Preferential Neuronal Loss in Layer III of the Medial Entorhinal Cortex in Rat Models of Temporal Lobe Epilepsy

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We recently described a pronounced neuronal loss in layer III of the entorhinal cortex (EC) in patients with intractable temporal lobe epilepsy (Du et al., 1993a). To explore the pathophysiology underlying this distinct neuropathology, we examined the EC in three established rat models of epilepsy using Nissl staining and parvalbumin immunohistochemistry. Adult male rats were either electrically stimulated in the ventral hippocampus for 90 min or injected with kainic acid or lithium/pilocarpine. Animals were observed for behavioral changes for up to 6 hr and were killed 24 hr or 4 weeks after the experimental treatments. At 24 hr, all animals that had exhibited a bout of acute status epilepticus showed a consistent pattern of neuronal loss in the EC in Nissl-stained sections. Neurodegeneration was most pronounced in layer III of the medial EC at all dorsoventral levels. A few surviving neurons were frequently present in the lesioned area. An identical pattern of nerve cell loss was also seen in the EC of rats killed 4 weeks following the treatments. This lesion was completely prevented by an injection of diazepam and pentobarbital, given 1 hr after kainic acid administration. Immunohistochemistry demonstrated a relative resistance of parvalbumin-positive neurons in layer III of the medial EC. Taken together, these experiments indicate that prolonged seizures cause a preferential neuronal loss in layer III of the medial EC and that this lesion may be related to a pathological elevation of intracellular calcium ion concentrations.

[Key words: calcium-binding protein, entorhinal cortex, kainic acid, neurodegeneration, pilocarpine, seizures, temporal lobe epilepsy]

Comprehensive clinical and pathophysiological studies have revealed a unique and stereotyped syndrome that accounts for the majority of cases of intractable epilepsy, the mesial temporal lobe epilepsy syndrome (reviewed by Wieser et al., 1993). The patients often experience status epilepticus or prolonged febrile

convulsions in early life and then, after a latency period of several years, begin to suffer from repetitive temporal lobe seizures. These seizures are difficult to control with medications, but can often be abolished by surgical intervention. Histological examination of hippocampal specimens from these patients frequently reveals Ammon's horn sclerosis, with loss of neurons and gliosis in CA1, CA3, and in the dentate hilus (Sommer, 1880; Margerison and Corsellis, 1966; Babb and Brown, 1987; Bruton, 1988). Although it is still not clear whether and how these neuropathological changes are linked to the cause of chronic temporal lobe epilepsy, Ammon's horn sclerosis is commonly accepted as a hallmark of the disease. Moreover, it is widely believed that Ammon's horn sclerosis can be caused by status epilepticus (Meldrum and Bruton, 1992), although this idea is still somewhat controversial (reviewed in Lothman and Bertram, 1993).

In examining specimens obtained at the time of surgery for medically intractable temporal lobe epilepsy, we noted a characteristic pattern of neuronal loss, along with gliosis, in layer III of the entorhinal cortex (EC) (Du et al., 1993a). Notably, some neurons in the lesioned area appeared to be relatively resistant to degeneration. This distinctive neuropathology, which was most prominent in the anterior portion of the medial EC, led us to hypothesize that this lesion is implicated in the pathophysiology of temporal lobe epilepsy.

Since the study of human brain specimens has obvious limitations, we decided to examine the putative seizure-related damage to the EC, in particular layer III, in three animal models of chronic epilepsy. Our experiments included the systemic administration of chemical agents [kainic acid (KA) or lithium/pilocarpine] and electrical stimulation of the ventral hippocampus (Ben-Ari et al., 1980; Honchar et al., 1983; Turski et al., 1983; Lothman et al., 1989, 1990). A common theme of the three models is a period of acute status epilepticus followed weeks later by recurring spontaneous hippocampal seizures and by hippocampal neuropathology that resembles Ammon's horn sclerosis (Cavalheiro et al., 1982, 1991; Ben-Ari, 1985; Olney et al., 1986; Bertram et al., 1990; Lothman et al., 1990; Du et al., 1993b).

Two time points, 24 hr and 4 weeks after the experimental treatments, were chosen to assess histopathological changes. In addition, to test the causal relationship between prolonged seizures and neuronal loss, pharmacological intervention with anticonvulsants to arrest status epilepticus was applied to a group of rats that had received KA. Since the resistance of neurons to seizure-related destruction has been linked to their ability to

Received Feb. 27, 1995; revised May 1, 1995; accepted May 8, 1995.

We gratefully acknowledge the excellent technical support of Mr. John Williamson and the expert secretarial assistance of Mrs. Joyce Burgess. This work was supported by USPHS Grants NS 16102 (to R.S., E.W.L., and F.D.) and NS 21671 (to E.W.L.), a grant from the Epilepsy Foundation of America (to F.D.), and a Fulbright Senior Scholarship (to T.E.).

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^aWe are saddened by the passing of our esteemed colleague, Dr. Eric W. Lothman, and dedicate this article to his memory.

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buffer intracellular free calcium (Scharfman and Schwartzkroin, 1989; Sloviter, 1989), our analysis of Nissl-stained material was combined with an immunohistochemical evaluation of the EC using an antibody against the calcium-binding protein, parvalbumin. Taken together, these experiments were expected to provide a better understanding of the causes and mechanisms of the preferential neuronal loss in layer III of the human EC.

A preliminary account of this work has appeared in abstract form (Eid et al., 1993).

Materials and Methods

Animals

Male Sprague-Dawley rats (250–300 gm), kept at a 12 L:12 D cycle with free access to food and water, were used in all experiments. There were three experimental groups: (1) animals that experienced self-sustaining limbic status epilepticus (SSLSE) precipitated by electrical stimulation, (2) animals with status epilepticus precipitated by KA, and (3) animals with status epilepticus precipitated by lithium/pilocarpine. Control animals received either saline injections or a hippocampal electrode implantation.

Treatment paradigms

SSLSE. The experimental procedure to produce SSLSE has been described previously (Lothman et al., 1989, 1990). Briefly, under ketamine-xylazine anesthesia, bipolar electrodes were stereotactically implanted in the CA3 subfield of the left ventral hippocampus (AP: 3.6 mm behind bregma, L: 4.9 mm, DV: 5.0 mm below dura; incisor bar: +5.0 mm). One week after surgery, the experimental animals received a continuous electrical stimulation that lasted 90 min. The animals were killed 24 hr ($n = 6$) or 4 weeks ($n = 6$) following the induction of SSLSE, and their brains were processed for histological analysis (cf. below). Implanted rats that received no electrical stimulation and were sacrificed 4 weeks after electrode implantation served as controls ($n = 6$).

Injection of chemoconvulsants. KA, pilocarpine hydrochloride, lithium chloride, and (–)scopolamine methyl nitrate were purchased from Sigma Chemical Co. (St. Louis, MO). Diazepam and sodium pentobarbital were obtained from Hoffmann-La Roche (Nutley, NJ) and Abbott Laboratories (Chicago, IL), respectively. All other chemicals used were purchased from Sigma. Drugs were dissolved in 0.9% NaCl (saline) and injected subcutaneously (s.c.). Nineteen rats received a dose of 10 mg/kg KA (24 hr time point: $n = 9$; 4 week time point: $n = 10$). For lithium/pilocarpine treatment (24 hr time point: $n = 11$; 4 week time point: $n = 6$), rats were first injected with lithium (127 mg/kg) and, 24 hr later, with scopolamine (1 mg/kg) followed after 30 min by pilocarpine (30 mg/kg) (Honchar et al., 1983; Turski et al., 1983). Since prolonged convulsions substantially increases mortality in these animals, all lithium/pilocarpine-treated rats described in this article also received an injection of diazepam (10 mg/kg) and sodium pentobarbital (30 mg/kg) 4 hr after the onset of clonic forelimb convulsions (Olney et al., 1986; Cavalheiro, personal communication). Control rats were injected (s.c.) with saline ($n = 6$), KA (6 mg/kg; $n = 5$), or lithium (127 mg/kg) and, 24 hr later, scopolamine (1 mg/kg) ($n = 5$).

Administration of anticonvulsants. Separate groups of KA-treated animals received a s.c. injection of diazepam (10 mg/kg) and pentobarbital (30 mg/kg) either 1 hr after the administration of KA ($n = 8$) or 1 hr after the onset of clonic forelimb convulsions ($n = 9$). All animals were killed 24 hr after KA administration.

Tissue preparation

After a survival time of 24 hr or 4 weeks, all animals were deeply anesthetized with chloral hydrate (600 mg/kg, i.p.) and perfused via the

ascending aorta with 50–100 ml of saline followed by 500 ml of 0.1 M phosphate buffer (PB; pH 7.4) containing 4% paraformaldehyde. The brains were removed and postfixed in the same fixative for 4 hr at 4°C. Following immersion in 0.1 M phosphate-buffered saline (PBS; pH 7.4) containing 20% sucrose for 48 hr (4°C), the brains were rapidly frozen and stored at –80°C.

Cryostat sections (30 μ m) were cut coronally through the anterior portion of the brain (including the dorsal hippocampus) and horizontally through the remainder of the brain, which contained the ventral hippocampus and the EC. Every first section of each series of five was mounted on gelatin-coated slides and was stained with thionin for histological analysis. In addition, representative horizontal sections from different levels along the dorsoventral axis of the EC were collected in cold PBS for immunohistochemical experiments.

Immunohistochemical procedure

A monoclonal antibody against parvalbumin (Sigma) was used. The immunohistochemical procedure has been described in detail previously (Du et al., 1993b). In brief, sections were incubated for 3 d at 4°C free-floating in PBS (0.01 M, pH 7.4) containing anti-parvalbumin (1:4000) antibody, 3% normal horse serum and 0.3% Triton X-100. Subsequently, the immunoreaction product was visualized according to the avidin-biotin complex (ABC) method using a standard ABC kit (Vectastain, Burlingame, CA) and diaminobenzidine as a chromogen. Immunostained sections were mounted on slides and counterstained with thionin.

Cell counting

To examine the possible differential susceptibility of parvalbumin-immunoreactive (–i) neurons to seizure-related neuronal damage, the numbers of parvalbumin-i neurons and Nissl-stained neurons were evaluated in layer III of the medial EC at three levels along the dorsoventral axis in normal and KA-treated rats. Neuronal counting was performed on immunostained sections counterstained with thionin with an ocular grid (0.0196 mm² per section). Per rat, two sections were chosen from each of the dorsal, middle, and ventral level (approximately corresponding to the first, third, and fifth levels, as illustrated in Fig. 3) of the EC. Six normal and six KA-treated rats were used for cell counting. Only neuronal profiles with a visible nucleus and/or a complete cell contour were counted. (Since the same counting method was used to estimate cell densities in both experimental and normal animals, common morphometric bias errors were assumed to be negligible in the context of the present comparative analysis.) On every section, counting was performed in two different fields in layer III of the medial EC. Subsequently, the cell numbers obtained from each rat were averaged for each of the three levels along the dorsoventral axis, and the mean numbers were used for statistical analysis.

Nomenclature

The parcellation and terminology of the rat EC used in this study was based on publications by Lorente de Nó (1933) and Witter et al. (1989). Thus, the EC was divided into a medial and a lateral portion in most horizontal planes (the most dorsal portion of the EC contains only the medial EC; cf. Caballero-Bleda and Witter, 1993, Fig. 4). In Nissl-stained horizontal sections, six layers can be identified in both the medial and the lateral EC based on the morphology, arrangement, and density of neurons. Layer I, the most superficial layer, contains very few neurons. Layer II is characterized by a thin assembly of relatively large neurons, which, in the lateral EC, are frequently arranged in clusters. Layer III is the widest layer of the EC and contains medium- to large-sized neurons. Layer IV is a thin layer containing both large and small neurons. Large neurons are often fusiform in shape and arranged parallel to the long axis of the lamina. Layer V consists of small, often intensely stained, neurons and has the highest neuronal density in the

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Figure 1. Photomicrographs of Nissl-stained horizontal sections (30 μ m) cut through the dorsal portion of the entorhinal cortex (EC) from a saline-treated (A), a SSLSE (B), a KA (C)- and a lithium/pilocarpine (D)-injected rat that survived for 24 hr after the treatment. E–H are higher magnifications of the areas outlined by the boxes in A–D, respectively. Loss of neurons is most pronounced in layer III of the medial EC in all three seizure models. Note surviving neurons in the degenerated area (indicated by arrowheads in F–H). The solid arrows in A–D point to the lateral limit of the EC and the open arrows indicate the approximate border between the medial (EC_m) and lateral (EC_l) EC. I–VI: layers of the EC. Scale bars: 500 μ m in D and also for A–C; 200 μ m in H and also for E–G.

Figure 2. Nissl-stained horizontal sections (30 μm) through the ventral level of the entorhinal cortex (EC) taken from the same rats as shown in Figure 1A–H. (cf. Fig. 1A–H for the definition of the labels and abbreviations). Note prominent neuronal loss in layer III in the most medial sector of the medial EC. Scale bars: 500 μm in D and also for A–C; 200 μm in H and also for E–G.

Table 1. Relative densities of parvalbumin-immunoreactive and Nissl-stained neurons in layer III of the rat medial entorhinal cortex: effect of KA treatment

	Dorsal level			Middle level			Ventral level		
	Parv.	Nissl	%	Parv.	Nissl	%	Parv.	Nissl	%
Normal rats									
#1	4.3	38.3	11.2	6.3	47.3	13.3	5.1	53.4	9.6
#2	5.0	42.5	11.8	4.8	45.8	10.5	3.8	53.5	7.1
#3	5.3	41.0	12.9	6.3	46.3	13.6	4.0	50.8	7.9
#4	5.5	42.3	13.0	5.5	46.3	11.9	3.5	50.5	7.9
#5	4.3	46.8	9.2	4.3	54.3	7.9	4.8	61.3	7.8
#6	5.0	45.5	11.0	6.3	45.0	14.0	3.5	54.8	6.4
Mean $\pm$ SEM	4.9 $\pm$ 0.2	42.7 $\pm$ 1.2	11.5 $\pm$ 0.6*	5.6 $\pm$ 0.4	47.5 $\pm$ 1.4	11.9 $\pm$ 1.0*	4.1 $\pm$ 0.3	54.1 $\pm$ 1.6	7.8 $\pm$ 0.4
KA-treated rats									
#1	3.0	22.3	13.5	5.5	22.5	24.4	3.5	19.0	18.4
#2	3.3	19.5	16.9	3.0	10.3	29.1	3.0	10.3	29.1
#3	2.5	3.3	75.8	4.8	6.5	73.8	3.8	8.8	43.2
#4	5.5	39.0	14.1	3.3	23.3	14.2	3.3	21.8	15.1
#5	4.5	35.8	12.6	4.8	29.3	16.4	4.0	28.5	14.0
#6	3.5	6.0	58.3	4.3	9.5	45.3	3.5	12.8	27.3
Mean $\pm$ SEM	3.7 $\pm$ 0.4	21.0 $\pm$ 6.0	31.9 $\pm$ 11.4	4.3 $\pm$ 0.4	16.9 $\pm$ 3.8	33.9 $\pm$ 9.2	3.5 $\pm$ 0.1	16.9 $\pm$ 3.1	24.5 $\pm$ 4.5
<i>p</i> -Value	0.037	0.005	0.104	0.034	<0.001	0.038	0.084	<0.001	0.004

Values represent the mean numbers of parvalbumin-immunoreactive (Parv.) and Nissl-counterstained (Nissl) neurons, and the percentage of parvalbumin-immunoreactive neurons in the total neuronal population in an area measuring 0.0196 mm² within layer III of the medial EC. Cell counts were performed on two sections from each of three levels (dorsal, middle, and ventral) of the EC per rat ($n = 6$ per group; see Materials and Methods for details). *p*-Values indicate the statistical significance in the differences between normal and KA-treated animals (two-tailed Student's *t* test).

* $p < 0.01$ as compared to the ventral level (one-way ANOVA followed by Dunnett's multiple comparison test).

EC. Compared to layer V, layer VI displays a relatively low density of neurons, which are rather heterogeneous in both shape and size.

Results

Behavioral observations

Behavioral observations were made in all animals for up to 6 hr after electrical stimulation or administration of the chemoconvulsants. The behavioral and electrographic events observed in the SSLSE group were as described before (Lothman et al., 1989). Thus, during many hours of electrographic status epilepticus animals showed decreased responsiveness and sporadic "limbic seizures" with head bobbing, chewing, and whisker twitching. Convulsive events were absent or rare. SSLSE therefore conforms to a condition of nonconvulsive status epilepticus, as corroborated by electrographic recordings. In agreement with previous reports (Schwob et al., 1980; Lothman and Collins, 1981; Nadler, 1981; Honchar et al., 1983; Clifford et al., 1987; Turski et al., 1989), rats that received KA showed behavioral changes similar to those displayed by animals treated with lithium/pilocarpine. Shortly after the treatments, the rats displayed a frozen posture, followed by automatisms such as head bobbing, sniffing, chewing, and blinking. These manifestations were often interrupted by increased locomotor activity and wet dog shakes. Approximately 30 min after the administration of lithium/pilocarpine or 90 min after the injection of KA, convulsive seizures commenced. Typically, seizure episodes were characterized by vigorous chewing and head bobbing, which developed into forelimb clonus and rearing. During clonic convulsions, many rats lost their balance and showed excessive salivation. Some animals also exhibited generalized clonic convulsions that affected the whole body and terminated in a tonic phase. Episodes of convulsive seizures recurred frequently, and in many

animals lasted to the end of the observation period. No behavioral seizures were observed in control animals.

Rats that received injections of KA and diazepam/pentobarbital fell asleep shortly after the anticonvulsive treatment. Upon awakening, no behavioral seizure activity was noted in these animals until the end of the observation period.

Histopathological observations: general comments

In the following description, disappearance of Nissl staining in neurons is equated with neurodegeneration, neuronal loss, or neuronal damage, though the loss of Nissl staining does not necessarily indicate the physical disappearance of dead cells. Notably, similar morphological alterations were found in the brains of rats that survived for either 24 hr or 4 weeks following the treatments. No neuronal loss was detected in any brain region of control rats. In agreement with prior studies (Schwob et al., 1980; Nadler, 1981; Honchar et al., 1983; Turski et al., 1983; Ben-Ari, 1985; Clifford et al., 1987; Du et al., 1993b), neurodegeneration was detected in several brain regions in SSLSE, KA- or lithium/pilocarpine-injected rats. Although subtle differences in the severity and location of these lesions were noticed among the three experimental models, neuronal damage in the hippocampus, the thalamus, and various neocortical regions was found to be a common feature in the brains of these animals. In addition, loss of neurons was consistently observed in layer III of the medial EC in all three models of epilepsy. In accordance with the purpose of the present study (cf. introductory paragraphs), only characteristics of this EC lesion are described in detail below.

Figure 3. Camera lucida drawings from Nissl-stained horizontal sections cut through the entorhinal cortex (EC) of a representative SSLSE, KA-, and lithium/pilocarpine-injected rat killed 24 hr after the treatment, showing typical neuronal loss in layer III at different levels along the dorsoventral axis. The shaded area represents the extent of the visible lesion along the medio-lateral axis in layer III. Arrows indicate the lateral limit of the EC, and arrowheads point to the approximate border between the medial (ECm) and lateral (ECI) EC. Note that the lesion in layer III is always present in and frequently restricted to the most medial sector of the medial EC.

Neuronal loss in the EC of SSLSE, KA-, and lithium/pilocarpine-injected rats

In Nissl-stained horizontal sections, loss of neurons was readily recognized in the EC of SSLSE, KA-, or lithium/pilocarpine-injected rats at 24 hr after the treatments. The pattern of neurodegeneration in the EC was similar in all three models (Figs. 1, 2). Therefore, the following description applies to all three experimental paradigms.

As illustrated in Figures 1 and 2, neurodegeneration was most

evident in layer III of the EC. In some cases, loss of neurons was also noticed in the deep layers (IV–VI), but neuronal damage was always more severe in layer III than in the deep layers. Cell counting in KA-treated rats revealed a significant decrease (51–68%) in the total neuronal population in layer III as compared to normal rats at all three levels examined (Table 1). However, a few surviving neurons were frequently observed in the degenerated sector of layer III, even in the cases with the most severe neurodegeneration (cf. Figs. 1, 2, and 6).

The preferential neuronal loss in layer III was found to occur in the medial EC, though the extent of neuronal damage in layer III along the mediolateral axis varied between cases (Figs. 1 and 2). Thus, in the majority of animals, loss of neurons was observed in the entire medial EC, with the most severe neurodegeneration in the most medial part of the region. In a few cases, neuronal loss was detected only in the most medial region of the EC.

Neuronal loss in the EC of SSLSE, KA-, or lithium/pilocarpine-injected rats was also assessed at different levels along the dorsoventral axis. Preferential loss of neurons in layer III was seen throughout the EC including both the dorsal and ventral portions (Figs. 1–3). The extent of the affected area within layer III tended to be greater towards the dorsal direction in most cases (cf. Fig. 3), but neurodegeneration appeared to be more severe in the ventral portion (cf. also Table 1).

The EC of SSLSE, KA-, and lithium/pilocarpine-injected rats killed 4 weeks after the induction of acute seizures showed a virtually identical pattern of neurodegeneration as that seen in rats perfused 24 hr after the experimental treatments (Fig. 4). Thus, both the preferential neuronal loss and the typical survival of a few neurons in layer III of the medial EC was evident in all rats that were sacrificed at 4 weeks. In addition to neuronal loss, gliosis, as indicated by the apparent increase in the number of Nissl-stained glial nuclei, was present in the degenerated layer III in most cases (see, for example, Fig. 4F,H). Moreover, the EC of most experimental rats was apparently shrunken, as noted in the horizontal plane.

Effects of diazepam/pentobarbital treatment on neuronal loss in the EC

Diazepam and pentobarbital, administered 1 hr after the injection of KA, abolished neurodegeneration in the EC in all rats studied. Thus, the density and morphology of neurons in the EC of these rats appeared identical to those observed in saline-injected controls (Fig. 5). Anticonvulsive treatment, when given 1 hr after the onset of KA-induced forelimb convulsions (i.e., approximately 2.5 hr after KA administration) prevented neuronal loss in the EC in seven of nine rats studied. Two rats in this group showed neurodegeneration in the EC, but neuronal loss was only noticed in the most medial region of layer III and was limited to the ventral portion of the medial EC (micrograph not shown).

Parvalbumin-immunoreactivity in the EC of normal, and KA- and lithium/pilocarpine-injected rats

In the EC of normal rats, parvalbumin-i was localized to a subpopulation of neurons, which were distributed throughout the region. The highest density of immunostained neurons was observed in layers II and III (Fig. 6A). In layer III, immunoreactive neurons accounted for 8–12% of the total neuronal population (cf. Table 1). Notably, the proportion of immunoreactive neurons in the total neuronal population in layer III was higher in the dorsal and middle than in the ventral level of the EC (cf. Table 1). The morphology of most immunoreactive neurons did not resemble that of pyramidal cells seen in Golgi preparations (Lorente de Nó, 1933). In layers II and III, many parvalbumin-i neurons had an oval perikaryon, with processes often arising from two poles of the cell body and coursing perpendicular to the long axis of the lamina. Immunoreactive neurons of this type were also observed in layer IV, but their cell bodies and processes were generally aligned parallel to the layer in the horizontal plane. Other parvalbumin-i neurons, including those in

layers V and VI, were frequently multipolar or round in shape, and their processes extended into different directions. In addition, a plexus of processes containing parvalbumin-i was noticed in layer III and particularly in layer II.

Examination of immunostained sections from SSLSE, KA-, or lithium/pilocarpine-injected rats (24 hr survival) revealed that the distribution pattern of parvalbumin-i neurons in the EC was similar to that seen in normal animals. In Nissl-counterstained sections, it was evident that many surviving neurons in the degenerated layer III were parvalbumin positive (Fig. 6B–D). Cell counting in KA-treated rats showed that the population of parvalbumin-i neurons was decreased, but the loss was relatively moderate (between 20% and 24%) and quantitatively similar at the three levels analyzed. Thus, the number of parvalbumin-i neurons relative to the total neuronal population in KA-treated rats increased by approximately threefold as compared to normal rats (Table 1). This indicated a relative resistance of parvalbumin-containing neurons to seizure-related damage.

Discussion

The principal findings of the present study are: (1) a preferential loss of neurons in layer III in the medial EC exists in three established rat models of chronic epilepsy, (2) prolonged seizures are likely to be responsible for neurodegeneration in the EC, and (3) neurons containing parvalbumin-i are relatively resistant to the pathophysiological processes that destroy the majority of neurons in layer III of the EC.

Prolonged seizures and preferential destruction of layer III neurons in the medial EC

The present study revealed in three rat models of chronic epilepsy a consistent and pronounced loss of neurons in layer III of the EC. The degeneration of layer III neurons, which was also briefly noted in earlier studies using KA (Nadler et al., 1978; Schwob et al., 1980), was permanent, since it was clearly demonstrable at 4 weeks after each of the experimental treatments.

Previous studies by several groups of investigators have shown that the brain damage following persistent electrical stimulation of the perforant path (Sloviter, 1983) or after the administration of KA or lithium/pilocarpine (Ben Ari et al., 1980; Olney et al., 1986; Sutula et al., 1992; Kelly and McIntyre, 1994) is seizure related. Thus, both KA- and lithium/pilocarpine-induced neuronal degeneration in several brain regions can be either prevented or reduced by treatment with anticonvulsants, including diazepam and pentobarbital. It has also been noted that sustained seizures, regardless of the method of seizure generation, consistently cause neuronal damage when they last more than 1 hr (Olney et al., 1986). In agreement with these studies, our experiments, using pharmacological intervention at two different time points following the administration of KA, indicate that neurodegeneration in layer III of the EC likely also resulted from prolonged seizures, which had persisted for more than 1 hr. Moreover, it appears that the duration of convulsive seizures necessary for producing neuronal damage in the EC is less than 4 hr, since anticonvulsive treatment at this timepoint did not prevent EC lesions in lithium/pilocarpine-treated rats. The idea that neurodegeneration in the EC is associated with seizures is further supported by the fact that a subconvulsive dose of KA (6 mg/kg, s.c.) or a combination of lithium and scopolamine caused neither behavioral seizures nor neuronal loss in the EC.

There are other reasons to believe that a common feature of

Figure 5. Nissl-stained horizontal sections (30 μ m) through the ventral level of the entorhinal cortex (EC) from a rat injected with KA followed by treatment with diazepam/pentobarbital 1 hr later (*A* and *C*) and a saline-treated control rat (*B* and *D*). *C* and *D* are high magnifications of the areas outlined by the boxes in *A* and *B*, respectively. Note the absence of neuronal loss in layer III of the medial EC in *A* and *C* (cf. also Fig. 2*C,G*). The solid arrows in *A* and *B* point to the lateral limit of the EC, and the open arrows indicate the approximate border between the medial (*ECm*) and lateral (*ECI*) EC. I–VI: layers of the EC. Scale bars: 500 μ m in *A* and *B*; 200 μ m in *C* and *D*.

the three models underlies the neuronal death in the EC. First, in spite of distinct differences in the trigger mechanisms (electrical stimulation or activation of kainate or muscarinic cholinergic receptors), a virtually identical pattern of neuronal loss in the EC was present in all three models. Second, neurodegeneration was always preceded by a bout of acute status epilepticus, which began approximately 1 hr after the treatment and lasted for several hours. Third, neurodegeneration in the EC in all three models took place within 24 hr, in agreement with previous reports that irreversible damage to nerve cells occurs very rapidly after an episode of status epilepticus (Olney et al., 1986; Auer and Siesjö, 1988; Ingvar et al., 1988). Finally, functional mapping experiments using 2-deoxyglucose autoradiography have previously shown an involvement of the medial EC in the status epilepticus in the SSLSE and the KA models (Lothman and

Collins, 1981; VanLandingham and Lothman, 1991). Multiple lines of evidence therefore strongly suggest that the lesion in layer III of the EC described here is a direct consequence of prolonged seizure activity and is independent of the method of initial seizure generation.

Mechanisms of seizure-related neurodegeneration in the EC

It is not immediately apparent why neurons in layer III of the medial EC are preferentially susceptible to prolonged seizure activity. Evidence from various seizure models suggests that seizure-related brain damage is excitotoxic in nature, i.e., that neuronal death is caused by an overactivation of membrane receptors for excitatory amino acids (Olney et al., 1986). In particular, the neuronal loss in the olfactory cortex, which occurs in several models of limbic seizures, has been linked to *N*-methyl-D-as-

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Figure 4. Photomicrographs of Nissl-stained horizontal sections (30 μ m) cut through the ventral portion of the entorhinal cortex (EC) from a saline-treated (*A*), a SSLSE (*B*), a KA (*C*)- and a lithium/pilocarpine (*D*)-injected rat that survived for 4 weeks after the treatment. *E–H* are higher magnifications of the areas outlined by the boxes in *A–D*, respectively. The pattern of neurodegeneration in the EC is virtually identical to that observed in rats killed 24 hr after the treatment (cf. Figs. 1 and 2). Note the large numbers of Nissl-stained glial nuclei, indicating gliosis in the degenerated layer III (*F* and *H*). The solid arrows in *A–D* point to the lateral limit of the EC, and the open arrows indicate the approximate border between the medial (*ECm*) and lateral (*ECI*) EC. I–VI: layers of the EC. Scale bars: 500 μ m in *D* and also for *A–C*; 200 μ m in *H* and also for *E–G*.

partate (NMDA) receptors based on the use of NMDA receptor antagonists as neuroprotective agents (Clifford et al., 1990). The notion of a critical involvement of NMDA receptors in the death of EC layer III neurons is also strongly supported by studies with the indirect excitotoxin aminooxyacetic acid (AOAA). When injected directly into the rat EC, this compound causes an episode of status epilepticus followed by a preferential neurodegeneration in layer III of the medial EC (Du and Schwarcz, 1992). This lesion is virtually identical to the EC neuropathology described in the present study and can be completely prevented by cotreatment with the NMDA receptor antagonist MK-801 (Eid et al., 1995).

The relative resistance of parvalbumin-containing neurons in layer III described here also suggests that excitotoxic mechanisms are responsible for seizure-related neurodegeneration in the EC. A rise in intracellular free Ca^{2+} concentrations is commonly believed to constitute an essential and critical component in the neurodestructive cascade initiated by excitatory amino acid receptor overactivation (Choi, 1988). This pathological Ca^{2+} accumulation is likely to be less pronounced in the presence of parvalbumin, which is capable of binding Ca^{2+} effectively (Blaustein, 1988). Similar arguments regarding endogenous neuroprotection have been made in another instance of seizure-related neurodegeneration, i.e., in the hippocampal CA1 region, where parvalbumin-positive neurons survive preferentially amidst highly vulnerable pyramidal neurons (Sloviter, 1989).

No detailed study of the distribution of NMDA receptors or the expression of NMDA receptor subunits has been performed in the rat EC. Currently available information does not indicate a particularly dense population of functional NMDA receptor on layer III neurons, but future studies with high cellular resolution and novel molecular probes for NMDA receptor subunits may yet help explain the preferential vulnerability of these cells to prolonged limbic seizures. An alternative possibility is that layer III neurons receive a disproportionately dense excitatory input in the limbic seizure circuit, and that excessive glutamate, released during status epilepticus, may preferentially bombard layer III neurons. Indeed, a putatively glutamatergic monosynaptic input from the presubiculum to the medial portion of layer III has been described (Köhler, 1985; van Groen and Wyss, 1990; Caballero-Bleda and Witter, 1993).

Functional implications of neuronal damage in EC layer III

Several lines of evidence indicate that the EC is involved in epileptogenesis. In animal experiments, electrolytic lesions, local administration of chemoconvulsants, or electrical stimulation of the EC can induce acute seizures in the hippocampus (Dasheiff and McNamara, 1982; Collins et al., 1983). Repeated electrical stimulation of the perforant path, the most prominent excitatory pathway from the EC to the hippocampus, not only elicits acute hippocampal seizures but also causes selective neuronal damage in the hippocampus (Sloviter, 1983). Moreover, a slowly developing loss of paired pulse inhibition, indicating disturbed GA-

BAergic neurotransmission and subsequent hyperexcitability, is detected in the hippocampus following EC lesions (Bekenstein and Lothman, 1991).

Clinical observations have also provided evidence that a dysfunction of the EC may be associated with intractable temporal lobe epilepsy (Deutch et al., 1991; Spencer and Spencer, 1994). For example, space-occupying lesions in parahippocampal regions including the EC can slowly give rise to temporal lobe seizures even when the hippocampus displays little or no Ammon's horn sclerosis (Kim et al., 1990; Lévesque et al., 1991; Spencer, 1994). Rutecki and colleagues (1989) have documented that stimulation of the EC in patients undergoing temporal lobectomy evokes a hippocampal response that is virtually identical to spontaneously occurring interictal spikes. Moreover, a relatively high susceptibility to electrical stimulation has been noticed in the EC of patients with temporal lobe epilepsy (Wilson et al., 1990). In patients with intractable temporal lobe epilepsy, removal of the EC during anterior temporal lobectomy appears to be essential for controlling complex partial seizures (Goldring et al., 1992), again suggesting that the EC may contain epileptic foci or the neuronal circuitry necessary for seizure activity. In this context, it is particularly noteworthy that prominent, preferential loss of neurons is found in layer III of the medial EC in some patients suffering from intractable temporal lobe epilepsy (Du et al., 1993a).

Anatomical studies support a role of the EC in the origination and propagation of limbic seizures. In rats (Steward and Scoville, 1976), cats (Witter et al., 1989), and monkeys (Witter and Amaral, 1991), two fiber systems project from the EC to the hippocampus. One projection, the perforant path, which constitutes the first portion of the classic trisynaptic circuit, originates from neurons in EC layer II and terminates on dentate granule cells, and has been central to many considerations regarding normal and abnormal hippocampal function (Andersen, 1975). A second, monosynaptic pathway arises from neurons in layer III of the EC and projects directly to the hippocampal CA1 and the subiculum. This pathway may correspond to that shown to have a lower threshold for excitation than the trisynaptic pathway (Yeckel and Berger, 1990) and may thus be involved in the pathophysiology of hippocampal seizures.

The neuronal loss in layer III of the EC described here will not only affect direct postsynaptic hippocampal targets, since the EC and the hippocampal formation have reciprocal connections (Köhler, 1986, 1988; Witter et al., 1989), which may form a loop with the potential for reverberatory activity (Lopes da Silva et al., 1990; Lothman et al., 1991; Paré et al., 1992; Jones, 1993; Spencer and Spencer, 1994). Thus, destruction of neurons in layer III is likely to affect neural communication between layers in the EC and to influence the hypothetical EC-hippocampal loop. In principle, an EC layer III lesion may have antiepileptogenic consequences similar to those elicited acutely by the "pharmacological lesion," which is caused by an injection of tetrodotoxin into the EC (Stringer and Lothman, 1992). Recent studies in our laboratories have shown, however, that the neu-

Figure 6. Photomicrographs of the medial entorhinal cortex (EC) at the middle level along the dorsoventral axis. Horizontal sections (30 μm) were taken from a normal (A), a SSLSE (B), a KA (C)-, and a lithium/pilocarpine (D)-injected rat that survived for 24 hr after the treatment. The sections were processed for parvalbumin immunohistochemistry, and were subsequently counterstained with thionin (see text for methodological details). In the normal rat EC (A), neurons containing parvalbumin-immunoreactivity (-i) are mainly present in layers II and III. Note that the majority of neurons in layer III disappeared after experimental treatments (B-D). In contrast, the distribution pattern and numbers of parvalbumin-i neurons in the degenerated area appear similar to those seen in A. I-VI: layers of the EC. Scale bars: 70 μm in D and also for A-C.

ronal loss described here is more likely to *promote* epileptogenesis. Thus, AOAA-induced lesions in layer III of the EC result in an upregulation of hippocampal excitatory amino acid receptors (Tamminga et al., 1994), and cause distinct hippocampal neuropathology (Du et al., 1994) and an increased excitability of hippocampal neurons (unpublished observation). It is therefore conceivable that overexcitation of and/or damage to EC layer III neurons, perhaps in concert with hippocampal dysfunction, may play a significant role in epileptogenesis.

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