. From this point of view, it may be useful to compare our present data in the rat with similar results from human brain.

In the human cerebrum, GAD activity increases from 5 to 19% of adult levels between 4–6 months of fetal age to birth. During the following 7 months GAD activity doubles to reach 38% of the adult level[6]. A similar increase occurs in the rat hippocampus during the second postnatal week when the number of GAD-positive neurons increases from 5 to 40% of adult levels. Although there are no data for the earliest age when GAD activity reaches adult levels in humans, it is known that for rats this occurs around 18 PND which is roughly equivalent to the second year of life in humans. Therefore, the analysis of the development of inhibitory circuits in the rat hippocampal formation during the second and third postnatal weeks may provide a better understanding of some developmental events during the late fetal–early postnatal and infant periods of humans. These data may be important for studying the environmental, hormonal and drug effects on the development of the human hippocampus during this period.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Dr. Wolfgang H. Oertel for providing the antiserum to GAD, Dr. Richard T. Robertson for advice and expertise with developing rat brains, and Margot Brundage and Yashoda Jhurani for photographic assistance. This work was supported by NIH Grant NS20228 and NSF Grant BNS 86-15579.

REFERENCES

1 Altman, J. and Das, G.D., Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats, *J. Comp. Neurol.*, 124 (1965) 319–336.
2 Amaral, D.G., A Golgi study of cell types in the hilar region of the hippocampus in the rat, *J. Comp. Neurol.*, 182 (1978) 851–914.
3 Amaral, D.G. and Dent, J.A., Development of the mossy fibers of the dentate gyrus. I. A light and electron microscopic study of the mossy fibers and their expansions, *J. Comp. Neurol.*, 195 (1981) 51–86.
4 Amaral, D.G. and Kurz, J., The time of origin of cells demonstrating glutamic acid decarboxylase-like immunoreactivity in the hippocampal formation of the rat, *Neurosci. Lett.*, 59 (1985) 33–39.
5 Anderson, K.J., Maley, B.E. and Scheff, S.W., Immunocytochemical localization of γ-aminobutyric acid in the rat hippocampal formation, *Neurosci. Lett.*, 69 (1986) 7–12.
6 Balázs, R. and Brooksbank, B.W.L., Certain biochemical aspects of the study of human brain development. In B.S. Henzel and R.M. Smith (Eds.), *Fetal Brain Disorders. Recent Approaches to the Problem of Mental Deficiency*, Elsevier, New York, 1981, pp. 67–96.
7 Bayer, S.A., Development of the hippocampal region in the rat. I. Neurogenesis examined with ^{3}H-thymidine autoradiography, *J. Comp. Neurol.*, 190 (1980) 87–114.
8 Cajal y Ramón, S., *Histologie du Système Nerveux de l'Homme et des Vertébrés. Tome II*, Maloine, Paris, 1911.
9 Coyle, J.T. and Enna, S.J., Neurochemical aspects of the ontogenesis of GABAergic neurons in the rat brain, *Brain Res.*, 111 (1976) 119–133.
10 Fisher, R.S., Levine, M.S., Adinolfi, A.M., Hull, C.D. and Buchwald, N.A., The morphogenesis of glutamic acid decarboxylase in the neostriatum of the cat: neuronal and ultrastructural localization, *Dev. Brain Res.*, 33 (1987) 215–234.
11 Gamrani, H., Onteniente, B., Seguela, P., Geffard, M. and Calas, A., Gamma-aminobutyric acid-immunoreactivity in the rat hippocampus. A light and electron microscopic study with anti-GABA antibodies, *Brain Res.*, 364 (1986) 30–38.
12 Gottlieb, D.I. and Cowan, W.M., Evidence for a temporal factor in the occupation of available synaptic sites during the development of the dentate gyrus, *Brain Res.*, 41 (1972) 452–456.
13 Harris, K. and Teyler, T., Evidence for late development of inhibition in area CA1 of the rat hippocampus, *Brain*

Res., 268 (1983) 339–343.
14 Hoch, D.B. and Dingledine, R., GABAergic neurons in rat hippocampal culture, *Dev. Brain Res.*, 25 (1986) 53–64.
15 Ishizuka, N., Krzemieniewska, K. and Amaral, D.G., Organization of pyramidal cell axonal collaterals in field CA3 of the rat hippocampus, *Soc. Neurosci. Abstr.*, 12 (1986) 1254.
16 Kosaka, T., Wu, J.-Y. and Benoit, R., GABAergic neurons containing somatostatin-like immunoreactivity in the rat hippocampus and dentate gyrus, *Exp. Brain Res.*, 71 (1988) 388–398.
17 Lauder, J.M., Han, V.K.M., Henderson, P., Verdoorn, T. and Towle, A.C., Prenatal ontogeny of the GABAergic system in the rat brain: an immunocytochemical study, *Neuroscience*, 19 (1986) 465–493.
18 Leblanc, M.O. and Bland, B.H., Developmental aspects of hippocampal electrical activity and motor behavior in the rat, *Exp. Neurol.*, 66 (1979) 220–237.
19 Léránth, C. and Frotscher, M., Cholinergic innervation of hippocampal GAD- and somatostatin-immunoreactive commissural neurons, *J. Comp. Neurol.*, 261 (1987) 33–47.
20 Lorente de Nó, R., Studies on the structure of the cerebral cortex. II. Continuation of the study of the Ammonic system, *J. Psychol. Neurol.*, 46 (1934) 113–177.
21 Lübbers, K. and Frotscher, M., Differentiation of granule cells in relation to GABAergic neurons in the rat fascia dentata. Combined Golgi/EM and immunocytochemical studies, *Anat. Embryol.*, 178 (1988) 119–127.
22 Lübbers, K., Wolff, J.R. and Frotscher, M., Neurogenesis of GABAergic neurons in the rat dentate gyrus: a combined autoradiographic and immunocytochemical study, *Neurosci. Lett.*, 62 (1985) 317–322.
23 Mugnaini, E. and Oertel, W.H., An atlas of the distribution of GABAergic neurons and terminals in the rat CNS as revealed by GAD immunohistochemistry. In A. Björklund and T. Hökfelt (Eds.), *Handbook of Chemical Neuroanatomy, Vol. 4, GABA and Neuropeptides in the CNS, Part I*, Elsevier, New York, 1985, pp. 547–553.
24 Oertel, W.H., Schmechel, D.E., Mugnaini, E., Tappaz, M.L. and Kopin, I.J., Immunocytochemical localization of glutamate decarboxylase in rat cerebellum with a new antiserum, *Neuroscience*, 6 (1981) 2715–2735.
25 Paxinos, G. and Watson, C., *The Rat Brain in Stereotaxic Coordinates*, Academic Press, Sydney, 1982.
26 Pokorny, J. and Yamamoto, T., Postnatal ontogenesis of hippocampal CA1 area in rats: I. Development of dendritic arborisation in pyramidal neurons, *Brain Res. Bull.*, 7 (1981) 113–120.
27 Ribak, C.E., Epilepsy hypothesis, *Science*, 238 (1987) 1292–1293.
28 Ribak, C.E., Hunt, C.A., Bakay, R.A.E. and Oertel, W.H., A decrease in the number of GABAergic somata is associated with the preferential loss of GABAergic terminals at epileptic foci, *Brain Res.*, 363 (1986) 78–90.
29 Ribak, C.E. and Roberts, R.C., The GABAergic synapses in the brain identified with antisera to GABA and its synthesizing enzyme, glutamate decarboxylase, *J. Electron Microsc. Tech.*, in press.
30 Ribak, C.E. and Seress, L., Five types of basket cell in the hippocampal dentate gyrus. A combined Golgi and electron microscopic study, *J. Neurocytol.*, 12 (1983) 577–597.
31 Ribak, C.E. and Seress, L., A Golgi-electron microscopic study of fusiform neurons in the hilar region of the dentate gyrus, *J. Comp. Neurol.*, 271 (1988) 67–78.
32 Ribak, C.E., Seress, L. and Amaral, D.G., The development, ultrastructure and synaptic connections of the mossy cells of the dentate gyrus, *J. Neurocytol.*, 14 (1985) 835–857.
33 Ribak, C.E., Seress, L., Peterson, G.M., Seroogy, K.B., Fallon, J.H. and Schmued, L.C., A GABAergic inhibitory component within the hippocampal commissural pathway, *J. Neurosci.*, 6 (1986) 3492–3498.
34 Ribak, C.E., Vaughn, J.E. and Saito, K., Immunocytochemical localization of glutamic acid decarboxylase in neuronal somata following colchicine inhibition of axonal transport, *Brain Res.*, 140 (1978) 315–332.
35 Seress, L. and Pokorny, J., Structure of the granular layer of the rat dentate gyrus. A light microscopic and Golgi study, *J. Anat.*, 133 (1981) 181–195.
36 Seress, L. and Ribak, C.E., GABAergic cells in the dentate gyrus appear to be local circuit and projection neurons, *Exp. Brain Res.*, 50 (1983) 173–182.
37 Seress, L. and Ribak, C.E., A combined Golgi-electron microscopic study of non-pyramidal neurons in the CA1 area of the hippocampus, *J. Neurocytol.*, 14 (1985) 717–730.
38 Seress, L. and Ribak, C.E., Basket cells of the dentate gyrus establish synaptic connections with granule cells in five day old rats, *Soc. Neurosci. Abstr.*, 14 (1988) 578.
39 Seress, L., Ribak, C.E., Peterson, G.M. and Oertel, W.H., Development of GABAergic neurons in the rat hippocampal formation, *Soc. Neurosci. Abstr.*, 10 (1984) 410.
40 Sloviter, R.S., Decreased hippocampal inhibition and a selective loss of interneurons in experimental epilepsy, *Science*, 235 (1987) 13–16.
41 Sloviter, R.S. and Nilaver, G., Immunocytochemical localization of GABA-, cholecystokinin-, vasoactive intestinal polypeptide-, and somatostatin-like immunoreactivity in the area dentata and hippocampus of the rat, *J. Comp. Neurol.*, 256 (1987) 42–60.
42 Somogyi, P., Hodgson, A.J., Smith, A.D., Nunzi, M.G., Gorio, A. and Wu, J.-Y., Different populations of GABAergic neurons in the visual cortex and hippocampus of cat contain somatostatin- or cholecystokinin-immunoreactive material, *J. Neurosci.*, 4 (184) 2590–2603.
43 Soriano, E., Cobas, A. and Fairén, A., Asynchronism in the neurogenesis of GABAergic and non-GABAergic neurons in the mouse hippocampus, *Dev. Brain Res.*, 30 (1986) 88–92.
44 Swann, J.W., Brady, R.J. and Martin, D.L., Postnatal development of GABA-mediated synaptic inhibition of rat hippocampus, *Neuroscience*, (1988) in press.
45 Wong, P.T.H. and McGeer, E.G., Postnatal changes of GABAergic and glutamatergic parameters, *Dev. Brain Res.*, 1 (1981) 519–529.