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Ganglioside Changes Associated with Temporal Lobe Epilepsy in the Human Hippocampus

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To understand better the molecular and cellular events associated with status epilepticus, a multifaceted analysis has begun on hippocampal tissues therapeutically removed from patients with temporal lobe epilepsy. In this first study, quantitative changes in major ganglioside species are reported, as well as the immunocytochemical localization on the ganglioside G_{D3} in epileptic human hippocampus. Although significant variations were found between patients, the pattern of change was consistent when compared to normal values obtained from an autopsied specimen and the literature. Total ganglioside content was reduced in epileptic hippocampi, which was attributable, in part, to pyramidal cell loss found in CA1 and CA3. In each case, the percentage of ganglioside G_{D3} was increased significantly, while ganglioside G_{D1a} decreased. The former change is probably associated with reactive astrocytosis and the latter with loss of neuronal dendrites. Immunocytochemical localization revealed G_{D3} in the stratum radiatum and the subgranular layer of the dentate gyrus. In these areas, G_{D3} was present in punctate structures and astrocytes. These findings indicate that G_{D3} increases in selected areas of the sclerotic hippocampus and is presumably related to localized accumulation of reactive glial cells. Since gangliosides have a high affinity for calcium and localized increase in extracellular calcium could disrupt normal neuronal function, the localized increase in G_{D3} may not only denote reactive glial cells but may contribute directly to the altered, hyperexcitable condition of epilepsy.

INTRODUCTION

Epilepsy is a major debilitating disease of the central nervous system (CNS) which is characterized by synchronized neuronal discharges [1]. Despite intensive research efforts, the cellular and molecular basis of epilepsy remains uncertain. We have begun a multifaceted analysis of human hippocampal tissue obtained from patients with electrically characterized temporal lobe epilepsy. By determining the biochemical and morphological features that characterize human epileptic tissue, we hope to identify the key factors that are responsible for promoting the hyperexcitable state. One possible factor is the glycolipid components of cell membranes.

Gangliosides are a family of sialic acid-containing glycolipids that are enriched in neuronal membranes [2–4]. These molecules participate in numerous cellular phenomena, e.g., growth, adhesion, membrane excitability, and functional interactions [5–7]. Numerous studies have shown that the relative amount of individual species of brain gangliosides is a direct indication of the structure, number, and types of neural membranes [8–10]. For example, G_{D1a} is associated with synaptic membrane densities [11–13]. Likewise, changes in ganglioside composition accompany pathological altera-

This paper is dedicated to our mentor, Dr. Gilbert H. Glaser.

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tions in neural tissues. For example, G_{D3} is often found in high concentrations in traumatized CNS tissue and is considered a marker for reactive glia in many neurological diseases [10,12,14,15,16]. Hence, an examination of gangliosides can provide important information on the health and cellular composition of brain tissue.

Studies from our laboratory [17–19] and the laboratories of others [20,21] have revealed significant ganglioside changes in various animal models of experimentally induced seizures. These changes reflect, to some extent, the pathological changes that are associated with epileptic brain. In humans as well as in animals, these pathological changes frequently involve neuronal loss and gliosis. In the hippocampus of an epileptic, this is referred to as "hippocampal sclerosis," which is characterized by gliosis and loss of neurons in the Sommer sector and end folium (Rose's area H1 and H3-5) [22–25].

One principal aim of the present study was to examine changes in ganglioside G_{D3}. We have previously shown that G_{D3} is closely associated with reactive, but not resting, glial cells [10,16,26,27]. In addition, G_{D3} is associated with a number of cells, such as Müller glial cells [15], undifferentiated neurons [14,28], and malignant transformed cells [29], which share a common property in being metabolically active. We have hypothesized that the membranes of these cells must be highly permeable to ions and metabolites [10,27]. Hence, reactive glial cells may have fundamental significance in regulating the extracellular environment in epileptogenesis [30]. To test this hypothesis, our first objective in this study was to test for the presence of G_{D3}-positive glia and determine their tissue distribution. The availability of brain tissue from human patients offers a unique opportunity to correlate the biochemical and immunohistochemical changes in ganglioside distribution that are associated with electrophysiologically and clinically defined epileptic tissue.

MATERIALS AND METHODS

Brain Tissues

Hippocampal gyri from epileptic patients were removed en bloc during surgery, and quickly cut into transverse slabs 1–2 cm wide. Individual specimens were either frozen on dry ice or fixed by immersion in 0.1 M phosphate buffer containing 2 percent formaldehyde. Frozen tissue slabs were used for both quantitative ganglioside analysis and immunocytochemical localization of G_{D3} on immediately adjacent tissue. Since histological preservation in fresh-frozen tissue is relatively poor, quickly fixed tissue was also examined by immunocytochemistry.

A normal control hippocampus was obtained with permission from a 26-year-old male who died suddenly with no known neurological symptoms. Comparable slabs of hippocampal tissue were obtained after autopsy and frozen on dry ice within 16 hours of death. Since gangliosides are relatively stable after death, the results of quantitative analysis were considered reliable. Immunocytochemical study was limited to fresh-frozen tissue from the hippocampus and was used only for comparative localization.

Ganglioside Quantitation

The amount of major gangliosides within samples was determined by methods described in detail elsewhere [31]. Briefly, a ganglioside-enriched fraction was obtained from each sample after chloroform/methanol extraction and DEAE column chromatography. Individual gangliosides were separated by thin-layer chromatography (TLC) [32], visualized by resorcinol, and quantitated with a densitometer.

Immunocytochemical Localization

To correlate biochemical analysis with immunocytochemical localization, fresh-frozen samples were first sectioned and the remaining adjacent tissue processed for ganglioside quantitation. Frozen specimens were sectioned on a cryotome (10 μm), mounted on glass cover slips coated with poly-1-lysine, and allowed to air dry for one hour. Sections were fixed in 0.1 M phosphate buffered saline (PBS), containing 4 percent formaldehyde for five minutes, and rinsed with PBS containing 0.3 percent gelatin. All subsequent steps were performed in PBS with gelatin.

Monoclonal antibody R24 was provided by Dr. K.O. Lloyd, Sloan-Kettering Institute, New York, and has known specificity for G_{D3} [33]. Polyclonal rabbit antibody against purified human glial fibrillary acidic protein (GFAP) was provided and characterized by Dr. L.F. Eng, Stanford University, Palo Alto, California. These primary antibodies were diluted 1:75 for anti-G_{D3} and 1:300 for anti-GFAP and applied to sections for 20 minutes. After rinsing, sections were incubated for 20 minutes in peroxidase-conjugated secondary antibody. Anti-G_{D3} was localized with goat anti-mouse IgG (Sigma) diluted 1:40, and anti-GFAP was localized with goat anti-rabbit IgG (Cappel), diluted 1:300.

Formaldehyde-fixed tissues were postfixed by immersion in 5 percent acrolein in phosphate buffer (PB) for one hour and sectioned on a vibrotome (50 μm). Sections were incubated in PB with 0.03 percent Triton-X100 for one hour, rinsed, and treated to block nonspecific stain. Sections were reacted at room temperature in the following reagents in PB at room temperature and rinsed after each step: 1 percent borohydride for ten minutes, 0.2 percent lysine and 0.1 percent gelatin for one hour, and 4 percent goat serum for 2–24 hours. Sections were incubated for 48 hours at 4°C with anti-G_{D3} diluted 1:200 or anti-GFAP diluted 1:600. After rinsing, sections were incubated for 48 hours with the complementary secondary antibodies diluted threefold more than for frozen cryotome sections. Peroxidase activity was visualized with diaminobenzidine/H_2O_2.

The quantitative ganglioside analysis and immunocytochemical studies were performed and evaluated independently in order to prevent bias.

RESULTS

Ganglioside Quantitation

The study was divided into two phases. In the initial phase, full transverse slabs of fresh-frozen tissue containing the entire hippocampal formation were assayed for ganglioside distribution. Four specimens from epileptic patients were assayed. The pattern of gangliosides separated on a TLC plate and treated with resorcinol is shown in Fig. 1. Quantitative results shown in Table 1 reveal significant differences between patients in the percentage of G_{D3}.

Immunocytochemical localization of G_{D3} in adjacent sections showed corresponding qualitative differences in the intensity of stain between specimens. The general pattern of stain, however, was similar in each case. Within the CA sub-regions, stain was most pronounced near the zones of pyramidal cell loss. The Sommer's sector region of CA1 showed at least partial loss of pyramidal cells in all cases (see Figs. 2,4A,6A), and typically was most heavily stained (see Figs. 3,4). Within CA2 and CA3, relatively less pyramidal cell loss was evident, and stain was less dense. By comparison, no pyramidal

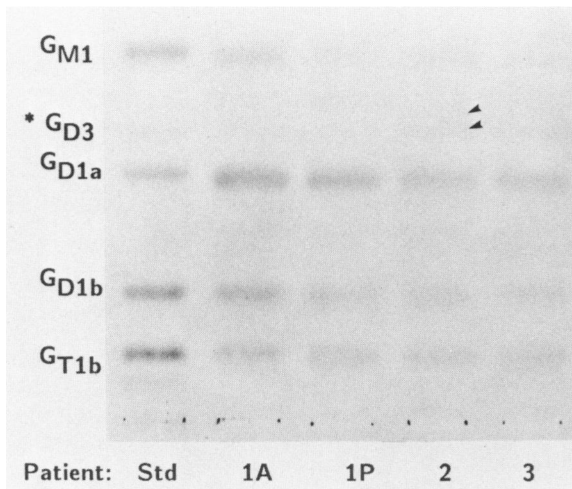

FIG. 1. Thin-layer chromatogram of extracted gangliosides from hippocampi of epileptic patients (**1–3**) and standard sample (**Std**). Each lane contained about 1 μg of sialic acid. The plate was developed with chloroform/methanol/0.02 percent $CaCl_2$ (55:45:10). Gangliosides were visualized with resorcinol-HCl agent. G_{D3} (*) is identified as two closely spaced bands between G_{M1} and G_{D1a}. Note the sample from Patient 2 has relatively more G_{D3} *(arrows)* compared to other samples. Two specimens were available from Patient 1, anterior (**1A**) and posterior (**1P**) portions.

cell loss was apparent in the prosubiculum, and no stain was detected above background.

Based on these findings, hippocampal specimens from a second set of patients were prepared for ganglioside quantitation. As shown in Fig. 2, the CA1 region was dissected into two approximately equal areas. The area designated "A" coincides with the region associated with the most common pyramidal cell loss (Sommer's sector). The area designated "B" was immediately adjacent and may include part of the prosubiculum where neuronal loss was less pronounced. Hippocampi from two patients and one normal autopsy were thus analyzed, and the results are shown in Table 2. The most striking differences were in the percentages of G_{D3}. In A and B regions from the normal control, G_{D3} was 2.9 percent. By comparison, G_{D3} was significantly higher in region A than region B in the two patients. This difference corresponds to the location of the pyramidal cell loss which was distinct in region A of both patients, but not apparent in region B.

In addition to G_{D3}, other consistent changes were evident in the ganglioside content

TABLE 1
Gangliosides from Hippocampus of Epilepsy Patients

	Patient			
	1(P)	1(A)	2	3
Total SA (μg/gdw)	1,902	1,077	1,037	909
G_{M1}	17.1	12.6	19.8	11.0
G_{D3}	5.4	6.1	13.6	7.7
G_{D1a}	32.0	35.1	23.8	31.8
G_{D2}	5.2	5.5	4.6	4.9
G_{D1b}	19.1	18.3	15.3	20.0
G_{T1b}	18.0	22.5	22.9	24.6

Gangliosides shown as percentage of total for each sample. Total sialic acid (SA) expressed as micrograms per gram dry weight. Samples from anterior (A) and posterior (P) regions of Patient 1 shown.

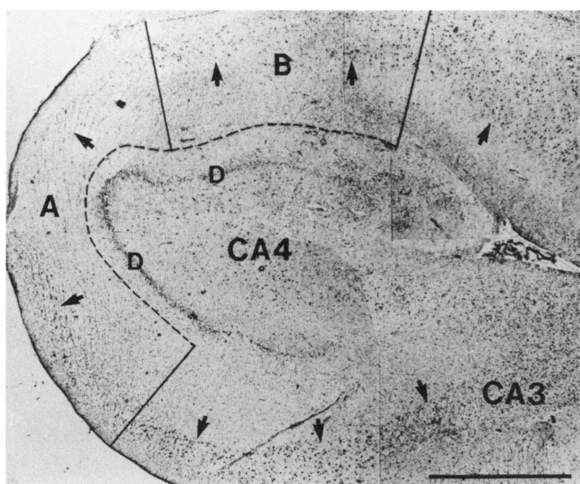

FIG. 2. Micrograph of transverse section of hippocampus from Patient 5 which shows typical pyramidal cell loss in Sommer's sector. Two sub-regions of Ammon's horn which were assayed for ganglioside content are illustrated. Region **A** includes Sommer's sector, and region **B** is the adjacent region with less pyramidal cell death. Arrows point to presumptive pyramidal cell layer. Dentate granule cell layer **D**. Bar = 2 mm.

of hippocampal tissue from epileptic patients (refer to Tables 1 and 2). The level of $G_{D1a}$was markedly lower in patient tissues and was inversely related to the level of G_{D3}. G_{M1} appeared to decrease moderately. The percentages of G_{D2}, G_{D1b}, and G_{T1b} were moderately elevated. Total ganglioside content within the tissue was markedly decreased in epileptic tissues. Most of these changes were noticeably evident in comparing sub-regions A and B from the two epileptic patients.

Immunocytochemical Localization

Based on immunocytochemical study of all patients' and control tissues, the distribution of G_{D3} associated with epilepsy was determined at the light microscopic level. In sections of patients' hippocampi, the overall intensity of G_{D3} immunoperoxidase stain varied from indistinct background levels to heavy localized deposits of reactivity. These qualitative variations correlated directly with the quantitative difference in G_{D3} levels determined by biochemical assay. No distinct stain was evident in hippocampus from normal tissue, and only moderate stain was present in sections

TABLE 2
Gangliosides from CA1 Region

			Epilepsy Patient			
	Normal		4		5	
Tissue Region	A	B	A	B	A	B
Total SA (μg/gdw)	2,127	2,705	1,133	1,684	1,642	2,046
G_{M1}	26.8	24.4	20.8	26.6	22.4	22.5
G_{D3}	2.9	2.9	9.6	4.3	4.4	2.8
G_{D1a}	41.2	43.2	26.9	28.7	29.1	34.6
G_{D2}	2.1	2.2	4.2	4.8	2.1	2.4
G_{D1b}	10.4	10.1	14.0	15.0	14.6	13.7
G_{T1b}	11.6	11.8	15.7	12.8	17.2	15.9

Gangliosides shown as percentage of total for each sample. Location of regions "A" and "B" are illustrated in Fig. 2. Total sialic acid (SA) expressed as micrograms per gram dry weight.

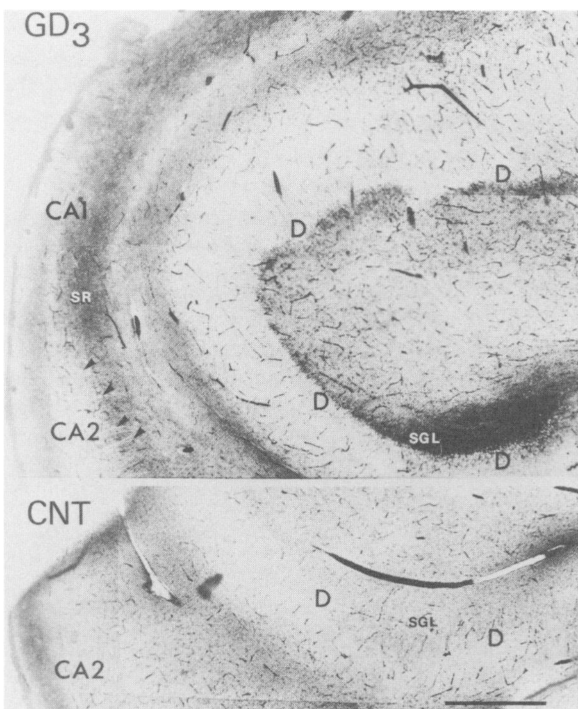

FIG. 3. Micrographs showing localization of G_{D3} in rapidly fixed human hippocampus. Control section (**CNT**) shows background and endogenous peroxidase activity in blood vessels. Specific stain is found in the subgranular layer (**SGL**) of the dentate (**D**). Apical dendrites of pyramidal cells *(arrows)* are seen in relief in **CA2** due to specific stain in the stratum radiatum (**SR**). No pyramidal cells are seen in this region of **CA1**. Bar = 1 mm.

from patients with low levels of G_{D3} increase. In cases of major increases of G_{D3}, a distinct and characteristic immunostaining pattern was evident. Since the immunocytochemical localization and quantitative analyses were performed independently with blind controls, these results indicated that the immunocytochemical localization method reliably reflects the changes in G_{D3} associated with the epileptic tissue. Whether the endogenous G_{D3} present in normal hippocampus may be masked to immunodetection or distributed relatively uniformly was not determined.

Despite the variation in stain intensity, a consistent pattern of localization was observed. In most cases, dense immunoperoxidase stain was localized in the subgranular layer (SGL) of the dentate and in parts of the stratum radiatum (SR) (Figs. 3 and 4). Lighter stain was apparent in adjacent regions of the stratum moleculare (SM). Within these regions, immunodetectable G_{D3} appeared in small, broadly scattered, punctate structures and in stellate cells that were identified by location, shape, and size as astrocytes (Fig. 5).

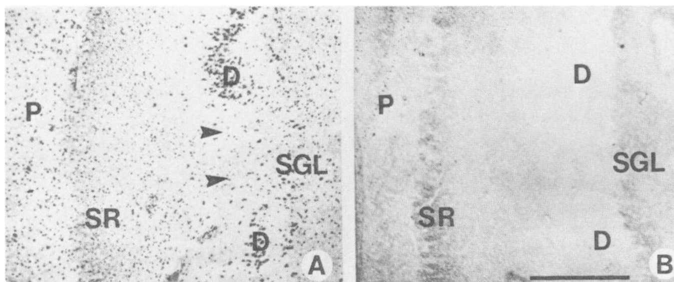

FIG. 4. Micrographs of adjacent fresh-frozen sections showing comparable areas of CA1 from Patient 2. Total pyramidal cell (**P**) loss in CA1 and major gaps *(arrows)* in granular cell layer of dentate (**D**). Immunoperoxidase stain for G_{D3} densely labels stratum radiatum (**SR**) and subgranular layer (**SGL**). Bar = 0.5 mm.

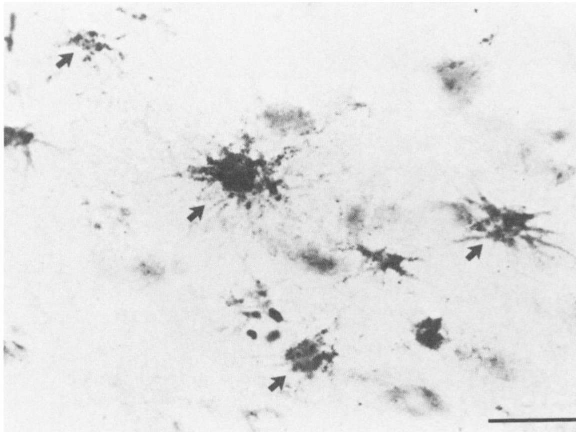

FIG. 5. Higher magnification micrograph showing cellular localization of G_{D3} in stratum radiatum of CA1. Specific reaction product is distributed in puncta scattered throughout tissue and appears to accumulate in astrocytes *(arrows)*. Bar = 40 μm.

Astrocytes stained by anti-GFAP were found throughout normal hippocampal tissue in patterns distinct for each area. For example, stellate astrocytes were located in the stratum moleculare (Fig. 6D). Within the dentate layer, cell bodies of large astrocytes were located in the subgranular layer, and many of their processes extended into the granule cell layer. In areas of neuronal loss, a massive increase in GFAP-positive processes was found (Fig. 6). The dense matrix of astrocytic processes in these sclerotic areas obscured the identification and morphology of individual astrocytes. Areas of astrocyte proliferation coincided in large part with the distribution of G_{D3} associated with epilepsy (Fig. 6).

DISCUSSION

The present study shows that increased levels of G_{D3} are present within localized regions of the hippocampal formation of epileptic patients. The amount of G_{D3} varied widely between patients and is correlated in part with the degree of pyramidal cell death. Immunocytochemically detectable G_{D3} is seen in dispersed punctate structures and associated with astrocytes. These findings support previous studies which indicate that G_{D3} is associated with neural degeneration and accompanying gliosis [28].

One important question is whether the localized increase of G_{D3} is a consequence or, rather, a causative factor of epilepsy. It is known that G_{D3}, as a divalent anionic glycolipid, binds calcium with high affinity [34,35]. An abnormal accumulation of G_{D3} in neural membranes may increase the local concentration of extracellular calcium, thereby altering a variety of critical homeostatic balances, and promote a hyperexcitable state. Based on the present positive finding, further experiments are being conducted on kindled cats as an experimental model. Initial results show an accumulation of G_{D3} in similar regions of the experimentally induced "epileptic" cat hippocampus [Holly JA, et al: unpublished results].

Other questions arising from this study include the ultrastructural distribution of G_{D3} in normal and epileptic hippocampi. If G_{D3} is involved in promoting neuronal hyperexcitability, then it should be distributed in association with neuronal membranes. The G_{D3}-positive punctate structures in epileptic tissue are similar in size to focal synapses and may represent astrocytic processes covering junctional contacts.

Also unknown is the distribution of G_{D3} in the normal hippocampus. In the normal human hippocampus assayed, G_{D3} was 2.9 percent of the gangliosides. Despite this

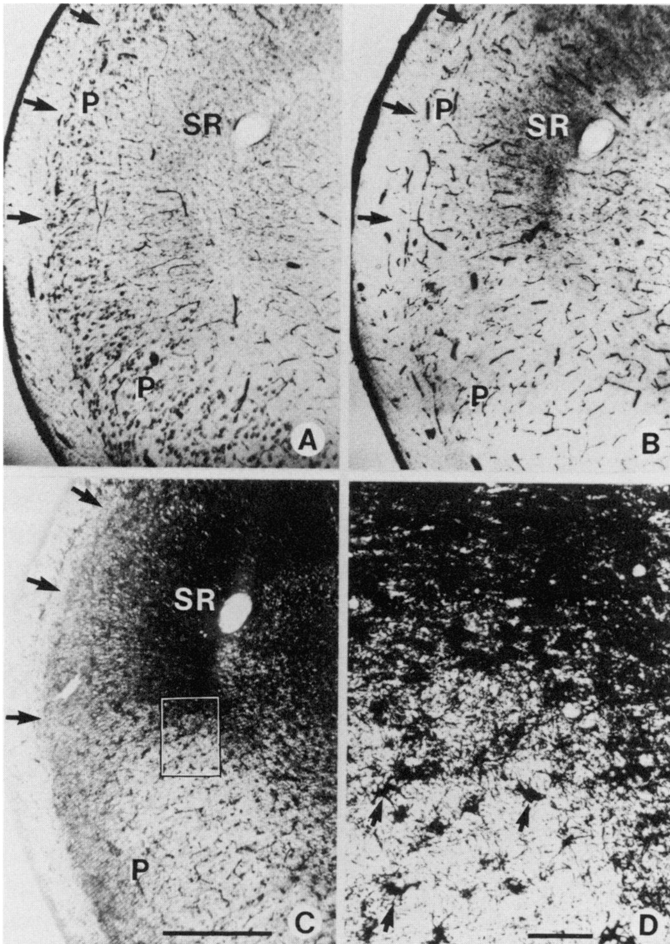

FIG. 6. Micrographs of adjacent vibrotome sections showing comparable areas of CA1 from Patient 4. **A** Relatively focal loss of pyramidal neurons (**P**) in CA1 *(arrowheads)*. **B** Immunoperoxidase stain for G_{D3} shows heavy label localized in stratum radiatum (**SR**) subjacent to pyramidal neuron loss. **C** Immunoperoxidase stain for GFAP shows stained astrocytes throughout tissue and dense gliosis in region of neuronal death, especially in stratum radiatum. Bar = 500 μm. **D** Higher magnification of area boxed in C, showing stellate astrocytes and transition from "normal" to gliotic regions. Bar = 70 μm.

substantial level of G_{D3} in normal tissue, immunocytochemical localization revealed no distinct stain above background. Similarly, other immunocytochemical studies of G_{D3} localization have been unable to show distinct localization in the adult brain of mice, despite its known biochemical presence [28,36]. G_{D3} has been shown to accumulate in the plasma membrane of oligodendrocytes during development [37]. Perhaps endogenous G_{D3} in normal adult tissue is masked from immunodetection.

The majority of immunodetectable G_{D3} associated with epilepsy was localized in two zones: the stratum radiatum of CA1, and the subgranular layer of the dentate. Neuronal death accompanying epilepsy is most common in CA1 and was clearly associated with increased levels of G_{D3} in this region. However, in most cases of epilepsy, relatively little neuronal death is found in the dentate granule cell layer or in CA4, which border the subgranular layer [22–25]. And yet, without substantial local neuronal death, the subgranular layer had increased levels of G_{D3}. Thus, G_{D3} accumulation within the hippocampus of epileptics may signify more than simply the presence of reactive astrocytes in zones of neuronal degeneration.

It is tempting to suggest that many forms of epilepsy are caused by an initial trauma leading to the generation of reactive astrocytes and accumulation of G_{D3}. The high

affinity of G_{D3} for calcium results in excessive calcium-dependent neural activities, which may include increases in transmitter release and protein phosphorylation. A cycle of neuronal hyperactivity and death followed by proliferation of G_{D3}-positive reactive glia may perpetuate the cycle.

Although speculative, this scheme is consistent with several lines of morphological and physiological evidence regarding temporal lobe epilepsy. Special attention is directed to the subgranular layer of the dentate where G_{D3} increases have been found in both epileptic human and kindled cat hippocampi. The dentate granule cells supply a major excitatory input into the hippocampus, the Schaffer collaterals, which have long been considered a primary candidate in promoting hippocampal hyperexcitability [38]. A dense layer of astrocytic cell bodies is present in the subgranular layer, and many of their processes are in close proximity to granule cell bodies and axons [39,40]. It is known that the principal neural transmitter of granule cells is glutamate, and that pharmacological agents which block glutamate activity inhibit ictal activity [41,42]. In addition, the injection of glutamate has been shown to cause neuronal death and gliosis, which is typical of epileptic hippocampal sclerosis [43]. Calcium has been implicated in epileptogenesis [44,45], and certain anticonvulsant drugs inhibit calcium-dependent protein phosphorylation [46]. Notably, Ca/calmodulin-dependent protein kinase is localized preferentially in the hippocampus and appears particularly abundant in the dentate [47]. *In vitro* experiments have shown that gangliosides can enhance Ca^{++}/calmodulin protein phosphorylation [48]. In conjunction with this process, long-term changes occur in Ca^{++}/calmodulin phosphorylation activity of kindled rat hippocampi [49]. The present results support this scheme, although other interpretations are possible. Future experiments, such as localized injections of G_{D3}, are planned to test the proposed G_{D3} mechanisms of epilepsy directly.

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