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NUMBER OF GABA IMMUNONEGATIVE AND GABA IMMUNOPOSITIVE NEURONS IN HUMAN EPILEPTIC TEMPORAL CORTEX*

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The number of neurons, both GABA immunopositive and immunonegative, was determined in temporal epileptic foci of 7 patients after temporal lobectomy, and compared to neuronal numbers in temporal cortex of two controls taken from tumor operated patients. The thickness of the cortex of the epileptic cortex diminished by about 10%, while the number of nerve cells decreased to 67% of that of the control value: it was 19.000/mm^3 vs. 28.000/mm^3 found in the control. This decline was due to cell degeneration, which, however, was more severe for non-GABAergic nerve cells. Accordingly, the proportion of the GABA-positive neurons in the othervise diminished neuronal population increased to 36.4% from the 32% control value. The number of GABAergic terminals, however, decreased even further, explaining the resulting disinhibition during epileptic seizures.

Keywords: Epilepsy – GABAergic neurons – numerical density – quantitative morphology

INTRODUCTION

Epilepsies, which comprise a diverse collection of disorders, affect about 0.8–1% of the population (1% in the U.S., see [4]). The most common form of epileptic syndrome (complex partial epilepsy) arises from an abnormality intrinsic to the temporal lobe, because its surgical resection eliminates epileptic seizures in most patients who are otherwise refractory to other, conventional medical treatment [20, 21]. In case of hippocampal sclerosis, which is causally connected to the development of temporal cortical local seizures, it was observed by Sloviter [17] that the first sign of sclerosis (and cell loss) is preceded by the development of hyperexcitability of the hippocampal nerve cells. This hyperexcitability accompanied by a marked neuronal loss leads to the hippocampal sclerosis. It was found also [9] that repeated, intense seizures cause a loss of recurrent, GABA-mediated inhibition of the dentale granule

* Dedicated to Professor György Ádám on the occasion of his 80th birthday.

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cells, indicating a preferential loss of GABAerg neurons in the same area. Paradoxically, however, it was also demonstrated [17] in experimental models that, in the course of the hippocampal hyperexcitability – caused neuronal loss, presumptive GABAergic nerve cells are more resistant to seizures, than other, GABA-negative neurons. The preferential preservation of GABA-immunoreactive neurons was also observed in speciemens from human hippocampal epilepsy [1].

More recently, contradicting previous suggestions, it was demonstrated by immunocytochemical quantitative studies [23] that there is a strong reduction in the number of inhibitory interneurons, especially in the hilar region, although the axon initial segment synapses from the surviving inhibitory (parvalbumin-containing) nerve cells are more numerous, than in the control. Other investigations of the epileptic foci in the cerebral cortex of human patients reveraled also a loss of not only inhibitory terminals but also their parent cell bodies, resulting in a loss of perisomatic inhibition and a simultaneous generation of recurrent seizures [3, 7, 8].

As a possible contribution to solve the paradox between the above-mentioned contradictory results, the aim of the present study was to estimate the number of both excitatory and inhibitory nerve cells, in the epileptic foci of human temporal cortex. For this purpose quantitative GABA-immunocytochemistry and classical histological methods were used.

MATERIALS AND METHODS

Cerebral cortical tissues containing epileptic foci were obtained from seven patients undergoing temporal lobectomy or anterial callosotomy following medically intractable seizures. Patients were operated in the National Neurosurgery Institute, Budapest. Control brain samples of non-epileptic temporal cortex were obtained from two patients with tumor in the centrotemporo-parietal area on the right side. Specimens were taken from the right medial temporal convolution.

The delay between the surgical removal and the beginning of the fixation was less than 20 min. Two-three mm thick slices were immersed for 16 hours into an ice-cold fixative containing 1% glutaraldehyde and 3.5% paraformaldehyde diluted in 0.2 M sodium phosphate buffer (PB) at pH 7.3.

Blocks of tissue were then further processed in one of three ways.

1. Non-osmicated blocks for postembedding immunohistochemistry were dehydrated and embedded in Durcupan ACM (Fluka) resin. Serial semithin sections (1 μm) were cut, mounted on slides coated with chrome-alum gelatin and kept in 56 °C thermostat overnight.

2. Osmicated blocks for postembedding immunohistochemistry [19] and for correlated electron microscopic examination were postfixed for 2–3 h in 1% OsO_4, dehydrated, and embedded in resin. Serial semithin sections (0.5 μm) were cut and mounted on slides coated with chrom-alum gelatin. To enhance the contrast for electron microscopy, 1% uranyl acetate staining was performed in 70% ethanol.

3. For pre-embedding immunocytochemistry blocks were sectioned on a Vibratome at 60 μm. Sections were collected and rinsed extensively for 2×10 mins in cold 0.01 M sodium phosphate buffered saline (PBS) at 7.3 pH. After incubation with 20% normal goat serum (NGS) to decrease background staining, the sections were incubated for 30 mins overnight at 4 °C in primary anti-GABA antibodies diluted to 1 : 5000 in PBS containing 1% NGS. The sections were then rinsed and incubated for 2 h in secondary biotinilated goat anti-rabbit immunoglobulins (B-GAR) diluted to 1 : 200 in PBS containing 1% NGS. This was followed by rinsing and by incubation with a solution of the highly specific sensitized avidin-biotin complex conjugated peroxidase (VECTASTAIN A, B, C) for 1 h at room temperature diluted in PBS (pH 7.8). After washing in PBS (pH 7.4) and in two changes of TB (pH 7.6) for 10 mins each, the sections were incubated in 0.5 mg/ml DAB diluted in 0.05 M TB with 0.01% H_2O_2 for developing peroxidase activity until obtaining the desired grade of intensification. After further washing the sections were postfixed with 0.5% OsO_4 dissolved in 0.1 M PB, dehydrated and mounted on slides in Durcupan.

For normal histological analysis and identifying the immunoreactive elements we drew the images of the light microscopical views of alternative semithin sections stained with toluidine-blue or postembedding GABA-method. The neurons magnified 40× were drawn via a drawing tube in the entire depth of cortex from at least 4 sections per specimen. The lamination of the cortex could be determined on the basis of profile morphology and cellular density with the help of a grid-pattern of rectangular sampling fields lying perpendicular to the pial surface with tangential sampling strata at 100 μm intervals. Morphometric semi-automatic analysis was made with the aid of an IBM computer assisted BIOQUANT IV program using [22] the following formula:

$$Nv = \frac{K\,(Na)^{3/2}}{\beta\,(Vv)^{1/2}}$$

where Na is the number of nuclear profiles per unit area, Vv is the volume (or area) fraction, K was chosen as 1.05, and = 1.39 which is a function of axial rations of the ellipsoidal nuclear cross-sections studied.

The numerical density was calculated in all layers for both the total and for GABA immunoreactive neural populations, and from these the proportion of GABA(+) interneurons has been established.

For electron microscopy and for postembedding immunocytochemistry [19] serial semithin sections were cut from Durcupan-embedded blocks. The slides were treated with sodium etanolate to etch the resin, and the osmium was removed by treatment with 1% sodium periodate. The serum was overlayered onto glass slides to reveal GABA immunoreactivity. Primary GABA-antiserum was used for 90 mins at a dilution of 1 : 1000, and colloidal gold coated secondary antibody (GAR-gold) was used for 90 mins under the same conditions.

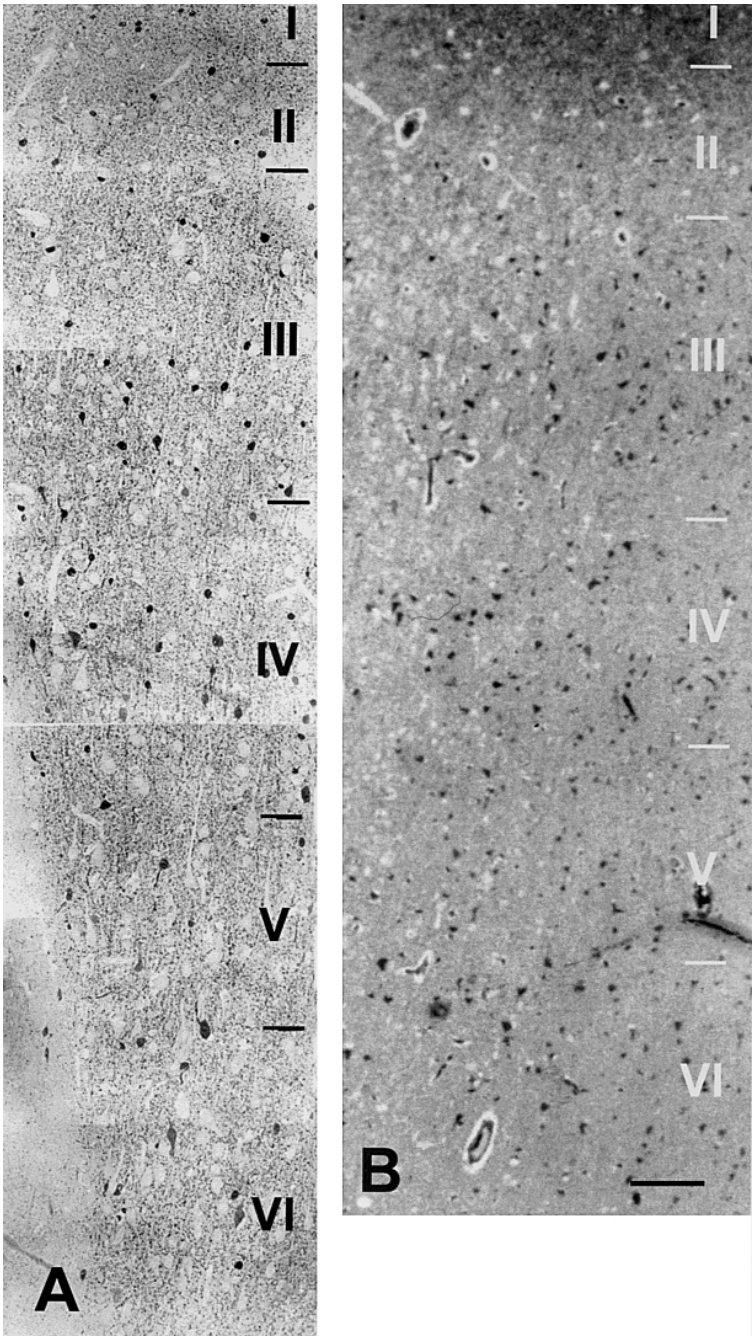


Fig. 1. Light microscopic view of the layers of temporal cortex in control (A) and in epileptic focus (B). Epileptic cortex is thinner, and black-stained GABA-positive nerve cells are practically absent in layers I and II. Both specimens were GABA-immunostained. Scale bar: 100 μm

RESULTS

The average thickness of the temporal cortex in the controls was 2.75 mm. In comparison (Fig. 1), the cortex in the epileptic foci was found to be somewhat (~ 10%) atrophic. GABA-immunocytochemistry revealed furthermore, in the same atrophic tissue, an almost complete disappearance of GABA-immunopositive cells in the Ist and IInd layers of epileptic cortex. The relatively rich punctuation from III to VI layers in the epileptic cortex was due, in addition to GABA-positive nerve cells, to dense debris of degenerating neurons. To distinguish between immunostained perikarya and degenerating neurons, and also to differentiate between astroglial cells and non-reacting, immunonegative nerve cells, higher magnification of light microscopic preparations was utilised (Fig. 2). Comparing neighbouring sections, stained with toluidine-blue (Fig. 2A) or GABA-immunoreaction (Fig. 2B) it was observed, that similarly to control specimens, surviving and healthy-looking pyramidal neurons in the epileptic cortex were surrounded by immunopositive GABA-cells and their terminals, the latter showing up as small immunoreactive puncta. At the same time, many pyramidal (Fig. 3C) or non-pyramidal (Fig. 3D) neurons in the epileptic cortex, have been observed to exhibit clear signs of degeneration. Astroglial cells in

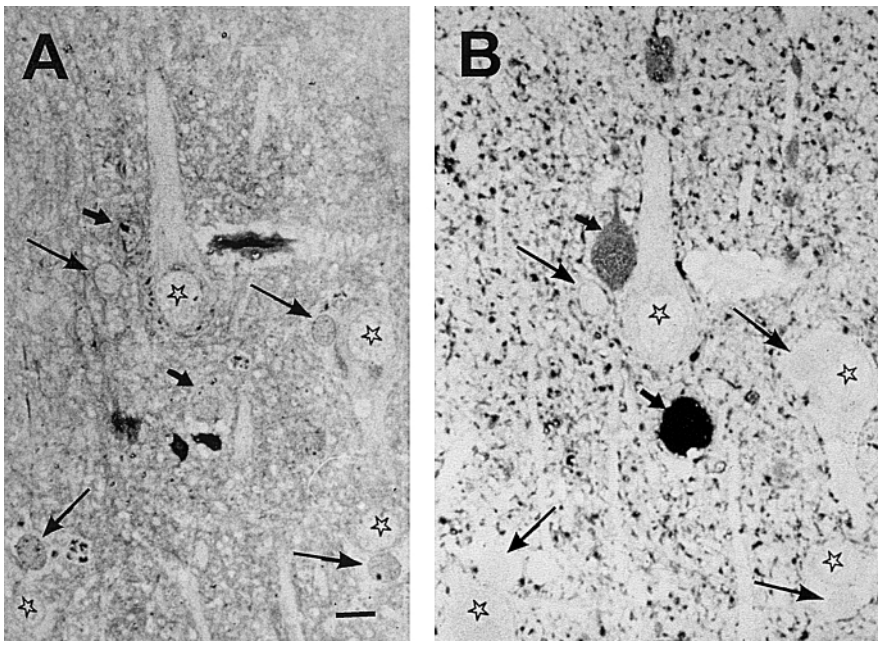


Fig. 2. Higher power light microscope view of epileptic temporal cortex, layer V, in toluidine blue-stained section (A) and the neighbouring section treated by GABA-immunostaining (B). Short, thick arrows indicate GABA-positive neurons, stars show GABA-negative nerve cells and long, thin arrows mark glial cells. Note, that small puncta in "B" represent GABA-positive processes + terminals. Scale bar: 10 μm

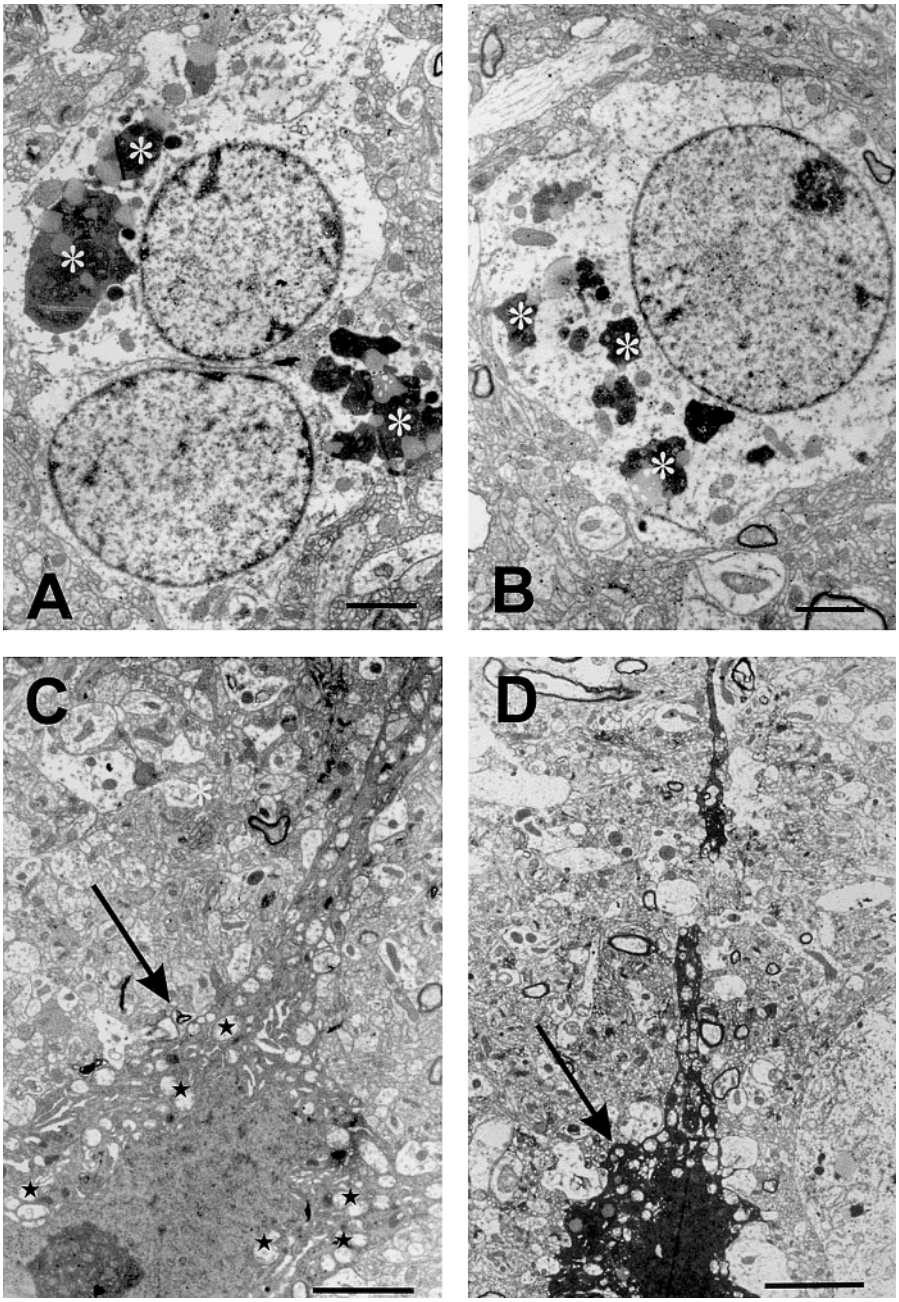


Fig. 3. Electron microscopic demonstration of astroglia cells (A+B) and degenerating neurons (C+D) in temporal epileptic cortex. Stars in A+B indicate various inclusions and debris of degenerating cells; small stars in "C" show vacuolization in degenerating nerve cell. Arrows in C and D show early (C) and late (D) form of degeneration. Scale bar: 2 μm

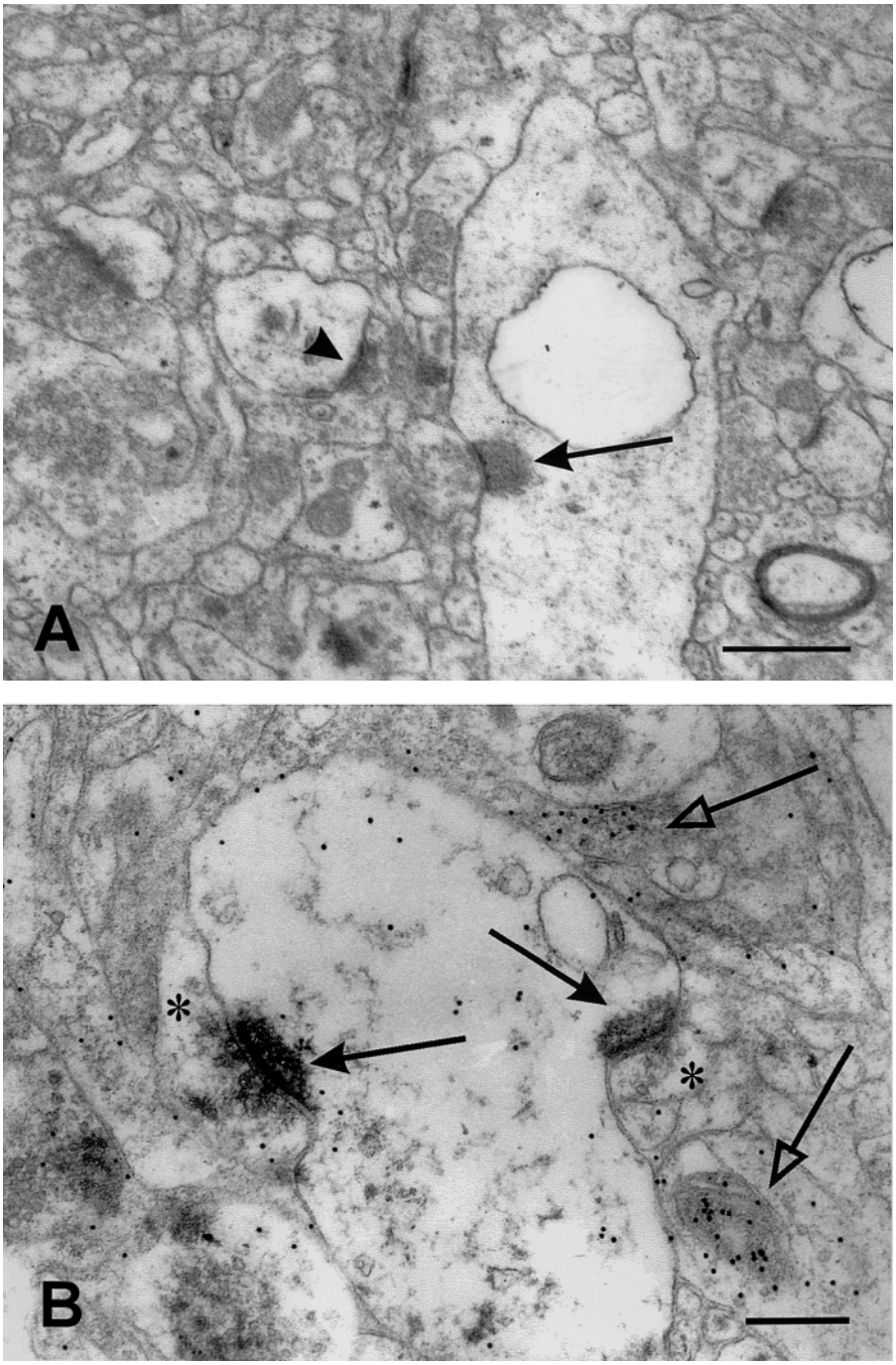
these samples have been also seen to exhibit an increased metabolic activity indicated by an accumulation of intracellular lipoid inclusions and other, cellular debris (Figs 3A and B) as a clear sign of their participation in the removal of degenerating neurons.

For quantitation, only healtly, well-identified GABA+ and GABA-nerve cells were considered, while degenerating neurons, as well as glia cell were excluded from the counting. The results of the quantification are shown in Table 1. The total cell number in the epileptic cortex varied between 12,000/mm^3 and 31,000/mm^3 but was 19,627/mm^3 in average. It means an 33% decrease of total cell number when compared to the 28,000/mm^3 cell density in the control cortex. This decline of nerve cell number in the epileptic cortex, however, was not proportional: while the percentage of GABA-immunopositive nerve cells was less then 32% in the control, in spite of an absolute decrease of GABA-immunopositive cells in the epileptic cortex, its proportion within the surviving cell population was higher (36.4%) than in the control. It indicates also that the decline in the number of non-GABAergic neurons in the epileptic cortex was both in absolute value, and also proportionally higher than that of the GABAergic, inhibitory neurons.

The electron microscopic investigation of the epileptic cortex has revealed that the density of GABA-positive terminals decreased significantly, and even when present, the GABA-immunolabeling was rather weak (Fig. 4B). Another characteristic morphological feature of synapses between presumambly GABAergic endings and dendritic processes (Fig. 4A–B) was that, opposite to axon endings containing pleomor-

Table 1
Numerical density of temporal cortical neurons and of GABA-immunoreactive neuronal subpopulation in seven epileptic and two control patients

No.	Sample	Nv total	NvGABA(+)	%GABA
		EPILEPTIC FOCI		
1.	temporal	17,302±1,832	5,639±249	32.6
2.	temporal	17,963±1,910	6,641±548	36.9
3.	temporal	19,476±2,320	7,021±1,034	36.0
4.	temporal	17,875±1,802	7,298±602	40.8
5.	temporal	21,017±1,917	7,545±1,002	35.9
6.	temporal	31,621±4,117	10,837±1,933	34.3
7.	temporal	12,136±996	4,705±301	38.8
		19,627±2,127	7,098±809	36.4
		CONTROL		
1.	temporal	29,766±1,805	9,493±405	31.9
2.	temporal	26,604±1,613	8,534±335	32.0
		28,185±1,709	9,013±370	31.95


A
B

←————

Fig. 4. A: Axodendritic synapse in epileptic cortex between a pleomorphic vesicle containing terminal and a dendritic process, exhibiting a huge accumulation of postsynaptic material (arrow). Arrowhead indicates "classical", axon-spine synapse, with a normal accumulation of postsynaptic density. Scale bar: 1 μm. – B: Postembedding GABA-immunostaining of epileptic cortex showing two synaptic contacts between GABA-immunonegative and GABA-positive postsynaptic dendrite. The huge accumulation of postsynaptic material (arrows) is opposite to small axon terminals practically devoid of GABA-staining. Note, the relatively, rich GABA-label (small gold particles) in other profiles (hollow arrow). Scale bar: 0.5 μm

phic-ovoid vesicles, we have observed a massive accumulation of postsynaptic material. Interestingly, even GABA-containing dendrites developed the huge dense accumulation of postsynaptic material, opposite to axon terminals containing pleomorphic vesicles. A specific feature of these terminals (Fig. 4B) was the appearent lack of their immunostaining with GABA-antibody. In other, classical axon-spine synapses, with an axon ending containing spheroid vesicles, no abnormal postsynaptic material accumulation was found, indicating that this type of synaptic arrangement is characteristic only and exclusively to synapses, where the presynaptic axon terminal or, *vice versa,* the postsynaptic dendrite, based on its morphology, belongs to GABAergic inhibitory neuron.

DISCUSSION

The development of epileptic seizure is accompanied, or caused by the breakdown of the otherwise harmonius equilibrium between excitatory and inhibitory processes. The cause of the resulting hyperexcitability may be either a prevalent excitation, or a decline in inhibitory activities [6, 13, 18]. Indeed, in earlier investigations an elevated level of intra- and extracellular pool of excitatory transmitters was observed in the brain both in experimental animal and human epilepsies [5, 10, 12, 16]. In patients investigated before temporal lobotomy, a decrease in the level of GABA and its synthetising enzyme, GAD has been found [6]. In accordance with the latter results, under experimental conditions a decrease in the number of both GABA cell bodies and GABAergic terminals was observed in epileptic focus [14, 15]. Our results, however, partly contradict the previous findings, at least in the human temporal epileptic cortex. The total number of cortical neurons in the epileptic foci declined to 67% (19,000/mm^3) of the control level (28,000/mm^3). At the same time, although the absolute number of GABA-immunopositive neurons was observed also to decrease, the degeneration and numerical decrease of non-GABAergic cortical neurons was more prevalent. Whereas in the control cortex 68% of the nerve cells belonged to GABA-negative population, its proportion in epilepsy declined to 63%. In contrast, the proportion of GABA-positive neurons increased to almost 37% of all surviving nerve cells. This means that although not all GABA-cells are preserved, non-GABAergic neurons are more vulnerable to epileptic seizures than are GABA-positive cells. That would mean that contrary to logical expectations, the breakdown

of equilibrium between exitatory and inhibitory processes, as expressed in the number of neurons of both types, would be a result of a strengthened inhibition, and not an increased excitation. This discrepancy might be explained partly by the present observation that the density of GABA-positive endings in the epileptic cortex decreased markedly. In addition, although immunocytochemistry is not a perfect quantitative method, the weak GABA-immunostaining of many terminals with morphology characteristic for "inhibitory" endings might be also indicative of weakend inhibitory activities.

Recently Bothwell et al. [2] reported that in temporal lobe epilepsy there is a significant atrophy of neocortical gray matter. Although these authors, in contrast to our present findings, did not report cell loss, they observed, paradoxically, a significant reduction in neuropil and its associated elements. Our ultrastructural observations indicate, that in addition to the reduction of neurophil, we have to count also with the disturbance of GABAergic transmission. This is underlined by the finding of the enlarged postsynaptic density both in GABA-positive and GABA-negative dendrites. This special morphological alteration of the postsynaptic density might be directly related to a change (upregulation?) of postsynaptic receptors. Indeed, Nusser et al. [11] have described a numerical increase of GABA-A receptors in hippocampal granule cells in an experimental model of temporal lobe epilepsy. Therefore, we suggest here, that in spite of the relatively large proportion of surviving GABAergic neurons in the human temporal cortex, the development of the seizures might be the result of the unproportional, marked reduction of GABAergic arborization and axonal endings. This suggestion, however, needs further quantitative morphological confirmation.

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REFERENCES

1. Babb, T. L., Pretorius, J. K., Kupfer, W. R., Crandall, P. H. (1989) Glutamate decarboxylase-immunoreactive neurons are preserved in human epileptic hippocampus. *J. Neurosci. 9,* 2562–2574.
2. Bothwell, S., Meredith, G. E., Phillips, J., Staunton, H., Doherty, C., Grigorenko, E., Glazier, S., Deadwyler, S. A., O'Donovan, C. A., Farrell, M. (2001) Neuronal hypertrophy in the neocortex of patients with temporal lobe epilepsy. *J. Neurosci. 21,* 4789–4800.
3. DeFelipe, J. (1999) Chandelier cells and epilepsy. *Brain 122,* 1807–1822.
4. Hauser, W. A., Hesdorffer, D. C. (1990) *Epilepsy: Frequency, Causes, and Consequences*. New York: Demos.
5. Lasley, S. M. (1991) Roles of neurotransmitter amino acids in seizure severity and experience in the genetically epilepsy-prone rat. *Brain Res. 560,* 63–70.
6. Lloyd, K. G., Bossi, L., Morselli, P. L., Munari, C., Rougier, M., Loiseau, H. (1986) Alterations of GABA-mediated synaptic transmission in human epilepsy. In: Delgado-Escueta, A. V., Ward, A. A., Woodbury, D. M., Porter, R. J. (eds) *Basic Mechanisms of the Epilepsies. Molecular and Cellular Approaches.* (Advances in Neurology, Vol. 44.) Raven Press, New York, pp. 1033–1044.

7. Marco, P., Sola, R. G., Pulido, P., Alijarde, M. T., Sanchez, A., Ramon y Cajal, S., DeFelipe, J. (1996) Inhibitory neurons in the human epileptogenic temporal neocortex. An immunocytochemical study. *Brain 119,* 1327–1347.
8. Marco, P., Sola, R. G., Cayal, S. R. Y., DeFelipe, J. (1997) Loss of inhibitory synapses on the soma and axon initial segment of pyramidal cells in human epileptic peritumoral neocortex: implications for epilepsy. *Brain Res. Bull. 44,* 47–66.
9. McNamara, J. O. (1994) Cellular and molecular basis of epilepsy. *J. Neurosci. 14,* 3413–3425.
10. Nadi, N. S., Wyler, A. R., Porter, R. J. (1987) Amino acids and catecholamines in epileptic focus from human brain. *Neurology 37,* 106.
11. Nusser, Z., Hajos, N., Somogyi, P., Mody, I. (1998) Increased number of synaptic GABA(A) receptors underlies potentiation at hippocampal inhibitory synapses. *Nature 395,* 172–177.
12. Olney, J. W. (1985) Excitatory transmitters and epilepsy-related brain damage. *Int. Rev. Neurobiol. 27,* 337–362.
13. Pitkanen, A., Matilainen, R., Holonen, T., Kutvonen, R., Hartikainen, P., Riekkinen, P. (1989) Inhibitory and excitatory amino acids in cerebrospinal fluid of chronic epileptic patients. *J. Neural Transm. 76,* 221–230.
14. Ribak, C. E., Harris, A. B., Vaughn, J. E., Roberts, E. (1979) Inhibitory GABAergic nerve terminals decrease at sites of focal epilepsy. *Science 205,* 211–240.
15. Ribak, C. E., Hunt, C. A., Bakay, R. A. E., Ortel, W. H. (1986) A decrease in the number of GABAergic somata is associated with the preferential loss of GABAergic terminals in epileptic foci. *Brain Res. 363,* 78–90.
16. Sherwin, A. L., Vernet, O., Dubeau, F., Olivier, A. (1991) Biochemical markers of excitability in human neocortex. *Can. J. Neurol. Sci. 18,* 640–644.
17. Sloviter, R. S. (1987) Decreased hippocampal inhibition and a selective loss of interneurons in experimental epilepsy. *Science 235,* 73–76.
18. Sloviter, R. S. (1991) Permanently altered hippocampal structure, excitability and inhibition after experimental status epilepticus in the rat: The "dormant basket cell" hypothesis and its possible relevance to temporal lobe epilepsy. *Hippocampus 1,* 41–66.
19. Somogyi, P., Hodgson, A. J., Chubb, I. W., Penke, B., Erdei, A. (1985) Antiserum to gamma amino butyric acid. II. Immunocytochemical application to the central nervous system. *J. Histochem. Cytochem. 33,* 240–248.
20. Spencer, S. S., Spencer, D. D., Williamson, P. D., Mattson, R. H. (1982) The localizing value of depth electroencephalography in 32 patients with refractory epilepsy. *Ann. Neurol. 12,* 248–253.
21. Walczak, T. S., Radtke, R. A., McNamara, J. O., Lewis, D. V., Luther, J. S., Thompson, E., Wilson, W. P., Friedman, A. H., Nashold, B. S. (1990) Anterior temporal lobectomy for complex seizures: evaluation, results, and long-term follow-up in 100 cases. *Neurol. 40,* 413–418.
22. Weibel, E. R., Gomez, D. M. (1962) A principle for counting tissue structures on random sections. *J. Appl. Physiol. 17,* 343–348.
23. Wittner, L., Maglóczky, Zs., Borhegyi, Zs., Halász, P., Tóth, Sz., Erőss, L., Szabó, Z., Freund, T. F. (2001) Preservation of perisomatic inhibitory input of granule cells in the epileptic human dentate gyrus. *Neuroscience 108,* 587–600.