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Axon Terminals of GABAergic Chandelier Cells Are Lost At Epileptic Foci

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Axon terminals of chandelier cells were analyzed in monkeys with cortical focal epilepsy produced by alumina gel to determine if this type of GABAergic terminal is lost at epileptic foci. These terminals form a dense plexus with the axon initial segments of pyramidal neurons, especially those in layers II and III. Axon initial segments of pyramidal neurons were traced for at least 40 μm in serial thin sections and beyond this point were observed to become myelinated. In single sections, 10–15 axon terminals were found to form symmetric synapses throughout the entire length of the axon initial segments from nonepileptic preparations and were observed to synapse with only these structures and not adjacent dendrites or spines. In epileptic cortex, the axon initial segments of pyramidal neurons were apposed by glial profiles that contained clusters of filaments typical of reactive astrocytes. Only a few, small axon terminals were observed to form symmetric synapses with these axon initial segments. Thus, the chandelier cell axons appeared to degenerate in epileptic cortex. The highly strategic site of GABAergic inhibitory synapses on axon initial segments suggests that they exert a strong influence on the output of pyramidal cells. The near absence of these chandelier cell axons in epileptic foci most likely contributes to the hyperexcitability of neurons.

INTRODUCTION

It is now apparent that in some varieties of epilepsy, a severe deficit occurs in the cortical GABAergic system. Numerous studies of experimental models that resemble post-traumatic epilepsy have shown a loss of GABAergic terminals in epileptic foci[22–24]. A quantitative assessment at the electron microscopic level has indicated that this loss is preferential for GABAergic, symmetric synapses[22]. Biochemical studies also support this finding in that indices for other neurotransmitters were not reduced as severely as those for GABA[1,2,34]. These findings in animal models have received corroboration from biochemical results of human epileptic foci[16]. Therefore, a loss of GABAergic terminals appears to be a hallmark of epileptic foci.

The initial immunocytochemical study on the morphology of cortical GABAergic neurons revealed an abundant variety of stained cell types distributed throughout all layers of cortex[21]. This study localized the synthesizing enzyme of GABA, glutamate decarboxylase (GAD). Although most of these neurons were classified as aspinous and sparsely spinous stellate cells, subsequent Golgi studies have revealed major subclasses of this category based on cell body shape, dendritic orientation and axonal distribution[13,20,29,30]. One cell type, the basket cell, was suggested to be GABAergic because its axonal plexus that forms pericellular nests around pyramidal cells in layer V was GAD-positive in immunocytochemical preparations[5,12,21,23]. This identification was given further support from recent studies that utilized colchicine-treated immunocytochemical preparations that displayed the cell bodies of this neuronal type[11,12]. Since the terminals of this plexus form symmetric axosomatic synapses, they are readily quantified and were shown to be reduced by 80% at epileptic foci[22]. These data indicated that a basket cell deficit occurs at epileptic foci and as a result the layer V pyramidal cells at such sites are probably more hyperexcitable than normal.

The present study was undertaken to determine if another cortical GABAergic cell type was reduced in function at epileptic foci. The cell type chosen for this analysis was the chandelier cell. Szentágothai and

Arbib[33] first described this neuron in Golgi preparations. This neuron is characterized by a small soma with aspinous dendrites in layers II and III. The chandelier cell's axon forms numerous vertical chains that appear to be apposed to pyramidal cell apical dendrites in light microscopic preparations[18,33]. However, Somogyi[29] subsequently demonstrated in electron microscopic preparations that these axons form symmetric synapses with the axon initial segments of pyramidal cells in layers II and III. Further studies have confirmed these electron microscopic observations in numerous species and cortical regions[6,20,30] including the hippocampus[31]. The morphology of these terminals and the localization of GAD-positive reaction product within these terminals[8,20,21,32] have indicated that they are GABAergic. These data suggest that chandelier cell axon terminals exert a strong inhibitory influence on the output of pyramidal cells. A loss of such a plexus of axons might also contribute to the hyperexcitability of neurons in an epileptic focus.

MATERIALS AND METHODS

The present study utilized specimens obtained from 3 of the 5 experimental monkeys from a previous study[23]. All of the monkeys had received alumina gel applications to the left cerebral hemispheres to produce seizure foci. Two of these monkeys (animals 3 and 5 from Ribak et al.[23]) received intracortical injections directly into both pre- and postcentral gyri. The remaining experimental monkey (animal 2 from Ribak et al.[23]) had an injection of alumina gel limited to the subarachnoid space in the area of the central sulcus. Electrocorticography of all experimental animals verified epileptic foci[23] and subsequently, the monkeys were fixed by intracardiac perfusions of a mixture of two aldehydes. The fixative solution contained 4% paraformaldehyde, 0.1% glutaraldehyde, and 0.002% $CaCl_2$ in a 0.12 M phosphate buffer.

Blocks of cortical tissue were obtained from the epileptic focus and the homologous area in the contralateral nonepileptic cortex. All blocks were sectioned on a Sorvall TC-2 tissue sectioner at a thickness of 150 μm. Specimens that contained the entire cortical thickness were cut from these sections. These specimens were postfixed in 2% OsO_4 for 1 h, dehydrated in ethanol and embedded in Epon. For light microscopy, semithin 1 μm sections were cut

from the embedded specimens and stained with 0.05% toluidine blue. Specimens were oriented in the ultramicrotome to obtain sections that yielded the longest segments of apical dendrites. The adjacent thin sections from these specimens usually had the most number of identified axon initial segments. These sections were then stained with uranyl acetate and lead citrate and examined on 1 × 2 mm formvar-coated slot grids with the electron microscope.

RESULTS

All observations were obtained from electron microscopic preparations of monkey sensorimotor cortex. The analysis in the present study was limited to the axon initial segments of pyramidal cells in layers II, III and V. Since the findings from the nonepileptic hemisphere were similar to those described in a previous study of normal primate sensorimotor cortex[28], they will be presented first. Then, the data obtained from epileptic cortex will be compared with the data from the nonepileptic cortex.

Nonepileptic cortex

A previous study[22] from this laboratory described the features of nonepileptic monkey sensorimotor cortex in electron microscopic preparations. Descriptions of layer V pyramidal neurons and adjacent neuropil regions were provided. Briefly, layer V pyramidal neurons have multiangular-shaped somata formed by an array of basal dendrites, a single apical dendrite and a single axon usually located between the basal dendrites. Much of the soma is occupied by a large, rounded nucleus whose nucleoplasm contains a relatively homogeneous sprinkling of electron-opaque chromatin and a centrally located nucleolus[19,22]. Pyramidal neurons contain numerous organelles in the perikaryal cytoplasm including both free ribosomes and cisternae of granular endoplasmic reticulum. Terminals that form axosomatic synapses with these neurons make only symmetric synapses. Pyramidal neurons in layers II and III are somewhat smaller than those in layer V, but they share the same ultrastructural characteristics. Since our previous ultrastructural study[22] provided details on the somata and dendrites of pyramidal neurons and the axon terminals that formed synapses with these structures, the present study will involve primarily an analysis

of the synaptic contacts with axon initial segments of pyramidal neurons.

The axon initial segment arises from the axon hillock region of the neuronal cell body. Peters et al.[19] provide a complete description of the features of axon initial segments. Such features will briefly be described and attention will be given to those structures and relationships that appear different in the epileptic cortex.

Axon initial segments usually appear at the base of the pyramidal cell body (Fig. 1). The boundary between these two portions of the neuron is apparent at low magnification because the cisternae of the granular endoplasmic reticulum or Nissl bodies that reside within the soma do not enter the axon. The axon hillock region is conical, whereas the axon initial segment has a constant cylindrical shape that is maintained throughout its length. This latter feature stands in contrast to dendrites which usually have different cross-section diameters over their entire length. The most distal part of the initial segment is characterized by the paranodal region of a myelin sheath (Figs. 1 and 2). These major features of axon initial segments of cortical pyramidal cells are often observed in a single thin section that is properly oriented perpendicular to the pial surface.

Axon initial segments are identified by two other characteristics. One of these is the increased number and aggregation of microtubules, and the other is the dense undercoating of the axolemma (Fig. 3). Both features are typical for axon initial segments and allow for their identification in sections where they may not be continuous with the cell body. Other organelles frequently observed in initial segments include cisternae of agranular endoplasmic reticulum, neurofilaments, elongated mitochondria, occasional free ribosomes and cisternal organelles.
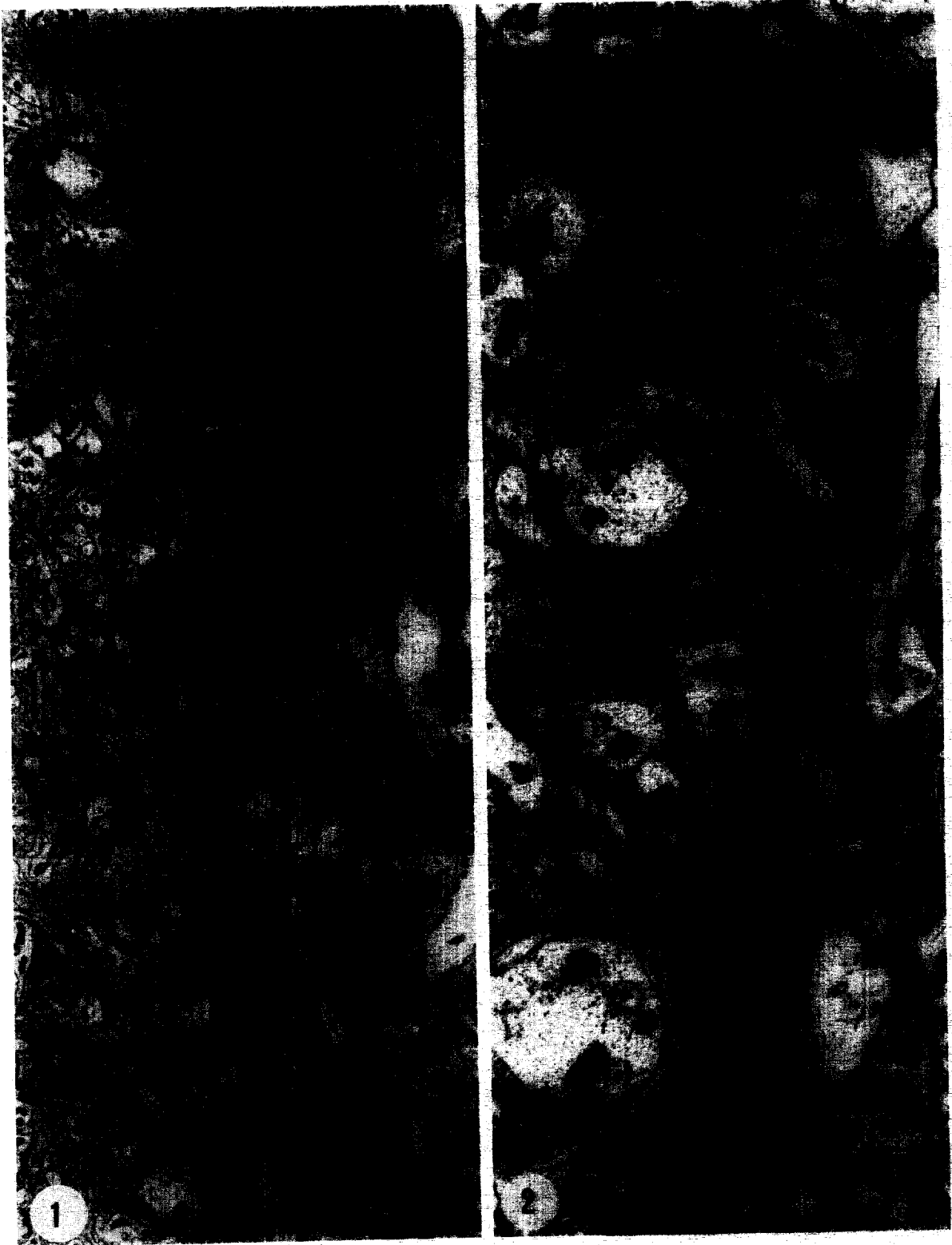
The terminals that contact axon initial segments of pyramidal cells in layers II and III form symmetric synapses (Fig. 3). These terminals that form synapses with axon initial segments do not appear to contact other structures in the adjacent neuropil. They are average in size, contain pleomorphic vesicles and usually one or two mitochondria. In single sections, 10–15 of these axon terminals form synapses throughout the entire length of the axon initial segments obtained from nonepileptic preparations. In contrast, the initial segments of layer V pyramidal

cells have somewhat fewer terminals that synapse with their initial segments. These findings are consistent with a previous quantitative description of axon initial segments of pyramidal neurons in primate sensorimotor cortex[28]. In that study, Sloper and Powell[28] reported that the total length of an axon initial segment of a pyramidal cell in layers II or III received 13.4 synapses, on average, while pyramidal cells in layer V were contacted by 4.8 synapses per axon initial segment. It is important to note that these terminals are not distributed evenly along the length of the axon initial segment. A gradient exists in that fewer terminals are present adjacent to the proximal axon and more are found associated with the distal portion of the axon initial segment. This distribution is similar to the one described for axons of chandelier cells studied in combined Golgi-electron microscopic preparations[8,20,29,30].

Epileptic cortex

Thin sections of cortical tissue from the epileptic focus display similar structures to those observed in the nonepileptic cortex. Pyramidal neurons are readily identified as well as axon hillocks and initial segments that arise from the bases of their somata (Figs. 4 and 5). Most axon initial segments have a normal appearance with the typical fasciculation of microtubules and dense undercoating (Figs. 4, 6 and 7). However, an occasional one displays a swelling in its most distal portion where numerous mitochondria have accumulated (Fig. 4). When this occurs, another dilation is found more distally in the myelinated part of the axon beyond the paranodal region. Although the internal structures of most axon initial segments from epileptic cortex appear normal, the adjacent structures differ from the normal in two ways: (1) a loss of terminals that form symmetric synapses; and (2) an increase in gliosis.

Most of the axon terminals that normally form symmetric synapses with the axon initial segments of pyramidal neurons in layers II, III and V are absent (Figs. 5–7). The few remaining terminals are much smaller than normal, and the number of these terminals per axon initial segment for a single section ranges from 0 to 3 for the 20 axons examined in this study. In addition, the axon hillock region of these same neurons displays a similar loss of terminals that form symmetric synapses. The smaller pyramidal neurons

in layers II and III of ten have no terminals that form synapses wth their axon initial segments. However, the larger pyramidal cells in layer V including two examined Betz cells have the highest number of initial segment terminals and this finding probably results from the much larger size of their axon initial segments.

The second difference observed in these epileptic preparations is the increased number of astrocytic processes that lie adjacent and orient parallel to the axon initial segments. Most of these astrocytic processes are packed with numerous filaments (Fig. 7). Such processes are found adjacent to the somata of these same pyramidal cells as described previously[22]. Often, the same astrocytic process will appose a portion of the cell body, the axon hillock and a 10 μm length of the axon initial segment (Figs. 5 and 6). Although this orientation preference for the longitudinal axis of the axon initial segment is observed most frequently (Fig. 7), some glial processes are oriented transverse to this axis (double arrow in Fig. 4). This apposition of glial processes is usually only one process thick. However, multiple layers of glial processes are found adjacent to the larger axons of Betz cells.

DISCUSSION

The major finding of this electron microscopic study is that most of the axon terminals which form symmetric synapses with axon initial segments of pyramidal neurons are lost at epileptic foci in monkeys. Although previous studies have demonstrated a loss of axosomatic synapses in epileptic foci[3,7,22,35], this report is the first to document a loss of these initial segment symmetric synapses that are formed by terminals mainly derived from the chandelier cell (Fig. 8).

Previous studies that utilized a combined Golgi–electron microscopic method have shown that axon terminals forming synapses with layer II–III pyramidal neuron axon initial segments arise from a specific cortical neuronal type, the chandelier cell[6,20,29,30]. Although a similar study of the chandelier cell has not been made in the monkey sensorimotor cortex, it is likely that this cell exists in this region because Somogyi et al.[30] have demonstrated chandelier cells in the motor cortex of cat and in the monkey's visual cortex. In addition, the distribution of axon terminals alongside the axon initial segments of pyramidal cells in layers II and III of the monkey sensorimotor cortex[28] is very similar to the distribution of chandelier cell axons. Taken together, these data indicate that most of the axon terminals that synapse with axon initial segments of pyramidal neurons arise from chandelier cells.

Recent results from immunocytochemical studies which localize the GABA synthesizing enzyme, GAD, have indicated that chandelier cells are GABAergic because most terminals that form symmetric synapses with axon initial segments contain GAD immunoreactivity[8,11,20]. In fact, Freund et al.[8] have provided direct evidence for this notion by combining Golgi impregnation with immunocytochemistry in the same preparation. In one fortuitous case from cat visual cortex, they demonstrated that 11 Golgi-impregnated boutons derived from a single chandelier cell contained GAD-positive reaction product. The highly strategic site of these GABAergic synapses on axon initial segments suggests that they exert a strong inhibitory effect on the pyramidal neurons which they contact. The large loss of these GABAergic synapses in epileptic foci indicates a significant reduction of inhibition because GABA has an inhibitory action on cortical neurons[14,15]. Therefore, a probable result of this loss is a hyperexcitability of neurons at the epileptic focus.

These findings are consistent with our previous results that demonstrated a severe loss of GAD-positive axon terminals at sites of aluminum gel-induced epilepsy[23]. In addition, they complement a previous electron microscopic study of axosomatic and axo-

Fig. 1. Electron micrograph of a layer III pyramidal soma and axon initial segment from a normal, nonepileptic hemisphere. The soma displays a round nucleus (N) and several cisternae of granular endoplasmic reticulum (E). The axon hillock (H) is formed at the base of the soma and gives rise to the axon initial segment (arrows) which can be followed to the point where it acquires a myelin sheath. This axon is contacted by numerous terminals that form symmetric synapses (see Fig. 2). ×5000.

Fig. 2. Enlargement of the distal portion of the axon initial segment shown in Fig. 1. Numerous terminals (T) are located alongside this axon and many form symmetric synapses (arrows). This axon becomes myelinated at the bottom of the photomicrograph. ×12,500.

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dendritic symmetric synapses in the same monkey preparations[22]. This latter study[22] showed an 80% loss of axosomatic symmetric synapses and a 50% loss of axodendritic synapses in cortical layer V of epileptic foci. Similar reductions in these two types of synapses were also observed in the superficial cortical layers where the present study was undertaken. It is interesting to note that the loss of symmetric synapses with axon initial segments was more similar to the larger reduction of axosomatic synapses than the smaller loss of axodendritic symmetric synapses. Therefore, the most severe loss of GABAergic, symmetric synapses at epileptic foci suggests a degeneration of two cortical GABA cell types, basket and chandelier cells.

The terminals of basket and chandelier cells have some similarities. They send their axons to the soma and axon initial segment, respectively, of pyramidal neurons, and these two sites are most strategic for the control of action potential generation. Thus, both cell types can dramatically influence the activity and output of the cortical projection neurons. Second, these two types of terminals average more than one mitochondria per terminal. This fact has been documented in many previous studies for chandelier cell terminals (see also Fig. 3) and in a quantitative study for the basket cell terminals[22]. Thus, the chandelier cell axonal plexus that forms initial segment symmetric synapses may provide a tonically active inhibition of cortical projection neurons in a way proposed originally for the pericellular basket cell axonal plexus[22]. Further support for this similar function of these two cell types arises from their similar degree of malfunction in epileptic foci. Although a thorough quantitative assessment was not made in the present study, one can conclude from the electron microscopic data of initial segment synapses in epileptic foci that they are reduced in magnitude to a similar amount as the axosomatic synapses as previously reported[22].

It is likely that the chandelier cells have degenerated in these epileptic foci because most of their axon terminals are not present. Other evidence to support this notion is derived from a report that demonstrated neuronal degeneration in Fink–Heimer preparations of alumina gel-treated epileptic monkeys[9]. A common sequel to degeneration of neurons is the hypertrophy and proliferation of glia[10]. The results of the present study showed an increase of glial profiles adjacent to the axon initial segments of pyramidal neurons at epileptic foci. In fact, the same glial profile was often found to appose portions of the soma, axon hillock and axon initial segment (Fig. 6). These findings are consistent with previous results that showed up to a 50% increase in glial proliferation at epileptic foci and indicated degeneration of neurons[3,7,10,22,24,35]. A likely cause of this proliferation of glia alongside axon initial segments in the alumina gel model of epilepsy is the loss of GABAergic axon terminals derived from chandelier cells.

The results of this study add further support for the GABA hypothesis of epilepsy that suggests a reduction of GABA-mediated inhibition in the brain may cause focal epilepsy[25]. Biochemical studies of cortical epileptic foci from humans and animal models have shown a selective loss of GAD activity, GABA concentration and GABA receptor binding[1,2,16,34]. These data are consistent with the results from immunocytochemical studies that show a reduction of GAD-containing terminals at epileptic foci[4,23]. Meldrum[17] and Krnjević[15] have summarized in review chapters the physiological and pharmacological data that show a GABA mechanism is involved in epileptic activity. Therefore, a selective reduction of GABA-mediated inhibition appears to be a hallmark of cortical epileptic foci. A possible cause for this loss of GABAergic neurons at epileptic foci is hypoxia[22,26]. Further studies must determine if the

Fig. 3. Enlargement of another axon initial segment from a nonepileptic hemisphere. The two characteristic features of axon initial segments are shown: a fasciculation of microtubules (M) and a dense undercoating that is continuous with the coating over a pinocytotic vesicle (V). Three terminals are shown to form symmetric synapses (arrows). ×25,000.

Fig. 4. Electron micrograph of an axon initial segment of a layer III pyramidal neuron from an epileptic focus. The axon hillock (H) is located at the top of this micrograph and the initial segment (arrows) is included in its entirety up to and including the point (arrowheads) at which it acquires a myelin sheath. Both proximal and distal to this point, the axon displays slight dilations that feature many disoriented mitochondria. Numerous large profiles of reactive astrocytes (A) are found near this axon. ×5000.
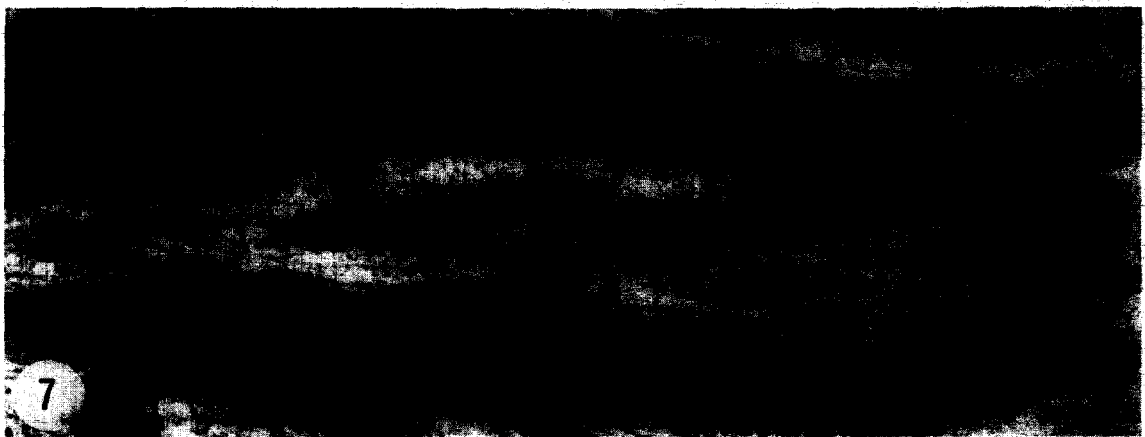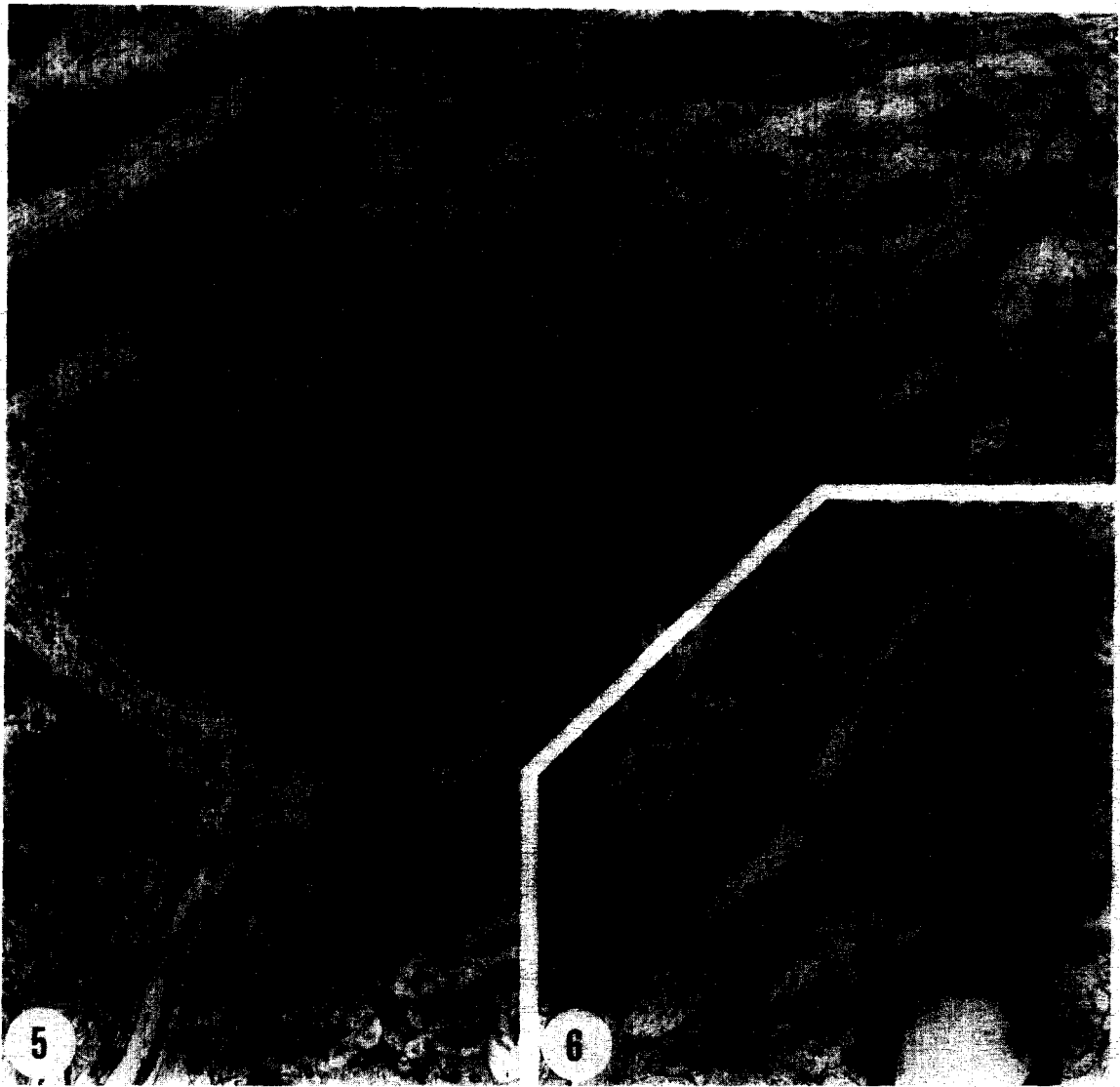
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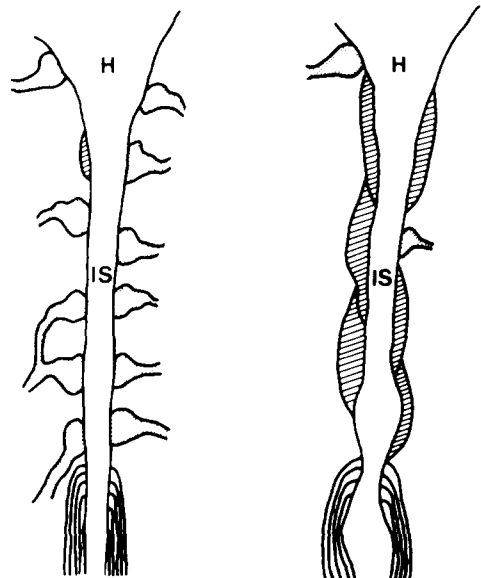
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| ▨ GLIA | ▢ TERMINALS THAT FORM SYMMETRIC SYNAPSES |

GABA hypothesis is responsible for other types of epilepsy, such as those with genetic causes.

Fig. 8. Summary diagram that shows the differences observed for axon initial segments from nonepileptic and epileptic preparations. A reduction in the number of terminals that form symmetric synapses and an increase in glia are the two major differences. Some axon initial segments also display dilations of their distal portions. Such a loss of inhibitory, GABAergic terminals could contribute to the hyperexcitability of the neurons at epileptic foci (see Discussion).

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Fig. 5. Another electron micrograph from epileptic cortex that shows a layer III pyramidal soma, proximal apical dendrite (AP) and axon initial segment (arrows). The soma contains a large nucleus (N) and a perikaryal cytoplasm that contains the typical organelles as well as an abundance of lipofuscin granules. The initial segment is apposed to a glial profile that also contacts the axon hillock and soma. ×13,000.

Fig. 6. Enlargement of the initial segment (IS) and adjacent reactive glial process (A) shown in Fig. 5. ×23,000.

Fig. 7. Enlargement of a distal portion of another axon initial segment (IS) from a layer III pyramidal neuron from an epileptic preparation. This axon displays fasciculation of microtubules (M) and a dense coating beneath the axolemma (arrow). Profiles of reactive astrocytes (A) that contain filaments lie adjacent to this axon initial segment. Axon terminals that form symmetric synapses are not present. ×28,000.

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