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JARI NISSINEN

Characterization of a Rat Model of Human Temporal Lobe Epilepsy

Doctoral dissertation

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Department of Neurobiology
A.I. Virtanen Institute for Molecular Sciences
University of Kuopio



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ABSTRACT

Epilepsy is the second most common neurologic disorder, and among epilepsies temporal lobe epilepsy (TLE) is the most common form of symptomatic epilepsy. Symptomatic TLE generally develops in three phases: initial -insult → epileptogenesis → epilepsy (i.e., spontaneous seizures). Several cellular and molecular alterations occur in epileptic brain, including neuronal loss, neurogenesis, and axonal sprouting. Therefore, understanding the mechanisms involved in seizure development and the subsequent degenerative process is crucial for designing new drug treatment strategies for TLE. The development of specific tools (i.e., experimental models) is one of the first steps toward this understanding.

The aim of this study was to develop an epilepsy model that would mimic different aspects of human TLE, including symptomatology of spontaneous seizures, neuropathology, and behavioral impairment. The developed model, in which spontaneous seizures occurred in the majority of the animals, was used to study whether mossy fiber sprouting contribute to the generation of spontaneous seizures in epileptic rats. Pharmacologic manipulation of the epileptogenesis using two different antiepileptic drugs with different mechanisms of action was also studied. In addition, the efficacy of four antiepileptic drugs that are used in human TLE patients was examined in this model.

The main findings of the present study were: 1) After status epilepticus as an initial insult, 87% of stimulated animals developed spontaneous seizures after an approximately 1-month latency period. 2) Histopathologic findings resembled those observed in human TLE patients. 3) At the time of epilepsy diagnosis, there was increased sprouting of granule cell axons in epileptic animals. Sprouting can occur, however, without spontaneous seizures. 4) Modulation of epileptogenesis with the gamma-aminobutyric acid (GABA)-ergic drug vigabatrin did not prevent the development of epilepsy. Modulation of epileptogenesis with Na⁺-channel blocker lamotrigine did not prevent the development of epilepsy. 5) Rats with focal epilepsy responded to the same compounds used to treat seizures of focal onset in humans.

In summary, the developed experimental epilepsy model for chronic TLE in rats provides a valuable tool to investigate the evolution of chronic epilepsy and develop new pharmacological approaches for prevention and treatment of human TLE.

National Library of Medicine Classification: WL 385, WL 340, QY 58

Medical Subject Headings: epilepsy, temporal lobe; disease models, animal; epilepsy/pathology; seizures; mossy fibers, hippocampal; hippocampus/pathology; amygdala; neurons; behavior; memory; status epilepticus; epilepsy/drug therapy; anticonvulsants; rats

To my family

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Kuopio, May 2006

Jari Nissinen

ABBREVIATIONS

AED	antiepileptic drug
AM	amygdala
BLA	basolateral amygdala
CBZ	5H-dibenz [b,f] azepine-5-carboxamide; carbamazepine
DMSO	dimethylsulfoxide
EC	entorhinal cortex
EEG	electroencephalogram
ESM	2-ethyl-2-methylsuccinimide; ethosuximide
VGB	λ -vinyl λ -aminobutyric acid; vigabatrin
HAFD	high amplitude and frequency discharge
HC	hippocampus
ILAE	International League Against Epilepsy
KA	kainic acid
LTG	GI 267119X; 6-(2,3-dichloro-phenyl)-1,2,4-triazine-3,5-diamine; lamotrigine
NMDA	<i>N</i> -methyl-D-aspartic acid
PP	perforant pathway
PTZ	pentylene-tetrazole
SE	status epilepticus
SPRD	sprague-dawley
SRS	spontaneous recurrent seizures
TLE	temporal lobe epilepsy
VPA	2-propylpentanoic acid, valproic acid

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by Roman numerals **I-V**.

- I.** **Nissinen J**, Halonen T, Koivisto E, Pitkänen A. A new model of chronic temporal lobe epilepsy induced by electrical stimulation of the amygdala in rat. *Epilepsy Research* (2000) 3 (2-3):177-205.
- II.** **Nissinen J**, Lukasiuk K, Pitkänen A. Is mossy fiber sprouting present at the time of the first spontaneous seizures in rat experimental temporal lobe epilepsy? *Hippocampus* (2001) 11(3):299-310.
- III.** Halonen T, **Nissinen J**, Pitkänen A. Chronic elevation of brain GABA levels beginning two days after status epilepticus does not prevent epileptogenesis in rats. *Neuropharmacology* (2001) 40(4):536-550.
- IV.** **Nissinen. J**, Large CH, Stratton SC, Pitkänen A. Effect of lamotrigine treatment on epileptogenesis: an experimental study in rat. *Epilepsy Research* (2004) 58(2-3):119-132.
- V.** **Nissinen J**, Pitkänen, A. Effect of antiepileptic drugs on spontaneous seizures in epileptic rats. *Epilepsy Research* (2006) Manuscript.

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1. INTRODUCTION

Epilepsy occurs in approximately 1% of the human population and is one of the most common neurologic disorders. Epilepsy may continue throughout life, and therefore epileptic patients require life-long medical care. Temporal lobe epilepsy (TLE) is the most prevalent form of symptomatic (acquired) epilepsy. Symptomatic TLE generally develops in three stages: 1) In many patients, the cause of the seizures (initial insult) can be identified, such as head trauma, status epilepticus (SE), stroke, or brain infarction. 2) The initial insult triggers a cascade of events (epileptogenesis) during which several neurobiologic changes occur, including cell death, neurogenesis, gliosis, axonal and dendritic plasticity, and molecular reorganization of cellular membranes and extracellular matrix. The duration of epileptogenesis varies significantly between individuals, ranging from years to tens of years. 3) Epileptogenesis culminates in the appearance of spontaneous seizures (epilepsy). Despite intensive medical care with modern antiepileptic drugs (AEDs), approximately 50% of patients continue to have seizures.

The lack of efficacy of AEDs on seizures makes the development of new and better AEDs imperative. Therefore, new treatment strategies that not only suppress ongoing seizures, but also alleviate the cascade of events, including the initial insult and neurodegeneration, must be developed.

Experimental animal models are crucial to develop a better understanding of symptomatic TLE and the mechanisms linked to the epileptic process. There are currently several experimental animal models (i.e., acute and chronic seizure models). Acute seizure models, including maximal electroshock and pentylenetetrazole (PTZ), are mainly used by the pharmaceutical industry. With these models it is easy to screen hundreds of candidate molecules in a short time. Chronic epilepsy models, however, in which spontaneous seizures occur provide a more realistic view of seizure development. Chronic models can be used to study all of the contributing factors, including the initial insult, latency phase preceding epilepsy, and spontaneous seizures.

The aim of this thesis was to develop a chronic epilepsy model that would mimic human TLE, including the histopathology, development of spontaneous seizures, and response of seizures to AED treatment similar to that in human TLE patients. Moreover, the contribution of granule cell axon sprouting and consequent neuropathology to seizure development at the time of epilepsy diagnosis was studied. In addition, the efficacy of two AEDs on epileptogenesis was examined.

2. REVIEW OF THE LITERATURE

2.1 Epilepsy

2.1.1 Definition of epilepsy

Epilepsy, like seizures, is a symptom of abnormal brain function. According to the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE) (ILAE, Fisher et al., 2005), an epileptic *seizure* is a transient occurrence of signs and symptoms due to excessive or abnormally synchronous neuronal activity in the brain. During a seizure, neurons fire abnormally and there are often alterations in consciousness, behaviour, emotion, motor function, and sensation. Brain disorders with an enduring predisposition to generate epileptic seizures and the neurobiologic, cognitive, psychologic, and social consequences of this condition are called *epilepsy*. The definition of epilepsy requires the occurrence of at least *one* unprovoked epileptic seizure (ILAE, Fisher et al., 2005). Many physiologic disturbances of the brain can provoke seizures, however, without being epilepsy. For example, the seizures observed during alcohol withdrawal syndrome are often similar to those observed in experimental epilepsy models, such as kindling models (Brailowsky and García, 1999).

2.1.2 Epidemiology and classification

The prevalence rate (proportion of a population affected with epilepsy) is 4 to 8 per 1000 population and the incidence rate (number of new cases of epilepsy in a population over time) is 30 to 50 per 100000 population (Annegers, 1997). The cumulative incidence (the proportion of a population developing epilepsy over a specified time) measures risk and is used as a summary of occurrence (Annegers, 1997). The risk of epilepsy from birth through age 20 is 1% and reaches 3% by age 75. Epilepsy is the second most common neurologic disorder after migraine, affecting approximately 2% of the population in Europe (Andlin-Sobocki et. al., 2005). In Finland, the total prevalence of epilepsy in those aged 18 to 65 in 2004 was approximately 33000 cases.

The classification of epileptic seizures is based on the proposal by the Commission on Classification and Terminology of the ILAE (1981, 1989). According to this proposal, seizures are classified according to seizure type and electroencephalogram (EEG) characteristics. First, epileptic seizures are classified as *partial* or *generalized*. During partial seizures, the first clinical and EEG changes indicate activation of neurons limited to part of one cerebral hemisphere. A partial seizure is classified on the basis of whether or not consciousness is impaired during the seizure. Consciousness is not impaired during *simple partial* seizures, but is

impaired during *complex partial* seizures. Both simple and complex partial seizures can further develop into secondarily generalized seizures. If both cerebral hemispheres are involved in seizure induction, the seizures are called *generalized*. Generalized seizures can further be divided into either convulsive or non-convulsive seizures: absence, myoclonic, clonic, tonic, tonic-clonic, and atonic seizures.

Epilepsy syndromes can be classified according to the underlying etiology as *idiopathic* (not associated with brain lesions) or *symptomatic* (consequence of a known or suspected disorder of the brain) epilepsy. According to Annegers (1997), several insults such as brain trauma, central nervous system infections, cerebrovascular disease, and brain tumors increase the incidence of epilepsy. *Probably symptomatic* (previously cryptogenic) epilepsies refer to a disorder whose cause is unknown or occult. Based on epidemiologic data in European populations and the United States, the most frequent type of seizures in humans are complex partial seizures with or without secondary generalization, which occur in approximately 40% to 50% of all patients with epilepsy (Hauser et al., 1993).

2.1.3 Temporal lobe epilepsy (TLE)

TLE is the most common form of symptomatic epilepsy (Engel, 1996). The temporal lobe consists of the hippocampus and adjacent anatomically related cortex, including entorhinal, perirhinal, parahippocampal cortices; subicular complex; amygdala; and lateral cortex (Squire and Zola-Morgan, 1991). Seizures arising from these structures usually produce symptoms such as the sensation of epigastric rising, emotional changes (mostly fear), and occasionally olfactory or gustatory hallucinations (Engel, 1996). TLE might develop following a variety of neurologic insults occurring early or later in life, such as complex febrile convulsions, intracranial infections, head trauma, stroke, tumor, and SE (Mathern et al., 1996). TLE develops in three phases; (1) *an initial precipitating insult* of the brain such as head trauma, SE, or stroke (Mathern et al., 1996) initiates a cascade of events; (2) *epileptogenesis (latency phase)*, during which several parallel processes (either molecular or structural) occur, culminating in spontaneous recurrent seizures; (3) *epilepsy*, which can be easily controlled or become drug-refractory over time (Jutila et al., 2002). These neurobiologic changes include neuronal loss, axonal and dendritic plasticity of surviving neurons, neurogenesis, gliosis, and molecular reorganization in the cellular matrix and membranes (Jutila et al., 2002). Some of these processes proceed in parallel and might even continue after the appearance of spontaneous seizures.

A conspicuous feature of TLE is that it is associated with hippocampal sclerosis, which is found in approximately 60% to 70% of patients with intractable TLE (Jutila et al., 2001; Kälviäinen and Salmenperä, 2002). Hippocampal sclerosis in TLE patients is normally characterized by the loss of pyramidal neurons and subsequent gliosis in the CA3 and CA1 regions of the hippocampus. Furthermore, there is neuron loss in the hilus of the dentate gyrus, and extrahippocampal pathology, especially in the amygdala and entorhinal cortex (Blümcke et al., 2002, Wieser, et al., 2005). Neuronal loss includes either glutamatergic (60%-70% loss of granule/pyramidal cells) or gamma-aminobutyric acid (GABA)-ergic inhibitory neurons (somatostatin and neuropeptide Y) (Thompson, 1998). Neuroanatomically, memory processing is associated with these temporal lobe structures, and therefore memory deficits represent the major cognitive impairment in focal epilepsies in which these structures are directly affected, as in TLE (Helmstaedter, 2002).

2.1.4 Status epilepticus (SE)

Status epilepticus (SE) is a medical and neurologic emergency associated with significant morbidity and mortality. The most widely accepted definition of SE is more than 30 min of either continuous seizure activity or intermittent seizures without full recovery of consciousness between seizures (Waterhouse and DeLorenzo, 2001). Clinically, epileptic seizures lasting at least 5 min continuously or two or more discrete seizures between which consciousness shows an incomplete recovery should be treated as a threatening SE (Lowenstein and Alldredge, 1998; Käypähoito suositus, 2005). The frequency of SE cases in United States is approximately 102000 to 152000 per year, and 55000 deaths are associated with SE (Lowenstein and Alldredge, 1998). SE accounts for approximately 7% of all epilepsy cases per year (Lowenstein, 1999). The incidence of SE in adults in a German study was 15.8 per 100000 people and epilepsy was previously diagnosed in approximately 50% of these patients (Knake et al., 2001). A population- based Finnish study in children (< 16 years) indicated that the annual incidence rate of SE was 47.5 of every 100000 episodes, and mean seizure duration was 42.5 min (Metsäranta et al., 2004). Twelve to thirty percent of adult patients present SE as the first symptom of epilepsy. The probability of developing epilepsy is 41% within 2 years following acutely precipitated SE compared with 13% for those with acute symptomatic seizures but no SE (Hesdorffer et al., 1998).

A history of epilepsy is the strongest single risk factor for generalized convulsive SE which is the most common form of SE in adults (Fountain, 2000). Other risk factors include low AED levels, young age, genetic predisposition, and acquired brain insults. Fever is a very

common risk factor in children, as is stroke in adults (Fountain, 2000). Neuropathologic and imaging studies revealed neuronal damage due to SE in the hippocampus, amygdala, piriform cortex, thalamus, cerebellum, and cerebral cortex (Du et al., 1993; Nohria et al., 1994; Jutila et al., 2002).

Patients with SE require immediate and intensive medical care. A recent proposal for the treatment of SE patients in Finland was published in *Duodecim* (Käypähoito suositus, 2005). The therapy for SE currently consists of drugs that stop seizures (e.g., benzodiazepines, phenytoin, barbiturates). If SE is refractory, barbiturates or benzodiazepines (e.g., thiopental, midazolam, propofol) are used to induce anesthesia to stop seizure activity.

2.1.5 Treatment of TLE

In humans, TLE that develops after a brain damaging insult characterized by recurrent complex partial and secondary generalized seizures is often difficult to treat with AEDs (Browne and Holmes, 2001). The currently available AEDs provide a seizure control in up to 70% of patients with epilepsy (Shorvon, 2004). The first choice of AED is based on its efficacy against a specific seizure type (Perucca, 2001), tolerability, and safety (Browne and Holmes, 2001). Different outcome measures are used to assess the efficacy of drug treatment. The duration and percentage of patients entering remission (complete seizure control), and the reduction or increase in seizure number (frequency) is used as measure of efficacy of the drug (Mattson, 2002). The outcome is most often expressed as the percentage of patients achieving a 50% or greater reduction in seizure number (frequency). There can also be a change in behavioral seizure severity due to drug treatment (Mattson, 2002).

Chronic administration of AEDs is the treatment of choice for epilepsy. Patients might be treated with different AEDs with different mechanisms of actions. AEDs work mainly through affecting glutamate, GABA, and ion-channels (Na^+ , Ca^{2+}) (Table 1).

TABLE 1. The main mechanisms of action of antiepileptic drugs.

Drug	Glutamate antagonism	GABA modulation	Na⁺-channel blocker	Ca²⁺-channel blocker
Carbamazepine			+	
Ethosuximide				+
Felbamate	+			
Gabapentin		+		
Lamotrigine			+	+
Phenobarbital		+		
Phenytoin			+	
Primidone		+		
Tiagabine		+		
Topiramate	+	+	+	+
Valproate		+	+	+
Vigabatrin		+		

The + indicates effective. Abbreviations: GABA, gamma-amino-butyric acid; Na⁺, sodium; Ca²⁺, calcium.

Most studies indicate that tonic-clonic seizures in humans are better controlled than partial- type seizures (Elwes et al., 1984; Kwan and Brodie, 2000, 2001). Clinically established standard AEDs, including carbamazepine (CBZ), phenytoin, primidone, phenobarbital, and valproate (VPA), are effective against simple and complex partial seizures, secondarily generalized, and primarily generalized tonic-clonic seizures (Löscher, 1994; Löscher and Schmidt, 1997; Mattson, 2002). Non-convulsive absence seizures, however, are treated with VPA and ethosuximide (ESM) (Löscher, 1997; 2002; Mattson 2002). Newer AEDs, such as felbamate, gabapentin, lamotrigine (LTG), levetiracetam, topiramate and vigabatrin (VGB), are clinically effective against both partial and generalized seizures. LTG also has an effect against absence-type of seizures. The preferred drugs for first line monotherapy in TLE, however, are CBZ, oxcarbazepine, VPA, and LTG (Mattson et al., 1985; Mattson et al., 1992; Brodie et al., 1995; Bill et al., 1997).

Despite significant advances in epilepsy therapy over the past few decades, approximately 30% of patients with epilepsy are not adequately controlled or their seizures are refractory to drug treatment (Regesta and Tanganelli, 1999; Kwan and Sander, 2004). The response to the first choice of drug is critical and therapy is highly predictive of long-term

outcome (Kwan and Brodie, 2000). An excellent seizure control is achieved in only 20% to 30% of patients. If treated, these patients become seizure-free following the first or second monotherapy with moderate doses, which can be successfully withdrawn after a seizure-free period. Some patients (20%-30%) only achieve remission with continued AED treatment. In patients in whom seizures continue despite medication, epilepsy might become worse and further decline cognitive function (Äikiä et al., 1995).

Many patients with drug-refractory epilepsy will eventually be treated with multiple drugs without any clear benefit (Kwan and Brodie, 2000). The mechanism underlying this multidrug resistance in epilepsy is not well understood, but might include alterations in AED targets in the epileptic brain tissue, reduced AED penetration to the seizure focus, and neuropathologic brain alterations such as hippocampal sclerosis (Regesta and Tanganelli, 1999; Kwan and Brodie, 2002; Volk et al., 2005). Cell loss in the hilus and elsewhere might be associated with persistent changes in composition and receptor function (Brooks-Kayal et al., 1998), or ion-channels (Bender et al., 2003; Ellerkmann et al., 2003).

2.2 Experimental epilepsy models

To better understand the mechanisms involved in seizure-initiation (precipitating injury), epileptogenesis, and spontaneous recurrent seizures, and to study new treatment options, several experimental models mimicking different aspects of the epileptic process have been developed. These experimental models are valuable tools to better understand the pathophysiology of TLE and to allow us to (1) examine the nature of different injuries (trauma, stroke, SE) that might contribute to the subsequent development of epilepsy; (2) observe different events that might contribute to the disease process (during epileptogenesis) subsequent to an injury, but prior to the onset of spontaneous seizures; and (3) study the chronically epileptic brain in detail using either physiologic, pharmacologic, molecular, or anatomic techniques. Moreover, experimental models are essential for developing new treatment strategies or to test the efficacy of new AEDs in epilepsy. Experimental seizure/epilepsy models can be divided into two categories, acute or chronic, according to their ability to induce seizures or epilepsy.

2.2.1 Acute seizure models

Almost 70 years ago, Merritt and Putnam (1937) introduced the maximal electroshock method, which is one of the most widely used methods together with the PTZ model for acute seizures in normal brain. The maximal electroshock method identifies agents with activity

against generalized tonic-clonic seizures. It is mainly used for drug studies, to determine whether a drug prevents the spread of seizure discharges through neural tissue. In the original method (Merritt and Putnam, 1937), in cat, an electrical current was administered via scalp and mouth electrodes. The stimulator consisted of a 45-V battery, discharging through a commutator operated by a motor and through a 50-Ohm potentiometer. The amount of current required to produce a tonic-clonic seizure was recorded (baseline convulsive threshold) and then the test was performed again some hours after the administration of a test drug (Shorvon, 2004). Currently, the maximal electroshock method test involves either bilateral corneal or transauricular electrodes via which electrical stimulation (mice 50 mA; rat 150 mA) induces tonic hind-limb extension and flexion followed by clonus (Walker and Fisher, 2004).

The PTZ test is also used to discover drugs with efficacy against non-convulsive absence or myoclonic seizures (Löscher, 2002; Walker and Fisher, 2004). The absence of seizures induced by PTZ (85 mg/kg in mice and 56.4 mg/kg in rats) suggests that the test substance raises the seizure threshold.

Between 1975 and 1995, the National Institute of Health AED program screened 16000 chemicals using the maximal electroshock method and PTZ tests. Of these 16000 compounds, 2700 had some AED activity, 130 were evaluated in further studies, 11 entered clinical trials, 6 failed to obtain approval, and 1 reached the market (felbamate) (Shorvon, 2004).

Convulsants with which acute seizures can be induced include a variety of GABA_A-related substances (bicuculline, picrotoxin), glutamic acid decarboxylase inhibitors (isonicotinehydrazide, 3-mercaptopropionic acid, allylglycine), excitatory amino acid-related substances (kainic acid, KA; N-methyl-D-aspartate, homocysteine, quisqualic acid), and acetylcholine-related substances (pilocarpine; PILO) (Velíšek, 2006).

2.2.2 Chronic seizure models

A variety of animal models in which seizures occur spontaneously have been developed to investigate TLE (Löscher, 1997, Coulter et al., 2002). The ethical and experimental limitations of human studies make appropriate animal models of epilepsy essential. The ideal model should be easily and efficiently produced (low mortality rate and high percentage of animals with spontaneous seizures), and should have the behavioral, electrographic, and anatomic characteristics of human TLE. Chronic models of epilepsy can be divided into *models of acquired* (symptomatic) epilepsy and *models of genetic* (idiopathic) epilepsy. In symptomatic epilepsy, epileptic conditions are most often induced by either

chemical substances (KA, PILO) or electrical methods in previously healthy (non-epileptic) animals (Löscher, 2002). Models of electrical induction of epilepsy include kindling, and models in which spontaneous seizures develop after SE include electrical stimulation of the hippocampus, amygdala, or other limbic brain areas (Mazarati et al., 2006). The epileptic process is best examined in SE models where epileptogenesis is triggered by the methods mentioned above. Arterial occlusion models of brain infarction (Kharlamova et al., 2003; Karhunen et al., 2006; unpublished data) and posttraumatic epilepsy (Pitkänen et al., 2006) also lead to the development of epilepsy, and therefore expand the number of models available for studying the etiology of human symptomatic epilepsy.

2.2.2.1 Kindling

Kindling, the phenomenon by which repeated application of an initially subconvulsive electrical stimulation via chronically implanted electrodes produces a clear progressive change in response over daily stimulations, is considered to be a chronic model of TLE (Goddard et al., 1969). The progression begins on the first day with a brief, low frequency electrographic afterdischarge at the electrode tip, which is associated with little behavioral response (early stage 1 and 2 seizures are primarily associated with facial and oral activity, including ipsilateral eye closure and blinking, followed by head bobbing and drooling/salivation; Racine, 1972). The complexity of the kindling phenomenon, however, begins to appear as the response evolves over days, resulting eventually in the triggering of long, high- frequency afterdischarges associated with strong convulsive seizures (stage 5; forelimb clonus with rearing and falling; Racine, 1972). Early stages of kindling represent a model for partial seizures. With daily repetition, however, kindling comes to model complex partial seizures with secondary generalization. The most sensitive areas for kindling are all limbic areas and many forebrain areas. The most dramatic effect is achieved by stimulating temporal lobe structures, such as the amygdala and adjacent cortices, including piriform, perirhinal, insular, and entorhinal cortices (Mohapel et al., 1996). If daily kindling is repeated over many weeks and months, approximately 90 to 100 kindled seizures are needed before animal progress to the stage of spontaneous seizures (Sutula and Ockuly, 2006). In approximately 20% to 25% of the rats kindled at the perforant path or amygdala will develop spontaneous convulsive seizures of stage 4 or greater (Racine, 1972; Michalakis et al., 1998) (see Table 4). According to Michalakis et al. (1998), spontaneously seizing animals do not show signs of tissue damage, and there are no histologic differences between spontaneously seizing and non-spontaneously seizing animals in the hilus of the dentate gyrus. One kindled seizure, however, with an afterdischarge duration of

82 seconds might induce DNA fragmentation of the cell nuclei bilaterally in the dentate gyrus (Bengzon et al., 1997). After 150 stage-5 seizures, there is a progressive reduction of neurons in the CA1-CA3 region of the hippocampus, the dentate hilus, and entorhinal cortex (Cavazos et al., 1991). Kindled animals have shown impaired spatial memory function depending on the stimulation site (Sutula et al., 1995; Gilbert et al., 2000). Kindling offers the advantage that seizures can be easily elicited.

2.2.2.2 Models based on status epilepticus (SE)

In contrast to the kindling model of epilepsy, SE is easier to produce, but expression of the sequelae is more variable. Most often, high doses of chemical convulsants (KA, PILO) are injected systemically (subcutaneously, intraperitoneally) or are applied to specific brain sites such as the amygdala or hippocampus (see Tables 1 and 2). In addition, SE can be induced by sustained electrical stimulation to specific sites of the brain such as the perforant path, the ventral hippocampus, and the amygdala (see Table 3).

Injection of chemical substances or sustained electrical stimulation of the brain induces SE and spontaneous seizures will occur after a period of 1 month (see Tables 1-3). In addition, morphologic changes that occur in the hippocampus following SE induced by either a chemical convulsant or electrical stimulation are often similar to those observed in human TLE. The disadvantage of the post-SE model is that SE can be difficult to control, and therefore a substantial proportion of the animals die during the SE phase (see Tables 1-3). By modulating the duration of SE, however, it is possible to reduce the number of animals developing epilepsy and to reduce the mortality rate (Klitgaard et al., 2002; Pitkänen et al., 2005).

In contrast to electrical models, surgery (electrode implantation) is not required for chemical models. To reliably confirm electrical seizure activity during SE and to evaluate the manifestations of SE, implantation of electrodes is recommended.

2.2.2.2.1 Kainic acid (KA) -induced SE

KA is a structural analogue of glutamic acid (Coyle, 1983). KA, which in Japanese means “monster from the sea or ghost of the sea”, was originally isolated from the seaweed (*Kai-Nin-Sou*) *Digenea simplex*, which was used in Japanese folk medicine as an ascaridial preparation. The KA model of epilepsy has been used since it was reported by Olney and coworkers (1974) that KA induces epileptiform behavior in rats after intracerebroventricular application. Systemic or intracerebral application of KA induces seizures in rats (Tanaka et al.,

1992), cats (Tanaka et al., 1985), mice (Kleinrok et al., 1980), baboons (Menini et al., 1980), and rabbits (Franck and Schwartzkroin, 1984).

KA is thought to interact with a postsynaptic KA-type glutamate receptor, thereby depolarizing all neurons with these receptors for a prolonged period. High-affinity KA binding sites are located in the stratum lucidum of CA3, and the pyramidal layers of CA1 and CA3 (Sperk, 1994), which are also the most sensitive areas for kainate action.

KA is mainly administered by intraperitoneal, intravenous, subcutaneous, intracerebroventricular, intrahippocampal, or intra-amygdalar injections. Treatments can be given as a single large dose (normally 8-15 mg/kg), as several low doses (2.5-5.0 mg/kg) that are repeated until the animals develop SE (Hellier et al., 1998), or as local administration at doses of 0.1 to 3.0 µg per hemisphere (Leite et al., 2002) (see Table 2). After systemic injection of KA, limbic seizures that originate from the hippocampus will rapidly spread to the lateral septum, amygdaloid complex, subicular complex, entorhinal cortex, and extralimbic areas (Ben-Ari et al., 1981). Within 5 min after systemic injection of KA, rats exhibit a catatonic posture with staring. Nine minutes after KA injection, the first epileptic signs are detected in the EEG (spikes or bursts) (Tuunanen et al., 1999), followed by wet-dog shakes approximately 20 min after KA injection. Later, animals show some motor signs (sniffing, masticatory movements head nodding, rearing, and loss of postural control). Approximately 1 hour after KA injection, the onset of continuous epileptiform spiking activity, which is considered to be a marker for the onset of SE, occurs. Separate recurrent seizures will become more prominent, culminating in SE that might last for several hours (Ben-Ari et al., 1981; Tuunanen et al., 1999). Previous studies demonstrated that KA-treated rats and cats develop spontaneous motor seizures (Tanaka et al., 1992; Hellier et al., 1998). First epileptic seizures occur approximately after 4 to 77 days after the KA injection (see Table 2). Seizure frequency in the chronic period varies considerably among epileptic animals (Hellier et al., 1998). Some animals present with only a low number of seizures and other animals have daily seizures. Most of the behavioral seizures in the post-KA model are stage 3 to 5 seizures (generalized) according to Racine's scoring scale (1972).

The KA model of TLE has many neuropathologic similarities to human TLE. In both humans and experimental animals, neuronal damage is confined to the hippocampal formation, parahippocampal region, amygdaloid complex, and piriform cortex (Schwob et al., 1980; Ben-Ari, 1985; Hudson et al., 1993; Pitkänen et al., 1998; Tuunanen et al., 1999). In the KA model, the hilus, CA3 and CA1 regions (Schowb et al., 1980) of the hippocampus, layer III of the entorhinal cortex (Du et al., 1993, 1995), and especially the lateral and basal nuclei of the amygdala (Tuunanen et al., 1999; Yilmazes-Hanke, et al., 2000) are damaged, similar to what is

observed in human TLE. In both the KA model and human TLE, the granule cell layer seems to be very resistant to damage following epileptic insult (Houser, 1992).

2.2.2.2.2 Pilocarpine (PILO)-induced SE

Pilocarpine, a cholinergic muscarinic type agonist induces seizures in rodents following systemic or intracerebral (intracerebroventricular or focal application) administration (Turski et al., 1989). Pilocarpine induced seizures have been demonstrated in Wistar (Fujikawa, 1996; Furtado et al., 2002) and Sprague-Dawley rats (André et al., 2003) as well as in mice (Cavalheiro et al., 1996; Borges et al., 2003). PILO can be administered as one large dose (normally 320-400 mg/kg) or, if lithium (3 mEq/kg; corresponds to 127 mg/kg) is given 24 hours previously, by a smaller dose (30 mg/kg)(see Table 1). Lithium pre-treatment, followed by several low doses of PILO, efficiently produces SE with a lower mortality compared to higher dose injections of PILO (Gliem et al., 2001). In the PILO model, subcutaneous administration of methylscopolamine (1 mg/kg) is normally used to reduce the peripheral effects associated with the autonomic activation caused by PILO (Cavalheiro et al., 2006).

PILO produces several behavioral alterations including staring spells, facial automatisms, salivation, piloerection, and behavioral automatisms, such as stereotypic scratching, grooming, sniffing, and chewing, resembling stage 1 or 2 kindled seizures. These are followed by limbic motor seizures that develop approximately 30 min after PILO injection. Stage 4 or 5 seizures start 10 min later and seizure activity progresses to limbic SE that lasts for several hours (Cavalheiro et al., 2006). Normally, SE is allowed to continue for 90 min, after which seizure activity is stopped with diazepam to reduce otherwise high mortality in PILO model (Cavalheiro et al., 2006).

First epileptic seizures occur approximately after 2 to 75 days after the PILO injection (see Table 3). Seizure frequency in the chronic period varies considerably among epileptic animals (Cavalheiro et al., 2006). Some animals present with only low seizure frequency throughout the several weeks of follow-up, whereas other animals may have daily seizures, or even clusters of several seizures in a short follow-up period. Most of the behavioral seizures in the PILO model are stage 3 to 5 seizures (generalized) according to Racine's scoring scale (1972).

The amygdala, thalamus, olfactory cortex, hippocampus, neocortex, and substantia nigra are the most sensitive regions for epilepsy-related damage following PILO-induced convulsions (Turski et al., 1989; Fujikawa, 1996). PILO-induced SE impairs cognitive function as assessed in a Morris water-maze task (Hort et al., 1999).

2.2.2.2.3 Electrical induction of SE

One of the first studies by McIntyre and coworkers (1982) in previously kindled animals demonstrated that if the amygdala was continuously stimulated with a 60-Hz sine wave, 50 μ A peak-to-peak for 60 min, 22/35 (63%) of the stimulated animals developed SE. Without drug intervention, rats spontaneously recovered from SE within 10 to 24 hours. The mortality rate in that model was only 14%. The afterdischarge threshold (focal electrical seizure activity measured on EEG) remained high throughout the 2-week follow-up time in animals that developed SE. If SE was stopped 30 min after SE induction, the afterdischarge threshold increased in intensity 1 day after SE induction, but decreased over time, nearly to within the normal range within 2 weeks. If SE was stopped 4 hours after the induction of SE the afterdischarge threshold remained elevated at 30% above baseline, but returned to baseline within 2 weeks. Two weeks after SE induction, there was neural damage in the olfactory bulb, amygdala, entorhinal cortex, hippocampus (CA1 region), and thalamus (McIntyre et al., 1982).

Thereafter several studies have indicated that SE can be induced by either high- (Handford and Ackerman, 1993) or low-frequency (Cain et al., 1992) stimulation of limbic structures. Stimulation can be focused on the perforant path (Shirasaka and Wasterlain, 1994; Mazarati et al., 1998; Gorter et al., 2003), ventral hippocampus (Bertram and Cornet, 1993, 1994), and amygdala (Brandt et al., 2004). There is differential sensitivity of the temporal lobe structures in SE induction. Mohapel and coworkers (1996) studied the sensitivity of different amygdaloid nuclei (basolateral, central, and medial), and the perirhinal and piriform cortices for SE induction. They demonstrated that the most sensitive area for SE induction is the basolateral amygdala, all animals (100%) developing SE. Fifty-five percent of animals stimulated in the central nucleus, 40% of animals stimulated in medial nucleus, 25% of animals stimulated in perirhinal cortex, and 0% of animals stimulated in the piriform cortex developed SE. According to Handford and Ackermann (1993) caudoventral hippocampus is another sensitive area for SE induction.

At the beginning of electrical stimulation of the perforant path (Mazarati et al., 1998), and ventral hippocampus (Bertram, 1997), the response from the dentate gyrus or amygdala is driven by electrical stimuli. Stimulus-independent epileptiform potentials appear within 15 min. The initial behavioral responses include motor arrest and facial myoclonus (Mazarati et al., 2006). Over time, seizure severity increases, animals occasionally express stage 3 or 4 or 5 kindled-like seizures. By the end of the stimulation, animals show continuous stimulus-independent seizure activity. During SE, high-amplitude and frequency discharges (HAFDs) (electrographic seizure episodes) occur that are separated with spikes and short bursts (see

Figure 1). Later, periodic epileptiform discharges occur. In animals in which SE is induced electrically, there is neuronal damage throughout the hippocampus. Particularly vulnerable are hilar neurons, and pyramidal neurons in the CA1 and CA3 regions (Bertram, 1997; Mazarati et al., 2006). Additional neuronal damage is also observed in the piriform cortex and thalamus (Bertram, 1997; Bertram and Scott, 2000).

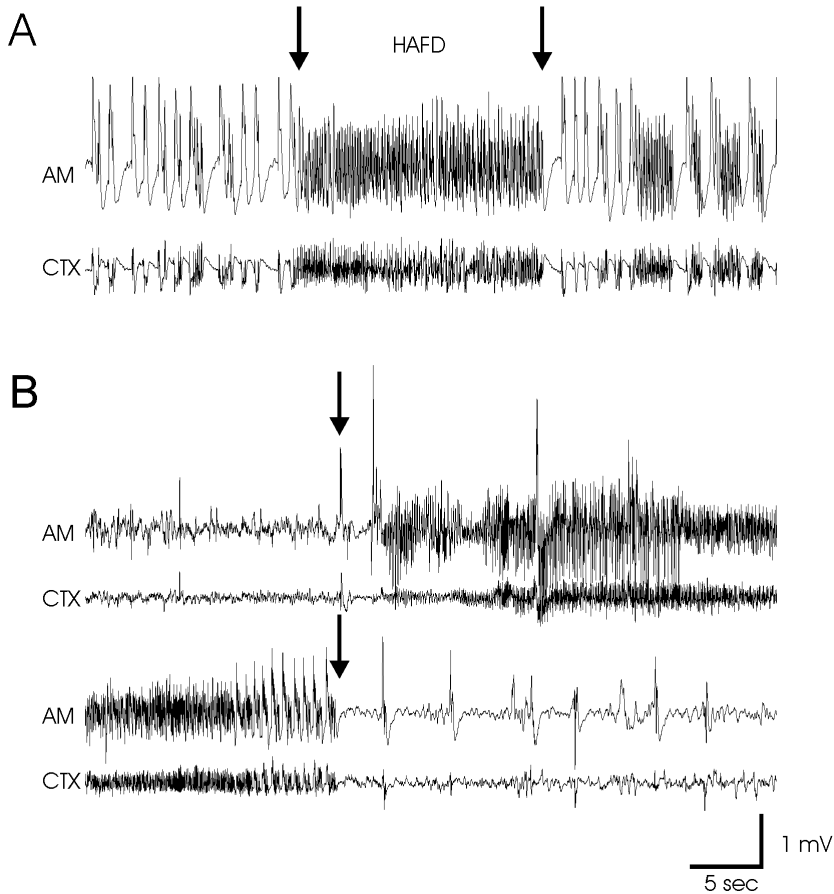


FIGURE 1. (A) An example of an electrographic recording from the ipsilateral amygdala (AM) and contralateral cortex (CTX) during SE (induced by electrical stimulation of the amygdala) demonstrating the high-amplitude and frequency discharge (HAFD) recorded 5 min after the end of electrical stimulation of the amygdala. Solid arrows depict the start and the end of the HAFD lasting 18 seconds. (B) An example of a spontaneous seizure (start and end indicated with solid arrows) that was recorded 3 months after the induction of SE. The seizure lasted for 47 seconds and was classified as generalized (stage 3) based on behavioral manifestations. Video recording indicated that animal had bilateral forelimb clonus during the seizure.

TABLE 2. An example of studies using kainic acid -induced SE as a model for symptomatic TLE.

Animal/Strain (Weight)	Dose (mg/kg)	SRS onset (days)	Animals with epilepsy (%)	Reference
Rat/Wistar (220-250g)	1 µg (intraHC)	9.8 (5-15)	20	Cavalheiro et al. (1983)
Rat/SPRD	14 (i.v.)	10-28	52	Cronin et al. (1988)
Rat/SPRD (60d)	10-12 (i.p.)	34 (4-121)	44	Stafstrom et al. (1992)
Cat	1 µg (intraAM)	14-21	n.d.	Tanaka et al. (1992)
Cat	4-12 µg (intraHC)	7-10	n.d.	Tanaka et al. (1992)
Rat/SPRD (220-250g)	0.7 µg (intraAM)	17-25	n.d.	Tanaka et al. (1992)
Rat/SPRD (280-320 g)	0.8 µg (intraAM)	n.d.	59	Mascott et al. (1994)
Rat/SPRD (150-250g)	5 (once/h: 3-4 h) (i.p.)	77±38	85-97	Hellier et al. (1998)
Rat/SPRD (250-350g)	0.4 µg (intraHC)	n.d.	41	Bragin et al. (1999)

Abbreviations: AM, amygdala; HC, hippocampal; i.p., intraperitoneal; i.v., intravenous; n.d., not determined; SPRD, Sprague-Dawley; SRS, spontaneous recurrent seizure.

TABLE 3. An example of studies using pilocarpine -induced SE as a model for symptomatic TLE.

Animal/Strain (weight)	Dose (mg/kg)	SRS Onset (days)	Animals with epilepsy (%)	Mortality (%)	Reference
Rat/Wistar (250-280g)	380 (s.c.)	4-44	100	40	Cavalheiro et al. (1991)
Rat/SPRD (300-350g)	350 (i.p.)	18	20	0	Liu et al. (1994)
	380 (i.p.)	16.9	100	31	Liu et al. (1994)
	400 (i.p.)	14.7	100	70	Liu et al. (1994)
Rat/Wistar (150-250g)	350 (i.p.)	5 - 34	100	30	Mello et al. (1993)
Rat/Wistar (220-250g)	320-350(i.p.)	5 - 45	n.d.	30	Cavalheiro et al. (1994)
Rat/Wistar (150-250g)	320-350 (i.p.)	6 - 50	100	n.d.	Mello et al. (1996)
Rat/Wistar (50-60d)	350 (i.p.)	17.8	100	27	Priel et al. (1996)
Rat/SPRD (75-100g)	300 (i.p.)	12 - 39	79	n.d.	Isokawa et al. (1996)
Rat/Wistar (200-250g)	2.4 mg/μl (hilus)	2-30	71	0	Furtado et al. (2002)
Rat/Wistar (200-225g)	LiCl 127 mg/kg +10 mg/kg (i.p.) every 30 min	10 - 75	83 - 100	7 - 44	Glien et al. (2001)
	LiCl 127 mg/kg + 30 mg/kg (i.p)	12 - 72	78	45	Glien et al. (2001)
Rat/Wistar (225-250g)	LiCl 127 mg/kg + 25 mg/kg (s.c.)	12 - 32	100	56	Rigoulot et al. (2003)
Rat/SPRD (300-350g)	LiCl 127 mg/kg + 25 mg/kg (s.c.)	25 ± 7	76	45	Detour et al. (2005)

Abbreviations: AM, amygdala; HC, hippocampal; i.p., intraperitoneal; n.d., not determined; s.c., subcutaneous; SPRD, Sprague-Dawley; SRS, spontaneous recurrent seizure.

TABLE 4. An example of studies using electrical stimulation induced -SE as a model for symptomatic TLE.

Animal/Strain (weight)	Stimulation site (area)	Stimulation time (min)	Stimulation parameters	SRS onset (days)	Animal with epilepsy (%)	Reference
Rat/SPRD (200-225g)	hippocampus (ventral)	90	1 ms square-wave pulses, (biphasic) at 50 Hz, 10 s trains (400 μ A) every 11 s	8 - 38	91	Bertram and Cornett (1993)
Rat/SPRD (200-225g)	hippocampus (ventral)	90	1 ms square-wave pulses (biphasic), at 50 Hz 10 s trains (400 μ A) every 11 s	n.d.	100	Bertram and Cornett (1994)
Rat/Wistar (400-500g)	angular bundle (PP)	24h	0.1 ms paired pulses at 25 Hz + single stimuli (10 s train of 0.1 ms pulses) at 20 Hz every min	3-4 (wk)	50%	Shirasaka and Wasterlain (1994)
Rat/Wistar (12-14wk)	angular bundle (PP)	30	0.1 ms square-wave pulses at 20 Hz 10 s trains (20V) every min together with 2 Hz continuous stimulation	20 - 32	n.d.	Mazarati et al. (2002a)
Rat/SPRD (200-230g)	BLA amygdala	25	1 ms square-wave pulses (biphasic) at 50 Hz, 100 ms trains (700 μ A) every 0.5 s	49 (23-99)	79	Brandt et al. (2004)

Abbreviations: BLA, basolateral amygdala; Hz, hertz; ms, millisecond; n.d. not determined; PP, perforant-path; SPRD; Sprague-Dawley; SRS, spontaneous recurrent seizure; wk, week.

TABLE 5. An example of studies using over kindling as a model for symptomatic TLE.

Animal/Strain (weight)	Stimulation site (brain area)	Kindling parameters	SRS onset (days)	Animals with epilepsy (%)	Reference
Cat	amygdala	monopolar sine waves, at 60 Hz for 1 s, 1 stimulation/day	76-293	43	Hiyoshi et al. (1993)
Rat/Long-Evans (300-500g)	angular bundle (PP) amygdala	1 ms, square wave pulses, at 60 Hz for 1 s, 3 stimulations/day	n.d.	50	Michalakis et al (1998)
Rat/Wistar (200-220g)	piriform cortex (area tempesta)	1 ms, monophasic square-wave pulses, 50 Hz for 1 s, at interval of 1 min	n.d.	10	Potschka et al. (2000)
	piriform cortex (central)	1 ms, monophasic square-wave pulses, 50 Hz for 1 s, at interval of 1 min	n.d.	0	Potschka et al. (2000)
Rat/Wistar	BLA amygdala	1 ms, monophasic square-wave pulses 50 Hz for 1 s, at interval of 5 h	n.d.	50	Brandt et al. (2004)

Abbreviations: BLA; basolateral amygdala; Hz, hertz; ms; millisecond; n.d. not determined; PP, perforant-path; s, second; SRS, spontaneous recurrent seizure.

2.2.3 Models for post-stroke and post-traumatic epilepsy

Recent developments in animal modelling have produced new experimental models for symptomatic epilepsy. In these models spontaneous seizures are triggered by ischemic stroke (Kelly et al., 2006; Karhunen et al., 2006) or traumatic brain injury (Pitkänen et al., 2006). Kharlamov and co-workers demonstrated that 50% of adult (4-20 month old) animals subjected to infarction of the left sensorimotor cortex with the photosensitive dye Rose-bengal developed focal seizures with or without a behavioral change. The latency to the first seizure occurred approximately 107 days after the infarction. Middle cerebral artery occlusion is the standard vascular occlusion technique used in models of cerebral infarction (Longa et al. 1989). Combined middle cerebral artery and common carotid artery occlusion for 3 hours result in the development of spontaneous seizures in 100% of the animals (Kelly et al., 2006). Latency to the first seizure varies from 2 to 4 weeks post-lesioning; seizure frequency varies from 1 to 2 seizures per week, seizure duration varies from 6 to 60 seconds, and behavioral seizures are mainly generalized. In the middle cerebral artery occlusion model with endothelin-1 (Karhunen et al., 2006, unpublished data), spontaneous seizures are detected only in 6% of lesioned animals during a 12- month follow-up. Forty-seven percent of lesioned animals, however, experience early seizures, which are a known risk factor for post-stroke epilepsy. After lateral fluid percussion- induced traumatic brain injury, 50% of lesioned animals develop epilepsy during a 12-month follow-up (Pitkänen et al., 2006) and lesioned animals develop seizures 7 to 8 months post-trauma. Seizure frequency is approximately 0.3 seizures per day and typically seizures are generalized lasting 100 seconds. Neuronal damage in the hippocampus is bilateral and the density of mossy fiber is increased in lesioned animals.

2.3 Epileptogenesis

During epileptogenesis, a large number of probably parallel neurobiologic changes occur that are potential targets for therapeutic intervention. These changes include neuronal loss (Lewis, 2005), neurogenesis (Hattiangady et al., 2004), gliosis (Dawody and Thom, 2005), axonal (Cronin and Dudek, 1988) and dendritic plasticity, inflammation (Vezzani, 2005), and molecular reorganization in cellular membranes and extracellular matrix (Jutila et al., 2002; Perosa et al., 2002; Pitkänen, 2002). The molecular mechanisms underlying these alterations are, however, unknown. There is a loss of neurons in the dentate gyrus and hippocampus proper in approximately 60% to 70% of the histologic specimens obtained from patients undergoing surgery for drug-refractory TLE (Babb and Pretorius, 1996). Neuronal damage occurs in both

the glutamatergic cells (granule cells and mossy cells in the dentate gyrus; pyramidal cells in the hippocampus proper) and GABAergic inhibitory neurons in the dentate hilus and in CA1 pyramidal region, which control the excitability of principal cells. Neurons that contain somatostatin and neuropeptide Y are particularly vulnerable (Thompson et al., 1998). The loss of inhibitory interneurons is presumed to be a key factor underlying the increased excitability of the epileptic hippocampus.

Both in human TLE and in rodent models of limbic epilepsy, dentate granule cells undergo extensive remodelling, including the appearance of granule cells in ectopic locations within the dentate gyrus, reorganization of granule cell axons (mossy fibers), and the dispersion of granule cells (Parent et al., 1997; 2005). Dentate granule cell neurogenesis is a process that normally occurs throughout life (Gage et al., 1998). Many newly born cells arise from the subgranular proliferation zone, a lamina between the granule cell layer and the hilus. Seizure-induced neurogenesis occurs after brief seizures (Bengzon et al., 1997) or seizures that are more prolonged, such as after PILO-(Parent et al., 1998) or KA-induced SE (Gray et al., 1998), or after electrical stimulation of the perforant path (Parent et al., 1998). Kindling also increases granule cell neurogenesis (Scott et al. 1998). The developing neurons in the dentate gyrus have a granule cell phenotype, and project aberrantly to both the CA3 pyramidal cell region and the dentate inner molecular layer of the hippocampus (Parent et al., 1997). These aberrant projections might contribute to the abnormal hyperexcitability in the epileptic dentate gyrus.

Mossy fiber sprouting is one of the most extensively studied forms of synaptic plasticity, perhaps because it involves such extensive structural changes within the dentate gyrus, and occurs not only in experimental models but also in TLE patients (Cavazos et al., 1991; Houser et al., 1990). Mossy fiber sprouting refers to changes in the terminal projections of axons of dentate gyrus granule cells, which are called the mossy fibers. Normally, granule cell axons innervate hilar interneurons or the apical dendrites of CA3 pyramidal neurons. Both in human and experimental TLE, mossy fibers develop axon collaterals that terminate in an abnormal location, such as the inner molecular layer of the dentate gyrus, probably because of the death of their normal target neurons in the hilus and CA3 during the epileptic process; or innervate basal dendrites of CA3 pyramidal cells (Shetty et al., 2005). The inner molecular layer also contains the dendrites of granule cells and inhibitory neurons (Buckmaster et al., 2002). This aberrant mossy fiber sprouting is thought to be an excitatory phenomenon; sprouted fibers form new excitatory connections between granule cells (Wuarin and Dudek, 1996; Scharfman et al., 2003). Also, recurrent excitation occurs among granule cells and has been linked to prolonged population spike activity after perforant pathway stimulation (Patrylo et al.

1999). However, there is some evidence that sprouted mossy fibers might enhance inhibition, and anatomic data suggest that sprouted mossy fibers also innervate inhibitory neurons (Kotti et al., 1997) as part of an inhibitory feedback mechanism.

Mossy fiber sprouting can occur after various experimental manipulations such as in the KA (Leite et al., 1996), PILO (Mello et al., 1993; Priel et al., 1996), kindling (Cavazos et al., 1991; Elmer et al., 1996; Garcia-Cairasco et al., 1996), electroconvulsive shock (Gombos et al., 1999; Vaidya et al., 1999), tetanus toxin (Anderson et al., 1999), PTZ (Golarai, et al., 1992), and alumina gel (Ribak et al., 1998) models, in mutant rats with spontaneous seizures (Amano et al., 1999), and in humans with TLE (Bahh et al., 1999; Houser et al., 1990). Mossy fiber sprouting occurs following lesions due to trauma (Santhakumar et al., 2001; Pitkänen et al., 2006), stroke (Arvidsson et al., 2001), and ischemia (Liu et al., 1998). Kindling experiments demonstrate a strong correlation between mossy fiber sprouting and the development of kindling (Cavazos et al., 1991). The prevention of mossy fiber sprouting with a protein synthesis inhibitor, however, does not block the development of spontaneous seizures in PILO and KA-models of epilepsy (Longo and Mello, 1997; 1998).

Axons of granule cells that have aberrant terminal projections, also dendrites might undergo plastic changes. Human and experimental animal studies indicate reduced local spine degeneration (reduced number of dendritic spines) (Isokawa et al., 1998). Also, in the perforant-path stimulation model, there is evidence for the formation of granule cell basal dendrites that are not normally present in adult hippocampus (Spigelman et al., 1998).

Recent data indicate a role for the increased number of astrocytes expressing glial fibrillary acidic protein following neuronal damage. Astrocytes in the sclerotic hippocampus might directly influence excitability through altered water homeostasis and K^+ buffering (de Lanerolle et al., 2005). Neurons in the brains of individuals with focal epilepsy exhibit sustained discharges, called paroxysmal depolarization shifts. Unexpectedly, new evidence has indicated that glutamate release from glia can generate these events and might serve to synchronize neuronal activity (Rogawski, 2005).

2.4 Antiepileptogenic effects of AEDs

The effects of different AEDs on epileptogenesis have been widely studied in rodent models of chronic epilepsy (see Table 6). Several AEDs have been studied following both chemically (e.g. pilocarpine and kainic acid) and electrically (e.g. hippocampus or perforant path) induced SE (Mikati et al. 1994, Bolanos et al., 1998; Andre et al., 2001; Cilio et al., 2001; Prasad et al., 2002; Mazarati et al., 2002a; Capella and Lemos, 2002). In these studies, the time

interval between SE and the beginning of drug treatment has varied from 10 minutes to 24 hours. Therefore, the effect of AED on epileptogenesis after SE may vary dramatically. For example, the effect of phenobarbital on epileptogenesis has shown variable results depending on the SE model and beginning of drug treatment. Phenobarbital treatment started 1-4 h (electrical stimulation of hippocampus) or 24 h (kainic acid) after the SE induction has shown different efficacy on epileptogenesis (Prasad et al. 1998; Bolanos et al., 1998). Among phenobarbital treated animals, there was a clear benefit to early treatment; only 10% of animals in 1-h treatment group developed epilepsy compared to 46% (2h group) and 55% (4 h group). However, if treatment started 24 h after SE induction in KA-model (Bolanos et al., 1998) it did not have antiepileptogenic effect. Seizures were observed in 69% of animals treated with phenobarbital compared to 55% of control animals (Bolanos et al., 1998).

TABLE 6. An example of preclinical trials with AEDs in status epilepticus models.

AED	Model	Beginning of medication	Dose (mg/kg)	Duration of treatment	Epileptogenesis/ Disease modification	Reference
Carbamazepine	Pilocarpine	24 h after SE induction	120 mg/kg/d	56 d	Epileptogenesis (± 0) Seizure frequency (\downarrow) Seizure duration (\downarrow)	Capella and Lemos (2002)
Fluorofelbamate	PP-stimulation	10 min after SE induction	200 mg/kg	1 dose	Epileptogenesis (± 0) Progression of epilepsy (\downarrow) Seizure frequency (\downarrow)	Mazarati et al. (2002a)
Gabapentin	Kainic acid	24 h after SE induction	400 mg/kg/d 200 mg/kg/d	30 d 10 d	Epileptogenesis (\downarrow)	Cilio et al. (2001)
Phenobarbital	Kainic acid	24 h after SE induction	70 mg/kg/d	P36-P153	Epileptogenesis (n.d.) Seizure frequency (± 0)	Mikati et al. (1994)
	Kainic acid	24 h after SE induction	70 mg/kg/d	P36-P75	Epileptogenesis (± 0) Seizure frequency (± 0)	Bolanos et al. (1998)
	HC-stimulation	1 - 4 h after SE induction	80 mg/kg	1 dose	Epileptogenesis (\downarrow)	Prasad et al. (2002)
Valproate	Kainic acid	24 h after SE induction	1200 mg/kg/d	P36-P75	Epileptogenesis (\downarrow)	Bolanos et al. (1998)
Vigabatrin	Li-Pilocarpine	10 min after	100 mg/kg + 250 mg/kg	Days 1-6 Days 7-45	Epileptogenesis (± 0) Seizure frequency (± 0)	Andre et al. (2001)

Abbreviations: (± 0) no effect; (\downarrow) decrease; HC: hippocampal; Li, lithium; PP, perforant path; SE, status epilepticus. Modified from Pitkänen and Kubova, 2004.

3. AIMS OF THE STUDY

The aim of this study was to develop an experimental epilepsy model in rat that mimics different aspects of human TLE, including the occurrence of spontaneous seizures, neuronal damage, and mossy fiber sprouting. The contribution of mossy fiber sprouting to seizure generation was studied. Also, the effects of two different AEDs against epileptogenesis were investigated. In addition, the effects of different AEDs against spontaneous seizures in an amygdala stimulation model were examined.

The series of studies was aimed at answering the following specific questions:

1. Do spontaneous seizures develop as a consequence of SE induced by electrical stimulation of the lateral nucleus of the left amygdala in rat? **(I)**
2. How does the neuronal pathology of epileptic animals compare with that in patients with TLE? **(I)**
3. Is mossy fiber sprouting associated with the generation of spontaneous seizures in epileptic brain? **(II)**
4. Can epilepsy be prevented or modified by augmenting brain GABA concentration with VGB treatment and can epilepsy be prevented or modified by LTG treatment? **(III, IV)**
5. Are AEDs that are effective in human TLE effective in epileptic animals in an experimental TLE model? **(V)**

4. MATERIALS AND METHODS

Schematic diagram of different study designs is presented in Figure 2.

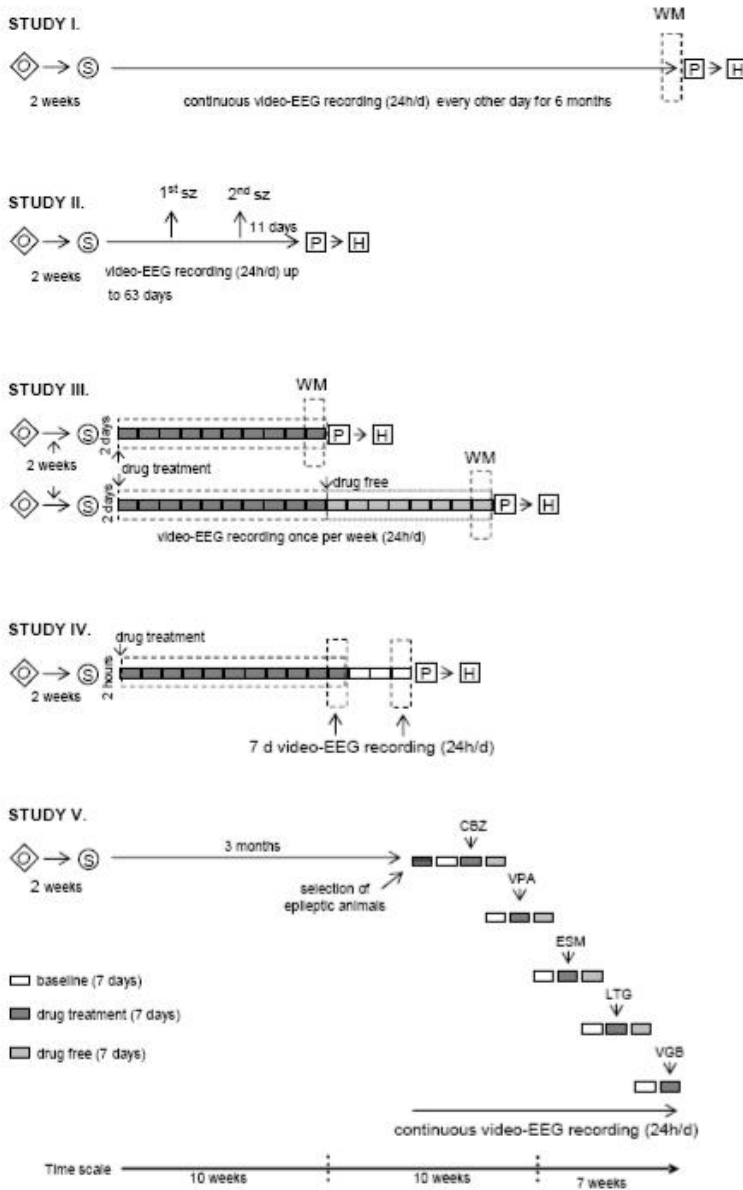


Figure 2. Schematic diagram of different study designs. Abbreviations: CBZ, carbamazepine; ESM, ethosuximide; H, histology; LTG, lamotrigine; O, surgical operation; P, perfusion; S, electrical stimulation; VPA, valproic acid; VGB, vigabatrin; WM, Morris water maze.

4.1 Animals

Adult male Harlan Sprague-Dawley (n=209, The Netherlands) rats weighing 275 to 390 g were individually housed in cages in a controlled environment (constant temperature, 22 ± 1 °C, humidity 50-60, lights on 07:00 to 19:00). Animals had free access to standard food pellets and fresh water. All animal procedures were approved by the Institutional Animal Care and Use Committee of University of Kuopio and by the Provincial Government of Kuopio, Finland, and conducted in accordance with the guidelines set by the European Council Directives 86/609/ETY. Administration of drugs was conducted by the guidelines set by good laboratory practice (Diehl et al., 2001). All attempts were made to minimize the number of experimental animals and their suffering.

4.2 Electrode implantation (I-V)

For placement of the recording and stimulation electrodes, animals were anesthetized by an intraperitoneal injection (6 ml/kg) of a mixture of sodium pentobarbital (58 mg/kg), chloral hydrate (60 mg/kg), magnesium sulfate (127.2 mg/kg), propylene glycol (40%), and absolute ethanol (10%), and placed in a Kopf stereotaxic frame (bregma and lambda at the same horizontal level). Self made electrodes: bipolar wire electrode (wire: Franco Corradi, Milano, Italy; diameter 0.127 μ m) connected with gold-plated pins (Plastics One Inc.) were implanted into the left lateral nucleus of the amygdala for stimulation and EEG recording. A pair of stimulating electrodes located 3.6 mm posterior, 5.0 mm lateral to bregma. The electrode tip was located 6.5 mm ventral to the surface of the brain (Paxinos and Watson, 1986). To record the spread of electrographic activity in the ipsilateral hippocampus, a bipolar electrode was implanted into the hilus of the left hippocampus (coordinates: 4.1 mm posterior, 2.6 mm lateral and 3.7 mm ventral to bregma) (**study I**). One cortical electrode (3.0 mm anterior and 2.0 mm lateral to bregma) was implanted contralaterally into the skull above the right frontal cortex to record the spread of the EEG activity. Two other screw electrodes were implanted bilaterally over the cerebellum to serve as reference and ground electrodes (10.3 mm posterior and 2.0 mm lateral to bregma) (**studies I-V**).

4.2 SE induced by electrical stimulation of the amygdala (I-V)

SE was induced by stimulating the lateral nucleus of the left amygdala electrically. Stimulation consisted of a 100-ms train of 1-ms biphasic square-wave pulses delivered at 60 Hz, every 500 ms, 400 μ A (peak to peak). The stimulation equipment comprised of an A300

Pulsemaster Stimulator (World Precision Instruments, WPI; Sarasota, FL) connected with two A360 Constant Current Stimulus Isolators (WPI), and shielded cables (Plastics One Inc., Roanoke, VA). Animals were connected to the system via a 6-channel commutator (Plastics One Inc.). First, each rat was stimulated continuously for 20 min. Thereafter, stimulation was interrupted, and the stage of SE was checked. If the behavior of the animal did not reveal clonic SE within 20 min (continuous electrographic epileptiform spiking and recurrent clonic seizures), stimulation was continued for another 5 to 10 min. The behavior of the animal was checked again after 5 to 10 min. Once the criterion of SE was achieved, stimulation was resumed. Maximum stimulation time did not exceed 40 min.

4.4 Antiepileptic drug treatments

4.4.1 Treatment with vigabatrin (VGB) (III, V)

Study III: VGB (Hoechst Marion Roussel, Strasbourg, France), dissolved in physiologic saline (0.9% NaCl) at a concentration of 200 mg/ml, was administered via subcutaneously implanted osmotic minipumps (75 mg/kg/d; Alza Corp., Palo Alto, CA). In this study, rats were treated with VGB for 10 weeks. Control rats (unstimulated and stimulated control rats) were treated with an equal volume of 0.9% NaCl.

Study V: VGB, dissolved in physiologic saline (0.9% NaCl) at a concentration of 125 mg/ml (2 ml/kg), was administered intraperitoneally (250 mg/kg/d) once a day for 5 days.

4.4.2 Treatment with lamotrigine (LTG) (IV, V)

Study IV: LTG (provided by Glaxo Wellcome, UK) dissolved in DMSO at a concentration of 10 mg/ml (2 ml/kg), was injected intraperitoneally at a dose of 20 mg/kg. Thereafter, LTG, suspended in distilled water at a concentration of 10 mg/ml (2 ml/kg), was administered intragastrically (20 mg/kg) once a day for 2 weeks. After 2 weeks, LTG was injected intragastrically at a dose of 10 mg/kg. Altogether, treatment was continued for 11 weeks. Control animals were treated with equal volumes of DMSO and distilled water.

Study V: LTG, suspended in 0.1% carboxymethyl cellulose at a concentration of 5 mg/ml (2 ml/kg) was administered by intragastric (10 mg/kg) injection twice a day for 7 days.

4.4.3 Treatment with carbamazepine (CBZ), valproic acid (VPA), and ethosuximide (ESM) (V)

CBZ (Sigma # C-4024, St. Louis, MO, suspended in 0.1% carboxymethyl cellulose CM-52, Whatman, Maidstone Kent, UK), was administered intragastrically 3 times a day (40 mg/kg, 20 mg/ml, 2 ml/kg) at 8-h intervals (total daily dose 120 mg/kg) for 7 days.

VPA (Sigma # P-4543, dissolved in 0.9% saline), was administered intraperitoneally twice a day (300 mg/kg, 2 ml/kg), at 12- hour intervals (total daily dose 600 mg/kg) for 7 days.

ESM (Sigma # E-7138, dissolved in 0.9% saline), was administered intraperitoneally twice a day (200 mg/ml, 2 ml/kg,) at 12-hour intervals (total daily dose 400 mg/kg) for 7 days.

4.5 Detection of electrographic seizures (HAFDs) during SE (I-IV)

During and after the induction of initial SE, electrographic seizure activity was recorded using video-EEG recording systems from Stellate Monitor System (Montreal, Canada) or Nervus (Taugagreining, Iceland).

Electrophysiologic characterization of SE was performed to quantify the severity and duration of SE based on video-EEG recordings during stimulation and for at least 16 hours thereafter. SE severity was assessed by counting the number and duration of HAFDs, which are typically associated with behavioral seizures. An HAFD was defined as a discharge that lasted at least 5 seconds, with a frequency of at least 8 Hz, and amplitude at least two times higher than the baseline EEG in the amygdala or cortex (see Figure 1A).

4.6 Detection of spontaneous seizures (I-V)

The development of epilepsy (e.g., appearance of spontaneous seizures) was studied using video-EEG recording systems from Stellate Monitor System or Nervus. The occurrence of spontaneous seizures was determined based on analysis of the EEG data.

Study I: Two groups of stimulated animals (8 animals in each group) were recorded 24 hours per day. Each individual animal was recorded every other day for 6 months.

Study II: (*Experiment I*) Appearance of spontaneous seizures was monitored (24 hours/day) until the animals developed a second spontaneous seizure and 11 days thereafter (up to 63 days). (*Experiment II*) Appearance of spontaneous seizures was monitored every other day (24 hours/day) for 60 days.

Study III: The development of spontaneous seizures in each animal was monitored once a week (24 h at a time) for 10 or 18 weeks.

Study IV: The appearance of spontaneous seizures was studied 10 weeks after the induction of SE (24 hours/day) for 7 days. The second 7 days video-EEG recording was performed 13 weeks after SE (i.e., 2 weeks after LTG wash-out).

Study V: Epileptic animals for the AED trials were selected 3 months after SE during which the EEG activity of the animals was recorded for 3 to 4 days (24 hours/day). Thereafter, 8 animals were recorded for baseline-, titration-, drug treatment-, and wash out-periods.

4.7 Morris water-maze (I, III)

Spatial learning and memory of the animal was tested in the Morris water-maze. Animals were tested on 7 consecutive days. A circular plastic tank (150 cm diameter x 75 cm high) was filled with water ($19 \pm 1^{\circ}$ C). The size of the submerged platform, onto which the rat could escape, was 10 x 10 cm. The location of the platform was alternated daily so that on odd days (days 1, 3, 5, and 7) the platform was in the 1st quadrant of the tank, and on even days (days 2, 4, and 6) in the opposite side of the tank (3rd quadrant). The tank was illuminated with spotlights. After finding the platform, the animals stayed on the platform for 10 seconds, after which the rat was given time (60 seconds) to rest before next trial. If the rat did not find the platform in 60 seconds, the animal was placed on the platform for 10 seconds. **(Study I)** The daily protocol consisted of four 60-seconds trials. Training the rats to locate the platform in water-maze started 6 months after the induction of SE. **(Study III)** Daily protocol consisted of five 60-second trials. Training of the rats to locate the platform in the water-maze was started either 9 or 17 weeks after the induction of SE.

4.8 Perfusion for histology (I-IV)

Animals were deeply anaesthetized by intraperitoneal injection (6 ml/kg) of a mixture of sodium pentobarbital (58 mg/kg) and chloral hydrate (60 mg/kg). Thereafter, animals were intracardially perfusion-fixed according to the silver-sulphide method for Timm-staining described by Sloviter (1982). The brains were fixed with 0.37% sulphide solution (30 ml/min) for 10 min followed by cold ($+4^{\circ}$ C) 4% paraformaldehyde (30 ml/min) in 0.1 M phosphate buffer (pH7.4) for 10 min. The brains were removed from the skull, post-fixed in a 4% paraformaldehyde solution for 4 h, and cryoprotected in a solution containing 20% glycerol in 0.02 M KPBS (pH 7.4) for 24 h and stored at -70° C until processed. Time points when the animals were perfused were: **(Study I):** 6 months; **(Study II):** 20-63 days (11 days after the second seizure, *Experiment I*) and 60 days (*Experiment II*); **(Study III):** 10 and 18 weeks; and **(Study IV):** 14 weeks after the induction of SE.

4.9 Tissue sectioning (I-IV)

The brains were cut in the coronal plane with a sliding microtome in a 1-in-4 series at a thickness of 50 μm (**Study I**), or in a 1-in-5 series at a 30- μm thickness (**Studies II-IV**). After cutting, sections were stored in a cryoprotectant tissue collecting solution (30% ethylene glycol, 25% glycerol in 0.05 M sodium phosphate buffer) at -20°C until processed.

4.10 Histologic stainings

4.10.1 Nissl-staining (I-IV)

To identify the cytoarchitectonic boundaries, distribution, and severity of neuronal damage, and the location of the stimulation electrode, the first series of sections was used for Nissl-staining with thionin.

4.10.2 Timm-staining (I-IV)

To analyze synaptic reorganization (mossy fiber sprouting), an adjacent series of sections was processed using Timm histochemistry. Staining was performed in the dark as follows: working solution that contained gum arabic (300 g/l), sodium citrate buffer (25.5 g/l citric acid monohydrate and 23.4 g/l sodium citrate), hydroquinone (16.9 g/l), and silver nitrate (84.5 mg/l) was poured into the staining dish that contained the slides with sections. The sections were developed until an appropriate staining intensity was attained (60–75 min). The slides were rinsed in tap water for 30 min and placed in 5% solution of sodium thiosulfate for 12 min. Finally, sections were dehydrated through an ascending series of ethanol, cleared in xylene, and cover-slipped with DePeX mounting medium (BDH, Laboratory Supplies, England).

4.10.3 Somatostatin immunohistochemistry (III)

To analyze the seizure-induced neuronal damage to somatostatin-immunoreactive neurons in the hilus immunohistologic staining for somatostatin was performed. A 1-in-5 series of free floating sections was collected from the tissue collecting solution and stained using the avidin-biotin technique. The sections were washed three times (10 min each time) in 0.02 M potassium phosphate buffered saline (KPBS), pH 7.4. To block non-specific binding, the sections were incubated in a solution containing 10% normal horse serum (NHS) and 0.5% Triton X-100 in 0.02 M KPBS for 2 hours at room temperature. The primary incubation was performed in the 1:8000 dilution of monoclonal mouse anti-somatostatin antibody obtained from MCR Regulatory Peptide Group (University of British Columbia, Canada), 0.5% Triton

X-100, 1% NHS in 0.02 M KPBS at pH 7.4, at +4°C for 3 days. The sections were washed three times (10 min each) in 0.02 M KPBS containing 2% NHS. Thereafter, the sections were incubated in a solution containing horse biotinylated anti-mouse IgG (Vector, BA-2000; Burlingame, CA), 1% NHS and 0.3% Triton X-100 in 0.02 M KPBS, pH 7.4 for 1 hour at room temperature. The sections were washed twice as described above and incubated for 45 min at room temperature in avidin-biotin solution (Biomedica, No.11001-1, Super ABC kit, Foster City, CA) in 0.02 M KPBS, pH 7.4. The sections were washed two times (10 min each) with 2% NHS in KPBS. They were then recycled into the secondary antibody solution for 45 min. After two 10-min washes in KPBS, they were recycled to avidin-biotin-complex for 30 min, and again washed in KPBS three times (10 min each time). The sections were then reacted with diaminobenzidine (0.05% diaminobenzidine and 0.04% H₂O₂ in 0.02 M KPBS, pH 7.4) and washed three times 10 min in KPBS. The sections were then mounted on gelatin-coated slides, dried overnight at 37°C and intensified with OsO₄ and thiocarbohydrazide according to the method of Lewis et al. (1986).

4.11 Analysis of the material

4.11.1 Analysis of SE severity and duration (I-IV)

Severity and duration of the SE analysis was based on the electrographic seizure activity. The duration of SE was assessed by measuring the time between the first and last HAFD. Moreover, total number, total duration of HAFDs, and the mean duration of individual HAFDs in each animal were analyzed.

4.11.2 Analysis of electrographic and behavioral seizures (I-V)

Analysis of spontaneous seizures was based on video-EEG recording data. For video-EEG recordings, animals were placed in plastic boxes and recorded continuously (24 h/d) with video-EEG. An infrared light-sensitive camera and an infrared light were positioned above the cages. Infrared light was used to allow for video monitoring of the animal behavior during the dark cycle. A wide angle lens permitted video-taping of up to eight animals at the same time. Data was collected on videotapes (time lapse video: by using slower recording speed it was possible to record 24 hours to one 3-hour cassette) and the data was stored on digital data storage files. (**Study I**) During the first 100 days, all video-EEG-files were analyzed, and thereafter every other video-EEG-file was analysed. (**Studies II-V**) All recorded video-EEG-files were analysed. An epileptic seizure was defined as a paroxysmal discharge with clear start and end with rhythmic, repetitive waveform that lasted at least 5 seconds (see Figure 1B).

Analysis of behavioral seizures was based on video recordings. The video recording system was time-locked with the digital EEG. If an electrographic seizure was detected, the severity of behavioral seizure activity was classified according to a slightly modified Racine's scale (1972): Stage 0, electrographic seizure without any detectable motor manifestation; Stage 1, mouth and face clonus, head nodding; Stage 2, clonic jerks of one forelimb; Stage 3, bilateral forelimb clonus; Stage 4, forelimb clonus and rearing; and Stage 5, forelimb clonus with rearing and falling. For the purpose of assessing the percentage of partial seizures or secondarily generalized seizures of the total number of seizures, behavioral seizures were classified into two categories: (1) partial seizures (Stages 0–2) or (2) secondarily generalized seizures (Stages 3–5).

4.11.3 Distribution and severity of neuronal damage (I-IV)

4.11.3.1 Nissl- staining (I, III, IV)

Microscopic analysis was performed using a Leica DMRB (Leitz, Wetzlar, Germany) microscope with brightfield optics.

The severity of neuronal damage in various temporal lobe structures was scored using semiquantitative analysis of cell death in thionin-stained sections. In **study I**, the scoring scale was as follows: Score 0, no damage; Score 1, less than 50% neuronal damage; Score 2, greater than or equal to 50% neuronal loss. Neuronal damage was assessed 6 months after the induction of SE. In **study III**, the scoring scale was as follows: Score 0, no damage; Score 1, less than 10% neuronal damage; Score 2, damage comprises 10% to 50% of neurons in area of interest; Score 3, lesion comprises more than 50% of neurons. Neuronal damage was assessed either 10 or 18 weeks after the induction of SE. In **study IV**, the scoring scale was as follows: Score 0, no damage; Score 1, less than 20% neuronal damage; Score 2, damage involving 20% to 50% of neurons in an area of interest; Score 3, lesions involving more than 50% of neurons. Neuronal damage was assessed 14 weeks after the induction of SE.

4.11.3.2 Somatostatin immunohistochemistry (III)

The number of somatostatin immunoreactive neurons in the hilus throughout the rostrocaudal extent of the hippocampus (section thickness 30 μm , 1-in-5 series) was calculated manually at a magnification of 200 X with the aid of an ocular grid starting at a level of 4.4 mm posterior to bregma according to atlas of Paxinos and Watson (1986). The number of neurons was counted in 5 consecutive sections taken at 300- μm intervals. The area of the hilus in the same sections was then measured using a Quantimet 570 Image Analysis System (Leica

Cambridge Ltd., Cambridge, England). Both the ipsilateral and contralateral sides of the hippocampus were examined.

4.11.3.3 Analysis of mossy fiber sprouting (I-IV)

Mossy fiber sprouting was analyzed in each section along the septotemporal axis of the hippocampus. Sections were analyzed using scoring scale according to Cavazos et al. (1991) as follows: Score 0, no granules; Score 1, sparse granules in the supragranular region and in the inner molecular layer; Score 2, granules evenly distributed throughout the supragranular region and inner molecular layer; Score 3, almost a continuous band of granules in the supragranular region and in the inner molecular layer; Score 4, continuous band of granules in the supragranular region and inner molecular layer; Score 5, confluent and dense laminar band of granules that covers most of the inner molecular layer, in addition to the supragranular regions. Both the ipsilateral and contralateral sides of the hippocampus were analyzed. **(Study I)** The mossy fiber sprouting was assessed from the supra- and infragranular regions separately starting at the level at which the suprapyramidal and infrapyramidal blades of the granule cell layer form a continuous band of cells. More temporally, sprouting was scored separately in the dorsal mid-portion and ventral mid-portion of the dentate gyrus where the granule cell layer of the septal and temporal ends merge and forms an easily identifiable and standardized ‘oval-shaped’ layer. Both the ipsilateral and contralateral sides of the hippocampus were examined. **(Studies II-IV)** The sprouting was assessed separately from the ‘tip’, ‘mid’, and ‘crest’ portions of the granule cell layer by starting at the level at which the suprapyramidal and infrapyramidal blades of the granule cell layer form a continuous band of cells. More temporally, sprouting was scored separately in the dorsal mid-portion and ventral mid-portion of the dentate gyrus where the granule cell layer of the septal and temporal ends merge and forms an easily identifiable and standardized ‘oval-shaped’ layer. Both the ipsilateral and contralateral sides of the hippocampus were examined. Assessment of neuronal damage and mossy fiber sprouting was conducted blind with respect to the treatment status of the animal and group.

4.12 Stereologic cell counting (II)

Systematic random sampling method (stereology) was used to assess damage to hilar cell neurons on both the ipsilateral and contralateral sides of the hippocampus. The system was composed of a motorized stage of the microscope system that was under computer control (NeuroLucida Morphometry System, MicroBrightField, Colchester, VT). Counting was performed throughout the section. The hilar fields in every histologic section were examined at

evenly spaced x-y intervals (70 x 70 μm septally, 150 x 150 μm temporally). For each x-y step, neuronal counts were derived from a known fraction of the total area, using an unbiased counting frame (25 x 25 μm septally and 30 x 30 μm temporally). Neuronal densities were calculated by dividing the number of neurons by the area.

4.13 Analysis of Morris water maze (I, III)

Animal spatial learning and memory was tested in Morris water-maze. The escape latency (time taken to find the submerged platform), swimming speed, and the total swim path distance (distance travelled by the animal) were measured.

4.14 Photographs (I-IV)

High power photographs were taken with a Leica DMRB (Leitz) camera system and low power photographs with a Nikon 6 x 9 cm system (Nikon, Tokyo, Japan).

4.15 Statistics (I-V)

All statistical analyses were performed with SPSS for Windows (6.0.1 or v.9.0 or v.10.0) and StatView (v.4.0 or v.4.5) for Macintosh. Differences in EEG parameters of SE and spontaneous seizures were tested with Mann-Whitney *U*-test. Differences in the development of epilepsy were tested using Fisher's exact test or Pearson χ^2 -test. Performance in Morris Water maze task was analysed using ANOVA with repeated measures followed by the Mann-Whitney *U*-test as a *post hoc* analysis. Differences in the mossy fiber density between different rat groups were tested by the Kruskal-Wallis test followed by the Mann-Whitney *U*-test as a *post hoc* test. The severity of neuronal damage in different structures of the temporal lobe between different animal groups was analysed using the Mann-Whitney *U*-test. Differences between the stimulation and contralateral sides were analysed using the Wilcoxon Signed rank-test. The Spearman correlation coefficient was used to examine the correlation between neuronal damage and mossy fiber sprouting. Seizure frequency between baseline, treatment, and post-treatment periods was analyzed using the Wilcoxon signed rank test. Change in seizure frequency between different treatment groups was tested by the Kruskal-Wallis-test followed by the Mann-Whitney *U*-test as a *post hoc* test. A *p*-value of less than 0.05 was considered statistically significant.

5. RESULTS

5.1 Seizure characteristics and development of epilepsy (I, II)

(Study I) After unilateral electrical stimulation of the left amygdala, 13/15 of animals developed spontaneous seizures within the 6-month follow-up period. The first spontaneous seizures occurred approximately 1 month (range 6-85 d) after the stimulation. The individual seizure number varied extensively between animals. The maximum number of seizures found was 1137 and the lowest number 1. According to individual seizure number data there were two different populations of epileptic animals: animals with either frequent or rare seizures during the 6-month follow-up. Of 13 epileptic animals, 31% (4/13) had frequent seizures and 69% (9/13) had rare seizures. Latency to the first seizure was shorter in animals with frequent seizures compared to animals with rare seizures ($p < 0.05$, 11 vs. 63 days). The number of HAFDs did not correlate with time to first seizure ($p > 0.05$).

The analysis of behavioral seizure severity indicated that there was a change in behavioral seizure type during the 6-month follow-up. During the first 10 weeks, seizures were mostly secondarily generalized (Stage 3-5 according to the modified Racine's scoring scale). During the last 16 weeks, most of the seizures (77%) were partial (Stage 0-2). Behavioral seizure type did not differ between animals with frequent and rare seizures within the first 10 weeks. Thereafter, in animals with frequent seizures, 80% of all seizures were partial compared to 4% of seizures in animals with rare seizures.

The mean duration of all seizures was 49 s (range 7-252 s). The mean duration of partial seizures was 44 s (range 7-232 s) and secondarily generalized seizures lasted 61 s (range 16-252 s). Partial seizures became shorter over time ($p < 0.001$), but the duration of generalized seizures remained constant over the follow-up period.

Distribution analysis of seizures within a day indicated that 57% of all seizures occurred between 07:00 and 19:00, when the house light was on in the animal room. More detailed analysis did not indicate any difference in diurnal occurrence of partial and secondarily generalized seizures ($p > 0.05$, respectively). There was no difference in diurnal occurrence of seizures between animals with frequent (57% of partial and 54% of generalized seizures) or rare (63% of partial and 69% generalized) seizures. Seizures peaked between 17:00 to 18:00.

Additional video-EEG recording (via hilar electrode) was performed 3 to 5 months after the SE induction to analyze the spreading of the EEG activity and independent seizure generation in the hippocampus. Those recordings revealed that most of the seizures had a diffuse onset emerging from the amygdala and hippocampus at the same time.

In this study, we used the number of HAFDs (see Figure 1A) and the duration of individual HAFDs as well as the duration of SE; that is, the time between the first and last HAFD as a measure of SE severity. We did not find any difference in the number of HAFDs, the duration of individual HAFDs, or the time between the first and last HAFD between the frequent and rare seizure groups (all $p > 0.05$).

In **study II**, seizure development was assessed by continuous video-EEG recording (24 h/d) every day until the animal was killed for histology. The more intense recording paradigm revealed that it took an average of 17 days (range 8-51) after SE induction to detect the first spontaneous seizure. The second seizure (time of epilepsy diagnosis) was observed 23 ± 15 days (range 8-54) after SE induction. Altogether, 14 of 16 (88%) animals were considered epileptic. Of these 14 animals, 13 expressed at least 2 spontaneous seizures during the 63-day follow-up. One animal had only one seizure, but interictal spiking activity was evident on the EEG. In five of the epileptic animals, 36% had more than 1 seizure per day and were considered as animals with “frequent seizures”. Nine epileptic animals (64%) had less than 1 seizure per day and were considered to be animals with “rare seizures”. Two animals did not have spontaneous seizures during the 63-day follow-up.

5.1.1 Effect of VGB on seizures and epilepsy (III)

VGB treatment, which was started 2 days after the induction of SE, did not prevent the development of epilepsy. The severity of SE did not differ between treatment groups as assessed by the number of HAFDs, total duration of HAFDs, and duration of SE (all $p > 0.05$). Therefore, the initial insult, which would affect later outcome, did not differ between the treatment groups.

VGB treatment did not affect the appearance of the first spontaneous seizure. The first spontaneous seizure was detected after an average of 24 days (range 9-58) in the saline-treated control group and 23 days (range 9-44) in the VGB group after the induction of SE. During the drug treatment period (weeks 0-10), spontaneous seizures were observed in 6/11 (55%) of controls, and 8/11 (73%) of VGB-treated animals. By the end of the 18-week follow-up period, 10/11 (91%) of VGB- treated animals had spontaneous seizures.

The daily seizure frequency during the drug treatment period (weeks 0-10) or after the drug treatment period (weeks 11 -18) did not differ between saline-treated controls or VGB-treated animals. In saline-treated control animals ($n=11$) the mean seizure frequency was 1.5 ± 1.0 seizures per day and in VGB group ($n=11$) 5.6 ± 2.7 seizures per day. After discontinuation of the treatment (during weeks 11-18) the mean daily seizure rate increased to 12.2 ± 8.2

seizure per day in saline-treated control animals (n=4) and to 11.5 ± 6.0 seizure per day in VGB-treated animals (n=7) ($p>0.05$).

During the first 10 weeks, 80% of seizures in saline-treated animals were generalized. In VGB-treated animals, only 19% of seizures were generalized. After discontinuation of VGB treatment or saline treatment in control animals (weeks 11-18), 25% of the seizures were generalized in the VGB group. In saline-treated control animals, 24% of seizures were generalized during weeks 11 to 18. The mean percentage of generalized seizures per rat did not differ between groups ($p>0.05$).

VGB-treatment did not have any effect on seizure duration during the drug treatment period (weeks 0-10). The mean seizure duration was 63 s in saline-treated animals and 68 s in VGB-treated animals ($p>0.05$). After discontinuation of VGB or vehicle treatment (weeks 11-18), seizure duration did not change.

5.1.2 Effect of LTG on seizures and epilepsy (IV)

During the drug treatment period, LTG decreased the number of HAFDs ($p<0.01$) compared to the vehicle group. It also decreased the duration of HAFDs compared to the time period before drug treatment ($p<0.05$).

LTG treatment, which was started 2 hours after the induction of SE, did not prevent the development of epilepsy. In the vehicle group, 13/14 (93%) of animals and all 14/14 (100%) animals treated with LTG (they were still on medication) expressed seizures during the first week follow-up 10 weeks after the induction of SE (no difference between the groups, $p>0.05$). During the second follow-up (2 weeks later) 12/14 (86%) of LTG-treated animals expressed spontaneous seizures. In the vehicle group, 13/14 (93%) of animals expressed spontaneous seizures (no difference between the groups; $p>0.05$). Altogether (both recordings combined), 13/14 (93%) in vehicle-treated animals and 14/14 (100%) LTG-treated animals expressed spontaneous seizures. During both follow-ups, seizure frequency and duration did not differ between the groups ($p>0.05$) or within the groups between the follow-ups ($p>0.05$). During the first follow-up period (10 weeks after SE induction), vehicle-treated animals had 12 seizures per day compared to LTG-treated animals, which had 9.8 seizures per day. During that time, LTG-treated animals were still on medication. Seizure duration was not affected by LTG treatment ($p>0.05$). During the second follow-up (13 weeks after the SE induction), vehicle-treated animals had 15.2 seizures per day compared to 12.9 seizures per day in LTG-treated animals (no difference between groups).

There was a difference in behavioral seizure severity in LTG-treated animals between the follow-ups. Behavioral seizure severity increased from 2.1 (follow-up 1, animals were on medication) to 3.5 (follow-up 2) ($p < 0.01$). Also, the percentage of generalized seizures increased during follow-up 2 compared to follow-up 1 (from 55% to 87%, $p < 0.05$).

The number of animals developing severe epilepsy (>1 seizure per day) did not differ between the groups ($p > 0.05$) or within groups between follow-up ($p > 0.05$). Altogether, in both groups 64% (9/14) of animals expressed severe epilepsy (no difference between groups). In the vehicle group, seizure duration and severity did not differ between follow-ups ($p > 0.05$). In the LTG-treated animals during follow-up 1, when the animals were still on medication, seizures were shorter (34 ± 13 s) compared to follow-up 2, when no drug was present (57 ± 6 s) ($p < 0.05$). Also, drug treatment lowered behavioral seizure severity from 3.4 to 1.4 ($p < 0.05$).

5.2 Neuronal damage in the temporal lobe (I-IV)

5.2.1 Hippocampal pathology at the time of epilepsy onset (II)

At the time of the epilepsy diagnosis (that is after the second spontaneous seizure) septal and temporal hilar neuron numbers were bilaterally reduced in epileptic rats compared to age-matched control animals. In septal hilus neuron number was reduced by 21% in epileptic rats. Thirty-six percent of epileptic animals (4/11) had increased neuronal damage compared to age-matched control animals. The remaining epileptic animals had neuronal damage within the normal range. In the temporal hilus neuronal damage was observed in 73% of epileptic animals. Sixty-two percent of neurons at the temporal end of the hippocampus were remaining compared to age-matched control animals. In 27% of the epileptic animals, the neuron number in the temporal hippocampus, as well as in animals that did not develop spontaneous seizures was within the expected range.

5.2.2 Hippocampal pathology in epileptic animals (I, III, IV)

Six months after the induction of SE 92% (12/13) of epileptic animals had bilateral neuronal damage in the amygdala (**study I**). The most damaged areas were the lateral nucleus, the parvocellular division of the basal nucleus, the accessory basal nucleus, the ventral part of the central division of the medial nucleus, layer III in the anterior cortical nucleus, the posterior cortical nucleus, the amygdalohippocampal area, the periamygdaloid cortex, and the nucleus of the olfactory tract. Bilateral hippocampal damage was detected in 46% (6/13) of epileptic animals, and it was more severe septally than temporally. The more severely damaged areas were the hilus and CA1 subfield. Granule cell damage was not detected in any of the epileptic

animals. Subiculum and entorhinal cortex damage was observed in 54% and 92% of epileptic animals, respectively. In the entorhinal cortex, the most damaged areas were the amygdalo-entorhinal transition area and dorsal intermediate entorhinal subfield. There was no damage detected in the piriform cortex.

Despite of the chronic treatment with VGB for 10 weeks after SE (**study III**), there were increased neuronal damage in the hilus, CA3, CA1, subiculum and layer III of the caudal entorhinal cortex in comparison with unstimulated control animals. The neuronal damage was evident both ipsi- and contralaterally. The neuronal damage progressed during the 8-weeks follow-up in the vehicle-treated controls, when compared to the first follow-up period (weeks 0-10) in CA3, subiculum, and EC (III) areas (both, $p < 0.05$). In the VGB-group, however, neuronal damage did not change over time.

Loss of somatostatin immunoreactive neurons was increased ipsilaterally in VGB-treated animals both at 10 and 18 weeks after SE induction compared to control ($p < 0.05$). In the contralateral hemisphere, the number of somatostatin immunoreactive neurons did not differ between VGB-treated and corresponding stimulated animals.

Chronic treatment with LTG (**study IV**) for 11 weeks did not prevent the SE-induced neuronal damage in the hippocampus. Total damage in septal hippocampus (combined damage in septal hilus, CA3c, and CA1) did not differ between vehicle and LTG-treated groups. In both groups, however, septal damage was significantly higher than that in the sham group, both ipsilaterally and contralaterally. The damage in the temporal hippocampus (combined damage in temporal hilus and CA1) was also significantly increased compared to the sham group (however, there was no difference between vehicle and LTG groups).

5.3 Mossy fiber sprouting (I-IV)

5.3.1 Mossy fiber sprouting at the time of epilepsy onset (II)

In *experiment I* in which animals were perfused 20 to 63 d after the SE induction (e.g., 11 days after the second seizure) had increased density of mossy fibers in all septal and temporal areas in comparison with controls (see Table 7). Animals that did not have spontaneous seizures (n=2) had increased mossy fiber sprouting in tip (n=2) and mid (n=1) portion of inner molecular layer of the dentate gyrus. .

TABLE 7. Mossy fiber sprouting and neuron density in different groups in *Experiment I*.

<i>Septal end</i>	Control (n=14)	No epilepsy (n=2)	Epilepsy (n=14)
Tip	2.2 ± 0.3	3.1 ± 0.1	3.8 ± 0.6**
Mid	1.4 ± 0.4	2.1 ± 0.0	2.6 ± 0.9***
Crest	1.8 ± 0.4	2.2 ± 0.2	2.9 ± 0.8***
Neuron density	713 ± 116	580 ± 21	560 ± 135**
<i>Temporal end</i>	1.3 ± 0.3	2.0 ± 0.0	3.5 ± 1.0***
Neuron density	1517 ± 184	1225 ± 106	940 ± 261***

Statistical significance: ** p<0.05, *** p<0.001 compared to control group.

TABLE 8. Mossy fiber sprouting in different groups in *Experiment II*.

<i>Septal end</i>	Control (n=14)	No epilepsy (n=20)	Epilepsy (n=10)
Tip	1.8 ± 0.5	2.5 ± 0.5***	3.7 ± 0.8***, ###
Mid	1.0 ± 0.3	1.5 ± 0.4***	2.7 ± 0.9***, ###
Crest	1.1 ± 0.3	2.0 ± 0.6***	3.1 ± 1.0***, #
<i>Temporal end</i>	1.1 ± 0.3	1.6 ± 0.4	3.1 ± 1.0***, ###

Statistical significance: *** p<0.001 compared to control group; ### p<0.001 compared to no epilepsy group.

Also, in *experiment II* (60 d after SE) epileptic animals had more intense mossy fiber sprouting compared to non-epileptic animals in the tip (p<0.001), mid (p<0.001), and crest (p<0.01) of the dentate gyrus. The mossy fiber sprouting was not, however, associated with the occurrence of spontaneous seizures, since in some of the epileptic animals some degree of mossy fiber sprouting was detected as in age-matched control animals (see Table 8).

In *experiment I*, correlation analysis indicated that both ipsilaterally and contralaterally the mossy fiber sprouting in the temporal hippocampus density correlated with seizure frequency ($p<0.05$), and neuron density (ipsi, $p<0.01$; contra, $p<0.05$). There was also a correlation between the seizure frequency and hilar cell damage in the temporal end both ipsilaterally and contralaterally ($p<0.01$). Septally, however, mossy fiber sprouting did not correlate with seizure frequency ($p>0.05$). In *experiment II*, there was no correlation between the density of mossy fiber sprouting and seizure frequency ($p>0.05$). Mossy fiber sprouting, however, correlated with hilar cell damage in the temporal end ($p<0.05$). There was no correlation between seizure frequency and neuronal density in the temporal hippocampus ($p>0.05$).

5.3.2 Mossy fiber sprouting in epileptic animals (I, III, IV)

(Study I) All epileptic animals had aberrant sprouting of granule cell axons (that is mossy fiber sprouting) and sprouting was denser temporally than septally as assessed 6 months after the induction of SE. In ipsilateral septal hippocampus, animals with epilepsy had a 2.4-fold ($p<0.01$) increase and in contralateral hippocampus a 2.3-fold ($p<0.01$) increase in the density of mossy fibers compared to controls. In the dorsal and ventral mid portions of the hippocampus, the density of mossy fiber sprouting varied ipsilaterally from a 2.5 to 3.7-fold increase and contralaterally from a 2.9 to 3.8-fold increase (all $p<0.01$) compared to controls. There was no difference in the density of mossy fiber sprouting between animals with frequent and rare seizures in septal hippocampus both ipsilaterally and contralaterally ($p>0.05$). Animals with frequent seizures, however had a 1.8 fold ($p<0.05$) increased density of mossy fiber sprouting in the contralateral dorsal mid-portion compared with animals with rare seizures. Both in ipsilateral (1.7-fold) and contralateral (2.4-fold) ventral mid-portion, animals with frequent seizures had an increased density of mossy fibers compared to animals with rare seizures (both $p<0.05$).

After chronic treatment with VGB (**Study III**) for 10 weeks, mossy fiber sprouting was more dense in all animal groups compared to the unstimulated control animals (all $p<0.01$), both ipsilaterally and contralaterally. The density of mossy fiber sprouting, however, did not differ between VGB-treated animals and saline-treated control animals ($p>0.05$).

Chronic treatment with LTG (**Study IV**) for 11 weeks did not affect the development of mossy fiber sprouting. Both vehicle- and LTG-treated rats had an increased density of mossy fiber sprouting compared to unstimulated control animals. Ipsilaterally in vehicle-treated animals, mossy fiber sprouting increased 2.6-fold and in LTG-treated animals it increased 2.9-

fold compared to unstimulated control animals. In LTG-treated animals, the density of mossy fiber sprouting did not differ from that in vehicle-treated animals either in septal or temporal hippocampus. In the vehicle group, sprouting was more intense ipsilaterally than contralaterally. In the LTG group, the density was similar both ipsilaterally and contralaterally.

5.4 Spatial memory performance (I, III)

Epileptic animals had impaired spatial memory performance in the Morris water maze compared to control animals ($p < 0.001$). Animals with frequent seizures were even more impaired compared to animals with rare seizures ($p < 0.001$).

Animals that were treated with VGB for 10 weeks did not perform better than saline-treated control animals ($p > 0.05$). Both the saline-treated and VGB-treated animals had impaired memory performance compared to unstimulated control animals ($p < 0.001$).

5.5 Efficacy of antiepileptic drugs against spontaneous seizures (V)

The effect of four antiepileptic drugs, including CBZ, VPA, ESM, and VGB, against spontaneous seizures were studied. The results indicated that VPA, LTG, and VGB were the most effective compounds investigated, decreasing the mean seizure frequency by 83%, 84%, and 60%, respectively. In the VPA group, the percentage of rats with a greater than 50% decrease in seizure frequency was 100%, in the LTG group 88%, and in the VGB group 83%. In the CBZ group, 29% of animals had a more than 50% decrease in seizure frequency and, finally, in the ESM group, 38% of the animals had a more than 50% decrease in seizure frequency compared to baseline (no drug treatment). Two animals were seizure free, 20% of VPA-treated animals ($n=1$) and 14% of CBZ-treated animals ($n=1$). With other treatments seizure free animals were not detected. In total, only 5% of the rats became seizure free with any of the treatments.

VPA, CBZ, and ESM were the only drugs that reduced seizure duration. In the VPA-treated group, seizure duration was reduced by 61% (from 46 s to 18 s), in CBZ group 41% (from 56 s to 33 s), and in the ESM group 28% (from 57 s to 41 s; all $p < 0.05$).

6. DISCUSSION

The main aim of this thesis work was to develop an experimental animal epilepsy model that mimics human TLE with different aspects of epileptic process including initial precipitating insult, epileptogenesis, and finally spontaneous recurrent seizures. The newly developed model was used to elucidate whether epileptic animals express neuropathology similar to human TLE patients and whether sprouting of granule cell axons contribute to the development of epilepsy. Finally, the effects of different AEDs on epileptogenesis and spontaneous seizures in this model were tested.

The new model was based on SE induced by electrical stimulation of the lateral nucleus of the amygdala. SE further triggered epileptogenesis and lead to the development of spontaneous recurrent seizures within approximately 1 month. Pathological findings resembled those in human patients, epileptic animals had neuronal damage in the hippocampus and dense mossy fiber sprouting in the dentate gyrus. The mossy fiber sprouting did not, however, predict the severity of epilepsy. There was, however, a strong correlation between the hilar neuronal damage and mossy fiber sprouting.

Vigabatrin treatment started 2 days after SE and continued for 10 weeks thereafter did not prevent the development of epilepsy in rats. VGB treatment also did not have disease-modifying or neuroprotective effects. Although the initial insult was milder, LTG treatment started 2 h after SE induction, did not have any antiepileptogenic or neuroprotective effects.

Valproic acid (VPA), lamotrigine (LTG), and vigabatrin (VGB) effectively stopped the spontaneous seizures in the present model. These AEDs are known to be effective also in human TLE patients.

6.1 Methodologic considerations

In this thesis work, we used only SE (as an initial insult) that was induced by electrically stimulating the lateral nucleus of the amygdala for 20 to 40 min. Electrical stimulation-induced SE was chosen to avoid the toxic effects associated with chemical convulsants.

The amygdala was chosen as the stimulation site because it has a low threshold for electrically induced seizures (Mohapel et al., 1996). Stimulation was directed especially to the lateral nucleus of the amygdala because the lateral nucleus has the most widespread projections to the other amygdaloid nuclei, which facilitates the contribution of the other amygdaloid nuclei to seizure activity. In addition, in TLE patients, the amygdala is the site of seizure origin in 13% of patients with hippocampal atrophy and in 47% of patients with amygdala and hippocampal

atrophy (Spanedda et al., 1997). Consistent with human studies, Bertram (1997) reported that in a hippocampal electrical stimulation SE model of epilepsy, 59% of spontaneous seizures begin diffusely (hippocampus, amygdala), and 25% of seizures have their initial onset in the amygdala.

In this thesis work, we used long-term continuous (24 h/d) video-EEG recordings for seizure detection. There are several studies in which adequate follow-up of seizures have not performed. In the majority of studies, only video- or limited combined video-EEG-monitoring have performed (i.e., few hours per day or few days, instead of intensive recording for several weeks or months) (Leite et al., 1995; Priel et al., 1996; Longo et al., 1998). Without continuous video/EEG monitoring over several weeks or months it is not possible to judge whether rats without observed spontaneous seizures have developed epilepsy. The absence of observed seizures during extensive EEG/video-monitoring in a portion of the rats with SE might at least indicate that these animals represent a subgroup of rats with less progressive epilepsy compared to rats with observed seizures. Similar to other chronic animal models based on the induction of convulsive SE, the electrical stimulation model of the lateral nucleus of the amygdala shows considerable variability in regard to the onset, frequency, and severity of the spontaneous seizures (Gliem et al., 2002; Brandt et al., 2004; Dudek et al., 2006). There is also, however, considerable variability with respect to the seizures and neuropathology in human TLE patients (Wieser et al., 2004).

In all studies, we used chronically implanted electrodes that were made of stainless steel with nylon insulation. There is some evidence that chronic electrode implantation induces epileptiform field potentials (Niespodiaziany et al., 1999), which predisposes the brain to kindling by reducing the afterdischarge threshold (Löscher et al., 1995), and reduces local GABA turnover (Löscher et al., 1999). This effect might progress with increasing duration of implantation (Niespodiaziany et al., 1999). We did not detect spontaneous seizures, however, in sham-operated animals with chronically implanted electrodes.

The dose of each AED (**studies III-V**) was chosen based on previous reports from other experimental models in which the compound was shown to protect rats from fully kindled and/or spontaneous seizures and clinical trials in which antiepileptic effects were demonstrated (Cereghino, J.J. 1975; Leite and Cavalheiro, 1995; Halonen, et al., 1995; Petroff et al., 1995; Pitkänen et al., 1996; Otsuki et al., 1998; Halonen et al., 1999; Löscher et al., 2002; Stratton et al., 2003; Schwabe et al., 2004). According to literature and other experimental studies the VGB dose of 75 mg/kg/day increases brain GABA levels 2.3-fold (Pitkänen et al. 1999), whereas VGB 250 mg/kg/day increases GABA levels up to 3.1-fold (Halonen et al., 1991). This

data is consistent with data on drug-refractory patients (Petroff et al., 1995). The experimental protocol used in **study V**, however, did not allow us to measure AED levels in the brain or plasma. Therefore, it is not possible to directly compare our results with human data due to the fact that the pharmacokinetics of AEDs differ considerably between rats and humans (Löscher, 2002). For example, rodents eliminate AEDs more rapidly than humans; therefore, the doses that have to be administered to reach appropriate brain concentrations in rats are markedly higher than respective doses in humans (Löscher, 2002).

6.2 Clinical picture of seizures

6.2.1 Human data

In the present series of experiments, brain damaging insult was triggered with electrical stimulation of amygdala, which led to the development of SE, culminating in spontaneous seizures in all animals with SE after a latent period of 1 month. Consistent with human data from symptomatic epilepsy seizures, in the present model seizures develop after a sequence of events (i.e., initial insult followed by a latent phase) that are known to be associated with other brain damaging insults such as stroke and head trauma (Mathern et al., 1993).

A review of the literature revealed that the risk in humans to develop subsequent unprovoked seizure after SE varies depending on the study from 37% within 1 year and 56% within 3 years (Hauser et al., 1990). Consistent with previous studies, Hesdorffer and coworkers (1998) indicated that at the 10 year follow-up, the risk of unprovoked seizure was 41% for those with acute symptomatic seizure with SE. This is quite different compared to the present study (**study I**), in which 87% of stimulated animals developed subsequent seizures. The difference might be due to the fact that animals were not treated with drugs to stop SE and were allowed to recover spontaneously after SE. SE with long duration has been associated with poor outcome (defined as severe neurologic disability or death) (Lowenstein, 1999). There is evidence that if an average duration of SE is less than 2.4 hours (median 1 hour) the outcome is relatively good, whereas a substantially longer average duration (approximately 11 hours) of SE has a poorer outcome (Lowenstein, 1999). In addition, DeLorenzo and coworkers (1995) reported 22000 deaths following to 102000 SE cases per year leading to the mortality rate of 20%. The mortality rate of 20% among the stimulated animals is consistent with the human data.

Although it is difficult to specify the seizure duration that constitutes SE, the duration of a typical isolated seizure is well documented. Theodore and colleagues (1994) studied 120 secondarily generalized tonic-clonic seizures in adults with video-EEG telemetry. The average duration of seizures was 62 seconds, and they rarely exceeded 2 min. Results of our study are

consistent with that because the duration of partial and generalized seizures were 44 seconds and 61 seconds, respectively.

There are only a few studies regarding the change in seizure phenotype over the time in patients with partial onset seizures. The seizure phenotype may evolve over time in human patients. Tinuper and colleagues (1996) demonstrated that in the majority (84%) of patients with partial seizure onset secondarily generalized seizures disappeared during the course of disease at the age of 50. In 38% of patients, seizures became progressively less elaborate. In 55% of patients, however, the seizure parameters remained unchanged. Only in 8% of patients did the seizures worsen and become secondarily generalized. Our results are consistent with human studies since partial seizures became shorter over time. In addition, the behavioral seizure type changed from generalized to partial, especially in animals with frequent seizures.

Clinical studies indicate that seizures might have preferred daily times of occurrence (Shouse et al., 1996). In many forms of human epilepsy, seizures commonly occur during sleep or arousal or are more likely to occur during quiescent states (e.g., relaxed). Review of the clinical data revealed that 60% of partial seizures occurred between 07:00-19:00 (Quigg et al., 1998). Among patients with mesial TLE, the peak incidence of seizures occurred at 15:00. Our results are consistent with human data because the peak incidence of seizures in our animals occurs at 17:00. Interestingly, both human TLE patients and animal subjects demonstrated similar temporal distributions of seizures, that is, both groups had a peak incidence in late afternoon, and both had significantly more seizures during light than dark period. Rats are, however, nocturnal animals and have a sleep/wake cycle that is the opposite with those of diurnal humans. Therefore, it is possible that the sleep/wake cycle alone does not explain the similarities between the human and animal data (Quigg et al., 1998).

6.2.2 Comparison of seizure parameters with other experimental TLE models

In the present model, all animals with SE induced by electrical stimulation of the amygdala developed spontaneous seizures within 6 months follow-up, which is consistent with the percentage of animals developing epilepsy after different brain damaging insults. The percentage of animals with epilepsy in the post-KA model varies from 20% to 97% (Cavalheiro et al. 1983; Hellier et al. 1998), 20% to 100% in the post-PILO model (depending on the PILO dose used to induce SE), 50% to 100% after electrical stimulation of the hippocampus (Shirasaka and Wasterlain, 1994; Bertram and Cornett, 1994), 100% in the angular bundle stimulation model (Gorter et al., 2001), and from 10 % to 50% in different models of over-kindled animals (Michael et al. 1998; Potschka, 2000).

The latency to the first seizure seems to be comparable between experimental models. In the post-KA model, the latency varies from 8 to 84 days (Stafstrom et al., 1992), in post-PILO model it varies from 5 to 75 days (Mello et al., 1993; Glien et al., 2001), and after post electrical stimulation models it varies from 21 to 49 days (Shirasaka and Wasterlain, 1994; Brandt et al., 2003). These results suggest that the latency period required for epileptogenesis does not depend on the model used.

Also the duration of seizures is rather similar between models. The average seizure duration in our model was 49 seconds. In post-KA and post-PILO models the seizures last approximately 40 seconds (Leite et. al, 1990; Stafstrom et al., 1992), in electrical stimulation model of hippocampus seizures are longer ranging from 64 to 106 sec. (Bertram and Cornett, 1994). In the electrical stimulation model of the angular bundle, Mazarati et al. (1998) reported that seizures last for up to 120 seconds. In the post-PILO model Leite and coworkers (1990) and Arida and associates (1999) reported that the seizure duration does not change significantly over time. This is contrast to our results because the duration of partial seizures especially in animals with frequent seizures became shorter over time. Taken together, seizure duration between several experimental seizure models (post-SE models) is rather similar.

Behavioral seizure severity in our model changed over time from generalized to more partial seizures, especially in animals with frequent seizures. Consistent with our results, Gorter and coworkers (2001) reported similar changes in behavioral seizure severity from stage 4 or 5 behavioral seizures to stage 2 or 3 seizures 2 months after SE induced by electrical stimulation of the angular bundle. Seizures in post-KA and post-PILO models, however, resemble mostly stage 5 seizures and they do not change over time (Turski et al., 1983). Consistent with our results, behavioral seizure severity gradually increased over time in the hippocampal stimulation model (Bertram and Cornett, 1994). During the first 4 weeks, however, seizures were mainly partial, and after 12 weeks they tended to be more generalized.

The diurnal occurrence of seizures is also rather similar between different experimental models. In all models seizures tended to occur during the day (lights on period) compared to the night. In the hippocampal stimulation model, 63% to 67% of seizures occurred between 07:00-19:00 (Bertram and Cornett, 1994; Quigg et al., 1998), which is similar to post-PILO (64%)(Arida et al., 1999) and post-KA (64%)(Hellier et al., 1999) models. Our results are consistent with those demonstrating 57% of seizures during light period. In a post-KA model, Hellier and coworkers (1999) reported that 3 months after systemic injection of low dose KA, 64% of seizures were observed during the light period (between 07:00-19:00) and a similar effect of the light-dark cycle on seizure occurrence was observed in 60% animals that were

treated with KA 4 months earlier. They stated that the occurrence of spontaneous motor seizures depends primarily on the activity state of the animal, because 82% of seizures occurred during inactivity compared to an active state (18%). We cannot comment on the activity state of our animals because we did not analyze it at the time of seizure onset.

6.3 Neuropathology

6.3.1 Neuronal damage caused by the epilepsy

In the present series of studies, neuronal damage in the hippocampus was already evident at the time of the epilepsy diagnosis, which correlated with the degree of mossy fiber sprouting. Furthermore, there was a selective loss of hilar somatostatin immunoreactive neurons already at 10 weeks after the induction of SE and it became more severe over the time. After 6 months, bilateral damage, which was not dependent on the severity of epilepsy, was evident in all epileptic animals. VGB started 2 days after SE induction and LTG started 2 hours after SE induction did not alleviate neuronal damage in the hippocampus.

Hippocampal sclerosis is the most common neuropathologic finding in patients with medically refractory TLE. It is estimated that 60% to 75% of patients undergoing surgical treatment for TLE have hippocampal sclerosis (Babb and Pretorius, 1993). Neuronal damage also occurs in the amygdaloid complex (Kälviäinen et al., 1997) and in the cortical areas such as the entorhinal and perirhinal cortices (Salmenperä et al., 2000). The concern in epilepsy research has long been whether the damage is already present at the time of a epilepsy diagnosis, or whether it appears later in life as a consequence of recurring seizures. Clinical studies have shown that after SE progressive volume reduction of hippocampus does not exist within 1 year after SE, if patients are treated intensively for cessation of seizure activity (Salmenperä et al., 2000). However, in newly diagnosed focal hippocampal damage is already evident at the time of epilepsy diagnosis if assessed by MRI (Salmenperä et al., 2005) and it increases over time especially in patients with frequent seizures. There are, however, a limited number of studies comparing seizure disorder and the occurrence of neuronal damage. It is not known how many seizures are needed to produce hippocampal damage and how severe seizures cause neuronal loss. Our results indicated that there is a 21% to 38% decrease in hilar neuron number at the time of epilepsy diagnosis. Consistent with our data 22% neuronal loss was found in kindled rats already after 3 generalized tonic-clonic seizures. It became more severe after 150 seizures. Neuronal loss was evident in the CA1 and CA3 regions of the hippocampus and entorhinal cortex (Cavazos et al., 1994). Human studies have indicated that it might take 20

years and over 6500 seizures to cause 50% reduction in hippocampal volume (Kälviäinen et al., 1997; Salmenperä et al., 1998).

In the present studies, neuronal loss was assessed analysing the total number of neurons in the hilus and measuring the total neuronal density in the septal and temporal hilus. This approach has limited sensitivity, however, for detecting a minor loss of a subpopulation of vulnerable neurons (Tuunanen et al., 1997; Pitkänen et al., 2000). Consistent with previous studies using different epilepsy models, there was a clear loss of neurons in different locations in the temporal lobe in the majority of the epileptic animals. These findings are also consistently observed in KA- (Stafstrom et al., 1992), PILO- (Mello et al., 1993; Fujikawa, 1996), and hippocampal electrical stimulation models (Bertram, 1997; Mathern et al., 1997). Damage to the different amygdaloid nuclei is similar to that observed in the KA model (Tuunanen et al., 1996).

6.3.2 Mossy fiber sprouting

Mossy fiber sprouting is associated with TLE in humans (Hauser et. al., 1990; Houser, 1992; Mathern et al., 1997), and numerous animal models of experimental epilepsy (Tauck et al., 1985; Chronin and Dudek, 1988), and it has been suggested to be a candidate in seizure progression. Anatomical and physiological studies have documented that sprouted mossy fiber collaterals synapse predominantly onto granule cells (Kotti et al., 1997; Buckmaster et al., 2002), suggesting that granule cells form synapses between themselves and thus create an aberrant recurrent excitatory network. Our data, however, do not support the hypothesis that mossy fiber sprouting is related to progression of spontaneous seizures since part of the animals developed spontaneous seizures without mossy fiber sprouting. Our data is also consistent with the previous observations by Gorter and coworkers (2001) who demonstrated in angular bundle stimulation model that increased mossy fiber sprouting did not lead to an increase in seizure frequency. Interestingly, mossy fiber sprouting in the temporal end of the hippocampus was correlated with seizure frequency in newly diagnosed animals. The correlation was not significant if mossy fiber sprouting was assessed 60 days after the SE induction. Each of these changes, however, could contribute to altered circuitry within the dentate gyrus of humans with TLE and such alterations could influence seizure susceptibility within the hippocampal formation.

6.4 Drug treatment

6.4.1 Prevention of epileptogenesis

The current treatment of epilepsy is mainly focused on AED-induced seizure suppression after epilepsy has developed. Altering the underlying epileptogenic process in order to modify the course and prognosis of epilepsy would, however, be more beneficial for the patients leading to long-term outcome and better quality-of life.

A number of AEDs have been evaluated in preclinical (Löscher, 2002; Pitkänen and Kubova, 2004) and clinical trials (Tempkin, 2001) to test whether they would prevent epileptogenesis. But to date no drug has been shown to be effective in such trials. We tested the antiepileptogenic effects of two commonly used AED on our new model.

In this setting, VGB and LTG which have very similar clinical efficacies but completely different mechanisms of action did not have antiepileptogenic or disease-modifying effects if treatment was started after the induction of SE.

VGB is a second generation AED which is widely used for patients with partial onset seizures and infantile spasms (Chadwick et al., 1999). It increases brain GABA levels by inhibiting GABA transaminase (Petroff et al., 1996) thus decreasing the vulnerability to seizures. VGB may also decrease the concentrations of excitation-related amino acids in the brain, such as aspartate, glutamate and glutamine (Halonen et al., 1990; Petroff et al., 1995). VGB also has anticonvulsive properties in a kindling model (Schwabe et al., 2004) and KA-model of epilepsy (Halonen et al., 1995). After perforant pathway stimulation, VGB protected neurons from seizure induced damage in rat models of SE (Halonen et al., 1995; Pitkänen et al., 1996). Compensatory therapies for the defective GABAergic circuitries (death of specific somatostatin-containing GABAergic neurons) started 2 days after SE induction did not prevent epileptogenesis. It is possible that the time period selected for initiating treatment was not optimal in this study, because in our model extensive neuronal damage is apparent as early as 24 h after the induction of SE (Lukasiuk and Pitkänen, 1998). This is not likely, however, because André and coworkers (2001) used a 3-fold higher VGB dose that was started 10 min after PILO injection and continued for 45 days, but did not prevent the development of epilepsy despite marked neuroprotection in CA3, CA1, and in the hilar regions of the dentate gyrus. Therefore, it is evident that neuroprotection of principal cells in the dentate gyrus is not sufficient to prevent epileptogenesis. In line with our study, however, it is possible that areas which are not protected by VGB, such as piriform cortex and entorhinal cortex are responsible for seizure generation (Andre et al., 2001).

On the other hand, LTG, which does not affect presynaptic GABA release but attenuates the presynaptic release of glutamate by suppressing voltage-dependent sodium influx did not have any antiepileptogenic or disease-modifying effects. LTG suppresses partial seizures as well as generalized seizures, similar to phenytoin or CBZ, and it is also effective in the treatment of absence seizures (Coppola et al., 2004). LTG has previously been demonstrated to have anticonvulsive properties in experimental models of epilepsy, including amygdala- and hippocampal kindling (Otsuki et al., 1998; Stratton et al., 2003).

Our results demonstrated that LTG reduced the severity of SE by reducing the number of high amplitude and frequency discharges, but it did not completely block the seizure activity. Although LTG could reduce SE severity, it could not prevent induction of epileptogenesis. The behavioral outcome of seizures was attenuated compared to controls, thus indicating that LTG has antiepileptic effects in the present model. In contrast to our results, phenytoin, another Na⁺-channel blocker, is ineffective in suppressing SE at 30 min or 2.5 h after the induction of SE in a hippocampal stimulation model of epilepsy (Prasad et al., 2002). However, Walton and Treiman (1988) reported that phenytoin administered during early discrete seizures prevents secondary generalization of seizures; however, generalized electrographic ictal activity without behavioral symptoms continued.

Experimental studies have previously implicated that early treatment of SE may have both antiepileptic and disease-modifying effects. It is known, that the longer the SE is allowed to last before drug treatment is initiated, the more difficult it is to stop seizure activity (Klitgaard et al. 2002; Prasad et al., 2002; Pitkänen et al. 2005). Similarly, treatment with MK-801 (NMDA-receptor blocker) started immediately after SE induction efficiently reduced the damage in limbic regions, but this neuroprotection did not prevent the development of epilepsy (Brandt et al., 2003). In contrast to previous studies VPA (600 mg/kg/d) has shown antiepileptogenic and neuroprotective effects in KA-model (Bolanos et al., 1998) if treatment was started 24 h after the SE induction.

One of the earliest studies by Lemos and Cavalheiro (1995) demonstrated that if SE was stopped 30 min after induction with a combination of diazepam (10 mg/kg) and phenobarbital (30 mg/kg), the animals did not express spontaneous seizures during continuous video monitoring for 120 days. If SE is allowed to last for a longer time (1, 2, or 6 hours), however, the animals develop spontaneous seizures and the latency to the first seizure become shorter; 14 days (1 hour), 38 days (2 hour), and 52 days (6 hour). Also, the daily seizure frequency was higher in animals with short latency to the first seizure, similar to our finding. Prasad et al. (2002) reported that treatment of SE with phenobarbital (80 mg/kg) or MK-801 (4

mg/kg) 1 or 2 hours after SE induction (electrical stimulation of hippocampus) lowered the percentage of animals that developed epilepsy. Similarly, diazepam treatment started 2 h after the SE induction by electrical stimulation of amygdala (20 mg/kg and 10 mg/kg 6 hours later) decreased the percentage of animals with epilepsy compared to the group of animals in which SE treatment was started 3 hours later (Pitkänen et al. (2005). On the other hand, pregabalin treatment (50 mg/kg) started 20 min after the PILO injection and continued until killed delayed the development of epilepsy from 22 ± 9 days to 39 ± 18.8 days (Andre et al., 2003). It also decreased neuronal damage in the CA1, CA3, and the hilus of the dentate gyrus, and in the piriform cortex.

The drug treatment started after the SE may also modify the outcome of seizures. Mazarati and coworkers (2002b) demonstrated that seizure frequency was lower in animals treated with fosphenytoin (50 mg/kg), MK-801 (0.5 mg/kg), levetiracetam (200 mg/kg) or felbamate (200 mg/kg) compared to untreated animals. Also, CBZ treatment (120 mg/kg/d) started 1 day after PILO-SE and continued for 56 days resulted in a 2.5-fold decrease in seizure frequency compared to control animals (Capella et al., 2002). It also had effect on seizure duration; seizures became shorter. Similarly, atipamezole (a selective α_2 -adrenoceptor antagonist) started 1 week after SE induction and continued for 9 weeks reduced the number of daily seizures in comparison with vehicle group.

Clinical trials in humans indicated that in epileptogenesis triggered by an initial precipitating insult, like SE, stroke, and head trauma, there are no clear data showing that acute treatment of these disorders decreases the risk of epileptogenesis and development of epilepsy (Tempkin et al., 1998). A review of 11 well-controlled prophylaxis studies of post-traumatic seizures has indicated that in only 3 of them (27%) lowered seizure rates were observed after phenobarbital/phenytoin treatment. If only late seizures were considered, however, slightly higher seizure rates with phenytoin than with placebo were found in 3 of 6 (50%) phenytoin studies (Tempkin et al., 1998). The results indicated that AEDs suppress seizures while therapy is ongoing, that is in line with our results, but do not reduce the later risk for development of epilepsy once AED medication is stopped (Tempkin, 2001).

According to clinical and animal data regarding the antiepileptogenic and disease modifying effects of AEDs are controversial. There is no AED available that could totally prevent the development of epilepsy. Therefore, new treatment strategies, and methodology related to assess the outcome should be developed.

6.4.2 Efficacy of CBZ, VPA, LTG, ESM, and VGB in treatment of seizures

In the present study, we investigated whether AEDs with a different mechanism of action (Kwan et al., 2001) that are used to control epileptic seizures in human TLE patients. We also studied, whether ESM that is used to treat absence type of seizures in humans, and therefore should not be effective against focal onset/primarily generalized seizures, has effect on spontaneous seizures observed in the developed model.

Our results indicated that the response to different AEDs varied between complete seizure control to an even more severe outcome, which is consistent with the clinical situation in patients with TLE. Clinical data with newly diagnosed epilepsy patients with focal onset seizures indicates that a more than 50% decrease in seizure frequency is achieved in 40% to 80% of patients depending on the drug used (French, 1999; Kosteljanetz et al., 1979; Sobaniec et al., 2005). In the present study, a more than 50% decrease in seizure frequency was observed in a majority of the animals treated with VPA, LTG, and VGB. The response to CBZ, however, was somehow unexpected because only 29% of the animals responded to CBZ and CBZ is one of the drugs used as a first choice of treatment in human TLE patients (Brodie et al., 1995; Bill et al., 1997). Interestingly, in ESM group seizure frequency decreased in 63% of rats, and from those rats more than 50% decrease in seizure frequency was observed in 60% of the animals. Seizure duration was shortened by treatment with CBZ (from 56 s to 33 s), VPA (from 46 s to 18 s), and ESM (from 57 s to 41 s).

There are only a few previous studies that have addressed the efficacy of different AEDs against spontaneous seizures in experimental rat models of epilepsy (Leite and Cavalheiro, 1985; Glien et al., 2002, Brandt et al., 2002). In the pilocarpine model complete seizure remission was achieved in 38% of animals treated with CBZ (120 mg/kg/d) and in 75% of animals treated with VPA (600 mg/kg) (Leite and Cavalheiro, 1995). Moreover, the remaining animals also achieved good seizure control. In contrast to our results, they did not detect any major interindividual variation in seizure number between the animals. Further, Glien and coworkers (2002) demonstrated with levetiracetam and Brandt and associates (2004) with phenobarbital that 63% and 55% of treated animals benefit from treatment, respectively. Total remission of seizures, however, was achieved in only 25% and 50% of animals, respectively. Interestingly, 38% of levetiracetam and 10% of phenobarbital treated animals had an increased seizure number in response to drug treatment. The effect of ESM was somewhat unexpected, since we found decreased seizure frequency in 63% (5/8) of the animals. Because the remaining animals had substantially increased seizure frequency compared to baseline period, the group mean did not differ from baseline. Similar results have been found in study by

Leite and Cavalheiro (1995). Accordingly, our results are consistent with those of other studies regarding the effect of different drugs on spontaneous seizures. These results indicate that the pharmacologic efficacy of AEDs against spontaneous seizures in animal models of TLE (e.g., pilocarpine, electrical stimulation) is not model dependent.

High initial seizure frequency, varying from 0.43 to 56 seizures per day could explain why satisfactory seizure control could not be achieved. In fact, only in 7% of the animals, the initial seizure frequency was less than 1 seizure per day. Kwan and Sander (2005) reported that the factors for poor prognostic medical intractability include high initial seizure density, symptomatic etiology, and presence of structural cerebral abnormalities. Furthermore, the majority of seizures in untreated animals with frequent seizures are partial (stage 0-2) which are the most difficult to treat seizures in adults with epilepsy. Because we did not analyze the behavioral type of the seizure we cannot exclude the possibility that there were more partial seizures, especially in animals with frequent seizures and in those which did not respond to AED medication.

An important feature of medically intractable epilepsy is that most patients with drug-refractory epilepsy are resistant to several, if not all, AEDs, even though these drugs act by different mechanisms (Kwan et al., 2001; Sisodiya, 2003). Consistent to this, our study indicated that a majority of the animals did not reach a satisfactory level of seizure control, continuing to have seizures despite several drug treatments. Only two animals (one treated with CBZ and another with VPA) had a total remission of seizures.

Mechanisms underlying drug resistance might include alterations in the uptake of AEDs to the brain or targets of AEDs. Recent studies have suggested in rat but also in humans with TLE that multi-drug transporters (alteration in drug efflux) have an important role in the pharmacoresistance to AEDs (Sisodiya et al. 2002; Löscher and Potschka, 2002). Multi-drug transporters, like P-glycoprotein-1 located in capillary endothelial cells of the blood-brain barrier, restrict the brain uptake of many lipophilic drugs. This is a possible explanation for the fact that a majority of the animals with spontaneous seizures had a reduced response to various AEDs. These molecules have the ability to transport several AEDs, including phenytoin, CBZ (Rizzi, 2002), VPA (Huai-yun et al. 1998), and LTG (Potschka et al. 2002). Whether these mechanisms are involved in the response of rats to AEDs tested in the present study is not known. However, recent studies have demonstrated that P-glycoprotein is overexpressed after kainate-induced seizures in Wistar rats and C57BL/6 mice after both acute and chronic epileptic activity. (Zhang et al., 1999; Rizzi et al., 2002).

In addition, the AED targets, such as voltage-dependent ion-channels, transporters, or metabolic enzymes involved in release, uptake, and metabolism of neurotransmitters, might exhibit altered functions (Rogawski and Löscher, 2004) during epilepsy. Experimental data (Ellerkmann et al., 2003) suggest that changes in the expression of Na⁺-channel subunits (β 1 and β 2) might explain the altered pharmacosensitivity of Na⁺-channels. As mentioned above, other targets for AEDs, such as GABA-mediated inhibition, might be altered in intractable epilepsy. In epileptic rats, expression of the α 1 subunit decreases and expression of α 4 and δ subunit increases. This results in a higher sensitivity of GABA_A receptors to zinc (Coulter 2000) and therefore, decreased inhibition. In addition, GABA_A receptors might lose their sensitivity to augmentation by a benzodiazepine site 1 agonist, such as zolpidem (Cohen et al., 2003).

The experimental model developed in this thesis fulfills the three main characteristics of an ideal animal model. First, animals display reduced number of seizures when treated with antiepileptic drugs similar to human TLE patients (predictive validity). Second, animals developed epilepsy after an initial insult (symptomatic temporal lobe epilepsy) comparable to humans (face validity), and third, the mechanisms underlying epilepsy are rather similar compared to humans (constructive validity). Therefore, the experimental model developed in this thesis offers a new tool to study the efficacy of new and present AEDs against seizures in spontaneously seizing rats. Furthermore, model can be used to study antiepileptogenic and disease-modifying properties of compounds.

7. SUMMARY AND CONCLUSIONS

1. Electrical stimulation of the lateral nucleus of the amygdala triggered SE that lasted for several hours. Approximately 90% of animals developed spontaneous seizures. There was great variability, however, in the time to the first seizure and in seizure frequency between individual animals **(I)**.

2. Epileptic animals had histopathologic findings resembling those in human TLE patients. Neuronal damage in the amygdala, entorhinal, and piriform cortex was observed in the majority of the epileptic animals. Half of the epileptic animals also had damage in the hippocampus. Also, epileptic animals had variable levels of increased mossy fiber sprouting **(I,II)**.

3. Mossy fiber sprouting was present at the time of first spontaneous seizures. It did not, however, was associated with the severity of epilepsy. More dense mossy fiber sprouting indicates more severe hilar cell damage **(II)**.

4. The AEDs VGB and LTG did not prevent the development of epilepsy if treatment was started after the SE induction. They did not have disease modifying effects either **(III, IV)**.

5. VPA, LTG, and VGB were the most effective drugs against spontaneous seizures in the amygdala stimulation model of epilepsy. In all animals treated with VPA, LTG, and VGB, seizure frequency was decreased. Interestingly, CBZ, which is a drug of choice for human TLE patients, was not as effective as VPA, LTG, and VGB. Overall, these data suggest that rats with focal epilepsy respond to the same compounds that are used to treat seizures of focal onset in humans **(V)**.

In summary, the present studies provide new information regarding the chronic experimental epilepsy models and their usefulness in preclinical study protocols in designing new antiepileptogenic and antiepileptic compounds for the prevention and treatment of human TLE. Furthermore, these results extend our knowledge about mechanisms of SE, epileptogenesis and generation of spontaneous seizures that are potential targets for therapeutic interventions.

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APPENDIX

Original publications I-V

I

A new model of chronic temporal lobe epilepsy
induced by electrical stimulation of the amygdala

Nissinen J., Halonen T., Koivisto E., Pitkänen A.

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A new model of chronic temporal lobe epilepsy induced by electrical stimulation of the amygdala in rat

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Abstract

Spontaneous seizures are the hallmark of human epilepsy but they do not occur in most of the epilepsy models that are used to investigate the mechanisms of epilepsy or to test new antiepileptic compounds. This study was designed to develop a new focal epilepsy model that mimics different aspects of human temporal lobe epilepsy (TLE), including the occurrence of spontaneous seizures. Self-sustained status epilepticus (SSSE) lasting for 6–20 h was induced by a 20–30 min stimulation of the lateral nucleus of the amygdala (100 ms train of 1 ms, 60 Hz bipolar pulses, 400 μ A, every 0.5 s). Stimulated rats ($n = 16$) were monitored with a video-EEG recording system every other day (24 h/day) for 6 months, and every other video-EEG recording was analyzed. Spontaneous epileptic seizures (total number 3698) were detected in 13 of the 15 animals (88%) after a latency period of 6 to 85 days (median 33 days). Four animals (31%) had frequent (697–1317) seizures and 9 animals (69%) had occasional seizures (1–107) during the 6-months follow-up period. Fifty-seven percent of the seizures occurred during daytime (lights on 07:00–19:00 h). At the end of the follow-up period, epileptic animals demonstrated impaired spatial memory in the Morris water-maze. Histologic analysis indicated neuronal loss in the amygdala, hippocampus, and surrounding cortical areas, and mossy fiber sprouting in the dentate gyrus. The present data indicate that focal stimulation of the amygdala initiates a cascade of events that lead to the development of spontaneous seizures in rats. This model provides a new tool to better mimic different aspects of human TLE for investigation of the pathogenesis of TLE or the effects of new antiepileptic compounds on status epilepticus, epileptogenesis, and spontaneous seizures. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Animal model; Epileptogenesis; Seizure; Sprouting; Status epilepticus

1. Introduction

There are more than 100 seizure models currently available for epilepsy research (for review, see Fischer, 1989; Löscher, 1997). Seizures occur

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spontaneously in only a few of them. These models include the systemic or intracerebral injection of kainic acid (Chronin and Dudek, 1988; Mathern et al., 1993; Mascott et al., 1994; Hellier et al., 1998), systemic injection of pilocarpine (Priel et al., 1996), and electrical stimulation of the ventral hippocampus (Lothman et al., 1989; Bertram and Cornett, 1993; Mathern et al., 1997) or the angular bundle (Shirasaka and Wasterlain, 1994; Halonen et al., 1996). In these models, the induction of epileptogenesis is dependent on the occurrence and duration of self-sustained status epilepticus (SSSE). Electrographic seizures have also been described after brain ischemia (Rominj et al., 1994), cortical trauma (Prince and Jacobs, 1998), or after a large number of kindling stimuli (Hiyoshi et al., 1993).

There are multiple advantages of epilepsy models with spontaneous seizures. For example, in models of temporal lobe epilepsy (TLE) all phases of the epileptic process that are typical to human symptomatic TLE (initial insult → epileptogenesis → generation of spontaneous seizures) can be investigated. Also, many pathologic findings in spontaneously-seizing animals resemble the appearance of structural damage in human drug-refractory symptomatic TLE (Liu et al., 1994). The few details known regarding the seizure symptomatology in these models suggests that spontaneous limbic seizures in rats are broadly similar to those in human TLE (Bertram, 1997). Finally, a study using the pilocarpine epilepsy model indicates that the effect of antiepileptic drugs on spontaneous seizures predicts their effect on seizures in patients with TLE (Leite and Cavalheiro, 1995).

Recently, it has become evident that the period after epileptogenic insult such as status epilepticus is not 'silent' but includes, for example, activation of over 1000 genes (Nedivi et al., 1993; Rafiki, et al., 1998), acute and delayed neuronal damage (Magloczky and Freund, 1995; Fujikawa et al., 1998), and axonal (Mello et al., 1993) and dendritic (Spigelman et al., 1998) plasticity. There is a growing interest in the molecular and network changes associated with various initial insults and consequent epileptogenesis (Morrell and DeToledo-Morrell, 1999; Salazar and Ellenbogen, 1999). This interest has been further fueled by the

challenging goal of preventing epilepsy in subjects that are at elevated risk of developing epilepsy later in life after brain insults, such as status epilepticus, head trauma, stroke, prolonged complex febrile seizures, or brain infection (Hernandez and Naritoku, 1997; Pitkänen and Halonen, 1998). Achievement of these goals, however, will require appropriate modeling of different phases of the human epileptic process.

With the exception of genetic models, and the recently described perforant pathway stimulation model (Mazarati et al., 1998), the induction of SSSE includes an injection of a chemoconvulsant, or a long-lasting (> 1 h) or repeated electrical stimulation. The principal aim of the present study was to induce epileptogenesis culminating in spontaneous seizures by using a local manipulation of the brain that is nontoxic and is as short-lasting as possible. Here, we describe a new model of human TLE, in which epileptogenesis is induced by electrically stimulating the lateral nucleus of the amygdala for 20–30 min. After a latency period of approximately 1 month, animals express spontaneous seizures that continue to occur for the rest of the animals life. The occurrence of spontaneous seizures was followed by continuous video-EEG monitoring system for 6 months. The results of the present study indicate that the symptomatology of spontaneous seizures, neuropathology, and behavioral impairment of these epileptic animals closely resembles that found in human TLE. Thus, this model provides a useful and novel tool with which to better investigate the mechanisms of status epilepticus, epileptogenesis, and spontaneous seizures, as well as their prevention.

2. Materials and methods

2.1. Animals

Adult male Harlan Sprague–Dawley rats ($n = 28$; 320–390 g) were used in this study. The rats were housed in individual cages in a controlled environment (constant temperature, $22 \pm 1^\circ\text{C}$, humidity 50–60%, lights on 07:00–19:00 h). Animals had free access to food and water. All animal procedures were conducted in accordance

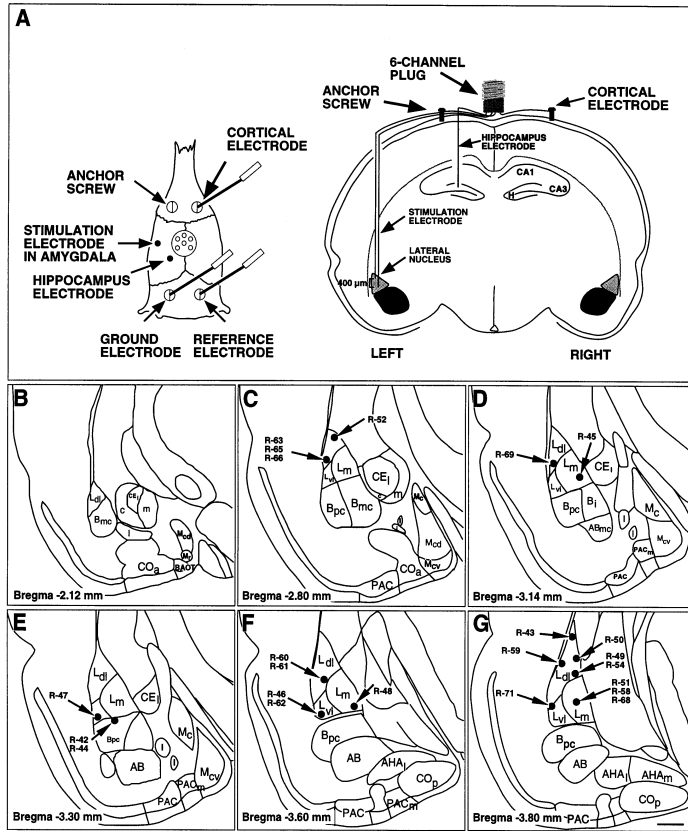


Fig. 1. (A) Schematic drawing illustrating the placement of stimulation and EEG-recording electrodes. A dorsal view of electrode locations is shown on the left and a coronal view on the right. A bipolar stimulation electrode was implanted into the lateral nucleus of the left amygdala. A monopolar electrode located in the ipsilateral hilus was used to record hippocampal seizure activity. A stainless steel screw was inserted in the skull over the contralateral frontal cortex to detect the generalization of electrographic seizure activity. (B–G) Line drawings of coronal sections from the rat amygdala arranged from rostral (B) to caudal (G) demonstrate the location of the tip of the stimulation electrode in the lateral nucleus of the amygdala of each rat included in the study. Rostrocaudal levels in relation to bregma are indicated for each section in the lower left-hand corner (according to Paxinos and Watson, 1986). Abbreviations: AB, accessory basal nucleus; AHA_l, amygdalohippocampal area, lateral division; AHA_m, amygdalohippocampal area, medial division; BAOT, bed nucleus of the accessory olfactory tract; B_i, basal nucleus, intermediate division; B_{mcc}, basal nucleus, magnocellular division; B_{pc}, basal nucleus, parvocellular division; CE_c, central nucleus, capsular division; CA1; CA1 subregion of the hippocampus; CA3; CA3 subregion of the hippocampus; CE_l, central nucleus, lateral division; CO_l, anterior cortical nucleus; CO_p, posterior cortical nucleus; H, hilus of the dentate gyrus; I, intercalated nucleus; L_{dl}, lateral nucleus, dorsolateral division; L_{ml}, lateral nucleus, medial division; L_{vl}, lateral nucleus, ventrolateral division; m, central nucleus, medial division; M_c, medial nucleus, caudal division; M_{cv}, medial nucleus, ventral portion of the central division; M_r, medial nucleus, rostral division; PAC, periamygdaloid cortex; PACm, periamygdaloid cortex, medial division. Scale bar = 500 μ m.

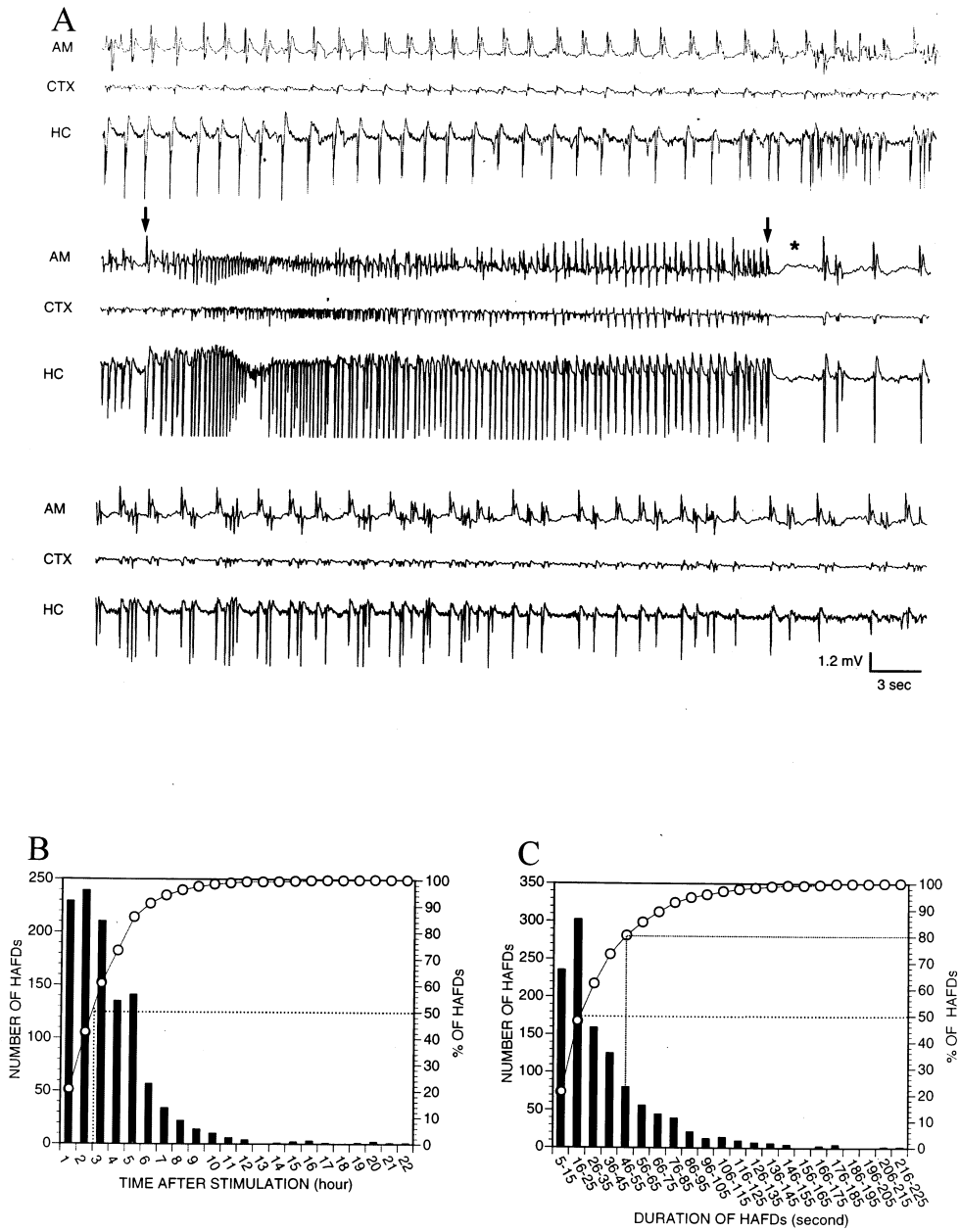


Fig. 2.

with the guidelines by the European Community Council Directives 86/609/EEC.

2.2. Implantation of stimulation and cortical EEG electrodes

For amygdala stimulation, a bipolar electrode (diameter 0.127 mm, dorsoventral distance between the tips 0.4 mm; Franco Corradi, Milano, Italy) was implanted into the lateral nucleus of the left amygdala; coordinates of the lower electrode tip, according to the rat brain atlas of Paxinos and Watson (1986) (3.6 mm posterior to bregma, 5.0 mm lateral to bregma) and 6.5 mm ventral to the surface of the brain, under deep sodium pentobarbital (60 mg/kg, i.p.) and chloral hydrate (100 mg/kg, i.p.) anaesthesia. The location of the electrodes was verified histologically at the end of the experiment, and only the animals with the correct location of the stimulation electrode in the lateral nucleus of the amygdala were included in this study (Fig. 1).

To record the spread of electrographic seizure activity to the ipsilateral hippocampus or the contralateral cortex, a bipolar electrode was implanted into the hilus (distance between the tips 0.8 mm; coordinates of the lower tip: 4.1 mm posterior, 2.6 mm lateral and 3.7 mm ventral to bregma) and a screw electrode (Plastics One Inc., Roanoke, VA) into the skull overlying the contralateral frontal cortex (coordinates: 3.0 mm anterior and 2.0 mm lateral to bregma). Two monopolar stainless steel screw-electrodes that were fixed to the skull symmetrically over the cerebellum with dental acrylate (Selectaplast CN, Dentsply DeTrey GmbH, Dreieich, Germany) served as ground and reference electrodes.

2.3. Afterdischarge threshold determination

To insure that the current used to stimulate the amygdala induced an afterdischarge (AD), the AD threshold of the amygdala was determined 1 week before the induction of SSSE (data not shown) and 0.5 to 1 h before the induction of status epilepticus. AD was defined as a high amplitude polyspike epileptiform activity in which at least five spikes could be identified. AD threshold was defined as the lowest intensity of current needed to elicit AD. AD duration was defined as the time period from the end of stimulus to the end of AD.

The stimulation for AD threshold determination consisted of a 1-s train of 1-ms biphasic square wave pulses delivered at 60 Hz using a A300 Pulsemaster Stimulator (WPI, Sarasota, FL, USA) connected to two A360 Constant Current Stimulus Isolators (WPI, Sarasota, FL, USA). To determine the amygdala AD threshold, rats were first stimulated at 50 μ A. If no AD was elicited, then the stimulus current was increased in 25- μ A increments to 100 μ A, then in 50- μ A increments. The interstimulus interval during the AD threshold determination was at least 1 min for all rats.

2.4. Induction and monitoring of SSSE by electrical stimulation of the amygdala

2.4.1. Stimulation paradigm and apparatus

Two weeks after surgery, the baseline EEG was recorded for at least 15 min from each rat. Thereafter, SSSE was induced by stimulating the lateral nucleus of the left amygdala for 20–30 min. The stimulation consisted of a 100-ms train of 1-ms

Fig. 2. A–C. (A) Electrographic recording from the ipsilateral amygdala (AM), contralateral cortex (CTX), and ipsilateral hippocampus (HC) during self-sustained status epilepticus demonstrating the high-amplitude and frequency discharge (HAFD, indicated with solid arrows) recorded 17 min after the end of 20-min amygdala stimulation. Three successive 50-s EEG epochs are shown in the upper, mid, and lower portions of the figure. The end of HAFDs was typically followed by brief (1–3 s) ‘flat periods’ on the EEG (asterisk). (B) Number of HAFDs (left y-axis) as a function of time after the end of amygdala stimulation. Altogether, 1122 HAFDs were included in the analysis. The curve with open circles shows the cumulative percentage of HAFDs over time (right y-axis). Approximately 61% of the HAFDs occurred during the first 3 h after the beginning of SSSE. (C) Number of HAFDs with different durations (left y-axis) during the status epilepticus. The curve with open circles shows the cumulative percentage of HAFDs (right y-axis) with different durations. Fifty percent of the HAFDs lasted for less than 25 s, and 80% of the HAFDs lasted less than 55 s.

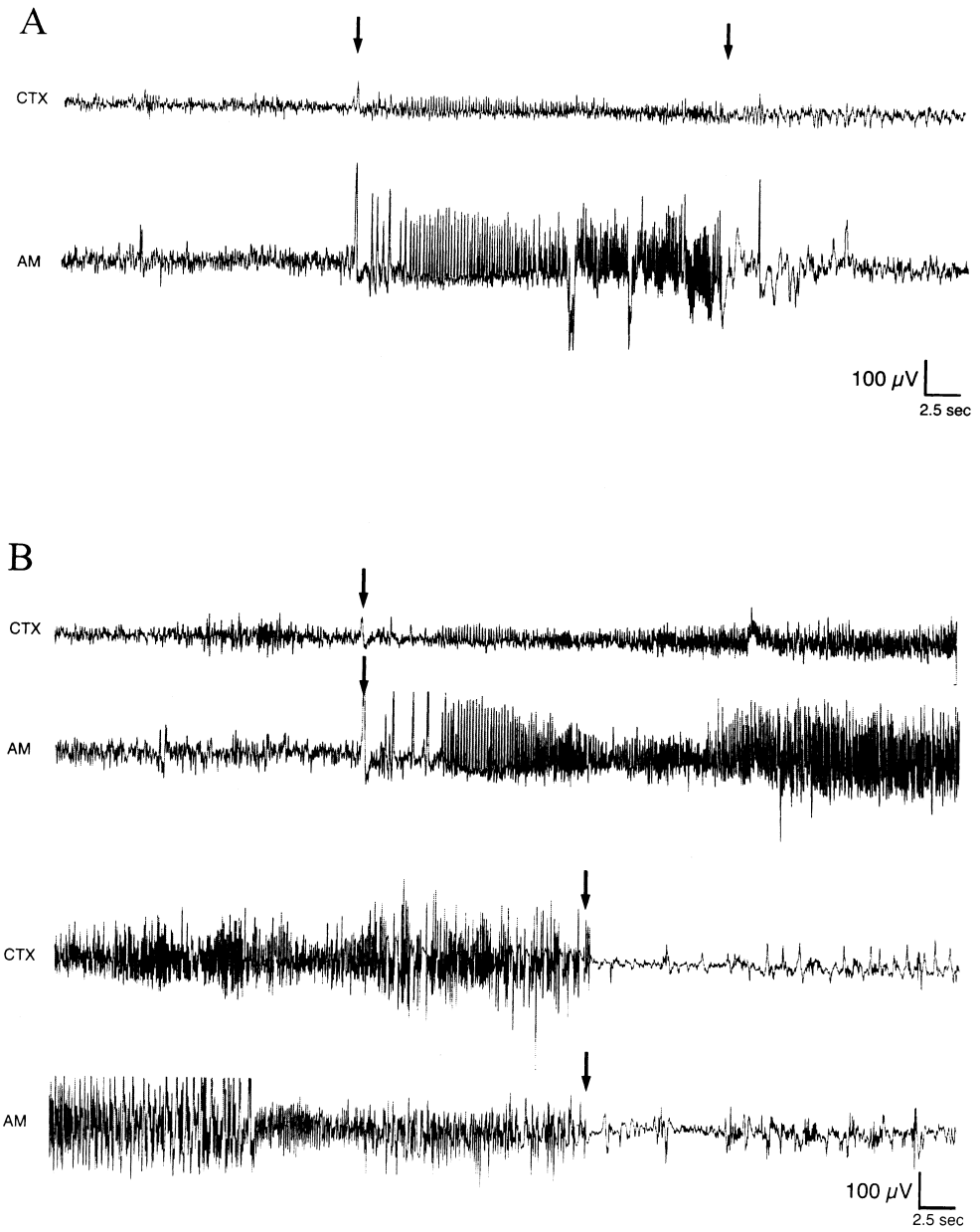


Fig. 3.

biphasic square wave pulses (400 μ A from peak to peak) delivered at 60 Hz every 0.5 s using A300 Pulsemaster Stimulator connected with two A360 Constant Current Stimulus Isolators, to which the animals were connected with a 6-channel commutator (Plastics One Inc.) and shielded cables. Development and duration of status epilepticus were monitored continuously for 48 h via amygdalar, hippocampal, and cortical electrodes using the Nervus EEG Recording System (Tautagreining, Iceland) connected with an ISO-1032 Amplifier (Braintronics, Netherlands), SVT-S3000P Hitachi Time Lapse 168 VCR (Japan), and Panasonic WV-CL350 Video Camera (Japan). The combined video-EEG monitoring system allowed simultaneous display of the EEG signal and video image on the screen.

2.4.2. Stimulation protocol

Each rat was stimulated continuously for 20 min. Thereafter, the stimulation was interrupted, and the behavioral and electrographic seizure activity of the animal was observed for 60 s. If the behavior of the animals revealed the presence of epileptic activity (head nodding/or limb clonus), the observation was continued for up to 5 min. If the animal did not meet the criterion of clonic status epilepticus (continuous electrographic epileptiform spiking and recurrent clonic seizures), stimulation was resumed and the behavior of the animal was checked again after 5 min. Once the criterion of SSSE was achieved, no further stimulation was given.

Altogether, 20 rats were stimulated. In 18 of these rats, the stimulation time was 20 min. Two rats, # R-43 and # R-60, were stimulated for 30 min. All that developed SSSE were included in the 6-months video-EEG follow-up. In

addition, eight electrode-implanted, unstimulated controls were included in the study. The control rats were video-EEG monitored at the end of study to investigate whether electrode implantation alone (and associated trauma) would induce epileptogenesis. These animals also served as controls for behavioral testing and histologic analysis.

2.5. Behavioral and electrographic monitoring of the appearance of spontaneous seizures

After amygdala stimulation, the animals were monitored for 48 h, and thereafter, every other day (24 h/d) with a combined video-EEG recording system for 6 months. EEG signals were recorded with a Stellate EEG Monitor System (sampling rate 200 Hz, high pass filter 1 Hz, and low pass filter 100 Hz), that was connected with two ISO-DAM 8 Amplifiers (WPI), to which the animals were connected with a 6-channel commutator and shielded cables. This system allowed the animals to move freely without twisting the cables. The behavior of the animals was recorded using a WV-BP312E Video Camera (Panasonic) that was positioned above the cages and connected with an SVT-S3000P Time Lapse 168 VCR (Sony) and PVC-145E Video Monitor (Sony). The video system was connected to the EEG recording system via a time code generator (MUL, TIM Electrode Inc.). Type 955 Infra Red Light (Videmech Ltd., Great Britain) was used at night to allow for video monitoring the animals behavior. A wide angle lens permitted video-taping of up to eight animals simultaneously. The manifestation of seizure activity was analyzed on DDS2-files and video tapes.

Fig. 3. (A) An example of a spontaneous seizure (between solid arrows) that was recorded 172 d after the induction of self-sustained status epilepticus in animal # R50. The seizure lasted for 25 s and was classified as partial (Score 1) based on behavioral manifestations that included mouth and facial clonus and head nodding. Electrographically, however, seizure activity was detected by both the ipsilateral amygdaloid and contralateral cortical electrodes. Moreover, seizure activity appeared in both electrodes simultaneously. (B) An example of spontaneous secondarily generalized seizure (Score 3; between solid arrows) in the same animal. The seizure occurred 30 min later than the partial seizure in panel A and lasted for 75 s. Video recording indicated that the animal showed mouth and facial clonus, head nodding, and bilateral forelimb clonus. Abbreviations: AM, amygdala, CTX, contralateral frontal cortex.

2.6. Analysis of EEG recordings and video tapes

2.6.1. Analysis of video-EEG recorded during status epilepticus

A conspicuous feature of EEG activity during status epilepticus was the occurrence of high-amplitude and frequency discharges (HAFDs), which were typically associated with behavioral seizures (Fig. 2A). The HAFD was defined as high-amplitude ($> 2x$ baseline) and a high-frequency (> 8 Hz), discharge in the amygdala or in the cortex (or both) that lasted for at least 5 s. As previously described by Treiman et al. (1990), the end of the electrographic seizure (HAFD) was marked by a brief (1–3 s) 'flat period' on the EEG (Fig. 2A).

2.6.2. Analysis of video-EEG recorded during epileptogenesis and established epilepsy

Each EEG file was analyzed manually by browsing the EEG on the computer screen. Although the manual analysis was laborious, manual analysis was performed instead of automatic analysis which detects only approximately 80% of the electrographic seizures (unpublished observation; see also Mascott et al., 1994).

If an electrographic seizure was observed, the behavioral severity was analyzed from the corresponding video-recording. An electrographic seizure was defined as a high-frequency (> 5 Hz), high-amplitude ($> 2x$ baseline) discharge either in amygdala or in the cortex (or both) that lasted for at least 5 s. The severity of behavioral seizures was scored according to a slightly modified Racine's scale (Racine, 1972). Score 0: electrographic seizure without any detectable motor manifestation; Score 1: mouth and face clonus, head nodding; Score 2: clonic jerks of one forelimb; Score 3: bilateral forelimb clonus; Score 4: forelimb clonus and rearing; Score 5: forelimb clonus with rearing and falling.

During the first 100 days, every video-EEG recording from each animal was analyzed to obtain a more reliable overview of the development of epilepsy and the frequency of spontaneous seizures during that time. Thereafter, every other video-EEG-file was analyzed to detect more spontaneous seizures.

2.7. Morris water-maze

Six months after the induction of status epilepticus, spatial learning and memory of the rats was tested in the Morris water-maze. Time between the last seizure and water maze testing was at least 1 h. If the animal had a seizure during testing, the test result of that day was rejected.

Testing was performed on 7 consecutive days. A circular plastic tank (120 cm diameter \times 75 cm high) was filled with water ($19 \pm 1^\circ\text{C}$). The pool was illuminated by spotlights. The daily test consisted of four 60-s trials. The location of the platform (10 \times 10 cm, onto which the rat could escape) was changed daily so that on days 1, 3, 5, and 7 the platform was in quadrant 1 and days 2, 4, and 6 in quadrant 3 (on the opposite side of the maze pool). After finding the platform, the rat was given 60 s to rest before the next trial. If a rat did not find the platform in 60 s, the animal was placed on the platform for 10 s. The escape latency, swimming speed, and the total swim path distance were measured.

2.8. Histology

2.8.1. Fixation and processing of tissue

The rats were perfused for histologic analysis immediately after finishing the Morris water-maze test. The animals were deeply anesthetized and perfused according to the following fixation protocol: 0.37% sulphide solution (30 ml/min) for 10 min followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (30 ml/min), $+ 4^\circ\text{C}$, for 10 min. The brains were removed from the skull and postfixed in buffered 4% paraformaldehyde for 4 h and then cryoprotected in a solution containing 20% glycerol in 0.02 M potassium phosphate buffered saline (KPBS) for 24 h. The brains were then blocked, frozen in dry ice and stored at -70°C until cut. The brains were sectioned in the coronal plane (50 μm , 1-in 4 series) with a sliding microtome. The sections were stored in a cryoprotectant tissue-collecting solution (TCS, 30% ethylene glycol, 25% glycerol in 0.05 M sodium phosphate buffer) at -20°C until processed. Adjacent series of sections were used for Nissl and Timm staining.

2.8.2. Nissl staining

To identify the cytoarchitectonic boundaries, the distribution and severity of neuronal damage and the location of stimulating electrode, the first series of 1-in-4 sections was stained for thionin. The severity of neuronal damage in various temporal lobe regions was scored as follows: Score 0 = no damage, Score 1 = less than 50% neuronal loss, Score 2 = greater than or equal to 50% neuronal loss.

2.8.3. Timm histochemistry

Synaptic reorganization (mossy fiber sprouting) was analyzed from sections stained with the Timm sulfide/silver method (Sloviter, 1982). For staining, all coronal sections (50 μ m, 1-in-4 series) where the hippocampus was present were mounted on gelatin-coated slides and dried at +37°C. Staining was performed in the dark according to the following procedure: working solution that contained gum arabic (300 g/l), sodium citrate buffer (25.5 g/l citric acid monohydrate and 23.4 g/l sodium citrate), hydroquinone (16.9 g/l), and silver nitrate (84.5 mg/l) were poured into the staining dish that contained the slides. The sections were developed until an appropriate staining intensity was attained (60–75 min). The slides were then rinsed in tap water for 30 min and placed in 5% solution of sodium thiosulfate for 12 min. Finally, sections were dehydrated through an ascending series of ethanol, cleared in xylene, and coverslipped with DePeX mounting medium (BDH, Laboratory Supplies, England).

Mossy fiber sprouting was analyzed along the septotemporal axis of the hippocampus. The septal end included the coronal sections between AP levels 2.3 and 6.0 mm posterior from bregma (Paxinos and Watson, 1986). The dorsal mid-portion and ventral mid-portion of the dentate gyrus included dorsal and ventral parts of the hippocampus where the granule cell layer of the septal and temporal ends becomes fused and forms an easily identifiable and standardized 'oval-shaped' layer (AP level 6.1–6.7 mm) posterior to bregma (see Fig. 10E,F). The density of mossy fiber sprouting was scored according to Cavazos et al. (1991): Score 0 = no granules,

Score 1 = sparse granules in the supragranular region and in the inner molecular layer, Score 2 = granules evenly distributed throughout the supragranular region and the inner molecular layer, Score 3 = almost a continuous band of granules in the supragranular region and inner molecular layer, Score 4 = continuous band of granules in the supragranular region and in the inner molecular layer, Score 5 = confluent and dense laminar band of granules that covers most of the inner molecular layer, in addition to the supragranular region.

Photomicrographs were taken with a Leica DM RB microscope equipped with a camera system. Low power photomicrographs were taken with a Nikon 6 \times 9 cm system.

2.9. Statistical analysis

Data were analyzed using SPSS for Windows (version 6.0.1) and Macintosh StatView 4.0 (non-FPU-version, Deneba Software, Berkeley, CA, USA). Changes in AD thresholds and seizure numbers between different animal groups were analyzed using the Mann–Whitney U-test. Morris water-maze data (latency) was evaluated with an analysis of covariance (ANCOVA), in which swimming speed was included as a covariate. Post-hoc analysis of the significance between different animal groups was carried out by Mann–Whitney U-test. Changes in seizure type and duration over time was evaluated using one way ANOVA. Changes in seizure frequency over time was analyzed using the Pearson Chi-Square-test. Changes in the proportions of seizure types between animal groups were evaluated using Mann–Whitney-U-test. A p value of less than 0.05 was considered statistically significant.

3. Results

A total of 28 rats were included in the study: eight unstimulated controls with electrodes implanted in the amygdala and 20 stimulated animals. Four of the 20 stimulated rats died within 24–48 h after the induction of status epilepticus.

In one of the stimulated animals, the stimulation electrode was located outside the amygdala, and therefore, the rat was excluded from the final analysis.

3.1. AD threshold and duration

3.1.1. AD threshold

At baseline, there were no differences in AD thresholds (mean \pm S.D.) between the control ($111 \pm 24 \mu\text{A}$) and epilepsy groups ($89 \pm 28 \mu\text{A}$, $P > 0.05$). Also, the AD thresholds in rats that developed frequent or rare seizures did not differ from each other at baseline (100 ± 41 and $84 \pm 22 \mu\text{A}$, respectively, $P > 0.05$).

3.1.2. AD duration

There were no differences in the AD duration between the control and epilepsy groups at baseline (20.1 ± 17.3 s vs 16.0 ± 9.8 s). Neither did the AD duration differ in rats that developed frequent seizures from that in rats with rare seizures (22.4 ± 8.9 s vs 13.2 ± 9.2 s).

3.2. Number and duration of HAFDs during SSSE

3.2.1. Appearance of HAFDs during SSSE

The mean number of HAFDs in epileptic animals during SSSE was 111 (range 70–154, median 106; Table 1). The first HAFD was observed within 5 min after the cessation of amygdala stimulation. Sixty-one percent of the HAFDs occurred during the first 3 h after the beginning of SSSE (Fig. 2B,C). The last HAFD occurred within 12 h in 6 of the 13 rats. Consequently, the duration of the SSSE (i.e. the time between the first and the last HAFD) varied between 330 and 1260 min (751 ± 363 min, median 607). There were no differences in the number of HAFDs between the animals that developed either frequent or rare seizures (Table 1). Also, the number of HAFDs did not correlate with the duration of the latency period before the appearance of the first spontaneous seizure ($r = 0.351$, $P = 0.442$ Pearson correlation coefficient; Fig. 4). The overall duration of SSSE did not differ between the rats with frequent or rare seizures.

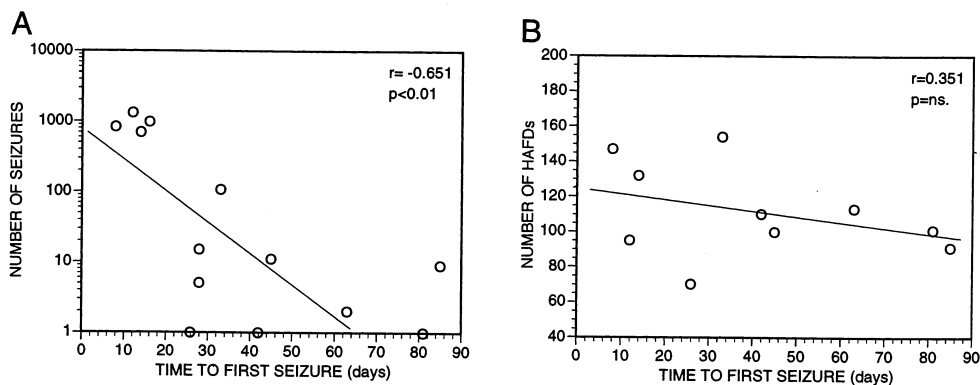


Fig. 4. A–B. (A) The relationship between the time to the appearance of the first spontaneous seizure after self-sustained status epilepticus (SSSE) and the number of spontaneous seizures that were recorded during the 6-month follow-up. Note that a short delay to the appearance of the first seizure predicts frequent spontaneous seizures. (B) Correlation between the time to first spontaneous seizure and the number of high amplitude and frequency discharges (HAFDs) during SSSE. Note that the severity of SSSE (number of HAFDs) did not predict the severity of epilepsy. Abbreviations: ns, not statistically significant; P , statistically significant (ANOVA); r , Pearson's correlation coefficient.

Table 1
Status epilepticus, epileptogenesis and spontaneous seizures in amygdala stimulated rats^a

Status epilepticus									
Rats with	HAFD			HAFD duration < 55 s		Total HAFD Duration (min)	Time to last HAFD (min)	Epileptogenesis	
	Number	Duration (s)	Range (s)	Number	% of all			Time to first seizure (days)	Seizure number (0–6 months)
Frequent seizures									
R47	–	–	–	–	–	–	–	16	975
R49	95	38 ± 30	6–172	77	81	60	674	12	1317
R50	147	27 ± 25	7–180	133	90	66	1260	8	827
R60	132	39 ± 28	10–127	112	85	85	435	6	697
Mean	128	34*	–	107	85	70	790	11*	954**
± S.D	27	28	–	28	5	13	425	4	267
Rare seizures									
R42	110	39 ± 33	6–154	82	75	72	540	42	1
R43	101	44 ± 31	7–128	69	68	75	500	81	1
R45	91	61 ± 43	9–306	52	57	92	1160	85	9
R46	–	–	–	–	–	–	–	28	5
R54	100	27 ± 30	5–151	90	90	45	330	45	11
R59	70	40 ± 36	5–185	61	87	46	1200	26	1
R66	154	35 ± 24	7–125	123	90	89	420	33	107
R69	–	–	–	–	–	–	–	28	15
R71	113	33 ± 21	5–117	97	86	62	990	63	2
Mean	106	39	–	82	78	68	737	48	17
± S.D	26	32	–	24	12	19	369	23	34
No spontaneous seizures									
R61	3	128 ± 23	100–144	0	0	6	20	0	0
R65	6	41 ± 48	9–137	5	83	4	30	0	0
Mean	5	69	–	5	83	5	25	0	0
± S.D	2	58	–	0	0	2	7	0	0

^a Abbreviations: HAFD, high amplitude and frequency discharge; S.D, standard deviation; no data available.

* $P < 0.05$.

** $P < 0.001$ compared to animals with rare number of seizures (Mann–Whitney U-test).

3.2.2. Duration of HAFDs

The distribution of the duration of HAFDs is shown in Fig. 4C. The mean duration of HAFDs was 37 ± 31 s (range 5–306 s, median 27). Eighty percent of HAFDs were shorter than 55 s in duration. There was no difference in the duration of HAFDs between the animals that developed frequent or rare seizures (Table 1).

3.3. Development and appearance of spontaneous seizures

Unless otherwise specified, the seizure number reported represents the number of seizures obtained by analyzing every other video-EEG recording during the 6-months follow-up period. Therefore, to estimate the total seizure number, the seizure number reported should be multiplied by four.

Spontaneous seizures were observed in 13 of 15 (88%) stimulated rats. From these 13 epileptic animals, 4 (31%) had frequent seizures (697–1317 seizures analyzed) and 9 (69%) had rare seizures (1–107 seizures analyzed) during the follow-up. Therefore, estimation of the total number of seizures in rats with frequent seizures varied between 2788 and 5268 and in rats with rare seizures between 4 and 428 during the 6-months follow-up period (Fig. 3).

To increase the sensitivity of detection of the time of appearance of the first spontaneous seizures in our animals, every video-EEG recording performed during the first 100 d (i.e. recordings from every other day) was analyzed. Based on that analysis, we recorded at least 2 spontaneous seizures from 13/15 animals. The latency from the SSSE to the appearance of the first spontaneous seizure varied between 6 and 85 days (mean 33, median 28). The latency to the appearance of the first seizures was shorter in animals that developed frequent seizures than in rats with rare seizures (11 ± 4 days and 48 ± 23 days, respectively, $P < 0.05$; Table 1). Once the seizures appeared, their frequency remained fairly constant during the last three follow-up months (one way ANOVA, $P > 0.05$; Fig. 5).

The behavioral appearance of the seizures changed during the 6-months follow-up period

(Pearson Chi-Square -test, $P < 0.001$). During the first 10 weeks, 79% of the seizures were second-

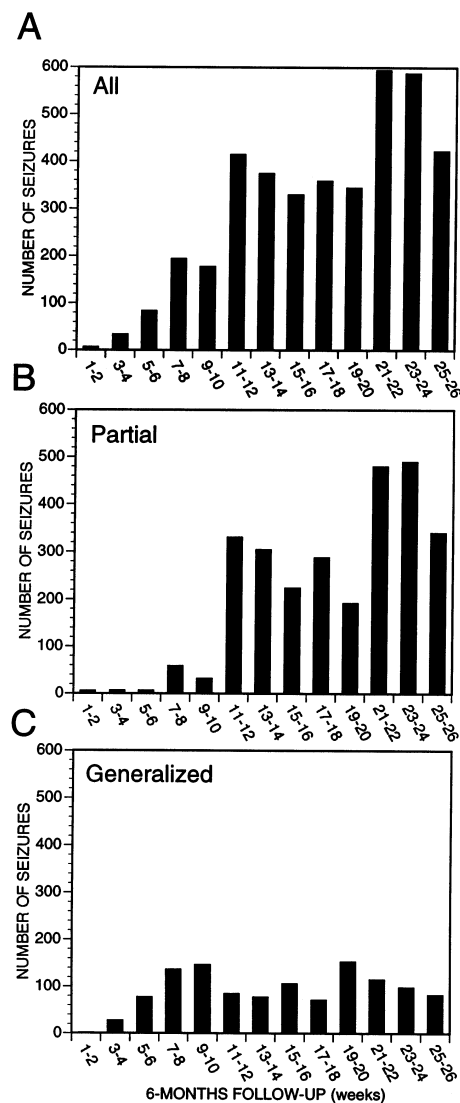


Fig. 5.

daily generalized (Score 3–5). Thereafter, most of the seizures (77%) were partial (behavioral seizure Score 0–2; Fig. 6A). There were no differences in the proportion of generalized seizures during the first 10 weeks between the rats with frequent or rare seizures (79% vs 70%, respectively; $P > 0.05$ Mann–Whitney U-test). Between the follow-up weeks 11 and 26, 80% of the seizures in the rats with frequent seizures were partial. Only 4% of the seizures in rats with rare seizures however, were partial (Fig. 7).

The mean duration of electrographic seizures assessed from EEG recordings was 49 ± 24 s (range 7–252, median 47). The mean duration of partial seizures (Score 0–2) was 44 ± 23 s (range 7–232, median 42). The mean duration of secondarily generalized seizures (Score 3–5) was 61 ± 23 s (range 16–252, median 59). The duration of secondarily generalized seizures stayed constant over the time. However, the duration of partial seizures shortened during the follow-up (one way ANOVA, $P < 0.001$; Fig. 6B).

Fifty-seven percent of the seizures appeared between 07:00 and 19:00 h (lights on) and 43% between 19:00 and 07:00 h (lights off; Fig. 8A) ($P = 0.5811$, one way ANOVA). When different seizure types were analyzed separately, 57% of the partial seizures and 56% of the secondarily generalized seizures appeared between 07:00 and 19:00 h (no difference compared to 19:00–07:00 h; $P > 0.05$, one way ANOVA) (Fig. 8B,C). There was no difference in the diurnal occurrence of seizures between the rats with rare or frequent seizures. That is, in animals with frequent seizures, 57% of the partial and 54% of the secondarily generalized seizures occurred during 07:00–19:00 h. In rats with rare seizures, 63% of the

Fig. 5. (A) Total number of spontaneous epileptic seizures recorded with video-EEG in all epileptic animals during the 6-mo follow-up divided into 2-week epochs. Seizures that appeared during handling were excluded ($n = 113$). The total number of seizures in the whole animal group increased up to 14 weeks. (B) Total number of partial seizures (Score 0–2) and (C) total number of generalized seizures (Score 3–5) during the 6-month follow-up period. Note that the progressive increase in seizure number during the first 14 weeks was because of an increase in the number of behaviorally partial seizures.

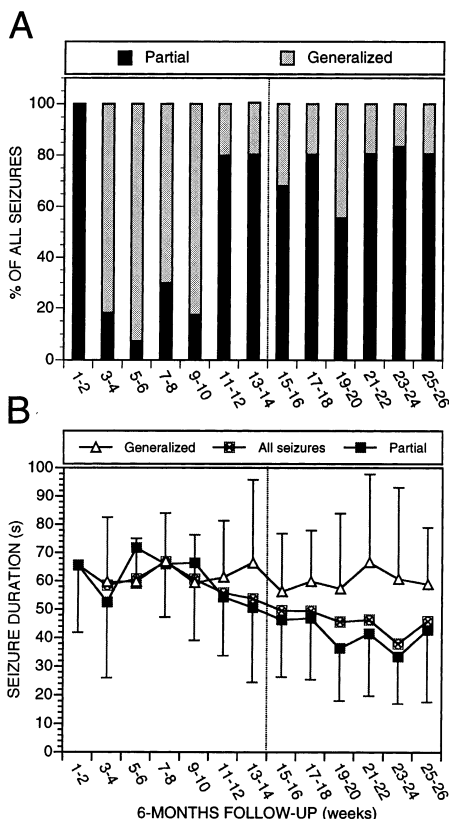


Fig. 6. (A) Proportion of partial and generalized seizures during the 6-mo follow-up period divided into 2-week epochs. During the first 10 weeks, most of the seizures were secondarily generalized, and thereafter, partial. The change in seizure type (partial vs generalized) during this time was statistically significant ($P < 0.001$, Pearson Chi-Square-test). Note that the bar demonstrating seizures during the first 1–2 weeks included data from three animals that did not have secondarily generalized seizures. (B) The mean duration of generalized seizures remained stable during the whole 6 month follow-up time ($P > 0.05$, one way -ANOVA). Data in both panels is based on the analysis of every other recorded video-EEG (total seizure number 3696).

partial and 69% of the secondarily generalized seizures occurred during 07:00–19:00 h. The peak seizure number appeared between 17:00 and 18:00 h.

To detect any independent seizure generation in the hippocampus, hippocampal EEG activity was recorded via hilar electrodes 3 to 5 months after the induction of SSSE. Altogether 268 seizures were recorded in seven animals (range of seizures recorded per animal was 3–80). Most of the seizures had a diffuse onset, arising from the amygdala and hippocampus simultaneously. In the recordings available, 20% of seizures appeared first in the amygdala and 9% in the hippocampus.

Control rats were video-EEG recorded for 24 h at the end of the 6-months follow-up period. Their EEG appeared normal.

3.4. Temporal lobe damage

The appearance of neuronal loss in thionin-stained preparations in various temporal lobe regions is summarized in Table 2. The amygdala was divided into various subregions according to Pitkänen et al. (1997a) and the hippocampal formation according to Amaral et al. (1995) and the entorhinal cortex according to Insausti et al. (1997). Typically, damage was equally severe ipsilaterally and contralaterally. The present study focused only on the temporal lobe damage. Substantial damage was also observed in other brain areas, including the thalamus (Nissinen, Halonen, and Pitkänen, in preparation).

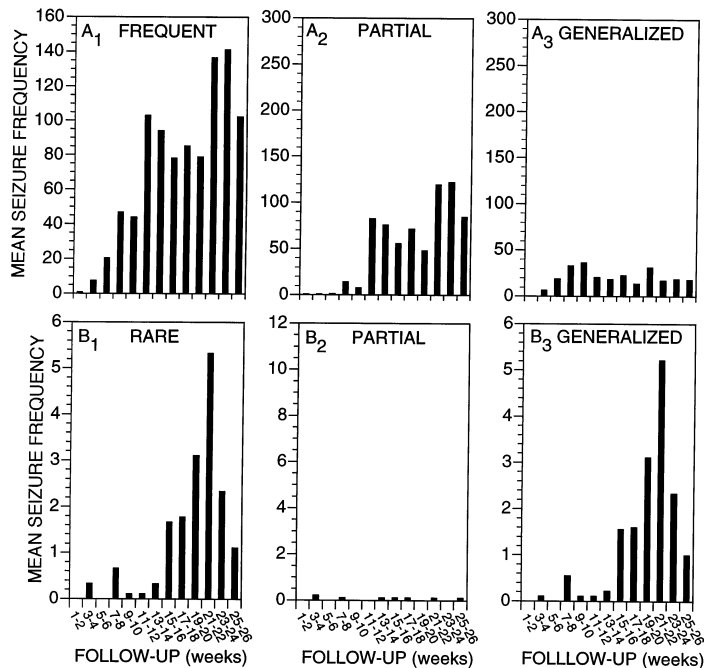


Fig. 7. Appearance of partial and generalized seizures in animals with either frequent or rare seizures during 6-month follow-up. Panel A₁-A₃ represents results from animals with frequent seizures. The majority of seizures (73%) were partial. Panel B₁-B₃ shows the mean seizure frequency in animals with rare number of seizures. The majority of seizures (96%) were secondarily generalized ($P < 0.001$ compared to rats with frequent seizures, Chi-square test).

3.4.1. Amygdala

The amygdaloid complex was damaged in 12 of 13 epileptic animals. In the amygdala, the lateral nucleus, the parvicellular division of the basal nucleus, the accessory basal nucleus, the ventral part of the central division of the medial nucleus, the anterior cortical nucleus (layer III), the posterior cortical nucleus, the amygdalohippocampal area, the periamygdaloid cortex, and the nucleus of the olfactory tract were the most damaged. The magnocellular division of the basal nucleus, caudal division of the medial nucleus, and the central nucleus were relatively well preserved in most of the cases (Fig. 9).

3.4.2. Hippocampus

Clear hippocampal damage was observed in 6 of 13 cases. The hilus and CA1 subfield were more often damaged than CA3. There was no clear granule cell loss in any of the chronically epileptic animals. The subiculum was damaged in 7 of 13 cases. No clear damage was observed in the pre-subiculum or the parasubiculum (Fig. 10).

3.4.3. Piriform cortex

Both the rostralateral and caudomedial aspects of the piriform cortex were damaged in 12 of 13 cases. Neuronal loss was observed in both layers II and III. The cortical area located between the piriform cortex and the entorhinal cortex, the amygdalopiriform transition area, was also damaged in 12 of 13 of the epileptic animals.

3.4.4. Entorhinal cortex

The entorhinal cortex was damaged in 12 of 13 epileptic rats. The DIE subfield was more often damaged than AE > DLE > VIE > CE subfields. In sections available for the analysis, there was no clear damage in the ME subfield. Damage was the most apparent in layer III and in layers V and VI (Fig. 11). There was also damage in other brain areas, for example, in the thalamus (manuscript in preparation).

3.5. Mossy fiber sprouting

In the septal end of the hippocampus, the density of Timm granules was higher in the epilepsy

group than in controls ($P < 0.01$). There were no differences observed between the ipsilateral or contralateral sides. Neither did the density of Timm granules differ between rats with rare or frequent seizures (Table 3).

Also in the dorsal mid-portion of the hippocampus, the density of mossy fiber sprouting was higher in epileptic animals (Table 3) than controls ($P < 0.01$) both ipsilaterally and contralaterally. Moreover, in the dorsal mid-portion of the contralateral hippocampus, the sprouting was more dense in animals with frequent seizures than animals with rare seizures ($P < 0.05$; Table 3).

In the ventral mid-portion of the hippocampus of epileptic animals (Table 3), the density of mossy fiber sprouting was increased compared to that in controls ($P < 0.01$). Moreover, there was a septotemporal gradient in sprouting: the density of Timm granules was heavier in the ventral mid-portion of the dentate gyrus than in the septal end ($P < 0.01$). Sprouting appeared more intense in rats with frequent seizures than in rats with rare seizures both ipsilaterally and contralaterally ($P < 0.05$).

Septal mossy fiber sprouting correlated with the severity of hilar cell damage both ipsilaterally, ($r = 0.76$, $P < 0.01$) and contralaterally ($r = 0.62$, $P < 0.05$). Also in the ventral mid-portion, the density of mossy fiber sprouting correlated with hilar cell damage both ipsilaterally ($r = 0.69$, $P < 0.05$) and contralaterally ($r = 0.84$, $P < 0.001$).

3.6. Water-maze performance

Fig. 12 shows the water maze performance in epileptic animals with frequent or rare seizures compared to control animals. Analysis of ANOVA indicated that animals with both frequent and rare seizures had a faster swimming speed compared to control animals ($P < 0.001$ and $P < 0.008$, respectively). Therefore, the escape latency was tested by ANCOVA using swimming speed as a covariate.

Six months after the induction of SSSE, water-maze performance of epileptic rats was impaired compared to that of controls (Fig. 12, $P < 0.001$; ANCOVA). Latencies were prolonged both in

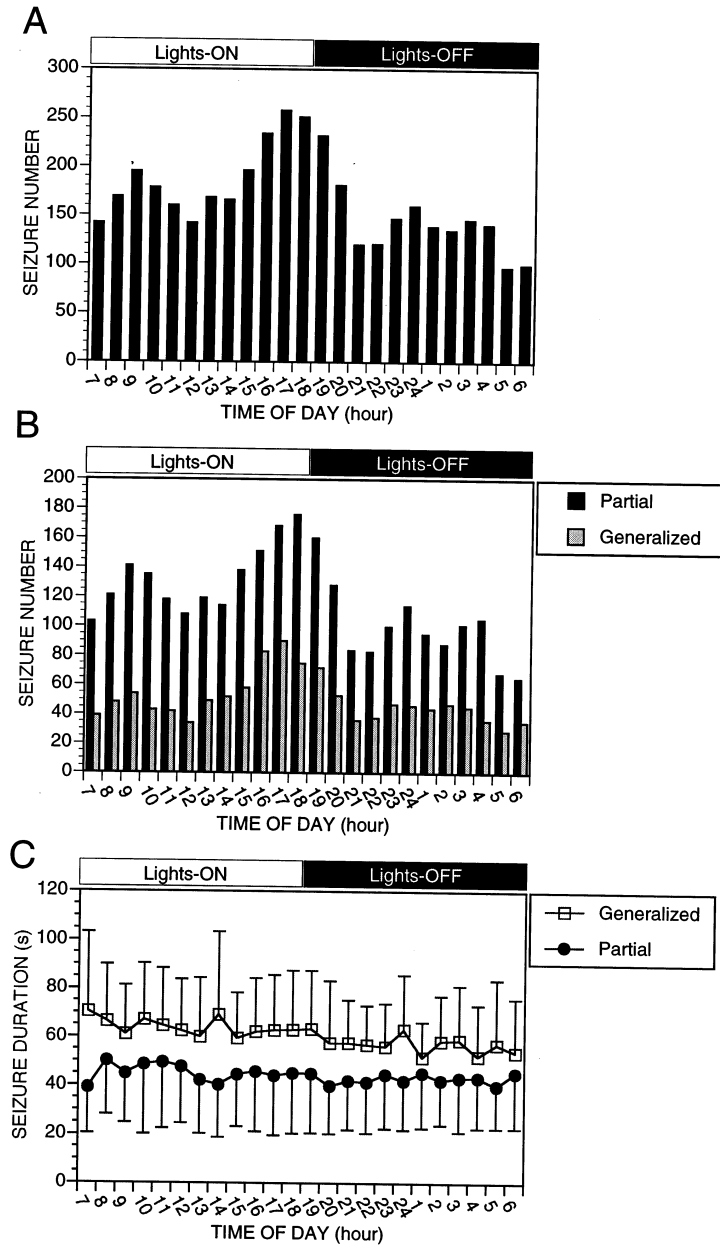


Fig. 8.

rats with frequent seizures ($P < 0.001$; ANCOVA) as well in rats with rare seizures ($P < 0.001$; ANCOVA). Moreover, rats with frequent seizures were even more impaired than were rats with rare seizures ($P < 0.001$; ANCOVA).

3.7. Characteristics of SSSE, histology, and behavior of animals that did not develop epilepsy after amygdala stimulation

Two of the stimulated animals did not develop spontaneous seizures. These rats had three and six HAFDs, which lasted for 69 s on average. The HAFDs appeared within 20 and 30 min from the end of the amygdala stimulation, respectively. The number of neurons (estimated by unbiased stereology) in the amygdala, hilus, and entorhinal cortex of these rats were within the range of mean \pm 2S.D. of that in controls (data not shown). Compared to controls, Timm scores were at normal levels both septally (ipsilaterally 1.0 and 1.0; contralaterally 1.0 and 1.0) as well as in the dorsal mid-portion (ipsilaterally 1.0 and 1.0; contralaterally 1.0 and 1.0) and ventral mid-portion of the hippocampus (ipsilaterally 1.0 and 1.0; contralaterally 1.0 and 1.3). The water-maze performance of the two animals was similar to that in controls.

4. Discussion

The aim of the present study was to develop an epilepsy model in which the epileptogenesis and appearance of spontaneous seizures is similar to that in human symptomatic TLE. That is, the initial insult is followed by a latency period, and thereafter, by spontaneous seizures. In the induction of SSSE, we wanted to avoid chemical substances with possible direct toxic effects on

neurons. Another goal was to minimize the length of electrical stimulation to avoid structural damage caused by the stimulation itself. Our study indicates that a 20–30 min unilateral electrical stimulation of the lateral nucleus of the amygdala in freely-moving rats is enough to induce SSSE that lasts for several hours. SSSE is followed by a latency period of approximately 1 month, after which the recurrent spontaneous seizures begin. In the following discussion, the clinical, pathologic, and behavioral features of the present model are compared with those in three other well-characterized and widely used models of TLE in adult rats. In these models, epileptogenesis was triggered by intraperitoneal injection of kainic acid (Stafstrom et al., 1992), intraperitoneal injection of lithium-pilocarpine (Leite et al., 1990; Cavalheiro et al., 1991), or by 90-min electrical stimulation of the ventral hippocampus (referred to later in the text as a hippocampus-stimulation model; Lothman et al., 1989, 1990). Finally, the appearance of epileptogenesis and epilepsy in the present model is compared to that in human TLE. This article is the first in a series of manuscripts in preparation, and focuses on the methodological description of the model.

4.1. Appearance of SSSE after electrical amygdala stimulation

The amygdala was chosen as a stimulation site because of its low threshold for electrically induced seizures (Goddard et al., 1969). Furthermore, the amygdala is the site of seizure origin in 7 to 13%, or is rapidly recruited in seizure activity in up to 70%, of patients with drug refractory TLE (Quesney, 1986; So et al., 1989; Spanedda et al., 1997). Stimulation was directed to the lateral nucleus. This was based on our previous tract-tracing studies indicating that the most wide-

Fig. 8. Diurnal distribution of spontaneous seizures. (A) Data represents all ($n = 3696$) EEG-recorded seizures from all 13 epileptic animals during the 6-month follow-up. The 12 h lights-on period is shown on the left-hand side of the figure, and the lights-off period on the right-hand side. Seizure number during lights-on and lights-off period was 188 ± 40 and 143 ± 36 (mean \pm S.D.), respectively. Note that the peak is around 17:00 h. (B) Appearance of partial ($n = 2509$) and secondarily generalized ($n = 1187$) seizures at different times of the day. The number of both partial and generalized seizures appeared to peak at 18:00 h and 17:00 h, respectively. (C) The duration (mean \pm S.D.) of partial or generalized seizures, however, did not fluctuate according to the light-dark cycle.

Table 2
Neuronal cell damage in different regions of the temporal lobe^a

Number and percentage of rats with damage						
Brain area	Ipsilateral	%	% with > 50% damage	Contralateral	%	% with > 50% damage
Amygdala						
Lm	12/13	92	92	12/13	92	85
Ldl	12/13	92	85	11/13	85	69
Lvl	12/13	92	92	11/13	85	77
Bmc	2/13	15	8	3/13	23	8
Bi	2/13	15	8	3/13	23	8
Bpc	12/13	92	69	11/13	85	69
ABmc	12/13	92	85	11/13	85	69
ABpc	12/13	92	69	10/13	77	77
Mr	6/13	46	0	4/13	31	0
Mcv	12/13	92	8	11/13	85	8
Mcd	3/13	23	0	2/13	15	0
Mc	2/13	15	0	1/12	8	0
COa	12/13	92	54	11/13	85	69
COp	12/13	92	85	11/13	85	85
AHA1	12/13	92	92	11/13	85	85
AHAm	12/13	92	8	10/13	77	31
PAC	12/13	92	69	11/13	85	77
PACm	12/13	92	69	11/13	85	77
PACs	12/13	92	77	11/13	85	77
Cec	0/13	0	0	0/13	0	0
Ce1	0/13	0	0	0/13	0	0
Ce1	0/13	0	0	0/13	0	0
Cem	1/13	8	0	1/13	8	0
NLOT	12/13	92	31	10/13	77	38
Hippocampus						
<i>Septal end</i>						
CA1	6/13	46	23	6/13	46	15
CA3c	1/13	8	0	1/13	8	0
CA3b	1/13	8	0	1/13	8	0
CA3a	2/13	15	0	2/13	15	0
Granule	0/13	0	0	0/13	0	0
Hilus	6/13	46	8	5/13	38	8
<i>Temporal end</i>						
CA1	4/13	31	8	4/13	31	0
CA3	1/13	8	0	1/13	8	0
Granule	0/13	0	0	0/13	0	0
Hilus	4/13	31	31	6/13	46	23
Subicular complex						
SUBs	6/13	46	0	6/13	46	0
SUBt	7/13	54	0	6/13	46	0
PreS	0/12	0	0	0/12	0	0
ParaS	0/12	0	0	0/12	0	0
Entorhinal cortex						
AE	12/13	92	23	11/13	85	15
DLE	6/13	46	0	5/13	38	0
DIE	12/13	92	8	11/13	85	8
VIE	3/13	23	0	0/12	0	0

Table 2 (Continued)

Number and percentage of rats with damage						
Brain area	Ipsilateral	%	% with >50% damage	Contralateral	%	% with >50% damage
ME	0/11	0	0	0/10	0	0
CE	2/12	17	0	1/10	10	0
Piriform cortex						
Pir(l)	12/13	92	85	11/13	85	85
Pir(m)	12/13	92	85	11/13	85	85
Apir	12/13	92	85	11/13	85	85

^a Abbreviations: All percentages were counted from the total population of epileptic animals. ABmc, accessory basal nucleus, magnocellular division; ABpc, accessory basal nucleus, parvicellular division; AE, amygdalo-entorhinal transition field; AHAl, amygdalohippocampal area, lateral division; AHAm, amygdalohippocampal area, medial division; Apir, amygdalo-piriform transition area; Bi, basal nucleus, intermediate division; Bmc, basal nucleus, magnocellular division; Bpc, basal nucleus, parvicellular division; CA1, CA1 subregion of the hippocampus; CA3a, CA3a subregion of the hippocampus; CA3b, CA3b subregion of the hippocampus; CA3c, CA3c subregion of the hippocampus; CE, caudal entorhinal subfield; CEc, central nucleus, capsular division; CEi, central nucleus, intermediate division; CEl, central nucleus, lateral division; CEm, central nucleus, medial division; COa, anterior cortical nucleus; COp, posterior cortical nucleus; DIE, dorsal intermediate entorhinal subfield; DLE, dorsolateral entorhinal subfield; Granule; granule cell layer of the dentate gyrus; Hilus, hilus of the dentate gyrus; Ldl, lateral nucleus, dorsolateral division; Lm, lateral nucleus, medial division; Lvl, lateral nucleus, ventrolateral division; Mc, medial nucleus, caudal division; Mcd, medial nucleus, dorsal portion of the central division; Mcv, medial nucleus, ventral portion of the central division; ME, medial entorhinal subfield; Mr, medial nucleus, rostral division; NLOT, nucleus of the lateral olfactory tract; PAC, periamygdaloid cortex; PACm, periamygdaloid cortex, medial division; PACs, periamygdaloid cortex, sulcal division; Pir(l), piriform cortex, lateral portion; Pir(m), piriform cortex medial portion; ParaS, parasubiculum; PreS, presubiculum; SUBs, subiculum, septal portion; SUBt, subiculum, temporal portion; VIE, ventral intermediate entorhinal subfield.

spread intra-amygdaloid projections originate from the lateral nucleus (Pitkänen et al., 1997b), thus facilitating the recruitment of parallel amygdaloid circuitries into seizure activity.

A 20 to 30 min stimulation given focally to the lateral nucleus of the amygdala was long enough to induce SSSE. Previously, a 90-min stimulation was used to evoke SSSE in the ventral hippocampus in the model of Lothman and colleagues (Lothman et al., 1990). Recently, Mazarati and coworkers (Mazarati et al., 1998) demonstrated that a 30-min stimulation of the angular bundle is long enough to induce SSSE and the later development of spontaneous seizures in adult rats. The current and the pulse train used to induce SSSE in the amygdala was comparable to that used to induce SSSE in the hippocampus (400 μ A, 50 Hz; 90 min; Bertram et al., 1998).

In the present study, 87% of all stimulated animals developed SSSE, a percentage that has been consistent between experiments (unpublished observation). In the kainate model, 93% (Stafstrom et al., 1992), in the pilocarpine model, 83% (Mello

et al., 1993), and in the hippocampus-stimulation model, 70% (Bertram and Cornett, 1993) of the rats developed SSSE. Therefore, the percentage of animals developing SSSE in different models is rather similar.

The HAFDs, which are the electrographic correlates of behavioral seizures started within 5 min after the cessation of amygdala stimulation. Sixty-one percent of HAFDs occurred during the first 3 h, and the mean time between the first and the last HAFD was approximately 12 h and the median 6 h. In the kainate model, the duration of status epilepticus is 8 to 11 h (Tuunanen, unpublished observation), in the pilocarpine model, 8 to 12 h (Leite et al., 1990; Cavalheiro et al., 1991, 1994), and in the hippocampus-stimulation model 6–12 h (Mathern et al., 1997). These data suggest that the duration of untreated SSSE in rats is independent of method of its induction. Interestingly, the mean duration of HAFDs was 37 s, and 80% of HAFDs were shorter than 55 s. Therefore, only 20% of HAFDs were longer than 55 s of the duration of discharge needed to induce

c-fos mRNA expression (Hosford et al., 1995). In other models, the duration of seizures during SSSE has not been analyzed in detail.

4.2. Appearance of spontaneous seizures after SSSE

In the present study, 87% of the stimulated animals developed spontaneous recurrent seizures after a latency period. In the kainate model, 45–59% of injected rats (Stafstrom et al., 1992; Mascott et al., 1994), in the pilocarpine model, 90–100% (Priel et al., 1996) and in the hippocampus-stimulation model, 91% of rats with SSSE (Bertram and Cornett, 1993) developed

spontaneous seizures. These estimations of percentages of animals with spontaneous seizures are obviously affected by the length of the follow-up in each study as well as whether the seizures were detected behaviorally or electrographically.

The latency period, that is, the time period from the SSSE to the appearance of the first spontaneous seizure varied between 6 and 85 days, the median being 33 days. Previously, in the kainate model, the mean latency to the first spontaneous seizure was 4.8 weeks (Stafstrom et al., 1992), in the pilocarpine model 4 to 44 days (mean 15 days) (Cavalheiro et al., 1991) and in the hippocampus-stimulation model from 8 to 38

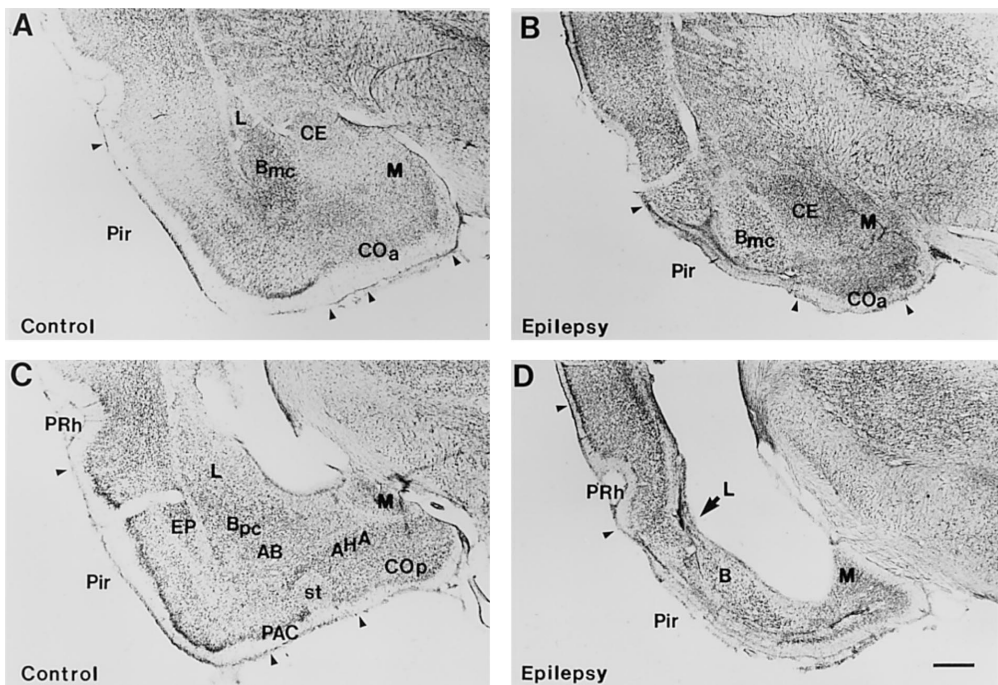


Fig. 9. Brightfield photomicrographs of coronal thionin-stained sections demonstrating damage in various amygdaloid nuclei. (A) Rostral amygdala in a control animal. (B) Rostral amygdala in an epileptic animal (rat # 46, 5 spontaneous seizures). The magnocellular division of the basal nucleus and the central nucleus were relatively well preserved 6 months after induction of epilepsy. Note the damage in the piriform cortex. (C) Caudal amygdala in a control animal. (D) Caudal amygdala in rat # 46. Note the severe damage to the lateral (arrow), basal, and accessory basal nucleus as well as to the posterior cortical nucleus. Arrowheads indicate the nuclear boundaries. For abbreviations, see legend to Fig. 2. Scale bar 500 μ m.

days (Bertram and Cornett, 1993). Therefore, in both chemical and electrical stimulation models the latency period required for epileptogenesis appears to be approximately 1 month.

In the present study, the frequency of spontaneous seizures in different animals varied from 0 to 125/days. In previous studies, Mello et al. (1993) reported that in the pilocarpine model behaviorally observed spontaneous seizures occurred with a frequency of 0.1–2.4 seizures/day. By counting the number of behavioral seizures, Cavalheiro et al. (1991) demonstrated that seizure frequency in the pilocarpine model was 2–4 seizures per animal per week (0.3–0.6 seizures/d). The higher number of daily seizures counted in the present study compared to previous observations might be related to the video-EEG recording method used for seizure monitoring, which is more sensitive than the visual observation of animals, particularly for partial seizures.

After the beginning of epilepsy, about one third of our epileptic rats had spontaneous seizures almost every day whereas two-thirds had only an occasional seizure. The proportion of generalized seizures did not differ between the animals with rare or frequent seizures during the first 10 follow-up weeks. During weeks 11–26, most of the seizures in animals with frequent seizures became partial. In rats with rare seizures, the percentage of partial seizures from all seizures (4%) did not change over time. We have no explanation for this finding. Also, we are not aware of any similar observations in other models of TLE. The two populations of rats, however, one with frequent seizures and the other with few seizures, provide a tool to test whether the molecular changes leading to epileptogenesis differ in these rat groups. Another advantage of the two populations of rats is the testing of antiepileptic efficacy of new compounds. Data obtained from rats with frequent seizures might predict the antiepileptic effect of drugs in human drug-refractory TLE better than data obtained from rats with rare seizures.

Altogether, 3696 seizures were analyzed and the mean seizure duration was 49 s. The data available on the seizure duration in other models indicate that in the kainate model the seizures lasted approximately for 40 s (Stafstrom et al., 1992),

and in the hippocampus stimulation model 63–105 s (Bertram and Cornett, 1994). Like the seizure type, the duration of partial seizures changed over time.

In the present model, 57% of all seizures occurred between 07:00 and 19:00 h and 43% during lights off. The frequency peak was 17:00 and 18:00 h. Recently, HELLIER and DUDEK (1998) reported that 62% of seizures in the kainate model occurred during lights on. In the hippocampus stimulation model, 67% of the spontaneous seizures are observed during lights on (Bertram and Cornett, 1994) and seizure frequency peaked at 16:00 h. Therefore, the diurnal occurrence of spontaneous seizures also appears rather similar in different models.

The amygdaloid complex sends substantial monosynaptic projections to various levels of the hippocampal formation (Pikkarainen et al., 1999). Therefore, the primary focus in the amygdala might induce the development of independent seizure focus in the hippocampus over the time. Based on the analysis of 268 seizures in seven animals, the electrographic seizure activity began in the hippocampus before the amygdala in only 9% of seizures. According to a study by Bertram and coworkers (Bertram, 1997), also in the hippocampus-stimulation model the independent seizure generation is rare, for example, in the amygdala or the piriform cortex. Rather, like in our model, the seizure onset appears ‘simultaneously’ at several recording sites. Most probably, our inability to detect focal seizure onset relates to the limited number of recording electrodes and the recording technique used. The identification of primary and secondary foci in spontaneous models of TLE requires further studies.

4.3. Histopathology

In the present study, histologic analysis of brains was performed 6 months after the SSSE. The bilateral distribution of damage appeared similar in all epileptic animals. The amygdala, the piriform cortex, the amygdalopiriform transition area, and the AE and DIE subfields of the entorhinal cortex were damaged in 92% of the epileptic rats. The amygdaloid and cortical dam-

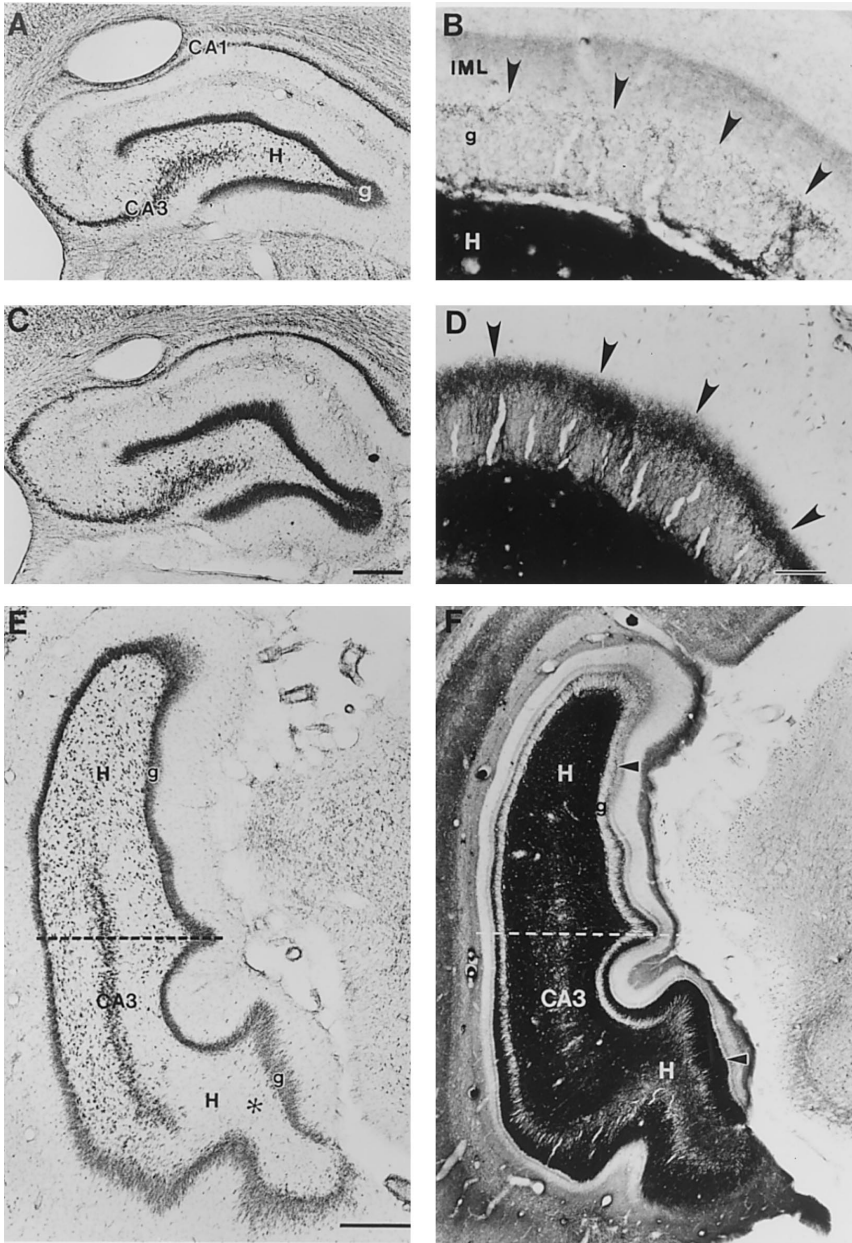


Fig. 10.

age are consistently found also in the kainic acid (Stafstrom et al., 1992) and pilocarpine (Mello et al., 1993; Fujikawa, 1996) models as well as in hippocampus-stimulation model (Bertram, 1997; Mathern et al., 1997) although many aspects of the extrahippocampal damage are not described in such detail as was the hippocampal damage. The distribution of damage in various amygdaloid nuclei in our model is similar to that after systemic kainic acid administration (Tuunanen et al., 1996). In both models, the magnocellular division of the basal nucleus, the central nucleus, and the caudal division of the medial nucleus are relatively well preserved compared to the other amygdaloid areas.

Damage to the hippocampal neurons was found in 62% of the epileptic rats. Although the analysis of neuronal damage in thionin-stained preparations might underestimate the occurrence of mild damage compared, for example, to the analysis in silver-impregnated sections, the structural damage appeared to be more severe in the amygdala and surrounding cortex than in the hippocampus. In our preparations, the septal end of the hippocampus was damaged in 54% and the temporal end in 38% of epileptic animals. If hilar cell damage occurred, over 50% neuronal loss was observed in 17% of the cases septally and 100% temporally. Based on our ongoing studies, one population of the damaged hilar cells is composed of somatostatin-immunoreactive neurons (Halonen et al., 1997). There was mossy fiber sprouting in 100% of the epileptic rats. In line with the severity of hilar cell damage along the septotemporal axis, sprouting appeared heavier temporally than septally.

There was a substantial variability in the density of sprouting, however, between the animals. Considering that all rats were sacrificed 6 months after SSSE, variability in axonal plasticity as a result of

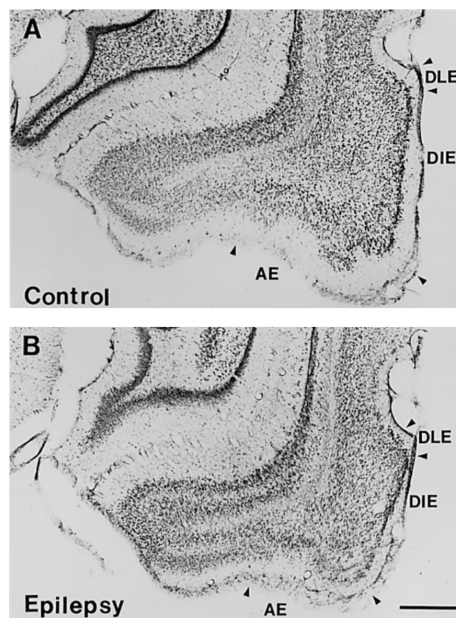


Fig. 11. A brightfield photomicrograph of a coronal thionin-stained sections demonstrating the damage in the entorhinal cortex. Note the neuronal loss in layer III of the dorsal intermediate subfield (DIE) of the entorhinal cortex and layers II–III of the AE subfield in panel B. Scale bar 500 μ m.

Fig. 10. Brightfield photomicrographs of coronal sections demonstrating the histologic damage in the hippocampus. (A) A thionin-stained section from animal # 60 (697 spontaneous seizures). Note the damage in the CA1 area in the septal end of the hippocampus. The hilus is relatively well preserved (B) An adjacent Timm-stained section from the same animal as in panel A. Note the mild mossy fiber sprouting in the supragranular region and inner molecular layer (arrowheads). (C) A thionin-stained section from animal # 69 (15 spontaneous seizures). Note the neuronal loss in the hilus and CA1 area of the septal hippocampus. (D) An adjacent Timm-stained section from the same animal as in panel C. Note the dense mossy fiber sprouting in the supragranular region and inner molecular layer (arrowheads). (E) A thionin-stained section from the mid-septotemporal portion of the hippocampus. The area dorsal to the dashed line was called in the text as 'dorsal mid-portion' and the area ventral to the dashed line as 'ventral mid-portion'. The section is from animal # 60 (697 spontaneous seizures). Note the neuronal loss in the hilus of the ventral hippocampus (asterisk). (F) An adjacent Timm-stained section of the same animal as in panel E. Note the dense mossy fiber sprouting in the supragranular region and inner molecular layer (arrowheads) in the ventral mid-portion of the dentate gyrus. In the dorsal-mid portion, the sprouting is lighter. Abbreviations: CA1 and CA3, corresponding subfields of the hippocampus; g, granule cell layer; H, hilus; IML, inner molecular layer. Scale bar = 500 μ m for panel A, C, E, and F. Scale bar = 50 μ m for panel B and D.

Table 3

The density of mossy fiber sprouting at the different septotemporal levels of hippocampus 6 months after the induction of status epilepticus^a

	Septal		Dorsal mid-portion		Ventral mid-portion	
	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral
Control (<i>n</i> = 8)	1.02 ± 0.05	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.24 ± 0.39	1.29 ± 0.31
Epilepsy (<i>n</i> = 13)	2.40 ± 1.16**	2.32 ± 1.02***	2.53 ± 1.40**	2.91 ± 1.71**	3.68 ± 1.79**	3.76 ± 1.86**
Frequent (<i>n</i> = 4)	2.71 ± 0.32**	2.83 ± 0.28**	2.97 ± 0.47**	4.10 ± 1.18**	5.13 ± 0.94**	5.45 ± 0.44**
Rare (<i>n</i> = 9)	2.26 ± 1.38**	2.09 ± 1.15**	2.31 ± 1.68*	2.32 ± 1.66* [#]	2.95 ± 1.69* [#]	2.32 ± 1.66* [#]

^a Density of sprouting is expressed as mean ± standard deviation. Number of rats (*n*) in each group is in parenthesis. Ipsilateral refers to the stimulation side.

* *P* < 0.05.

** *P* < 0.01.

*** *P* < 0.0001 compared to control.

[#] *P* < 0.05 compared to animals with frequent number of seizures (Mann-Whitney U-test).

a difference in survival time is ruled out. Factors that are associated with mossy fiber sprouting are currently being investigated. Whether the density of sprouting is associated with frequency of seizures or with the severity of hilar cell loss in rats with frequent seizures remains to be resolved. In addition to hilar cell damage, neuronal cell loss was also found in the CA1 in 54%, in the CA3 in 15%, and in the subiculum in 54% of the epileptic rats. Damage to these areas was also reported in animals expressing spontaneous seizures after kainic acid injection (Stafstrom et al., 1992) or pilocarpine injection (Liu et al., 1994; Fujikawa, 1996) or after hippocampal stimulation (Mathern et al., 1997). Finally, as was also reported for the kainate model (Pitkänen et al., 1995), the subiculum appeared more damaged than the presubiculum or parasubiculum.

Contribution of damage in the entorhinal cortex to seizure generation was demonstrated in several models (Du and Schwarcz, 1992; Du et al., 1995; Pitkänen et al., 1997a). Many of these studies, however, have focused on the caudomedially located CE subfield (Du et al., 1998). In our model, the CE subfield was damaged infrequently, whereas the more rostrally located subfields, AE and DIE, were damaged in 92% of the cases. The most distinct damage appeared in layer III. The lateral nucleus, which was the primary stimulation site in our model, was recently shown to provide a substantial input to layers III of the DIE

subfield (Pikkarainen et al., 1999). It cannot be concluded, however, that DIE damage directly relates to its monosynaptic connectivity with the stimulation site because the projection from the lateral nucleus to the VIE subfield is even heavier, and substantial damage in the VIE was observed in only 3 of 13 rats. Taken together, the rostrally located entorhinal subfields, which project to the temporal half of the hippocampus, were the most damaged in the present model.

The 20–30 min electrical amygdala stimulation, consequent SSSE, or recurrent seizures might have contributed to the damage observed in the present model. Ongoing studies demonstrate that the damage induced by stimulation itself is limited to the lateral nucleus and postrhinal cortex (Lukasiuk and Pitkänen, 1998). The damage, however, becomes more widespread and more severe the longer SSSE is allowed to continue (Lukasiuk and Pitkänen, 1998). A correlation between the severity of damage and the number of spontaneous seizures has not yet been demonstrated (Nissinen et al., 1997).

4.4. Spatial memory performance

Performance of rats in the Morris water-maze is sensitive to hippocampal damage, particularly if the lesion is in the septal end (Moser et al., 1993). In the present study, all animals were impaired in the water-maze, although only about 60% of them

had clear hippocampal damage. Whether the impaired spatial learning and memory in these animals was a result of the entorhinal damage remains to be clarified. Animals with spontaneous seizures are also impaired in fear-conditioning to tone task, which is sensitive to neuronal loss in the lateral and basal nuclei of the amygdala (Nissinen et al., 1998).

4.5. Comparison of epilepsy induced by amygdala stimulation with human TLE

Electrical amygdala stimulation elicits SSSE that culminates in the appearance of spontaneous seizures, that is, epilepsy after a latency period. Therefore, the sequence of events in the present model is identical to that in human symptomatic TLE associated with various brain insults such as head trauma, brain infection, or stroke (Mathern et al., 1993).

In humans, the risk of recurrent seizures after status epilepticus is 37% within 1 year and 56% within 3 year (Hauser et al., 1990). In the present study, 87% of animals who eventually developed epilepsy within 6 months had their first two spontaneous seizures within 8 weeks after SSSE. In

another recent study, in which rats were followed for 24 h/day until they developed at least 2 spontaneous seizures, 92% of the animals developed epilepsy within 8 weeks (unpublished). Therefore, the percentage of rats with spontaneous seizures peaks within a few months after SSSE. Whether the appearance of spontaneous seizures can be delayed or prevented by medication in the present model, and how well that predicts the prevention of epilepsy in humans remains to be studied.

Only a few studies have measured seizure duration in patients with drug-refractory TLE using video-EEG monitoring. Theodore et al. (1994) reported that generalized tonic clonic seizures typically last approximately for 1 min. Consistent with human studies, the mean duration of partial seizures was 44 s and secondarily generalized seizures 61 s in the present model. At the time of the first seizures, most seizures were secondarily generalized. After a longer follow-up, however, up to 80% of the seizures were classified as partial based on behavioral criteria (Racine, 1972). Recently, Tinuper et al. (1996) reported that in 84% of humans with partial onset seizures, secondarily generalized seizures disappear during the course of the illness at approximately the age of 50.

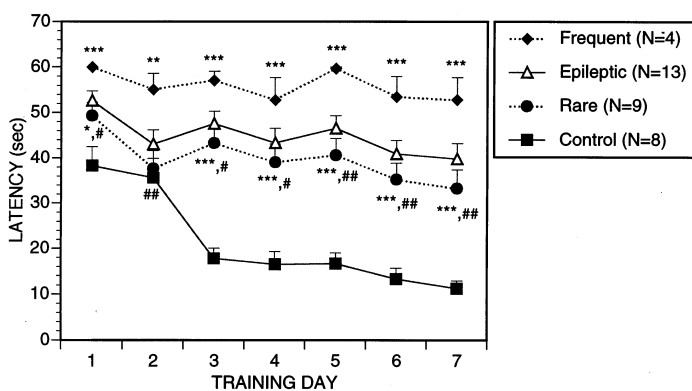


Fig. 12. Performance of epileptic animals in the Morris water-maze task. Epileptic animals were impaired in finding the platform compared to control animals ($P < 0.001$; ANCOVA). Latencies (time taken to find the submerged platform) were prolonged both in rats with frequent ($P < 0.001$; ANCOVA) or rare number of seizures ($P < 0.001$; ANCOVA). Moreover, rats with frequent seizures were even more impaired than the rats with rare seizures ($P < 0.001$; ANCOVA). Latency is expressed in $s \pm$ standard error of mean. Number of animals is in parenthesis. Statistical significances: ** $P < 0.01$, *** $P < 0.001$ compared to control; # $P < 0.05$, ## $P < 0.01$ compared to animals with frequent number of seizures (Mann–Whitney U-test).

Whether the change in seizure symptomatology in our rats is related to aging or whether it is a consequence of progressive alterations in the neuronal network remains to be studied. At this time, some animals have been followed for longer than 1 year and these animals still express spontaneous seizures. Otherwise, no spontaneous remissions in the occurrence of seizures were detected in our systematic 6-months follow-up period.

Approximately one third of the rats had a large number of seizures during the follow-up period and two-thirds had only occasional seizures. About 73% of the seizures in rats with frequent seizures were partial, whereas most of the seizures in rats with rare seizures were generalized. Human data indicate that approximately 20–30% of patients with TLE have frequent seizures despite polytherapy (Sander and Sillanpää, 1997). In line with our observations in the rat model, the appearance of partial seizures is a risk factor for a poor prognosis in humans (Tinuper et al., 1996).

The occurrence of seizures at different times of the day has recently received attention. Quigg et al. (1998) reported that in human TLE, the seizure frequency peaks at 15:00 h and that 60% of seizures appeared during light hours. In the present study, 57% of seizures were during light hours and the daily peak seizure frequency occurred around 17:00 h. The similarity of the time, at which seizure frequency peaks in rats and humans is somewhat unexpected considering that rats are less active than humans during lights-on period.

In this amygdala stimulation model, the most severe damage appeared in the amygdala and surrounding cortex. Amygdaloid damage included neuronal loss in nuclei that were also damaged in humans who died from status epilepticus, that is, the parvicellular divisions of the basal nucleus and the medial division of the lateral nucleus (for review, see Pitkänen et al., 1998). The entorhinal damage was the most severe in rostral subfields AE and DIE, which correspond to rostromedial subfields EO and ER in the human entorhinal cortex (Insausti et

al., 1995), and which were found to be damaged in human drug-refractory TLE (Du et al., 1993; Mikkonen et al., 1998).

Based on recent MRI studies, amygdaloid damage occurs in combination with hippocampal atrophy in 60–70% of patients with TLE (Pitkänen et al., 1998). In 67% of our rats with amygdaloid damage, the hippocampus was also damaged. The hippocampal damage included neuronal loss in the hilus as well as in CA1 and CA3, areas that have also been reported to be damaged in human TLE (Babb and Pretorius, 1993). A high percentage of amygdaloid damage in the present rat model compared to human TLE is obviously related to the fact that SSSE was induced by amygdala stimulation. Mossy fiber sprouting was present in all cases, which is also typical for patients with chronic symptomatic TLE (Houser et al., 1990).

5. Conclusions

Like the development of symptomatic TLE in humans, the epileptogenic process in our model has three major phases that succeed each other sequentially: initial insult→latency period or epileptogenesis→epilepsy. There are several other similarities between the present model and human symptomatic TLE: (1) the occurrence of spontaneous seizures after a latency period; (2) behavioral appearance, duration, frequency and diurnal distribution of seizures; (3) distribution and appearance of temporal lobe damage; and (4) memory and emotional (Nissinen et al., 1998) impairment that corresponds with structural damage. Also, a response of spontaneous seizures to antiepileptic medication (with vigabatrin) appears similar to that found in human TLE (Halonen et al., 1996). Therefore, the amygdala stimulation model of chronic TLE in rats provides a useful tool for studies aimed at understanding the mechanisms of status epilepticus, epileptogenesis, and generation of spontaneous seizures as well as testing new antiepileptogenic and antiepileptic compounds for the prevention and treatment of human TLE.

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II

Is mossy fiber sprouting present at the time of the
first spontaneous seizures in rat experimental
temporal lobe epilepsy?

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Is Mossy Fiber Sprouting Present at the Time of the First Spontaneous Seizures in Rat Experimental Temporal Lobe Epilepsy?

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ABSTRACT: The contribution of mossy fiber sprouting to the generation of spontaneous seizures in the epileptic brain is under dispute. The present study addressed this question by examining whether sprouting of mossy fibers is present at the time of appearance of the first spontaneous seizures in rats, and whether all animals with increased sprouting have spontaneous seizures. Epileptogenesis was induced in 16 rats by electrically stimulating the lateral nucleus of the amygdala for 20–30 min until the rats developed self-sustained status epilepticus (SSSE). During and after SSSE, rats were monitored in long-term by continuous video-electroencephalography until they developed a second spontaneous seizure (8–54 days). Thereafter, monitoring was continued for 11 days to follow seizure frequency. The density of mossy fiber sprouting was analyzed from Timm-stained preparations. The density of hilar neurons was assessed from thionin-stained sections. Of 16 rats, 14 developed epilepsy. In epileptic rats, the density of mossy fiber sprouting did not correlate with the severity or duration (115–620 min) of SSSE, delay from SSSE to occurrence of first (8–51 days) or second (8–54 days) spontaneous seizure, or time from SSSE to perfusion (20–63 days). In the temporal end of the hippocampus, the sprouting correlated with the severity of neuronal damage (ipsilateral: $r = -0.852$, $P < 0.01$ contralateral: $r = -0.748$, $P < 0.01$). The two animals without spontaneous seizures also had sprouting. Increased density of sprouting in animals without seizures, and its association with the severity of neuronal loss was confirmed in another series of 30 stimulated rats that were followed-up with video-EEG monitoring for 60 d. Our data indicate that although mossy fiber sprouting is present in all animals with spontaneous seizures, its presence is not necessarily associated with the occurrence of spontaneous seizures. *Hippocampus* 2001;11:299–310. © 2001 Wiley-Liss, Inc.

KEY WORDS: amygdala; epileptogenesis; hippocampus; plasticity; temporal lobe

INTRODUCTION

Mossy fibers are granule cell axons that normally innervate hilar cells and the apical dendrites of CA3 pyramidal cells (Frotscher et al., 1994). In

experimental and human temporal lobe epilepsy (TLE), mossy fibers sprout and innervate postsynaptic targets in abnormal locations, including the granule cell dendrites in the inner molecular layer of the dentate gyrus (rat: Tauck and Nadler, 1985; human TLE: de Lanerolle et al., 1989; Sutula et al., 1989; Houser et al., 1990; Babb et al., 1991) and basal dendrites of CA3 pyramidal cells in the hippocampus proper (Reprea et al., 1990; Reprea and Ben-Ari, 1992).

Several lines of evidence suggest that aberrant glutamatergic mossy fibers in the inner molecular layer are critically involved in seizure generation. Electron microscopic analysis indicates that sprouted mossy fiber terminals form asymmetric contacts with dendritic spines of granule cells (Reprea et al., 1993; Okazaki et al., 1995; Zhang and Houser, 1999). Via these contacts, granule cells can form excitatory circuitries with adjacent granule cells in the epileptic brain (Buckmaster and Dudek, 1999). Consistent with anatomic findings, electrophysiologic analysis of hippocampal slices from kainate-treated animals indicates that antidromic stimulation of granule cells evokes prolonged seizure-like bursts of action potentials, particularly when GABA-A receptor function is blocked with bicuculline (Cronin et al., 1992; Masukawa et al., 1992; Wuarin and Dudek, 1996). Further, local application of glutamate induces a robust increase in the excitatory postsynaptic potential (EPSP) frequency of granule cells (Wuarin and Dudek, 1996). These phenomena are observed only if mossy fiber sprouting is present. In addition to glutamate, mossy fibers contain Zn^{2+} , and its release during seizures can reduce GABA-A receptor mediated inhibition (Buhl et al., 1996). Consistent with slice studies, some observations in *in vivo* kainate and pilocarpine models of TLE in the rat suggest an association between the density of mossy fiber sprouting and the frequency of spontaneous seizures (Lemos and Cavalheiro, 1995; Buckmaster and Dudek, 1997). Genetically mutant mice with spontaneous seizures also have mossy fiber sprouting (e.g., star-

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gazer mutant, Qiao and Noebels, 1993). Perhaps the clearest evidence of an association between seizure number and the density of mossy fiber sprouting comes from studies in the kindling model, in which the density of sprouting increases with the number of electrically induced seizures (Cavazos et al., 1991). Finally, the reduction of mossy fiber sprouting by pretreatment with an NMDA antagonist (MK-801; McNamara and Routtenberg, 1995; Sutula et al., 1996), pentobarbital (McNamara and Routtenberg, 1995), phenobarbital (Sutula et al., 1992), calcineurin inhibitor (FK506; Moriwaki et al., 1996), or nerve-growth factor antibodies (van der Zee et al., 1995) is associated with prevention of or delay in kindling.

Recently, however, it was demonstrated that the prevention of mossy fiber sprouting by cycloheximide, a protein synthesis inhibitor, did not prevent the development of epilepsy despite preventing mossy fiber sprouting (Longo and Mello, 1997, 1998). Furthermore, anatomic findings indicate that sprouted mossy fibers also make synaptic contacts with inhibitory basket cells in the dentate gyrus, which is associated with the restoration of inhibition in chronically epileptic brain (Sloviter, 1992, 1999; Kotti et al., 1997). Association of mossy fiber sprouting with enhanced epileptogenesis was also questioned by Elmer et al. (1997), who found that rats with a higher density of mossy fiber sprouting became kindled at a slower rate than rats with a lower density of sprouting.

To address the controversial question of whether mossy fiber sprouting is associated with onset of TLE, we took advantage of our *in vivo* model of TLE in which the development and occurrence of spontaneous seizures can be followed up by a long-term video-electroencephalographic monitoring (Nissinen et al., 2000). Here, for the first time, we determined the density of sprouting at the time of occurrence of the first spontaneous seizures. We hypothesized that only animals with spontaneous seizures have mossy fiber sprouting, and that all animals with increased sprouting have spontaneous seizures. Some of the data were presented earlier at a congress report (Pitkänen et al., 2000).

MATERIALS AND METHODS

Experiment I

In the first experiment, the animals were followed after self-sustained status epilepticus (SSSE) until they developed a second spontaneous seizure, and 11 days thereafter (maximum follow-up was 63 days). Considering that epilepsy persists in these animals for the rest of their lives, the time period studied corresponds to a very early stage of the disease. Each time an epileptic rat was perfused for histology, a matched unstimulated control rat with an electrode implanted into the amygdala was also sacrificed.

Animals

Adult male Harlan Sprague-Dawley rats ($n = 38$; 320–390 g) were used. The rats were housed in individual cages in a controlled environment (constant temperature, $22 \pm 1^\circ\text{C}$, humidity 50–

60%, lights on 0700–1900 h). Animals had free access to food and water. All animal procedures were conducted in accordance with the guidelines set by the European Community Council Directive 86/609/EEC.

Implantation of stimulation and cortical electroencephalography (EEG) electrodes

Details of the methodology were recently published (Nissinen et al., 2000), and therefore, are only briefly summarized. For amygdala stimulation, a bipolar electrode (diameter, 0.127 mm; dorsoventral distance between the tips, 0.4 mm; Franco Corradi, Milan, Italy) was implanted into the lateral nucleus of the left amygdala under sodium pentobarbital (60 mg/kg, intraperitoneally (i.p.)) and chloral hydrate (100 mg/kg, i.p.) anesthesia. Only animals with the stimulation electrode in the correct location were included in this study. To record the spread of electrographic seizure activity to the contralateral cortex, a screw electrode (Plastics One, Inc., Roanoke, VA) was implanted into the skull overlying the contralateral frontal cortex. Two monopolar stainless steel screw-electrodes were fixed to the skull symmetrically over the cerebellum with dental acrylate (Selectaplus CN, Dentsply DeTrey GmbH, Dreieich, Germany) to serve as ground and reference electrodes.

Induction and monitoring of SSSE

Two weeks after surgery, SSSE was induced by stimulating the lateral nucleus of the left amygdala for 20 min. The stimulation consisted of a 100-ms train of 1-ms biphasic square wave pulses (400 μA from peak to peak) delivered at 60 Hz every 0.5 s, using an A300 Pulsemaster Stimulator connected with two A360 Constant Current Stimulus Isolators, to which the animals were connected with a six-channel commutator (Plastics One, Inc.) and shielded cables. Thereafter, the stimulation was interrupted, and the behavioral and electrographic seizure activity of the animal was observed for 60 s. If the behavior of the animals indicated epileptic activity (head nodding/limb clonus), the observation was continued for up to 5 min. If the animal did not meet the criterion of clonic status epilepticus (continuous electrographic epileptiform spiking and recurrent clonic seizures), stimulation was resumed and the behavior of the animal was checked again after 5 min. Once the criterion of SSSE was achieved, no further stimulation was given.

During the amygdala stimulation, the development and duration of status epilepticus were monitored continuously via amygdalar and cortical electrodes, using the Nervus EEG Recording System (Tautagreining, Iceland) connected with an ISO-1032 Amplifier (Braintronics, Netherlands), SVT-S3000P Hitachi Time Lapse 168 VCR (Japan), and Panasonic WV-CL350 Video Camera (Japan). After the stimulation period, the animals were followed with the Stellare system, as described below.

A conspicuous feature of the EEG activity during SSSE was the high-amplitude and frequency discharges (HAFDs), which were typically associated with behavioral seizures. HAFD were defined as high-amplitude ($>2 \times$ baseline) and high-frequency ($>8\text{-Hz}$) discharges in the amygdala and/or in the cortex that lasted for at least 5 s.

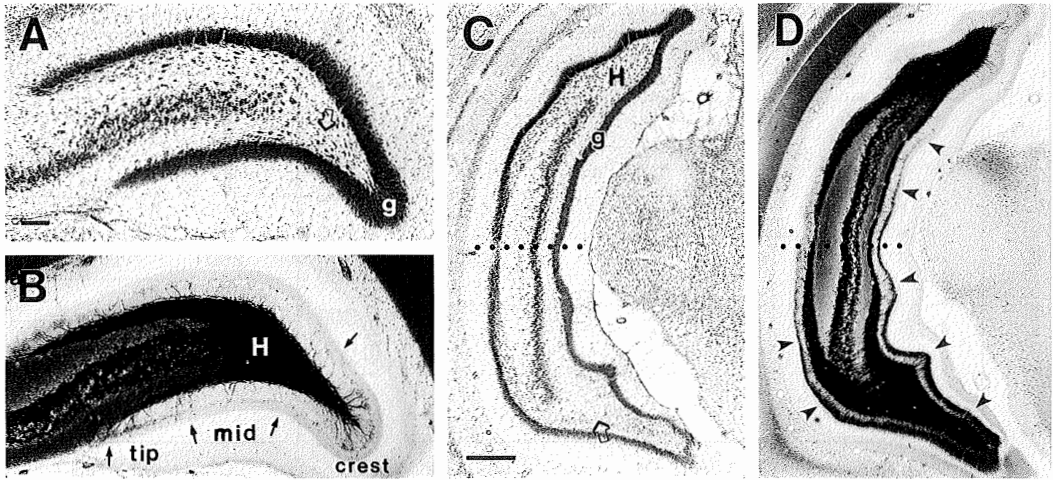


FIGURE 1. Bright-field photomicrographs demonstrating neuronal damage and mossy fiber sprouting in the septal and temporal ends of the hippocampus. **A:** Coronal thionin-stained section from the septal end of the hippocampus. Note loss of hilar cells (open arrow). **B:** Adjacent section stained using the Timm method to demonstrate mossy fiber sprouting. Sprouting was analyzed in the "tip," "mid," and "crest" portions of the supragranular region and inner molecular layer. **C:** Thionin-stained coronal section from the more

temporal aspect of the hippocampus. Note loss of neurons in the temporal hilus (open arrow). The area dorsal to the dotted line was referred to in the text as "dorsal mid-portion," and the area ventral to the dotted line as "ventral mid-portion." **D:** Adjacent Timm-stained section demonstrates mossy fiber sprouting in the temporal end of the hippocampus (arrowheads). g, granule cell layer; H, hilus. Scale bar in A, B = 100 μm ; in C, D = 500 μm .

Long-term behavioral and EEG monitoring of the appearance of spontaneous seizures

All rats that developed SSSE were followed up with continuous video-EEG monitoring. Electrode-implanted, unstimulated rats were included in the study as time-matched controls.

Monitoring was performed 24 h/day with a combined video-EEG recording system until the rats experienced two spontaneous seizures, and for 11 days thereafter to follow seizure frequency. (During that time, behavioral testing was also performed; data not shown). After stimulation, EEG signals were recorded with a Stellate EEG Monitor System (sampling rate 200 Hz, high-pass filter 1 Hz, low-pass filter 100 Hz) that was connected with two ISO-DAM 8 Amplifiers (World Precision Instruments), to which the animals were connected with a six-channel commutator and shielded cables. The behavior of the animals was recorded using a WV-BP312E Video Camera (Panasonic) that was positioned above the cages and connected with an SVT-S3000P Time Lapse 168 VCR (Sony) and a PVC-145E Video Monitor (Sony). The video system was connected to the EEG recording system via a time code generator (MUL, TIM Electrode, Inc.). Type 955 Infra Red Light (Videmech, Ltd., UK) was used at night to allow for continuous video monitoring of the animals' behavior. A wide angle lens permitted videotaping of up to eight animals simultaneously. The manifestation of seizure activity was analyzed on DDS2-files and video tapes.

Analysis of the occurrence of spontaneous seizures from the video-EEG recorded during epileptogenesis and established epilepsy

Each EEG file was analyzed manually by scanning through the EEG recording on the computer screen. If an electrographic seizure was observed, behavioral severity was analyzed from the corresponding video-recording. An electrographic spontaneous seizure was defined as a high-frequency (>5 Hz), high-amplitude (>2 \times baseline) discharge that lasted at least 5 s. The severity of behavioral seizures was scored according to a slightly modified Racine's scale (Racine, 1972): score 0, electrographic seizure without any detectable motor manifestation; score 1, mouth and face clonus, head nodding; score 2, clonic jerks of one forelimb; score 3, bilateral forelimb clonus; score 4, forelimb clonus and rearing; and score 5, forelimb clonus with rearing and falling.

Histology

The animals were perfused according to the Timm fixation protocol, and the brains were postfixed, cryoprotected, cut in 1-in-5 series (30 μm), and stored as described previously (Nissinen et al., 2000).

Mossy fiber sprouting was analyzed from sections stained using the Timm sulfide/silver method (Sloviter, 1982). Both the ipsilateral and contralateral hippocampus were analyzed. Density of sprouting was analyzed in each section along the septotemporal

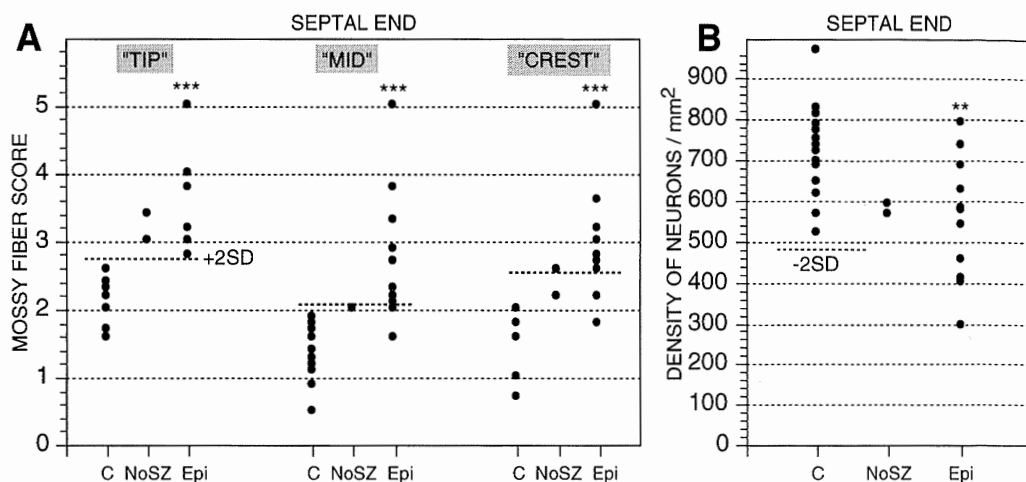


FIGURE 2. A: Mossy fiber sprouting in the septal end of the dentate gyrus in experiment I. Note that the stimulated nonepileptic rats also had increased sprouting (>2 SD compared to control mean, indicated with a dashed line) in the "tip" and "crest" portions.

B: Mean density of hilar neurons was reduced by 21% in epileptic rats. C, controls; Epi, epileptic rats; NoSz, stimulated nonepileptic rats; SD, standard deviation of the mean. Statistical significance compared to controls: $**P < 0.01$, $***P < 0.001$.

axis of the hippocampus (Fig. 1). In the septal end, sprouting was assessed separately from the "tip", "mid", and "crest" portions of the granule cell layer by starting at the level at which the suprapyramidal and infrapyramidal blades of the granule cell layer form a continuous band of cells (Fig. 1A,B). More temporally, sprouting was scored in the dorsal mid-portion and ventral mid-portion of the dentate gyrus, where the granule cell layer of the septal and temporal ends becomes fused and forms an easily identifiable and standardized "oval-shaped" layer (AP level 6.1–6.7 mm posterior to bregma; Fig. 1C,D). The density of mossy fiber sprouting was scored according to Cavazos et al. (1991): score 0, no granules; score 1, sparse granules in the supragranular region and in the inner molecular layer; score 2, granules evenly distributed throughout the supragranular region and the inner molecular layer; score 3, an almost continuous band of granules in the supragranular region and inner molecular layer; score 4, continuous band of granules in the supragranular region and in the inner molecular layer; and score 5, confluent and dense laminar band of granules that covers most of the inner molecular layer, in addition to the supragranular region. The mean of the scores (mean mossy fiber score) in all septal sections (i.e., sprouting in the (tip + mid + crest)/3) was calculated and used in the statistical analysis. The mean of scores in the dorsal mid- and ventral mid-temporal portions was calculated and used in analysis. Scoring was conducted blindly with respect to the treatment status of the animal.

To identify the cytoarchitectonic boundaries and the location of the stimulating electrode, and to assess the severity of neuronal damage, one series of sections was stained with thionin. Because mossy fiber sprouting has a septotemporal gradient, the density of hilar cells was estimated from the septal and

temporal ends of the hippocampus separately. In the septal end, neuronal density was assessed from three sections that were systematically sampled at 450- μ m intervals, starting at the level at which the suprapyramidal and infrapyramidal blades of the granule cell layer form a continuous band of cells. In the temporal end, neuronal density was assessed from two adjacent sections (150 μ m apart) at the level where the granule cell layer forms an "oval-shaped" layer (Fig. 1C). Therefore, neuronal damage was assessed from sections that matched those used for the analysis of sprouting.

Hilar cell density was estimated using a fractionator with Stereo Investigator software in a Neuro Lucida morphometry system (MicroBrightField, Colchester, VT), according to guidelines described by West et al. (1991). A color video camera (Hitachi HV-C20, Japan), interfaced with an Olympus BX50 microscope, was used to view sections on a high-resolution monitor, and neuroanatomic borders of the hilus were digitized under low-power magnification. Subsequent cell counting was confined within these borders. The sections were inspected according to a systematic random sampling scheme such that counts were derived from a known and representative fraction of the hilus. Specifically, the motorized stage of the microscope system was under computer control, and the hilar fields in every histologic section were surveyed at evenly spaced x-y intervals (70 \times 70 μ m septally, 150 \times 150 μ m temporally). For each x-y step, cell counts were derived from a known fraction of the total area, using an unbiased counting frame that was 25 \times 25 μ m septally and 30 \times 30 μ m temporally. Counting was performed throughout the section, avoiding the neurons that were in focus at the surface of the section. Neuronal nuclei were counted only as they first came into focus within each optical disector. Glia, iden-

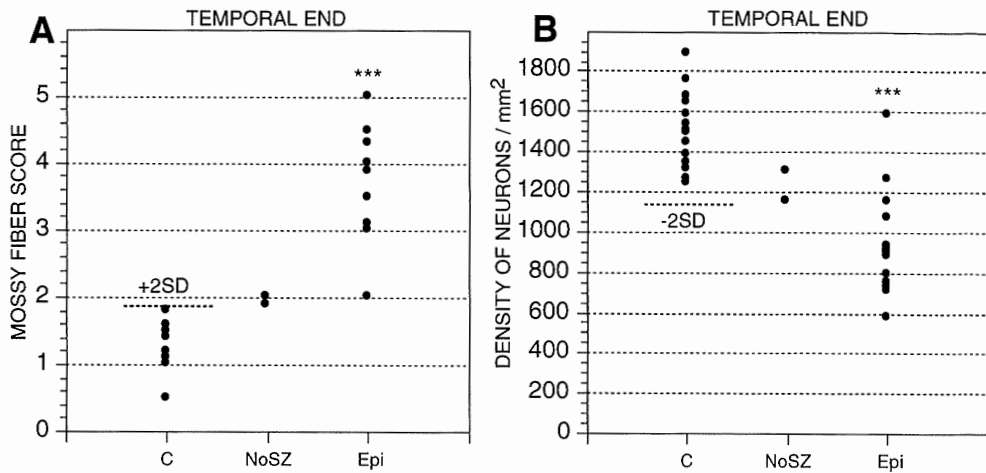


FIGURE 3. A: Mossy fiber sprouting in the temporal end of the dentate gyrus in experiment I. Note that the stimulated nonepileptic rats also had increased sprouting (>2 SD compared to control mean, indicated with a dashed line). B: Mean density of hilar neurons was

reduced by 38% in epileptic rats. C, controls; Epi, epileptic rats; NoSZ, stimulated nonepileptic rats; SD, standard deviation of the mean. Statistical significance compared to controls: $***P < 0.001$.

tified by size and cytologic characteristics, were excluded from the counts. Neuronal densities were calculated by dividing the neuronal number by the area. The mean density was used for the statistical analysis. Analyses were conducted blindly with respect to the treatment status of the animal.

Photomicrographs were taken with a Leica DM RB microscope equipped with a camera system. Low-power photomicrographs were taken with a Nikon 6×9 cm system.

Experiment II

To confirm and further investigate the data obtained in experiment I, SSSE was induced in another group of 30 rats, and each rat was monitored with video-EEG (every other day, 24 h/day) for 60 days. In addition, there were 14 electrode-implanted unstimulated controls. In contrast to experiment I, in experiment II SSSE was stopped with diazepam (15 mg/kg initially and 10 mg/kg 8 h later, i.p.) after the rats experienced 5 or fewer HAFDs ($n = 7$), 6–50 HAFDs ($n = 15$), or more than 50 HAFDs (or a maximum of 3 h after the beginning of SSSE; $n = 8$). Analyses of mossy fiber sprouting and neuronal densities were performed as in experiment I.

Discontinuation of SSSE at different time points was expected to provide data regarding the association of the duration of SSSE with the severity of sprouting and to produce more animals with SSSE but no spontaneous seizures. Further, because the survival time in each animal in experiment II was the same, we eliminated the effect of differential survival time on the variability in the density of mossy fiber sprouting between animals.

Statistical Analysis

Data were analyzed using a Macintosh computer and StatView 4.0 (non-FPU-version, Deneba Software, Berkeley, CA). Differences in the density of sprouting or hilar neurons between different animal groups were analyzed using the Kruskal-Wallis test. An increase in mossy fiber sprouting was defined as mossy fiber density greater than 2 standard deviations (SD) from the control mean. Post hoc analysis of significance between different animal groups was performed using the Mann-Whitney U-test. Correlations were assessed using the Spearman rank correlation test. A P value of less than 0.05 was considered statistically significant.

RESULTS

Experiment I

Of 16 stimulated rats, 13 had two or more spontaneous seizures. One rat had only one spontaneous seizure, but the EEG showed interictal spiking activity. Therefore, 14 of 16 rats were considered epileptic at the end of the study. The number of spontaneous seizures varied from 1–70 (median, 8). During the 11-day follow-up, the seizure frequency varied from 0–6.2/day (median, 0.5). Of the 14 rats, 5 had more than one seizure per day on average, and consequently, these animals belonged to a subgroup of animals with "frequent seizures". In 8 rats, the mean seizure frequency was less than 1/day during follow-up; they were classified into a subgroup of rats with "rare seizures".

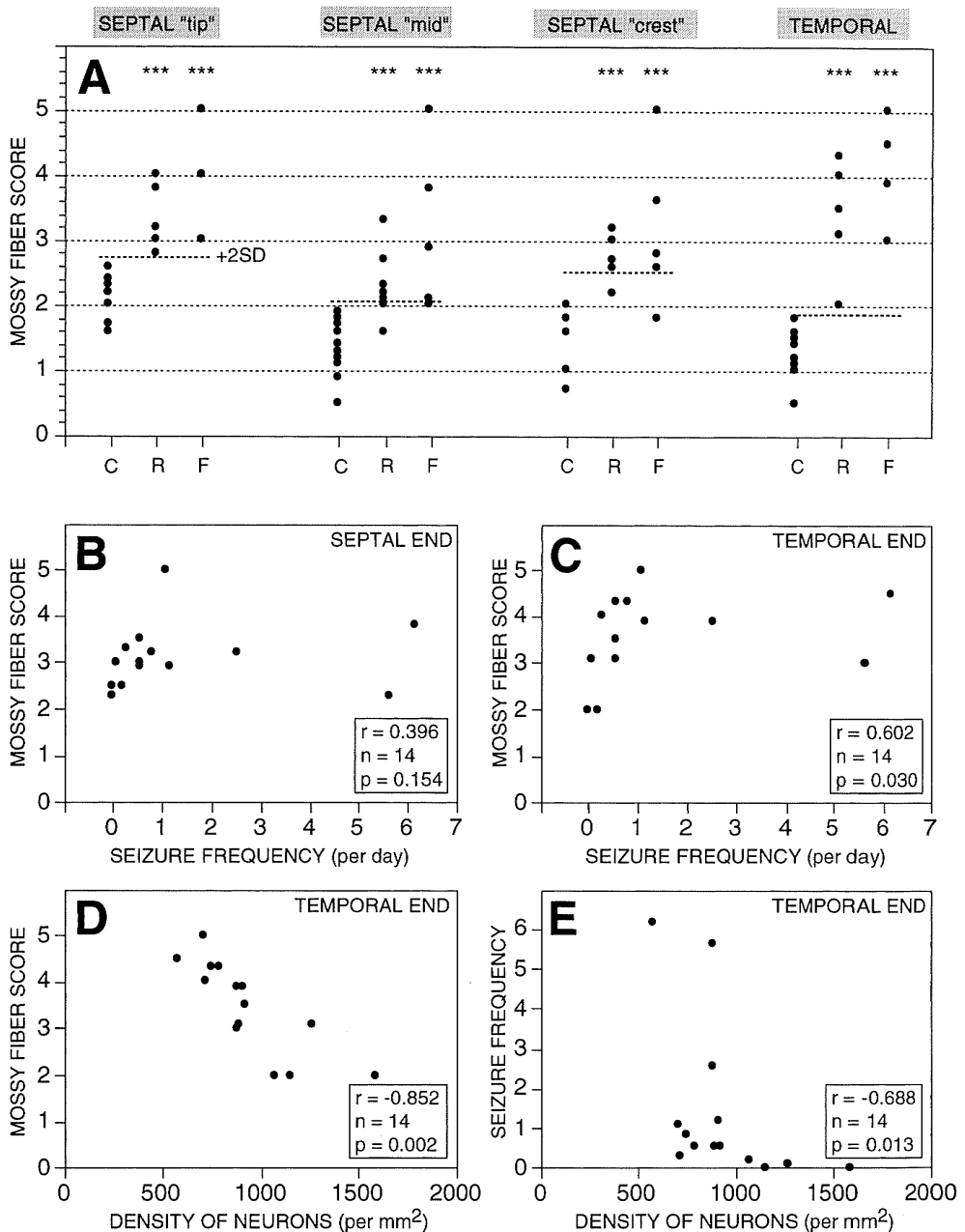


FIGURE 4. A: Mossy fiber sprouting in rats with "rare" (<1 seizure/day) or "frequent" (>1 seizure/day) seizures in different parts of the dentate gyrus in experiment I. Note the substantial overlap in density of sprouting between rats with "rare" or "frequent" seizures. Scattergrams show correlations (B) between seizure frequency and mossy fiber sprouting (mean mossy fiber score) in the ipsilateral septal end of the dentate gyrus, (C) between seizure frequency and mossy fiber sprouting in the ipsilateral temporal end of the dentate gyrus,

(D) between severity of hilar cell damage and mossy fiber sprouting in the temporal end of the dentate gyrus ipsilaterally, and (E) between neuronal density in the ipsilateral temporal hilus and seizure frequency. C, controls; n, number of rats; p, statistical significance; r, correlation coefficient (Spearman); R, "rare" seizures; F, "frequent" seizures; SD, standard deviation of the mean. Statistical significance compared to controls: *** $P < 0.001$.

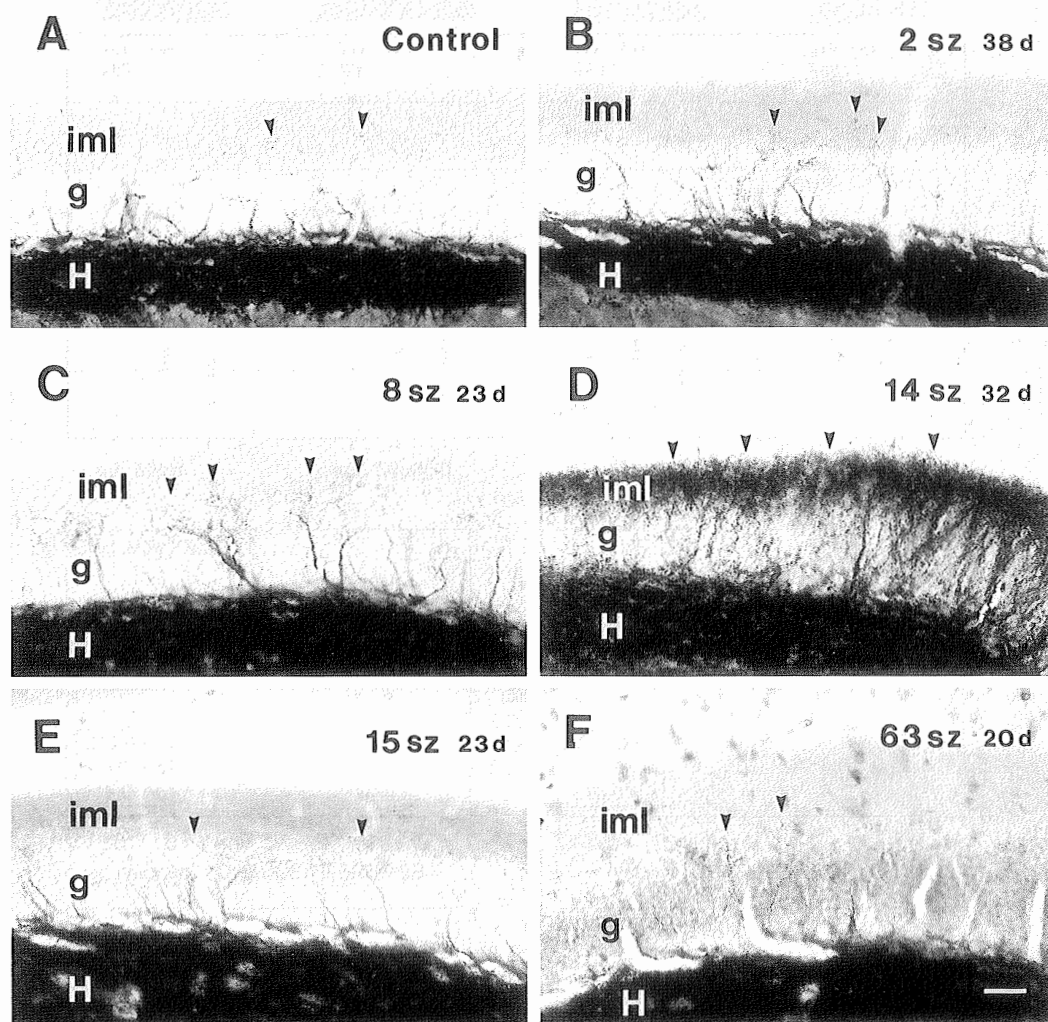


FIGURE 5. Bright-field photomicrographs from Timm-stained sections, demonstrating the density of mossy fiber sprouting (arrowheads) in different cases in experiment I. Photomicrographs were taken from the "mid" portion of the septal dentate gyrus. A: Control. B: Rat with two spontaneous seizures, perfusion 38 days after stimulation. C: Rat with eight spontaneous seizures, perfusion 23 days after stimulation. D: Rat with 14 spontaneous seizures, perfusion 32 days

after stimulation. E: Rat with 15 spontaneous seizures, perfusion 23 days after stimulation. F: Rat with 63 spontaneous seizures, perfusion 20 days after stimulation. Note the difficulty in predicting seizure activity based on the density of mossy fiber sprouting. Survival time did not correlate with density of sprouting. d, day; g, granule cell layer; h, hilus; iml, inner molecular layer; sz, seizure. Scale bar: 50 μ m.

Time to the first spontaneous seizure was 17 ± 13 days (range, 8–51 days; median, 11 days), and time to the second spontaneous seizure was 23 ± 15 days (range, 8–54 days; median, 17 days). Time from SSSE to perfusion varied from 20–63 days (mean, 39 ± 16 days; median, 35 days).

Ipsilateral side

In the epileptic rats, the mean density of mossy fiber sprouting was increased in the "tip" (3.76 ± 0.58), "mid" (2.62 ± 0.90), and "crest": (2.92 ± 0.76 ; $P < 0.001$) portions of the inner molecular

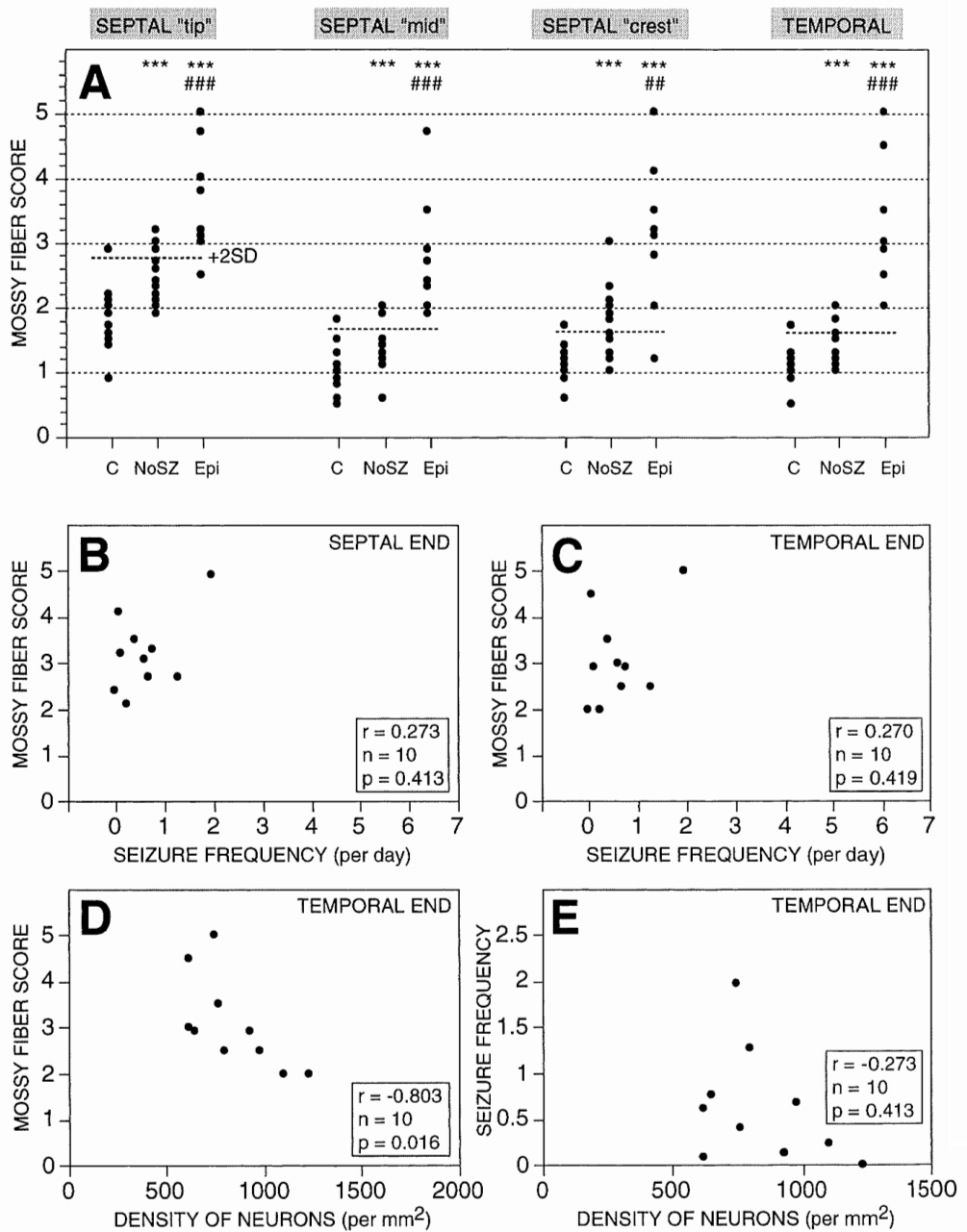


FIGURE 6

layer of the septal dentate gyrus, compared to controls (2.18 ± 0.29 , 1.37 ± 0.36 , and 1.79 ± 0.39 , respectively; Fig. 2A). Both of the stimulated nonepileptic rats had increased mossy fiber sprouting (>2 SD from the control mean) in the "tip" and one in the "mid" portion (Fig. 2A). The mean hilar cell density in epileptic rats was reduced by 21% compared to controls (560 ± 135 vs. 713 ± 116 , $P < 0.01$; Fig. 2B). In the two stimulated nonepileptic rats, neuronal density was within the ± 2 SD range of the control mean (Fig. 2B).

In the temporal half, the mean density of mossy fiber sprouting was increased in epileptic rats compared to controls (3.48 ± 0.98 vs. 1.29 ± 0.30 , $P < 0.001$; Fig. 3A). Also, in the two stimulated nonepileptic rats, the density of sprouting exceeded the >2 SD limit compared to controls (Fig. 3A). The mean hilar cell density was decreased by 38% in epileptic rats compared to controls (940 ± 261 vs. $1,517 \pm 184$, $P < 0.001$; Fig. 3B). In the two stimulated nonepileptic rats, the neuronal density was within the ± 2 SD range of the control mean (Fig. 3B). There was no difference in mean density of sprouting between rats with "mild" or "severe" epilepsy (Fig. 4).

In the epileptic rats, the density of mossy fiber sprouting did not correlate with the number of HAFDs during the SSSE or the total duration of SSSE (time between first and last HAFD). Also, there was no correlation between density of sprouting and latency to onset of first or second seizure, or the time to perfusion. In the temporal end, the mean density of sprouting ($r = 0.602$, $P < 0.05$; Fig. 4C) as well as hilar cell density ($r = -0.688$, $P < 0.05$; Fig. 4E) correlated with the total number of seizures that each animal had experienced (i.e., seizure frequency, because the follow-up time in each animal was 11 days). Further, the degree of sprouting correlated with hilar cell density ($r = -0.852$, $P < 0.01$; Fig. 4D). There were no such correlations in the septal end (Figs. 4B, 5).

Contralateral side

In epileptic rats, the mean density of mossy fiber sprouting in the contralateral septal or temporal hippocampus did not differ from that on the ipsilateral side. Also, the severity of neuronal damage did not differ between the contralateral and ipsilateral

sides. The density of sprouting was within the 2 SD limit of the control mean in 5 of 14 epileptic rats in the "mid" portion and in 4 of 14 rats in the "crest" portion of the septal hippocampus. Of 14 epileptic rats, 1 had normal sprouting density in the temporal end. Both of the stimulated nonepileptic rats had increased sprouting in the "tip" portion of the septal hippocampus and in the temporal hippocampus. The mean density of sprouting did not differ between rats with "mild" or "severe" epilepsy (data not shown).

There were no correlations between density of sprouting and number of HAFDs during SSSE, duration of SSSE, time to the first or second spontaneous seizure, or time to perfusion. As ipsilaterally, in the temporal end the mean density of sprouting ($r = 0.599$, $P < 0.05$), as well as the degree of hilar cell loss ($r = -0.689$, $P < 0.05$), correlated with the total number of seizures (i.e., seizure frequency). Further, the degree of sprouting correlated with neuronal density ($r = -0.748$, $P < 0.01$; Fig. 4).

Experiment II

The time from the SSSE to perfusion in experiment II was 60 days in all rats. Ten animals were considered epileptic. Twenty rats with SSSE did not develop epilepsy (no difference in duration of SSSE between epileptic and nonepileptic rat groups). The number of spontaneous seizures recorded during a 60-day follow-up (every other day) varied from 1–51 (median, 8). Therefore, the estimated total number of seizures varied from 2–102 (median, 16). The duration of epilepsy (time from second seizure to perfusion) varied from 15–53 days (mean, 35 ± 16 days; median, 41 days). Consequently, seizure frequency after occurrence of the second seizure varied from 0–2.0/day (median, 0.5/day).

As in experiment I, the mean density of sprouting in the ipsilateral hippocampus of the epileptic animals was higher compared to stimulated nonepileptic animals or controls ("tip", 3.70 ± 0.78 vs. 2.52 ± 0.48 vs. 1.81 ± 0.48 ; "mid", 2.71 ± 0.85 vs. 1.54 ± 0.40 vs. 1.04 ± 0.33 ; "crest", 3.08 ± 1.04 vs. 1.97 ± 0.56 vs. 1.13 ± 0.26 ; temporal end, 3.08 ± 1.00 vs. 1.60 ± 0.35 vs. 1.09 ± 0.27 ; Fig. 6A). As in experiment I, several stimulated nonepileptic rats had increased sprouting but no detected seizures (Fig. 6A). Moreover, the mean density of mossy fiber sprouting in stimulated nonepileptic animals was higher than in controls (Fig. 6A).

As in experiment I, in epileptic rats the density of mossy fiber sprouting (ipsilateral or contralateral, septal end or temporal end) did not correlate with the number of HAFDs during SSSE or the total duration of SSSE. Also, there was no correlation between density of sprouting and the latency to appearance of the first or second seizure. In experiment II, in which the follow-up of animals was approximately 3 weeks longer than in experiment I, there was no correlation between density of sprouting and total number of seizures that each animal had experienced, seizure frequency (Fig. 6B,C), or duration of epilepsy. Also, degree of hilar cell loss did not correlate with seizure frequency. As before, however, the degree of sprouting in the temporal end correlated with the severity of hilar cell damage both ipsilaterally ($r = -0.803$, $P < 0.05$; Fig. 6D) and contralaterally ($r = -0.724$, $P < 0.05$).

FIGURE 6. A: Mossy fiber sprouting in different parts of the dentate gyrus in experiment II. As in experiment I, many animals without seizures had abnormal sprouting. Scattergrams show the correlations (B) between seizure frequency and mossy fiber sprouting (mean mossy fiber score) in the septal end of the dentate gyrus, and (C) between seizure frequency and mossy fiber sprouting in the ipsilateral temporal end of the dentate gyrus. Unlike experiment I, the density of mossy fiber sprouting did not correlate with seizure frequency. D: Correlation between severity of hilar cell damage and mossy fiber sprouting in the ipsilateral temporal end of the dentate gyrus. E: Correlation between neuronal density in the ipsilateral temporal hilus and seizure frequency. C, controls; Epi, epileptic rats; n, number of rats; NoSz, stimulated nonepileptic rats; p, statistical significance; r, correlation coefficient (Spearman); SD, standard deviation of the mean. Statistical significance: *** $P < 0.001$ compared to controls; ## $P < 0.01$, ### $P < 0.001$ compared to nonepileptic stimulated rats.

DISCUSSION

The present study investigated whether the sprouting of granule cell axons (mossy fibers) is critical for the occurrence of spontaneous epileptic seizures. The following questions were addressed: 1) Do all animals with seizures have sprouting? 2) Does density of sprouting correlate with frequency of spontaneous seizures? 3) Do all animals with sprouting have seizures? We used our recently developed animal model that mimics all three phases of symptomatic TLE in humans; i.e., initial insult leading to epileptogenesis leading to epilepsy. To determine the time when the first spontaneous seizures occurred, animals were monitored long-term with video-EEG 24 h/day. Considering that epilepsy persists in these animals for the rest of their lives, the analyses performed 11 days or approximately 35 days after epilepsy diagnosis occurred at an early stage of the disease.

Mossy fiber sprouting is a typical pathologic finding in animals and humans with spontaneous seizures of temporal lobe origin (reviewed in Dudek et al., 1994). Because of methodologic challenges, however, the question remains as to whether sprouting is present at the time when the first spontaneous seizures appear, or whether it develops as a consequence of recurrent seizures. Data obtained in the present study indicated that all animals had increased mossy fiber sprouting in some portion of the dentate gyrus at the time of the first spontaneous seizures, i.e., the time of epilepsy diagnosis. The most clear sprouting was observed in the temporal end of the hippocampus, where all animals with seizures had increased sprouting (≥ 2 SD compared to control mean). Septally, sprouting was most pronounced in the "tip" of the granule cell layer, where all seizing animals had increased sprouting. In the "mid" and "crest" portions of the septal dentate gyrus, however, the density of mossy fibers in some seizing animals was similar to that in controls. The presence of sprouting at time of first seizures supports the idea that mossy fiber sprouting is a causative factor for seizure occurrence. Further analysis of the data, however, challenged this conclusion.

A detailed analysis of the distribution of mossy fiber sprouting in different parts of the hippocampus in two separate experiments revealed that animals with no spontaneous seizures had the same degree of abnormal sprouting as some of the chronically seizing animals. Similar observations were reported by Mello et al. (1993) in a pilocarpine model, and by Buckmaster and Dudek (1997) in a kainate model. However, interpretation of the results of these studies is compromised by the lack of continuous monitoring of electrographic seizure activity that might have resulted in underestimation of seizure number, particularly that of partial seizures. In a kindling model, relatively dense sprouting has been observed without spontaneous seizures (Cavazos et al., 1991). These data from different models of TLE suggest that sprouting occurs without spontaneous seizures. Considering that some of our nonseizing animals had the same density of sprouting as some epileptic animals, and that some of these animals might have developed seizures if the follow-up had been continued for an extended period of time, it appears reasonable to conclude that sprouting probably

precedes the occurrence of spontaneous seizures. Confirmation, however, will require a longitudinal follow-up of sprouting in vivo that is not possible using current technology. It is unlikely that sprouting in nonseizing animals was associated with the time of delay to perfusion, because similar findings were obtained in experiment II, in which all animals were perfused 60 days after stimulation. Finally, it is possible that our monitoring system did not detect a few seizures that the nonseizing animals with sprouting might have experienced. This is unlikely, however, because 96% of the seizures in animals with a low seizure frequency are secondarily generalized and detectable with cortical electrodes (Nissinen et al., 2000). Whether the absence of seizures in animals with sprouting was associated with low density of sprouting was thus assessed.

There was no correlation between density of sprouting and seizure frequency in epileptic rats in experiment II, even though there was such a correlation in experiment I. This might relate to the difference in the duration of follow-up in the two experiments. In experiment II, rats were followed for 35 days on average after the diagnosis of epilepsy, compared to 11 days in experiment I. Consequently, in experiment II the seizure frequency might have been more stabilized and therefore might more reliably reflect long-term seizure frequency, as supported by previous follow-up studies (Hellier et al., 1998; Nissinen et al., 2000; Pitkänen and Nissinen, 2000). Several previous studies, however, demonstrated an association between mossy fiber sprouting and increased excitability. In hippocampal slices cut from kainate-treated chronically epileptic animals, Wuarin and Dudek (1996) reported a correlation between the occurrence of seizure-like events and a proportion of granule cells connected through excitatory circuits. Further, Cavazos et al. (1991) reported that animals with a higher number of kindled seizures had a higher density of sprouting. One explanation is that the majority of seizures in our model originated in the amygdala, even though they readily spread to the hippocampus (Nissinen et al., 2000), and therefore the extrahippocampal alterations in the circuitry have a more critical role than mossy fiber sprouting in seizure initiation. Consistent with our observation, Buckmaster and Dudek (1997) did not observe a correlation between frequency of spontaneous seizures and mossy fiber sprouting in kainate-treated chronically epileptic animals. Mello et al. (1993) also reported a lack of a correlation in chronically epileptic animals in the pilocarpine model. Finally, sprouting in the present model as well as in the kainate and pilocarpine models is always bilateral, but the seizures might have a unilateral focal onset. This observation also argues against the role of sprouting in a unilateral seizure onset. Therefore, the association between mossy fiber sprouting and seizure susceptibility in hippocampal slices from epileptic animals or during the kindling procedure is not apparent when the association of density of sprouting with the occurrence of spontaneous behavioral and electrographic seizures is assessed in epileptic rats in vivo. One explanation for this discrepancy is that the observations in slices are performed under conditions in which GABAergic inhibition is masked. In *in vivo* models, however, GABAergic inhibition is not consistently reduced. Rather, the inhibition in the dentate gyrus is enhanced (Babb et al., 1989; Schwarzer and Sperk, 1995; Buckmaster and Dudek, 1997).

Mossy fiber sprouting is one form of axonal plasticity that might be an attempt of the brain to restore function following neuronal damage. Consistent with this idea, there was a correlation between density of sprouting and severity of hilar cell loss both ipsilaterally and contralaterally in the temporal end of the hippocampus. This observation is similar to findings of Buckmaster and Dudek (1997) and Pitkänen et al. (1999) in the kainate model. Unexpectedly, there was no association between duration of SSSE and sprouting, which probably relates to the fact that there is no correlation between neuronal density in the hilus and duration of SSSE. This is consistent with our earlier study in another series of rats, in which the total number of hilar neurons were estimated using stereologic cell counting (Nissinen et al., 2000; Nissinen and Pitkänen, unpublished findings). This is in contrast to the observations of Lemos and Cavalheiro (1995), who reported that sprouting and neuronal damage in the hilus were more pronounced in rats with a longer duration of status epilepticus. Our findings, however, are consistent with theirs in that SSSE has to last for at least 20 min before any damage is detected (Lukasiuk and Pitkänen, 1998). Furthermore, recent studies indicate that axonal sprouting in the epileptic brain is not limited to the dentate gyrus. Sprouting has also been detected in the damaged CA1 subfield of the hippocampus (Meier et al., 1992; Perez et al., 1996; Mikkonen et al., 1998) and the entorhinal cortex (Mikkonen et al., 1998). Sprouting might occur in all these locations in the same brain (Mikkonen et al., 1998). These observations support the view that the site of plastic response does not necessarily correspond to the cellular location of seizure onset.

Mossy fiber sprouting can be induced experimentally without any epileptic seizures. For example, long-term potentiation induces mossy fiber sprouting in the CA3 subfield of the hippocampus and in the inner molecular layer of the dentate gyrus (Adams et al., 1997). Also, lesions of the perforant path (Zimmer, 1973, 1974) or genetic mutations (prion protein null mouse, Colling et al., 1997) induce sprouting without any seizure activity. One study in humans without a history of epilepsy also demonstrated mossy fiber sprouting (Cassell and Brown, 1984).

Taken together, our data indicate that although mossy fiber sprouting is present in all animals with spontaneous seizures, its presence is not necessarily associated with the occurrence of spontaneous seizures.

Acknowledgments

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III

Chronic elevation of brain GABA levels beginning
two days after status epilepticus does not prevent
epileptogenesis in rats

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Chronic elevation of brain GABA levels beginning two days after status epilepticus does not prevent epileptogenesis in rats

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Abstract

Vigabatrin (VGB) treatment is neuroprotective in various models of status epilepticus (SE) and delays the development of kindling via mechanisms that are assumed to relate to the elevation of GABA levels in the brain. Here, we tested the hypothesis that a chronic elevation of brain GABA levels obtained by VGB treatment prevents the development of spontaneous seizures (i.e. epilepsy) following SE in rats. Self-sustained SE (SSSE) was induced by stimulating the lateral nucleus of the amygdala. Two days later, chronic VGB (75 mg/kg/day) or saline treatment was started *via* subcutaneous osmotic minipumps. The development of spontaneous seizures was monitored once a week (24 h at a time) using video-EEG recording. Rats were perfused for histology either at the end of the 10-week drug treatment, or later at the end of an 8-week drug-free follow-up period. Before perfusion for histology, spatial learning and memory perform was tested in the Morris water-maze. Spontaneous seizures were observed in 55% (6/11) of the saline-treated and 73% (8/11) of the VGB-treated rats during the 10-week treatment period. Seizure frequency, severity, and duration were similar in VGB-treated rats and controls during and after the drug-treatment period. VGB treatment did not decrease neuronal damage in various temporal lobe regions or mossy fiber sprouting. VGB treatment also did not attenuate spatial learning or memory impairments. These findings indicate that the augmentation of GABAergic neurotransmission by VGB does not prevent the development of epilepsy when treatment is started 2 days after SE. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Amygdala; Antiepileptic drug; Electroencephalogram; Epilepsy; Neuronal damage; Vigabatrin

1. Introduction

Previous data from animal and human studies indicate that the development of symptomatic temporal lobe epilepsy (TLE) is a process that includes three phases:

1. brain insult [e.g., head trauma, stroke, cerebral infection, prolonged febrile seizures, or status epilepticus (SE)]
2. epileptogenesis; and
3. spontaneous seizures or epilepsy (see Mathern et al., 1995b).

Initial brain insults, such as SE, induce several pathologic processes that may occur at different times during

the epileptic process. For example, neuronal damage and the activation of genes regulating cellular death or survival occur within hours to a few days after the insult, whereas the axonal and dendritic plasticity appear after many days, weeks, or months (see DeLorenzo and Morris, 1999). Some changes presumably favor functional recovery whereas others lead to the development of pathologic neuronal circuitries, and eventually to the appearance of spontaneous seizures. An important question to be answered is: How can the cascades of events following brain insults be controlled in such a way that the development of epilepsy is prevented without compromising recovery? Do antiepileptic drugs, designed to control seizures, have a role in the treatment regimen?

A few clinical trials have demonstrated that prophylactic treatment with carbamazepine, phenytoin, or phenobarbital does not prevent the development of epilepsy following head trauma (see reviews of Hernandez, 1997; Temkin et al., 1998). Furthermore, the occurrence of seizures in patients with brain tumors are not pre-

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vented by valproic acid (Glantz et al., 1996). The effects of drugs on epileptogenesis associated with brain damage have been investigated in rats by treating them after SE. Data available on these studies, however, are somewhat inconclusive. For example, chronic phenobarbital treatment following kainic acid-induced SE does not prevent epileptogenesis in rats (Mikati et al., 1994; Bolanos et al., 1998), whereas treatment with a high dose of valproic acid (1200 mg/kg/day) prevents the appearance of generalized behavioral seizures (Bolanos et al., 1998). The effect of valproate on the development of partial seizures, however, was not analyzed. The antiepileptogenic effects of other antiepileptic drugs in models where the epileptogenesis is preceded by brain damage are unknown. Candidate drugs for trials aimed at preventing epilepsy should:

1. affect the mechanisms underlying the development of a decreased seizure threshold;
2. attenuate neuronal damage following brain insult; and
3. delay the development of kindling, which is presumed to predict antiepileptogenic efficacy.

γ -Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain and has an important role in epileptogenesis. For example, loss of GABAergic neurons (Houser and Esclapez, 1996) and GABA_A receptor mediated inhibition (Shirasaka and Wasterlain, 1994) are associated with chronic epileptogenicity following SE. Recent studies demonstrated that the expression of various GABA_A receptor subunits is altered before the development of spontaneous seizures (Rice et al., 1996; Brooks-Kayal et al., 1998; Sperk et al., 1998). Drugs that augment GABAergic inhibition [e.g., benzodiazepines, barbiturates, vigabatrin (VGB) and tiagabine] are effective antiepileptics in experimental and human epilepsy (see reviews of Grant and Heel, 1991; Ticku and Kulkarni, 1992; Bourgeois, 1996; Homan and Rosenberg, 1996; Adkins and Noble, 1998). These compounds also have neuroprotective effects in models of SE if the treatment is started before or during SE (Fuller and Olney, 1981; Ault et al., 1986; Ylinen et al., 1991; Sutula et al., 1992; Halonen et al., 1995, 1996; Lemos and Cavalheiro, 1995; Pitkänen et al., 1996). Finally, GABAergic compounds suppress or delay the development of kindling (Shin et al., 1986; Schmutz et al., 1988; Dalby and Nielsen, 1997).

The enhancement of brain GABAergic function appears to:

- 1 restore impaired neuronal inhibition;
- 2 attenuate neuronal damage induced by SE; and
- 3 delay the development of kindling.

Thus, we hypothesized that the development of epilepsy

following SE would be delayed or prevented by chronically elevating brain GABA levels. With this goal in mind, SE was induced in rats by electrically stimulating the amygdala and VGB treatment was initiated using subcutaneous minipumps to elevate the brain GABA levels approximately two-fold. To mimic a clinical situation, prophylactic VGB administration was started 2 days *after* the end of SE. Chronic VGB treatment, however, failed to prevent the development of epilepsy following SE in rats.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats ($n=48$, 275–325 g) were used in the study. After implantation of electrodes, the rats were housed in individual cages at 19–21°C, 50–60% humidity, and lights on from 0700 to 1900 h. Standard food pellets and water were freely available. Animal procedures were conducted in accordance with the guidelines of the European Community Council directives 86/609/EEC.

2.2. Implantation of electrodes

The animals were deeply anesthetized intraperitoneally with a mixture of sodium pentobarbital (60 mg/kg) and chloral hydrate (100 mg/kg) and placed in a Kopf stereotaxic frame (lambda and bregma at the same horizontal level). A pair of stimulation electrodes (electrode tip separation: 0.5 mm) was implanted into the lateral nucleus of the left amygdala [atlas coordinates: 3.6 mm posterior, 5.0 mm lateral to bregma, and 6.5 mm ventral to brain surface according to Paxinos and Watson (1986)]. One stainless steel screw to serve as an epidural electrode was inserted into the skull above the right frontal cortex (3 mm anterior to bregma, 2 mm lateral to midline). Two stainless steel screws to serve as indifferent and ground electrodes were inserted into the skull bilaterally over the cerebellum. The electrodes were fixed with dental acrylate (Selectaplast CN, Dentsply DeTrey GmbH, Dreieich, Germany). Following surgery, the rats were allowed to recover for 14 days before electrical stimulation was started.

2.3. Induction of self-sustained status epilepticus (SSSE)

Afterdischarge threshold was assessed by stimulating the amygdala with a 1-s train of 60 Hz, 1-ms biphasic square-wave pulses starting from 50 μ A and increasing the current with 25 μ A increments to 150 μ A and then at 50 μ A increments to 400 μ A (peak to peak). Only those rats in which afterdischarges could be induced at

400 μ A or lower were included in the study. Self-sustained SE was induced as described previously (Nissinen et al., 2000). Briefly, the lateral nucleus of the amygdala was stimulated with 60-Hz trains (2 trains/s, each train lasting 100 ms) of 1-ms biphasic square-wave pulses at 400 μ A current (peak to peak). After 20 min of continuous stimulation, the stimulation was interrupted and the behavioral and electrographic seizure activity of the animal was observed for 60 s. If the behavior of the animals revealed the presence of epileptic activity (head nodding and/or limb clonus), the observation was extended up to 5 min. If the animal did not meet the criterion of beginning clonic SSSE (continuous electrographic epileptiform spiking and recurrent behavioral clonic seizures), stimulation was resumed and the behavior of the animal was checked again after 5 min. No further stimulation was given once the criterion of SSSE was achieved or if the rat did not develop SSSE in response to a 40-min stimulation.

2.4. Characterization of electrophysiologic seizures

In order to electrophysiologically characterize the severity and duration of SSSE, the seizure activity was recorded using a video-digital EEG system during the amygdala stimulation (Nervus EEG Recording System, Tautagreining, Iceland) and a video-digital EEG system (Stellate EEG Monitor System, Montreal, Canada) for 16 h thereafter. A conspicuous feature of EEG activity during SE was the occurrence of high-amplitude and high-frequency discharges (HAFDs), which are typically associated with behavioral seizures. HAFD was defined as a high-amplitude ($>2\times$ baseline) and a high-frequency (>8 Hz), discharge in the amygdala or in the cortex (or both) that lasted for at least 5 s. The number and duration of HAFDs was used as a measure of the severity of SE. The analysis included the number and total duration of HAFDs and the time elapsed to the appearance of the last HAFD.

After the initial SSSE, the rats were observed once a week using the video-EEG monitoring system (Stellate) for 24 h at a time. Each EEG file was analyzed manually by an experienced observer (T.H.) to detect the appearance of spontaneous seizures. Epileptic seizure was defined as a high frequency (>5 Hz) and high amplitude ($>2\times$ baseline) discharge, either in the amygdala or in the cortex (or both), that lasted for at least 5 s.

2.5. Characterization of behavioral seizures

Videotapes were viewed to rate the severity of behavioral seizures into two categories:

1. subclinical (i.e., seizure detected in EEG recordings only) or partial seizures (jaw clonus, head nodding, unilateral forelimb clonus); and

2. secondarily generalized seizures (bilateral forelimb clonus).

2.6. Preliminary experiment: antiepileptic effect of VGB on spontaneous seizures

To determine whether VGB treatment suppresses spontaneous seizures in the model used in the present experiment, we administered VGB into chronically epileptic rats that had been experiencing seizures for ca 4 months. Baseline seizure frequency and duration were monitored with continuous video-EEG recording for 7 days in seven vehicle-treated rats (saline administered *via* a subcutaneous Alzet minipump). Thereafter, minipumps were changed and the same animals were treated with VGB (75 mg/kg/day) for 7 days. After a drug-wash-out period of 2 weeks, rats were treated with a higher dose of VGB (250 mg/kg/day, *i.p.*, one dose) for 5 days.

2.7. Antiepileptogenic effect of VGB: chronic administration of VGB and saline

Rats were assigned to the following groups:

1. unstimulated, 10-week saline-treated controls (unstimulated controls);
2. stimulated, 10-week saline-treated controls (Sal-10 group);
3. stimulated, 10-week saline-treated controls, continued with 8-week follow-up period (Sal-10-FU-8 group);
4. stimulated, 10-week VGB-treated rats (VGB-10 group);
5. stimulated, 10-week VGB-treated rats, continued with 8-week drug-free follow-up period (VGB-10-FU-8 group).

Vigabatrin (Hoechst Marion Roussel, Strasbourg, France) was dissolved in 0.9% NaCl (200 mg/ml). Alzet 2ML2 osmotic pumps (Alza Corporation, Palo Alto, CA) were filled with either VGB solution or vehicle (0.9% NaCl). Two days (48 h) after the induction of SE, the rats were anesthetized and osmotic pumps were subcutaneously implanted in the back, slightly posterior to the scapulae according to the manufacturer's protocol. In the unstimulated control, SAL-10, and Sal-10-FU-8 groups, the pumps contained 0.9% NaCl and in the VGB-10 and VGB-10-FU-8 groups, VGB was delivered at 75 mg/kg/day. The pumps were replaced by a new one on every 14th day. After removing the pump, the volume of solution in the pump was checked in order to ascertain that the delivery rate had been correct. The drug treatment continued for 10 weeks. Thereafter the rats in the unstimulated control, SAL-10, and VGB-10 groups were perfused. The rats in the Sal-10-FU-8 and VGB-10-FU-

8 groups continued in the study for 8 more weeks without any further drug treatment.

The dose of VGB was based on our previous study in which chronic VGB treatment (75 mg/kg/day) increased hippocampal GABA levels 2.3-fold (Pitkänen et al., 1999), which corresponds to the increase of GABA levels in human brains during VGB treatment that is associated with a decrease in seizure frequency (Petroff et al., 1995, 1996). This dose does not produce vacuoles in the rat brain during chronic treatment as the higher doses (Gibson et al., 1990).

2.8. *Morris water-maze*

The spatial learning and memory performance of rats was tested using the Morris water-maze (HVS Image Ltd, Hampton, UK), as described previously (Halonen et al., 1996). Training the rats to locate the platform in the water-maze began either 9 weeks (groups: unstimulated controls, Sal-10 and VGB-10) or 17 weeks (groups: Sal-10-FU-8 and VGB-10-FU-8) after the induction of SE and was performed over 7 consecutive days (five 60-s swims/day). The location of the platform was alternated daily so that on days 1, 3, 5, and 7, the platform was in quadrant 1, and on days 2, 4, and 6, in quadrant 3 (the opposite side of the maze). The latency (time taken to find the platform on each trial), path length (distance traveled by the animal), and swimming speed were measured.

2.9. *Histological analysis of brain tissue*

The rats were intracardially perfused 10 weeks (unstimulated control, Sal-10, and VGB-10 groups) or 18 weeks (Sal-10-FU-8 and VGB-10-FU-8 groups) after the induction of SE according to the fixation protocol for Timm staining (Sloviter, 1982). The brains were cryoprotected in 20% glycerol in 0.02 M potassium phosphate buffer (pH 7.4) for 24 h, frozen in dry ice, and stored at -70°C . A one-in-five series of horizontal frozen sections (30 μm) was cut throughout the brain, using a sliding microtome. Sections were collected into tissue collection solution (TCS) and stored at -20°C until processed.

Severity of neuronal damage in the hilus, pyramidal cell layers of CA3 and CA1 subfields of the hippocampus, subiculum, and layer III of the caudal entorhinal subfield was semiquantitatively assessed in thionin-stained sections as follows: Score 0=no damage, Score 1=lesions involving <10% of neurons in the region of interest, Score 2=lesions involving 10–50% of neurons, Score 3=lesions involving >50% of neurons. The damage score for each region was obtained by scoring the severity of damage in each section (1-in-5) in which the region of interest was present and by calculating the mean. The sections were analyzed in a blinded manner

by an experienced researcher (A.P.). In addition, thionin-stained sections were used to determine the location of electrode tips.

To determine the seizure-induced damage to somatostatin-immunoreactive (SOM-ir) neurons in the hilus, sections were immunohistochemically stained for somatostatin and analyzed as described previously (Pitkänen et al., 1996) by starting at the level of 4.4 mm posterior to bregma according to the atlas of Paxinos and Watson (1986). The mean number of neurons was counted in five successive sections taken at 300 μm intervals.

To analyze mossy-fiber sprouting, Timm-staining was performed using the sulfide/silver technique as described by Sloviter (1982) and Pitkänen et al. (1999). The mean density of mossy fiber sprouting was analyzed from 10 successive sections (taken at 150- μm intervals) starting at the level 3.86 mm posterior to bregma. The density was assessed using a five-stage scoring scheme described by Cavazos et al. (1991).

2.10. *Statistical analysis*

Data were analyzed using SPSS for Windows (version 6.1.4). Nonparametric statistics were used because the distribution of values was not normal, ordinal values were used, or the number of animals in treatment groups was small. Severity of SSSE (the number and duration of HAFDs, the appearance of last HAFD) and the appearance and number of spontaneous seizures between different treatment groups were compared using the Mann–Whitney *U*-test. The number of epileptic rats between different groups was compared using the Pearson χ^2 -test. The Morris water-maze data between the groups was evaluated using analysis of covariance (ANCOVA) with swimming speed being a covariate. The severity of neuronal damage in different temporal lobe structures between the different treatment groups was compared using the Mann–Whitney *U*-test. The severity of neuronal damage between the stimulation and contralateral sides was compared using the Wilcoxon signed rank test. Spearman correlation coefficients were used to analyze significance of correlations between the neuronal damage in the hilus and mossy fiber sprouting in the dentate gyrus. A *P* value of <0.05 was considered significant.

3. Results

3.1. *Preliminary experiment*

3.1.1. *Anticonvulsant effect of VGB treatment on spontaneous seizures*

In chronically epileptic animals, the baseline mean seizure frequency was 30 ± 28 seizures per day (median

39; range 1–63). During VGB treatment of 75 mg/kg/day, mean seizure frequency was 25 ± 19 seizures per day (median 34; range 1–46: the last 5 days of monitoring were included in the analysis, during which the drug concentration had reached a therapeutic level). Analysis of seizure frequency in individual rats indicated that seizure frequency was reduced in three of the seven rats during VGB treatment (mean 29%; range 20–38%). Duration of seizures during VGB treatment (37 ± 8 s) was similar to that at baseline (43 ± 6 s).

Administration of VGB (250 mg/kg/day) reduced seizure frequency in four of six rats (one of the seven rats died) compared to baseline (mean 43%; range 11–68%) and in five of six rats compared to treatment with a lower dose of VGB (mean 56%; range 38–73%). The two rats that did not respond to VGB treatment had a low seizure frequency compared to responders (2 vs 17 seizures per day). Seizure duration (49 ± 16 s) was not affected by treatment with a higher dose of VGB.

As shown previously, the lower dose of VGB (75 mg/kg/day) administered via minipumps elevates hippocampal GABA levels 2.3-fold (Pitkänen et al., 1999) and 250 mg/kg/day of VGB (i.p.) elevates GABA levels 3.1-fold (Halonen et al., 1991).

3.2. Antiepileptogenic effects of VGB treatment

3.2.1. Animals

Electrodes were implanted in 48 rats and 40 of these rats were stimulated. In a previous study, the most important criteria for the development of spontaneous seizures was the appearance of at least 60 HAFDs during the SSSE (Nissinen et al., 2000). Thirty-five rats fulfilled the criteria of SSSE with at least 60 HAFDs. Seven of the stimulated rats, however, died from SE within 10 days. In addition, one rat in the VGB-10 group and one rat in Sal-10-FU-8 group died during the follow-up period (there was no difference in mortality between saline or VGB-treated animals). Thus, 26 stimulated and eight unstimulated rats survived to the end of the study. Four rats were excluded because the stimulation electrode was outside the lateral nucleus of the amygdala. Therefore, 30 rats went through all of the procedures and were included in the statistical analysis.

3.2.2. Severity of SSSE

Amygdala stimulation that lasted only 20–40 min was enough to produce SSSE that included electrographic spiking activity in the amygdala and cortex and intermittent seizure activity (HAFDs) accompanied by head nodding, forelimb clonus, and often rearing and falling. Severity of initial SSSE did not differ between the groups as assessed by the number of HAFDs (all saline treated rats: 129 ± 40 , range 68–212, median 127; all VGB-treated rats: 139 ± 40 , range 84–212, median 130), total duration of HAFDs (all saline treated rats:

63.7 ± 25.6 min, range 29.7–106.9 min, median 66.3 min; all VGB-treated rats: 61.4 ± 16.7 min, range 37.8–94.5 min, median 63.0 min), and the time between the beginning of SSSE and the last HAFD (all saline treated rats: 572 ± 209 min, range 311–1121 min, median 521 min; all VGB-treated rats: 544 ± 166 min, range 186–785 min, median 557 min). Amplitude and frequency of discrete spiking activity diminished gradually but continued in all animals for at least 16 h after the beginning of SSSE. It should be noted that the total number and duration of HAFDs did not differ between the Sal-10 and Sal-10-FU-8 groups, or between VGB-10 and VGB-10-FU-8 groups (data not shown). Therefore, for further analysis, the data from the two saline-treated groups were combined with each other as were the data from the two VGB-treated groups in order to evaluate the development of spontaneous seizures, seizure frequency, and seizure severity during the treatment period (0–10 weeks) after saline or VGB treatments.

3.2.3. Appearance of the first epileptic seizures

The emergence of spontaneous seizures was determined after evaluating the EEG data (Fig. 1). Unstimulated controls demonstrated normal background EEG throughout the study (Fig. 1A) whereas stimulated animals had intermittent interictal spike discharges (Fig. 1B). The first spontaneous seizures observed in EEG were observed 9–99 days following the induction of SE. The spontaneous seizures were either subclinical ($n=1/16$) or associated with behavioral seizure activity which could be seen in video recordings as a partial ($n=3/16$) or secondary generalized seizure ($n=12/16$). In saline-treated rats, the first spontaneous seizure was observed 24 ± 17 days (mean \pm SD; range 9–58 days; median 19 days) after the stimulation and in VGB-treated animals 23 ± 14 days (range 9–44 days; median 16 days) after the stimulation. In addition, two VGB-treated rats had the first observed seizure after the drug wash-out. When these rats were included, the first epileptic seizures in VGB-treated animals were observed 38 ± 34 days (mean \pm SD; range 9–99 days; median 22 days) after the stimulation. The mean latency for the first seizure did not differ between the control and VGB-treated groups.

During the drug treatment period (0–10 weeks), spontaneous seizures were observed in 6 of 11 (55%) saline-treated controls and 8 of 11 (73%) VGB-treated animals (Fig. 2A). Altogether, 91% (10/11) of the VGB-treated rats had spontaneous seizures during the study (0–18 weeks; $P=0.055$, compared to saline-treated controls). One VGB-treated rat that had seizures during the drug-treatment period did not have any seizure activity following drug withdrawal.

3.2.4. Seizure frequency

Altogether, 1816 seizures were recorded in the study. During the drug treatment period, saline-treated controls

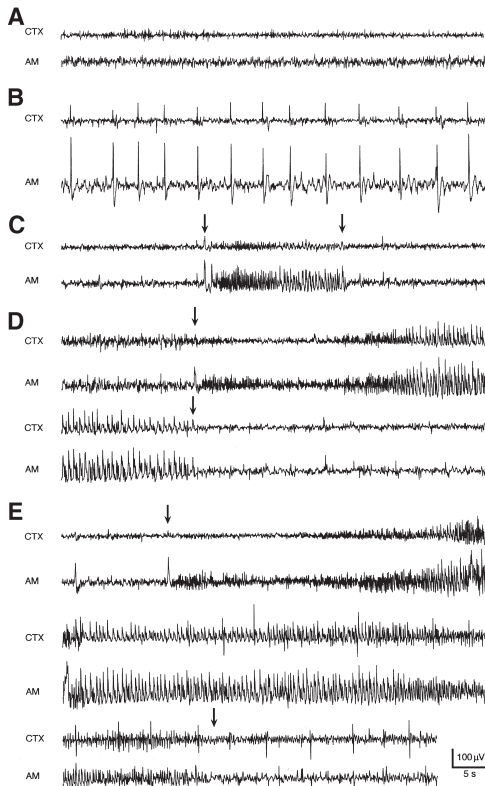


Fig. 1. Electroencephalographic (EEG) recording of spontaneous seizures. (A) Normal background EEG activity in the amygdala (AM) and contralateral frontal cortex (CTX) in an unstimulated rat (No. 76) observed 4 weeks after the implantation of electrodes. (B) EEG recording in rat No. 94 demonstrating the typical interictal spiking found intermittently in all stimulated rats. (C) Representative pattern of a subclinical seizure that was observed only in the EEG. Seizure in VGB treated animal (No. 97) occurred 52 days after SSSE, was observed by both AM and CTX electrodes and lasted for 24 s. The rat was asleep during the seizure. (D) An example of a spontaneous seizure that was recorded in the same VGB-treated animal (No. 97) 37 days after the induction of SSSE. The seizure lasted 62 s and was classified as a partial seizure based on behavioral manifestations (facial clonus and head nodding). The seizure activity was detected by both the AM and CTX electrodes. (E) An example of a spontaneous secondarily generalized seizure in the same animal 37 days after the induction of SSSE. The seizure started as a partial seizure and became generalized (bilateral forelimb clonus with rearing and falling) after 45 s, lasting 135 s.

had 165 seizures [1.5 ± 1.0 seizures/day (mean \pm SEM), range 0–115, $n=11$] and VGB-treated rats had 619 seizures [5.6 ± 2.7 seizures/day (mean \pm SEM), range 0–245, $n=11$]. The daily seizure frequency in 2-week blocks over the period of 1 to 10 weeks for combined control groups ($n=11$) and for combined VGB groups ($n=11$) is

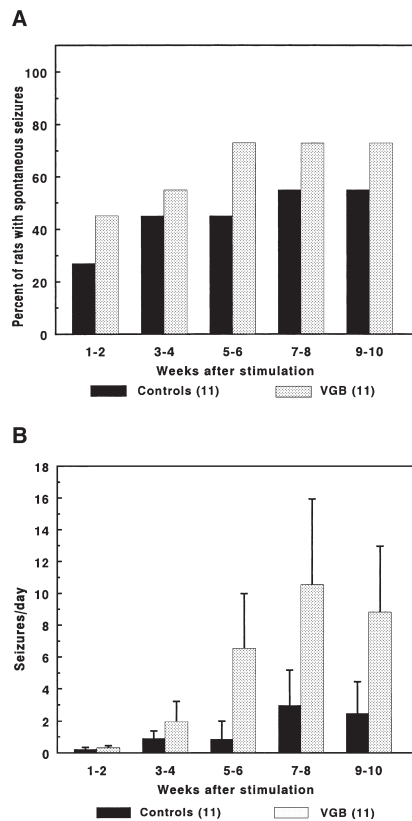


Fig. 2. (A) Cumulative percent of rats with spontaneous seizures in combined vehicle and combined VGB groups 1–10 weeks after SSSE. The mean interval from the stimulation until the first recorded spontaneous seizure was 24 ± 17 days (mean \pm SD) for saline-treated animals ($n=11$) and 23 ± 14 days for VGB-treated animals ($n=11$; no statistically significant differences between the groups, χ^2 -test). (B) Mean seizure frequency/day (\pm SEM) for 2-week blocks in combined vehicle ($n=11$) and combined VGB-treated ($n=11$) groups 1–10 weeks after SSSE. During the 10-week treatment period the mean seizure frequency in saline-treated controls was 1.5 seizures/day ($n=11$) and in VGB-treated rats, 5.6 seizures/day ($n=11$; no statistically significant differences between the groups, Mann-Whitney U -test).

shown in Fig. 2B. There was no difference in the seizure frequency between the groups during 1 through 10 weeks. After drug withdrawal (weeks 11–18), rats in the Sal-10-FU-8 group had 390 seizures [12.2 ± 8.2 seizures/day (mean \pm SEM), range 0–279, $n=4$] and rats in the VGB-10-FU-8 group had 642 seizures [11.5 ± 6.0 seizures/day (mean \pm SEM), range 0–318, $n=7$; no difference between the groups].

3.2.5. Severity of seizures

Severity of seizures varied between the animals. In addition, some rats had subclinical seizures that were observed only in the EEG (Fig. 1C), behaviorally partial seizures (Fig. 1D), and secondary generalized seizures (Fig. 1E). In VGB-treated rats, 19% of all seizures were generalized during the treatment period (0–10 weeks), whereas in saline-treated control groups 80% of seizures were generalized. After discontinuation of VGB treatment (between 11 and 18 weeks), 25% of all seizures were generalized in the VGB-10-FU-8 group and 24% in the Sal-10-FU-8 group. The mean percentage of generalized seizures/rat, however, did not differ between the groups during weeks 1 through 10 [$66.0 \pm 16.5\%$ (mean \pm SEM) for controls and $63.5 \pm 14.5\%$ for VGB-treated animals] or after discontinuation of VGB treatment (Sal-10-FU-8 group: $52.7 \pm 25.0\%$ and VGB-10-FU-8 group: $63.8 \pm 16.7\%$).

3.2.6. Duration of seizures

VGB-treatment did not affect the duration of seizures. Mean duration of seizures during weeks 1 through 10 was 63.4 ± 7.7 s (mean \pm SEM) for saline-treated control rats and 68.1 ± 5.1 s in VGB-treated rats. During the follow-up period of weeks 11 through 18, the duration of seizures in the Sal-10-FU-8 group was 65.0 ± 12.0 s and in the VGB-10-FU-8 group, 67.9 ± 8.0 s (no difference between the groups).

3.2.7. Morris water-maze

The VGB-10 group had a slower swimming speed compared to unstimulated controls and the Sal-10 group ($P < 0.001$, ANOVA). Therefore, the escape latency was tested using ANCOVA with swimming speed as a covariant. Ten weeks after the stimulation, both the Sal-10 and VGB-10 groups were impaired in finding the platform compared to the unstimulated controls (Fig. 3A, increased escape latency, $P < 0.001$). Moreover, the VGB-10 group was even more impaired than the Sal-10 group in the water-maze ($P < 0.001$). When the rats without spontaneous seizures (that is, rats without histologic damage observed in thionin preparations, see Section 3.2.8) in the Sal-10 group were excluded, however, there was no difference in the escape latencies compared to the seizing VGB-10 rats. Also, there was no difference when the escape latencies of the seizing Sal-10 and VGB-10 rats on the 6th and 7th testing days only were compared.

The VGB-10-FU-8 group had a slower swimming speed compared to the Sal-10-FU-8 group ($P < 0.01$, ANOVA). Eighteen weeks after the stimulation (8 weeks after the withdrawal of VGB treatment), the VGB-10-FU-8 group had a prolonged escape latency compared to the Sal-10-FU-8 group (Fig. 3B, $P < 0.05$). There was no difference when the escape latencies on the 6th and

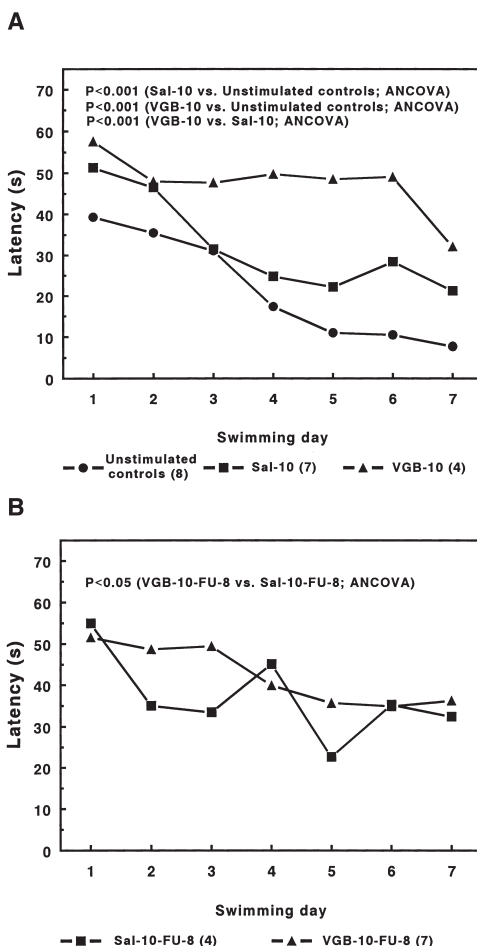


Fig. 3. Performance of different rat groups in the Morris water-maze task. (A) At the end of the treatment (9–10 weeks after SSSE), the Sal-10, and VGB-10 groups were impaired in finding the submerged platform compared to unstimulated control animals ($P < 0.001$, ANCOVA; analysis of the 6th testing day data only: unstimulated controls vs Sal-10 group, $P < 0.001$; unstimulated controls vs VGB-10 group, $P < 0.001$; Sal-10 group vs VGB-10 group, $P < 0.01$, and analysis of the 7th testing day data only: unstimulated controls vs Sal-10 group, $P < 0.001$; unstimulated controls vs VGB-10 group, $P < 0.001$). In addition, the VGB-10 group appeared more impaired than the Sal-10 group ($P < 0.001$). The difference was not significant, however, if the rats without seizures in the Sal-10 group (no histologic damage) were excluded (see text). (B) At the end of the drug-free follow-up period (17–18 weeks after SSSE), the VGB-10-FU-8 group was more impaired in finding the platform than the Sal-10-FU-8 group ($P < 0.001$; no difference in the escape latencies when the data from the 6th or 7th testing days only were compared between groups). Performance is expressed as the latency (s) to find the submerged platform. The number of animals is indicated in parentheses.

Table 1
Neuronal cell damage in the hilus, CA3, CA1, subiculum and layer III of the caudal entorhinal subfield in various treatment groups^a

Group	Hilus	CA3	CA1	Subiculum	EC Layer III
<i>Stimulation side</i>					
Unstimulated controls (8)	0	0	0	0	0
Sal-10 (7)	0.20±0.36	0±0	0.11±0.20	0.14±0.24	0.09±0.23
VGB-10 (4)	0.98±0.84**	0.33±0.38*.#	0.78±0.56**	0.70±0.51**.#	0.98±0.75**.#
Sal-10-FU-8 (4)	1.23±0.97**	0.25±0.33*.#	0.53±0.55**	0.68±0.46**.#	1.08±0.76**.#
VGB-10-FU-8 (7)	1.00±1.00*	0.51±0.66*	0.67±0.73*	0.67±0.70*	1.07±1.01*
<i>Contralateral side</i>					
Unstimulated controls (8)	0	0	0	0	0
Sal-10 (7)	0.16±0.21*	0.04±0.08	0.26±0.44	0.14±0.19*	0.16±0.31
VGB-10 (4)	0.93±0.87**	0.28±0.32*	0.73±0.56**	0.58±0.46**	0.95±0.72**
Sal-10-FU-8 (4)	1.12±1.03**	0.23±0.26*	0.48±0.46**	0.65±0.47**	0.98±0.77**
VGB-10-FU-8 (7)	0.79±0.90*	0.37±0.56*	0.63±0.65*	0.56±0.52*	0.84±0.89*

^a Abbreviations: EC, caudal entorhinal subfield; Sal-10, stimulated, 10-week saline-treated controls; Sal-10-FU-8, stimulated, 10-week saline-treated controls, continued with 8-week follow-up period; VGB-10, 10-week vigabatrin-treated group; VGB-10-FU-8, 10-week vigabatrin-treated rats, continued with 8-week drug-free follow-up period. Values are presented as mean±standard deviation. Number of animals is in parentheses. The severity of pyramidal cell damage differed between the groups in all areas studied on the stimulation and contralateral side ($P<0.05$, Kruskal-Wallis test) except in CA3 of the contralateral side.

* $P<0.05$.

** $P<0.01$ compared to unstimulated controls (Mann–Whitney U-test).

$P<0.05$ compared to the Sal-10 group (Mann–Whitney U-test). No differences were found between the stimulation and contralateral sides.

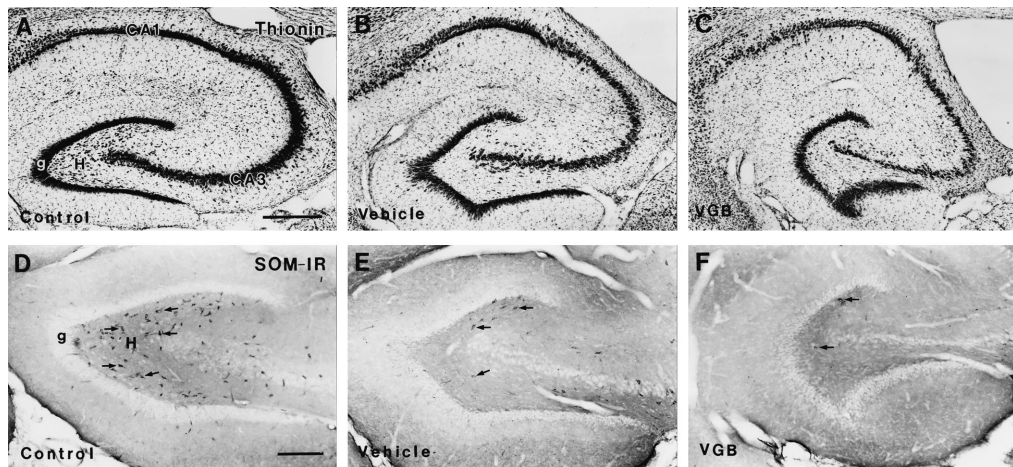


Fig. 4. Brightfield photomicrographs of horizontal sections stained for thionin (A–C) and somatostatin (SOM) immunohistochemistry (D–F) demonstrating the neuronal damage in the septal end of the hippocampus. (A) Unstimulated control animal (No. 113) without neuronal damage. (B) Vehicle-treated stimulated control (No. 109, Sal-10-FU-8 group, 394 seizures). Note the remarkable neuronal loss in the hilus of the dentate gyrus. Milder damage is also observed in the CA3 and CA1 subfields of the hippocampus. (C) VGB-treated animal (No. 112, VGB-10-FU-8 group, 20 seizures). Note the neuronal loss in the hilus of the dentate gyrus and also in the CA3 and CA1 regions of the hippocampus. (D) An adjacent SOM-immunostained section of the same unstimulated control animal as in (A). SOM-immunoreactive (SOM-ir) neurons in the hilus are indicated with arrows. (E) A SOM-immunostained section of the same vehicle-treated stimulated control animal as in (B). Note the loss of SOM-ir neurons in the hilus (54% of SOM-ir neurons left). (F) A SOM-immunostained section of the same VGB-treated animal as in (C). Note the loss of SOM-ir neurons in the hilus (21% of SOM-ir neurons left). Abbreviations: g, granule cell layer; H, hilus; VGB, vigabatrin. Scale bar=500 μ m for (A)–(C) and 250 μ m for (D)–(F).

7th testing days only were compared between the groups.

3.2.8. Neuronal cell damage in thionin-stained sections

Severity of neuronal damage in the hilus of the dentate gyrus, pyramidal cell layers of the CA3 and CA1 regions of the hippocampus, subiculum, and layer III of the caudal entorhinal subfield is summarized in Table 1. Representative examples of neuronal damage in the hippocampus of the vehicle and VGB-treated stimulated animals in comparison to unstimulated control rats are shown in Fig. 4A–C and in the layer III of the caudal entorhinal subfield in Fig. 5D–F. Unstimulated control rats had no damage in the regions analyzed. Also, there was no apparent neuronal loss in rats that were stimulated for 15–40 min but developed fewer than 15 HAFDs (these rats did not develop SSSE and therefore were excluded from the study; data not shown).

The Sal-10-FU-8 group, VGB-10, and VGB-10-FU-8 groups had marked neuronal damage in all analyzed brain regions (Table 1). In contrast, the mean score of neuronal damage in the Sal-10 group was statistically significant only in the contralateral hilus and subiculum ($P < 0.05$ compared to unstimulated controls; Table 1). This was because damage was observed in the thionin

preparations in only three of seven animals in the Sal-10 group (even though the duration of SSSE in these animals did not differ from that in rats with damage). Because the rats with damage were the same animals that had spontaneous seizures, we conducted an additional analysis between the seizing rats in the Sal-10 group ($n=3$) and the seizing rats in the VGB-10 group ($n=3$). This indicated that the seizing animals in the Sal-10 group had damage in the ipsilateral hilus, CA1, and subiculum as well as in the contralateral hilus, CA3, CA1, subiculum, and layer III of the entorhinal cortex ($P < 0.05$). In area CA1, the subiculum, and layer III of the entorhinal cortex, the seizing animals in the VGB-10 group still had more severe damage than the seizing animals in the Sal-10 group ($P < 0.05$).

3.2.9. Somatostatin-immunoreactive neurons

The number of SOM-ir neurons in the hilus is summarized in Table 2 and examples of SOM-ir-stained hilar sections of an unstimulated and stimulated animals are shown in Fig. 4D–F. The area of the hilus did not differ between the groups ($P > 0.05$). Therefore, the density of SOM-ir neurons per mm^2 was calculated and used in the statistical analyses. Rats that were stimulated via amygdala electrodes for 15–40 min but that did not develop SSSE, did not have a decrease in the density of SOM-ir

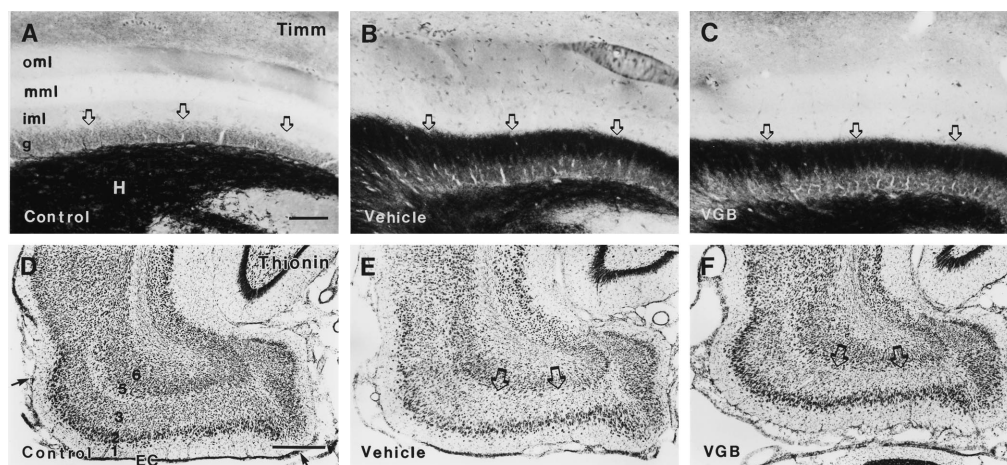


Fig. 5. Brightfield photomicrographs of horizontal sections demonstrating the extent of mossy fiber sprouting in the dentate gyrus (A–C) and the neuronal damage in the entorhinal cortex (D–F). (A) A Timm-stained section from the same unstimulated control animal as in Fig. 4A and D. Note the absence of Timm granules in the supragranular region and inner molecular layer. (B) Vehicle-treated stimulated control animal (the same as in Fig. 4B and E). Note the dense layer of sprouted mossy fibers in the inner molecular layer (open arrows). (C) VGB-treated animal (the same as in Fig. 4C and F). Note that the mossy fiber sprouting (open arrows) is as extensive as in the vehicle-treated rat (B). (D) A thionin-stained section demonstrating intact entorhinal cortex in the same unstimulated control animal as in (A). (E) Vehicle-treated stimulated control animal (the same as in B). Note the neuronal damage in the layer III of the caudal entorhinal subfield (open arrows). (F) VGB-treated animal (the same as in C). Note the similar neuronal damage in the layer III (open arrows) as in the vehicle-treated rat (B). Abbreviations: EC, entorhinal cortex; g, granule cell layer; H, hilus; iml, inner molecular layer; mml, mid molecular layer; oml, outer molecular layer; VGB, vigabatrin. Layers of the entorhinal cortex are indicated with Roman numerals. Scale bar=100 μm for (A)–(C) and 500 μm for (D)–(F).

Table 2
Density of SOM-ir neurons in the hilus and mossy fiber sprouting in the molecular layer of the dentate gyrus in different treatment groups^a

Group	SOM-ir neurons/mm ²		Timm score	
	Stimulation side	Contralateral side	Stimulation side	Contralateral side
Unstimulated controls	184.3±24.8 (6)	179.7±41.5 (8)	0.30±0.28 (8)	0.30±0.27 (8)
Sal-10	172.3±44.7 (6)	139.9±44.8 (7)	1.97±1.25 (7)**	1.89±1.12 (7)**
Sal-10-FU-8	97.4±76.4 (4)	97.2±65.7 (4)	4.25±2.29 (4)**	4.05±2.27 (4)**
VGB-10	114.9±30.8 (3)*	121.2±23.8 (4)*	3.25±1.73 (4)**	3.21±1.84 (4)**
VGB-10-FU-8	92.6±69.6 (5)*	112.7±75.4 (7)	3.76±2.02 (7)**	3.50±1.97 (7)**#

^a Abbreviations: Sal-10, stimulated, 10-week saline-treated controls; Sal-10-FU-8, stimulated, 10-week saline-treated controls, continued with 8-week follow-up period; VGB-10, 10-week vigabatrin-treated group; VGB-10-FU-8, 10-week vigabatrin-treated rats, continued with 8-week drug-free follow-up period. Values are presented as mean±standard deviation. Number of animals is in parentheses. The density of SOM-ir neurons/mm² differed between the groups on the ipsilateral side ($P<0.05$, Kruskal–Wallis test). There was also a significant difference in Timm scores between the groups on the stimulation side ($P<0.001$) and on the contralateral side ($P<0.001$).

* $P<0.05$ compared to unstimulated controls (Mann–Whitney U-test).

** $P<0.01$ compared to unstimulated controls (Mann–Whitney U-test).

$P<0.05$ compared to the contralateral side (Wilcoxon signed rank test).

neurons in the hippocampus (data not shown). Therefore, amygdala stimulation alone is not the cause of hilar damage in this model of epilepsy.

3.2.9.1. Stimulation side In the Sal-10 group, 93% of the SOM-ir neurons remained compared to the unstimulated controls (in the seizing rats in the Sal-10 group, 72%, $P<0.05$). In the Sal-10-FU-8 group, 53% of the SOM-ir neurons remained ($P=0.088$, compared to the unstimulated controls). In the VGB-10 group, the density of SOM-ir neurons was lower than in unstimulated controls (62% of SOM-ir neurons remaining, $P<0.05$). Also, in the VGB-10-FU-8 group, the density of SOM-ir neurons was reduced (50% remaining, $P<0.05$). The density of SOM-ir neurons did not differ between the VGB-treated rats and the corresponding stimulated control animals.

3.2.9.2. Contralateral side In the Sal-10 group, 78% of the SOM-ir neurons remained compared to the unstimulated controls (in the seizing rats in the Sal-10 group, 54%, $P<0.05$). In the Sal-10-FU-8 group, 54% of the SOM-ir neurons remained ($P=0.062$, compared to the unstimulated controls). In the VGB-10 group, the density of SOM-ir neurons was lower than in unstimulated controls (67% of SOM-ir neurons remaining, $P<0.05$). In the VGB-10-FU-8 group, the density of SOM-ir neurons was 63% of that in unstimulated rats. The density of SOM-ir neurons did not differ between the VGB-treated rats and the corresponding stimulated control animals. There were no differences between the stimulation and contralateral sides in any of the groups.

3.2.10. Mossy fiber sprouting (Timm staining)

The density of mossy fiber sprouting in the supragranular region and in the inner molecular layer of the dentate gyrus is shown in Fig. 5A–C and summarized in

Table 2. The rats that were stimulated for 15–40 min but that did not develop SSSE did not have increased mossy fiber sprouting (data not shown).

3.2.10.1. Stimulation side Both the saline-treated stimulated rats and VGB-treated rats had substantial amounts of mossy fiber sprouting ($P<0.01$ compared to unstimulated controls). The density of mossy fiber sprouting in VGB-treated animals did not differ from that in corresponding saline-treated stimulated controls.

3.2.10.2. Contralateral side Both the saline-treated stimulated controls and VGB-treated rats had an increased density of mossy fiber sprouting contralaterally ($P<0.01$, compared to unstimulated controls). In the saline-treated stimulated control groups, the density of mossy fiber sprouting did not differ between the stimulation and contralateral sides, whereas in the VGB-10-FU-8 group, the contralateral side had slightly less mossy fiber sprouting than the stimulation side ($P<0.05$). The density of mossy fiber sprouting in the VGB-treated animals did not differ from that in corresponding saline-treated stimulated animals.

3.2.10.3. Correlations between neuronal damage and mossy fiber sprouting Mossy fiber sprouting in the dentate gyrus of the stimulation and contralateral side correlated with the neuronal cell damage in the hilus (stimulation side: Sal-10+VGB-10 groups, $r=0.922$, $n=11$, $P<0.001$; Sal-10-FU-8+VGB-10-FU-8 groups, $r=0.778$, $n=11$, $P<0.01$; contralateral side: Sal-10+VGB-10 groups, $r=0.956$, $n=11$, $P<0.001$; Sal-10-FU-8+VGB-10-FU-8 groups, $r=0.931$, $n=11$, $P<0.001$).

4. Discussion

The present study tested the hypothesis that chronic elevation of brain GABA with VGB started 2 days after SE will prevent the development of epilepsy. This hypothesis was based on previous studies showing that in addition to its antiepileptic efficacy (see Grant and Heel, 1991), VGB pretreatment reduces hippocampal damage in rat models of SE (Ylinen et al., 1991; Halonen et al., 1995; Pitkänen et al., 1996). In addition, neuronal damage in post-ischemia-treated rats was reduced in short-term follow-up studies (Shuaib et al., 1996). Also, VGB effectively delays the development of kindling (Shin et al., 1986). Finally, as our observations indicate, the dose of VGB used in the chronic experiment (75 mg/kg/day) is anticonvulsant in approximately one-third of chronically seizing animals and elevates brain GABA levels 2.3-fold (Pitkänen et al., 1999), which is consistent with data on drug-refractory patients (Petroff et al., 1999). The results of the present study, however, indicate that a chronic increase in brain GABA levels by VGB treatment does not prevent the development of epilepsy in rats when VGB treatment is started 2 days after SE. Treatment with VGB does not attenuate neuronal damage in the temporal lobe or the development of mossy fiber sprouting in the dentate gyrus. Further, VGB does not reduce the impairment in spatial learning and memory.

4.1. Role of GABA in epileptogenesis

A substantial amount of data indicate that altered GABAergic neurotransmission has an important role in focal epileptogenesis and epilepsy. First, a subpopulation of hilar GABAergic neurons containing somatostatin or neuropeptide Y are lost in epileptogenic regions in experimental (Sloviter, 1987; Ylinen et al., 1991; Sperk et al., 1992; Schwarzer et al., 1995; Pitkänen et al., 1996) and human TLE (DeLanerolle et al., 1989; Mathern et al., 1995a). The damage occurs before the appearance of spontaneous seizures. Second, recent studies indicate that there are cell type, region, and model-specific changes in the expression of GABA_A receptor subunits in animal models of epilepsy (Rice et al., 1996; Schwarzer et al., 1997; Tsunashima et al., 1997; Brooks-Kayal et al., 1998; Poulter et al., 1999). Altered GABA_A receptor subunit expression in the dentate granule cells of pilocarpine-treated rats is observed at 24 h and precedes the onset of spontaneous seizures. Furthermore, the changes correlated with altered GABA_A receptor function, including larger GABA-evoked responses, decreased enhancement by zolpidem, and increased response to zinc blockade (Brooks-Kayal et al., 1998). Third, poor seizure control is associated with low GABA levels in the brain (Petroff et al., 1996). Finally, drugs that enhance GABAergic neurotransmission are effective

antiepileptics in experimental and human TLE (see reviews of Grant and Heel, 1991; Ticku and Kulkarni, 1992; Bourgeois, 1996; Homan and Rosenberg, 1996; Adkins and Noble, 1998). Based on these observations, we assumed that defective GABAergic function is one of the key elements in the pathology of epileptogenesis, and that consequently, enhancement of GABAergic neurotransmission could prevent epileptogenesis. Chronic treatment, however, with a dose of VGB that increases brain GABA levels 2.3-fold (Pitkänen et al., 1999) and corresponds to the increase in GABA levels in the brain of VGB-treated epileptic patients (Petroff et al. 1995, 1996), had no effect on epileptogenesis when started 2 days after SE. These data are consistent with those of Ander et al. (1999), which demonstrate that treatment with a higher dose of VGB (250 mg/kg/day) does not prevent epileptogenesis after pilocarpine-induced SE even though the increase in brain GABA levels is higher than that with a lower dose (2.3-fold vs 3.1-fold; Halonen et al., 1991; Pitkänen et al., 1999). Therefore, the GABA increase cannot compensate for the defect in GABAergic circuitries. It should be noted that in the VGB group, the delay to the appearance of the first spontaneous seizure tended to be longer than in the saline group. Also, there were fewer generalized seizures in the VGB group compared to the saline group. Neither one of these findings, however, reached statistical significance in the present study. Alternatively, reduced GABAergic function might not be a major determinant in epileptogenesis. In fact, recent data indicate that the number of neurons expressing GAD₆₅ and GAD₆₇ is increased following kainate- and pilocarpine-induced SE (Schwarzer and Sperk, 1995; Houser and Esclapez, 1996; Brooks-Kayal et al., 1998). Also, GABA_A receptor number and inhibitory responses might be enhanced in the dentate gyrus of kindled animals (Nusser et al., 1998). These findings, together with the present data, raise questions about the use of GABAergic enhancers in the prevention of epileptogenesis, as they might even increase the synchronization of cell firing and facilitate seizure generation in the maturing epileptogenic network (see Avoli, 1996).

4.2. Neuronal damage and development of epilepsy

Neuronal damage is proposed to serve as a signal for the induction of epileptogenesis following SE (see Wasterlain and Shirasaka, 1994). In the experimental model used in this study, extensive neuronal damage is apparent as early as 24 h after the beginning of SSSE (Lukasiuk and Pitkänen, 1998). Therefore, when VGB treatment was started 2 days after SSSE, GABA elevation did not interfere with the normal progression of SE that leads to acute neuronal damage. There is evidence that neuronal damage can also be delayed and occur several days after SE (Tuunanen et al., 1999). Recurrent

seizures might also contribute to the damage (Cavazos et al., 1994; Tuunanen et al., 1997; Tuunanen and Pitkänen, 2000). Previous studies indicate that VGB reduced SE-induced neuronal damage in rats if the treatment was started before perforant pathway stimulation (Ylinen et al., 1991; Pitkänen et al., 1996) or kainic acid injection (Halonen et al., 1995). In contrast, no substantial neuroprotection was observed if the treatment was started after kainic acid-induced SE (Pitkänen et al., 1999). Similar results have been reported with another GABAergic drug, phenobarbital. If phenobarbital is given before or during SE, it has neuroprotective effects (Fuller and Olney, 1981; Ault et al., 1986; Sutula et al., 1992; Lemos and Cavalheiro, 1995). In contrast, chronic phenobarbital treatment started 1 day after kainate-induced SE does not reduce hippocampal neuronal cell loss, but rather exacerbates it (Mikati et al., 1994). Therefore, the available data indicate that the enhancement of GABAergic function following SE fails to provide any substantial protection against delayed or seizure-induced damage and associated epileptogenesis.

Rats in the Sal-10 group had milder temporal lobe damage and fewer spontaneous seizures than the other groups. A less severe SSSE in the Sal-10 group would be the most logical explanation for milder damage. The behavioral and EEG measures, however, did not demonstrate any differences in the severity or duration of SSSE between the treatment groups. Identification of other factors associated with the severity of damage are currently under evaluation (Nissinen et al., 2000).

4.3. Mossy fiber sprouting and development of epilepsy

Consistent with failure to prevent the development of spontaneous seizures, the present results clearly demonstrated that VGB treatment does not decrease mossy fiber sprouting. It should be noted that even though the mossy fiber sprouting is temporally associated with the occurrence of spontaneous seizures (Tauck and Nadler, 1985; Lemos and Cavalheiro, 1995; Mathern et al., 1997), its functional significance in the development of spontaneous seizures is still under dispute (Sloviter, 2000). For example, the density of mossy fiber sprouting did not correlate with the length of the silent period (time from SE to the first spontaneous seizure); that is, seizures might occur before any substantial sprouting (Mello et al., 1993). Consistent with these observations, we observed the first spontaneous seizure in two rats (one vehicle-treated, one VGB-treated) within 2 weeks following SE and in eight rats (three vehicle-treated and five VGB-treated) within 3 weeks following SE. At that time, the intensity of sprouting is low (Nissinen and Pitkänen, unpublished). Furthermore, if mossy fiber sprouting is prevented by cycloheximide following pilocarpine-induced SE, spontaneous seizures and interictal

EEG events are unaltered in frequency and intensity (Longo and Mello, 1998). Finally, the mossy fiber sprouting correlated with the severity of hilar cell damage, and therefore, the coexistence of damage and sprouting makes it impossible to emphasize one over the other in seizure generation.

4.4. VGB treatment did not attenuate the impairment of spatial learning and memory

The Morris water-maze test is sensitive to the severity of hippocampal damage (Moser et al., 1993). It was previously demonstrated that the neuroprotective effects of VGB pretreatment in a perforant pathway stimulation model of SE were associated with a less impaired performance in the Morris water-maze task (Ylinen et al., 1991). In the present study, however, VGB-treated animals were more impaired in the spatial memory task than the saline-treated rats both at 10 and 18 weeks following the SSSE. Better performance in the Sal-10 group compared to the VGB-10 group might be related to the milder structural damage in the hippocampus of saline-treated rats. This was supported by data demonstrating that when the rats without seizures (no histologic damage observed in thionin preparations) were excluded from the Sal-10 group, there was no difference compared to the VGB-10 group. In contrast, neuronal damage in the VGB-10-FU-8 group was similar to that in the Sal-10-FU-8 group. Therefore, chronic VGB treatment did not restore the cognitive impairment observed following SE. It is also possible that VGB itself had an unfavorable effect on water-maze performance. This is unlikely, however, because in the present study, VGB treatment had been stopped 8 weeks before water-maze testing. Further, VGB treatment up to 150 mg/kg does not impair performance in the Morris water-maze (Mazurkiewicz et al., 1992).

5. Conclusions

VGB treatment that was started 2 days after SE did not prevent the development of the epilepsy. In addition, VGB treatment did not prevent hippocampal neuronal damage and the associated impairment in spatial learning and memory. Therefore, if a large amount of neuronal damage is already present in the brain at the time when drug treatment is started, chronic GABA elevation appears to be inefficient in preventing epileptogenesis. Whether treatments started with a shorter delay relative to the beginning of SE would provide a better outcome remains to be explored.

It is now evident that approaches that aim at preventing epileptogenesis using antiepileptic drugs that were designed to suppress spontaneous seizures have failed both in animals and humans, particularly when

epileptogenesis is triggered by an insult associated with brain damage. Present and previous studies aimed at augmenting the GABAergic system by increasing GABA levels or by stimulating GABA_A receptors using phenobarbital (Mikati et al., 1994; Bolanos et al., 1998) demonstrate that epileptogenesis is not prevented if the treatment is started 1–2 days after SE. Also, in human studies, phenobarbital alone or in combination with phenytoin did not prevent the development of epilepsy after traumatic brain injury (see Kuhl et al., 1990; Temkin et al., 1998). Similar results were reported for the sodium channel blockers, phenytoin (Temkin et al., 1990) and carbamazepine (Glötzner et al., 1983). Also, valproate treatment failed to prevent epileptogenesis after diagnosis of a tumor (Glantz et al., 1996) and might have even caused detrimental effects on recovery after traumatic brain injury (Temkin et al., 1997). In experimental animals, Bolanos et al. (1998) reported that sedative doses of valproate might reduce neuronal damage and prevent the development of generalized seizures following kainic acid-induced SE in rats. Data were based on behavioral screening of SE, however, and spontaneous generalized seizures without EEG recordings. As the present study indicates, however, the long-term EEG recordings provide a useful tool to avoid false negative results if behavioral seizures are subclinical or partial rather than secondarily generalized. The inefficacy of antiepileptic drugs reported in the present and previous studies provide a great challenge for epileptologists to seek novel strategies for the prevention of epilepsy without compromising normal recovery.

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IV

Effect of lamotrigine treatment on epileptogenesis: an experimental study in rat

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Effect of lamotrigine treatment on epileptogenesis: an experimental study in rat

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Abstract

Prevention of epileptogenesis in patients with acute brain damaging insults like status epilepticus (SE) is a major challenge. We investigated whether lamotrigine (LTG) treatment started during SE is antiepileptogenic or disease-modifying. To mimic a clinical study design, LTG treatment (20 mg/kg) was started 2 h after the beginning of electrically induced SE in 14 rats and continued for 11 weeks (20 mg/kg per day for 2 weeks followed by 10 mg/kg per day for 9 weeks). One group of rats ($n = 14$) was treated with vehicle. Nine non-stimulated rats with vehicle treatment served as controls. Outcome measures were occurrence of epilepsy, severity of epilepsy, and histology (neuronal loss, mossy fiber sprouting). Clinical occurrence of seizures was assessed with 1-week continuous video-electroencephalography monitoring during the 11th (i.e. during treatment) and 14th week (i.e. after drug wash-out) after SE. LTG reduced the number of electrographic seizures during SE to 43% of that in the vehicle group ($P < 0.05$). In the vehicle group, 93% (13/14), and in the LTG group, 100% (14/14) of the animals, developed epilepsy. In both groups, 64% of the rats had severe epilepsy (seizure frequency >1 per day). The mean frequency of spontaneous seizures, seizure duration, or behavioral severity of seizures did not differ between groups. The severity of hippocampal neuronal damage and density of mossy fiber sprouting were similar. In LTG-treated rats with severe epilepsy, however, the duration of seizures was shorter (34 versus 54 s, $P < 0.05$) and the behavioral seizure score was milder (1.4 versus 3.4, $P < 0.05$) during LTG treatment than after drug wash-out. LTG treatment started during SE and continued for 11 weeks was not antiepileptogenic but did not worsen the outcome. These data, together with earlier studies of other antiepileptic drugs, suggest that strategies other than Na^+ -channel blockade should be explored to modulate the molecular cascades leading to epileptogenesis after SE.

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Keywords: Anticonvulsant; Antiepileptic drug; Disease modification; Epilepsy; Mossy fiber sprouting; Spontaneous seizures; Status epilepticus

1. Introduction

Epidemiologic studies indicate that 41% of patients with status epilepticus (SE) develop epilepsy within the next 10 years (Hesdorffer et al., 1998). Based on

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experimental and clinical evidence, SE triggers a cascade of events that alter synaptic networks, including acute and delayed neuronal death, gliosis, neurogenesis, axonal and dendritic sprouting, and molecular reorganization of the extracellular matrix and cellular membranes (for review, see Jutila et al., 2002; Lukasiuk et al., 2003). These changes occur in parallel and/or serial fashion, and presumably underlie the development of spontaneous seizures. Reorganization begins during SE and can continue for days, weeks, or even months (for review, see Jutila et al., 2002). These data suggest that the long-term clinical and cognitive outcome of patients with SE might be improved by starting antiepileptogenic/neuroprotective treatment during SE and continuing it for some time thereafter.

There have been several previous attempts to prevent epileptogenesis in animal models using chronic drug treatment during the epileptogenic phase (for reviews, see Löscher, 2002; Pitkänen, 2002a). The compounds tested include antiepileptic drugs (AEDs), *N*-methyl-D-aspartic acid antagonists, immunosuppressants, caspase 3 inhibitors, and growth factors (Pitkänen, 2002b; Narkilahti et al., 2003). In most studies, a delay in the development of kindling has been used as a marker of antiepileptogenic effects. Despite the efficacy of some compounds on the kindling model, none of them has had an indisputable antiepileptogenic action in animal models with spontaneous seizures (Löscher, 2002; Pitkänen, 2002a) or in humans (Temkin, 2001). Also, no disease-modifying effects have been described. Recent studies by Prasad et al. (2002) and Nissinen et al. (2001) indicate that acute short-term administration of some AEDs, like phenobarbital or diazepam but not phenytoin, within the first 2 h after the beginning of SE might have beneficial effects on epileptogenesis. That is, fewer animals develop epilepsy (Prasad et al., 2002) and the epilepsy that develops is milder (Nissinen et al., 2001). The possibility of altering the epileptogenic process by modulating the initial epileptogenic insult at the acute phase with AEDs is appealing, particularly if the insult is associated with the occurrence of seizure activity.

Lamotrigine (LTG; 3,5-diamino-6-[2,3-dichlorophenyl]-1,2,4-triazine) blocks neuronal voltage-gated sodium channels in a use-dependent manner, which

is associated with a reduction in glutamate release from presynaptic terminals (Xie and Hagan, 1998). Previous studies reported that pre-treatment with LTG is neuroprotective in several *in vitro* (Longo et al., 1995; Ashton et al., 1997; Siniscalchi et al., 1998; Garthwaite et al., 1999; Pisani et al., 2001; Calabresi et al., 2000, 2003) and *in vivo* models of brain damage (Schulz et al., 1995; Shuaib et al., 1995; Casanovas et al., 1996; Yang et al., 1998; Lee et al., 1999, 2000; Farber et al., 2002), including SE (Maj et al., 1998). Importantly, treatment with LTG also has neuroprotective effects when started *during or soon after* brain damaging epileptogenic insults. For example, in a gerbil model of global ischemia, LTG attenuates hippocampal CA1 damage when administered immediately after reperfusion (Ward et al., 1995). In a rat model of focal ischemia, LTG administered within 1 h after medial cerebral artery occlusion significantly reduces the volume of cortical infarct (Smith and Meldrum, 1995). LTG is also neuroprotective after cardiac arrest in rats when administered either immediately after or within 5 to 8 h of ischemia (Crumrine et al., 1997; Kanthasamy et al., 1999). We recently reported that LTG has mild neuroprotective effects against SE-induced neuronal damage in rats even when administered 1 h after the beginning of SE (Halonen et al., 2001). Further, administration of 20 mg/kg of LTG, but not a lower dose (O'Donnel and Miller, 1991), prevents the development of kindling, which is a model of epileptogenesis in humans (Stratton et al., 2003). Finally, LTG has been suggested to reduce excitatory dendritic input to CA1 pyramidal neurons by activating the hyperpolarization activated cation current, I_h (Poolos et al., 2002). This is of interest because modification of I_h is proposed to be responsible for a lowered seizure threshold after experimentally induced hyperthermic seizures (Chen et al., 2001).

The present study examined whether an AED that is both neuroprotective and delays the development of kindling is antiepileptogenic in models in which spontaneous seizures develop after epileptogenic insult. LTG was administered to rats 2 h after the beginning of SE and the treatment continued for 11 weeks. Outcome measures were the occurrence and severity of epilepsy, hippocampal neuronal loss, and mossy fiber sprouting.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (300–361 g) were used. After electrode implantation, rats were housed in individual cages at a temperature of 19–21 °C, with humidity maintained at 50–60% and lights on 07:00–19:00 h. Standard food pellets and water were freely available. Animal care and experimental procedures were conducted in accordance with the guidelines of the European Community Council directives 86/609/EEC.

2.2. Induction of status epilepticus

Details of the induction of SE by electrical stimulation of the amygdala were described previously (Nissinen et al., 2000). Briefly, the animals were anesthetized, a pair of stimulation electrodes was implanted into the lateral nucleus of the left amygdala. Stainless steel screws inserted into the skull above the right frontal cortex and bilaterally over the cerebellum served as reference, indifferent, and ground electrodes. The rats were allowed to recover from the surgical operation for 14 days until the electrical stimulation was started.

2.3. Characterization of electrographic seizures

To quantify the duration and severity of SE, seizure activity was recorded using a digital video-electroencephalography (EEG) system during the amygdala-stimulation and for at least 16 h thereafter. The severity of SE was assessed by counting the number of high-frequency and high-amplitude discharges (HAFD), which are typically associated with behavioral seizures. The criteria for a HAFD were a discharge that lasted for at least 5 s, at a frequency of at least 8 Hz, and amplitude at least two times higher than baseline either in the amygdala or in the cortex (or both).

The number and severity of epileptic seizures was studied 10 weeks after the initial SE (rats still on LTG treatment). The rats were observed by continuous video-EEG monitoring for 7 days (24 h per day). The occurrence of spontaneous seizures was determined based on the analysis of EEG data. If there was an

electrographic seizure, the severity of the behavioral seizure was scored based on the video. The criterion for an epileptic seizure was a high-frequency (>5 Hz), high-amplitude (>2× baseline) discharge either in the amygdala or in the cortex (or both) that lasted for at least 5 s. The second 1-week recording was performed 13 weeks after SE (i.e. 2 weeks after LTG wash-out).

2.4. Characterization of behavioral seizures

During the stimulation and follow-up periods, rat behavior was recorded with a video camera that was time-locked with the EEG. Behavioral motor seizure activity was classified according to a slightly modified Racine's scale (Racine, 1972). Score 0: electrographic seizure without any detectable motor manifestation; score 1: mouth and face clonus, head nodding; score 2: clonic jerks of one forelimb; score 3: bilateral forelimb clonus; score 4: forelimb clonus and rearing; score 5: forelimb clonus with rearing and falling. For the purpose of assessing the percentage of partial seizures or secondarily generalized seizures of the total number of seizures, behavioral seizures were classified into two categories: (1) partial seizures (scores 0–2) or (2) secondarily generalized seizures (scores 3–5).

2.5. Administration of LTG or vehicle

Lamotrigine (GI 267119X, ST/62287; provided by Glaxo Wellcome, UK) was started 2 h after the beginning of SE. The first dose was 20 mg/kg i.p. Thereafter, LTG (20 mg/kg, p.o.) was given once daily for 2 weeks (at 10:00–12:00 h). After 2 weeks, LTG was administered at a dose of 10 mg/kg (p.o.) for the next 9 weeks. For intraperitoneal administration, LTG was dissolved in DMSO (dimethyl sulfoxide (10 mg/ml), #34943, Riedel-de Haën). For per os administration, LTG was suspended in distilled H₂O (dH₂O; 5 mg/ml). Treatment was continued for 11 weeks. Three different groups were included in the study.

- Unstimulated, vehicle-treated controls ($n = 10$, referred to as the sham group). Vehicle treatment was started 14 days after the electrode implantation (this time point corresponds to the beginning of vehicle or LTG treatment in stimulated animals). On the first day, rats received DMSO (2 ml/kg, i.p.). On the second day, and thereafter, rats were treated with

dH₂O (2 ml/kg, p.o.). After 11 weeks, the treatment was stopped. Rats were perfused for histology 16 weeks after the operation.

- Stimulated, vehicle-treated controls ($n = 14$, referred to as the vehicle group). Vehicle treatment was started 2 h after the beginning of SE. On the first day, rats received 2 ml/kg of DMSO (i.p.). On the second day, and thereafter, rats were treated with dH₂O (2 ml/kg, p.o.). After 11 weeks, the treatment was stopped. Rats were perfused for histology 14 weeks after the stimulation.
- Lamotrigine-treated group ($n = 14$, referred to as the LTG group). LTG treatment was started 2 h after the beginning of SE (on the first day, 20 mg/kg i.p.). On the second day and for 2 weeks thereafter, rats were treated with LTG (20 mg/kg per day, p.o.; at 1000–1200 h). After 2 weeks, the LTG dose was decreased and treatment was continued for the next 9 weeks with 10 mg/kg per day (p.o.). Rats were perfused for histology 14 weeks after the stimulation.

2.6. Histologic analysis of brain tissue

Fourteen weeks after the induction of SE, rats were deeply anesthetized and perfused intracardially according to fixation protocol for Timm staining (Sloviter, 1982) and processed as described previously (Narkilahti et al., 2003).

One series of sections was thionin-stained to characterize the cytoarchitectonic boundaries of various temporal lobe regions and to locate the electrode tips. These sections were also used to assess the severity of overall neuronal damage in the ipsilateral and contralateral hilus as well as in the pyramidal cell layers of CA3c and CA1 subfields of the hippocampus. Damage was semiquantitatively assessed in thionin-stained sections as follows: Score 0 = no damage, score 1 = lesions involving <20%, score 2 = 20–50%, score 3 \geq 50% of neurons in the region of interest. The damage score for each region was obtained by scoring the severity of damage in five successive sections (one-in-five, 150 μ m intervals), in which the region of interest was present and by calculating the mean. In addition to scoring each region separately, we also analyzed the differences in total septal damage = (septal HILUS + septal CA3c + septal CA1 damage scores), total temporal damage (temporal HILUS + temporal

CA1 damage scores), and total damage (total septal damage + total temporal damage).

Sprouting of granule cell axons, that is, mossy fiber sprouting, was analyzed from sections stained using the Timm sulfide/silver method (Sloviter, 1982). Both the ipsilateral and contralateral hippocampus were analyzed. The density of sprouting was analyzed in each section along the septotemporal axis of the hippocampus as described previously (Cavazos et al., 1991; Narkilahti et al., 2003).

Assessment of neuronal damage and mossy fiber sprouting were conducted blind with respect to the animal treatment group.

2.7. Statistical analysis

Data were analyzed using SPSS for Windows (version 9.0) or Statview 4.5 for Macintosh. Non-parametric statistics were used because the distribution of values was not normal, values were on the ordinal scale, or the number of animals in the treatment groups was small. Severity of SE (number of HAFDs, duration of individual HAFDs, total duration of HAFDs, percent duration of HAFDs of the total duration of SE, duration of SE), number and severity of spontaneous seizures, severity of neuronal damage, and density of mossy fiber sprouting between different treatment groups were compared using the Mann–Whitney U test. Severity of neuronal damage between the stimulation and contralateral sides was compared using the Wilcoxon signed rank test. Differences between treatment groups in the occurrence of epilepsy were tested using a χ^2 -test. A P -value of less than 0.05 was considered significant.

3. Results

3.1. Number of rats in different groups

Electrodes were implanted in 46 rats. Of these 46 operated animals, 3 died after the operation. Of the 43 remaining electrode-implanted rats, 10 were included in the unstimulated sham-group. The remaining 33 rats were stimulated and randomly assigned into the vehicle or LTG groups. Of 33 stimulated animals, 3 did not develop SE. Of 30 stimulated rats with SE, 2 died within 13 days (excluded from the analysis;

Table 1
Characteristics of status epilepticus in different treatment groups

Group	Number of HAFDs		Total number of HAFDs	Duration of individual HAFDs (s)		Mean duration of all HAFDs (s)	Total duration of HAFDs (min)	Percent duration of HAFDs	Duration of SE (min)
	Before treatment (0–2h)	After treatment (>2h)		Before treatment (0–2h)	After treatment (>2h)				
Vehicle (14)	64 ± 16	35 ± 23	99 ± 26	32 ± 7	26 ± 12	30 ± 8	48 ± 11	7.6 ± 2.7	695 ± 262
LTG (14)	65 ± 19	15 ± 19**	80 ± 23	32 ± 13	20 ± 14#	29 ± 9	37 ± 9**	12.5 ± 11.1	627 ± 484

Data are presented as mean ± standard deviation. Number of rats is shown in parenthesis. Abbreviations: HAFD, high-amplitude and frequency discharges. Percent duration of HAFDs refers to the percentage of HAFD duration from the total duration of SE.

** $P < 0.01$ compared to the vehicle group.

$P < 0.05$ compared to the time period before treatment (Mann–Whitney U test).

1 in the LTG group and 1 in the vehicle group). In addition, one rat in the vehicle group died at the end of the second video-EEG recording session (included in the analysis of SE and seizure parameters but not in the histology). Thus, 28 stimulated (14 in the vehicle group, 14 in the LTG group) and 10 unstimulated rats survived to the end of the study and were included in the statistical analysis. There was no difference in mortality between vehicle and LTG-treated animals.

3.2. Effect of LTG on the severity of SE

3.2.1. Number of HAFDs

To assess the treatment effect on HAFD number, we calculated the number of HAFDs at 0–2 h after the beginning of SE (prior to treatment) and >2 h after the beginning of SE (after treatment was started). The data are summarized in Table 1.

For the first 2 h, there was no difference between the groups in the number of HAFDs (Table 1). During the treatment period (>2 h after the beginning of SE), the LTG group had a lower number of HAFDs than did the vehicle group ($P < 0.01$; Table 1). The total number of HAFDs (all HAFDs recorded either before or after treatment) did not differ between groups (Table 1).

3.2.2. Duration of individual HAFDs

Before drug-administration, the mean duration of the individual HAFDs did not differ between groups (Table 1). LTG treatment had no effect on the duration of individual HAFDs (Table 1).

3.2.3. Total duration of HAFDs

The total duration of HAFDs (sum of all HAFDs) was shorter in the LTG group than in the vehicle group ($P < 0.01$; Table 1).

3.2.4. Percentage of HAFDs of the total duration of SE

There was no difference between the vehicle and LTG groups (Table 1).

3.2.5. Duration of SE

The duration of SE was calculated as the time interval between the first and last HAFD. There was no difference between groups (Table 1).

3.3. Spontaneous seizures in the vehicle or LTG-treated animals

3.3.1. Development of epilepsy

The percentage of rats with spontaneous seizures (i.e. epilepsy) did not differ between the vehicle and LTG groups in either follow-up period (Table 2).

3.3.2. Seizure frequency

Altogether, 4516 seizures were recorded from 28 rats during a total of 2-weeks continuous video-EEG recording. The data are summarized in Table 3.

3.3.2.1. Follow-up 1. During the first 1-week follow-up period 10 weeks after SE, the 14 vehicle-treated rats experienced 1173 seizures (mean seizure frequency (\pm S.D.) for each animal was 12.0 ± 13.7 seizures per day, range 0.0–42.7, median 6.1). The 14 LTG-treated rats (note that LTG treatment was ongoing) experienced 962 seizures (9.8 ± 11.3 seizures per day, range 0.4–32.7, median 4.9). The seizure frequency did not differ between the vehicle and LTG groups (Table 3).

Table 2
Number of animals with epilepsy in the different treatment groups

	Follow-up 1		Follow-up 2		All	
	Seizures		Seizures		Seizures	
	Yes	No	Yes	No	Yes	No
Vehicle	13/14 (93%)	1/14 (7%)	13/14 (93%)	1/14 (7%)	13/14 (93%)	1/14 (7%)
LTG	14/14 (100%)	0/14 (0%)	12/14 (86%)	2/14 (14%)	14/14 (100%)	0/14 (0%)
χ^2 -Test	n.s.		n.s.		n.s.	

Note that during follow-up 1 animals in the vehicle or LTG groups were still on treatment. During follow-up 2 animals were untreated. Abbreviations: LTG: lamotrigine; n.s.: statistically not significant.

Table 3
 Characteristics of spontaneous seizures in epileptic animals in different treatment groups

Group	Follow-up 1 (10 weeks after status epilepticus)				Follow-up 2 (13 weeks after status epilepticus)					
	Number	Frequency (seizure per day)	Duration (s)	Behavioral severity	Percent of generalized seizure	Number	Frequency (seizure per day)	Duration (s)	Behavioral severity	Percent of generalized seizure
Vehicle (13)	84 ± 97	12.9 ± 13.8	67 ± 28	2.8 ± 1.5	60 ± 43	104 ± 106	15.2 ± 15.2	64 ± 24	3.0 ± 1.5	63 ± 40
LTG (14 or 12) ^a	69 ± 79	9.8 ± 11.3	56 ± 36	2.1 ± 1.1	55 ± 41	85 ± 87	12.9 ± 12.4	63 ± 14	3.5 ± 0.9 ^{##}	87 ± 17 [#]

Note that during follow-up 1 animals in the vehicle or LTG groups were still on treatment. During follow-up 2 animals were untreated. Data are presented as mean ± standard deviation. Number of rats is shown in parenthesis. Abbreviations: LTG: lamotrigine; sz: seizure.

^a During the follow-up 1 14 rats and during the follow-up 2 12 rats had epilepsy.

[#] $P < 0.05$, compared to the follow-up 1 (Wilcoxon).

^{##} $P < 0.01$, compared to the follow-up 1 (Wilcoxon).

3.3.2.2. Follow-up period 2. During the second 1-week follow-up period 2 weeks later (after LTG wash-out, 13 weeks after SE), the seizure frequency did not differ between the vehicle and LTG groups. When the seizure frequencies were compared between the first and second follow-up periods, there were no differences in any of the groups (Wilcoxon signed rank test) (Table 3).

3.3.3. Severity of spontaneous seizures

Seizure severity was assessed by (a) measuring the duration of electrographic seizures, (b) scoring the severity of behavioral seizures, and (c) assessing the percentage of partial and generalized seizures of all seizures in each animal. The data are summarized in Table 3.

3.3.3.1. Duration of electrographic seizures. There was no difference between the vehicle and LTG groups. Also, the seizure duration did not differ between the first and second follow-up periods in any of the groups (Table 3; see, however, Section 3.3.5 and Table 5).

3.3.3.2. Severity of behavioral seizures. The mean behavioral seizure score did not differ between the vehicle and LTG groups. Also, there was no difference in the behavioral seizure score between the first and second recording sessions in the vehicle and LTG groups. The seizure score was increased in the second follow-up in the LTG group ($P < 0.01$; Table 3).

3.3.3.3. Percentage of secondarily generalized seizures of all seizures in each animal. The percentage of secondarily generalized seizures of the total seizure count was assessed in each animal. The mean

percentage of secondarily generalized seizures of all seizures did not differ between the vehicle and LTG groups in either follow-up period (Table 3).

Comparison of the percentage of secondarily generalized seizures in follow-up periods 1 and 2 indicated that, a higher percentage of seizures in the LTG group were secondarily generalized during follow-up period 2 (no LTG treatment) compared to follow-up 1 (LTG treatment; $P < 0.05$, Table 3).

3.3.4. Percentage of animals with no/mild (≤ 1 seizure per day) or severe (> 1 seizure per day) epilepsy

The number of animals in the “no/mild” or “severe” categories did not differ between the LTG and vehicle groups (Table 4).

3.3.5. Analysis of various seizure parameters in rats with severe (> 1 seizure per day) epilepsy

Animals with frequent seizures have a different clinical syndrome than animals with rare seizures (Nissinen et al., 2000). Therefore, we analyzed the data separately in rats with severe epilepsy. As shown in Table 5, the mean duration of spontaneous seizures in the first monitoring period was shorter in the LTG group (34 ± 13 s) compared to the vehicle group (59 ± 12 s; $P < 0.01$). After LTG washout, the seizure duration was prolonged in the LTG group (to 54 ± 6 s; $P < 0.05$) and the severity of behavioral seizures increased (from 1.4 ± 0.7 to 3.4 ± 0.9 ; $P < 0.05$).

3.4. Histologic analysis

3.4.1. Overall neuronal damage in thionin-stained sections

The severity of neuronal damage in the hilus of the dentate gyrus and the pyramidal cell layers of the

Table 4
Number of animals with severe (> 1 seizure per day) or no/mild (≤ 1 seizure per day) epilepsy in the different treatment groups

	Follow-up 1		Follow-up 2		All	
	Severe	No/mild	Severe	No/mild	Severe	No/mild
Vehicle	8/14 (57%)	6/14 (43%)	9/14 (64%)	5/14 (36%)	9/14 (64%)	5/14 (36%)
LTG	8/14 (57%)	6/14 (43%)	9/14 (64%)	5/14 (36%)	9/14 (64%)	5/14 (36%)
χ^2 -Test	n.s.		n.s.		n.s.	

Note that during follow-up 1 animals in the vehicle or LTG groups were still on treatment. During follow-up 2 animals were untreated. Abbreviations: LTG, lamotrigine; n.s.: statistically not significant.

Table 5
Effect of LTG on spontaneous seizures in rats with severe epilepsy (>1 seizure per day)

	Group	Follow-up 1	Follow-up 2
Number	Vehicle	144 ± 85 (8)	150 ± 98 (9)
	LTG	117 ± 73 (8)	113 ± 84 (9)
Frequency (per day)	Vehicle	21 ± 12 (8)	21 ± 14 (9)
	LTG	17 ± 10 (8)	16 ± 12 (9)
Duration (s)	Vehicle	59 ± 12 (8)	54 ± 10 (9)
	LTG	34 ± 13 (8)**	57 ± 6 (9)#
Behavioral severity	Vehicle	1.9 ± 1.0 (8)	2.3 ± 1.2 (9)
	LTG	1.4 ± 0.7 (8)	3.4 ± 0.9 (9)#

Note that during follow-up 1 animals in the vehicle or LTG groups were still on treatment. During follow-up 2 animals were untreated. Values are expressed as mean ± standard deviation. Number of rats is in parenthesis. Abbreviations: LTG: lamotrigine.

** $P < 0.01$, compared to the vehicle group (Mann–Whitney U test).

$P < 0.05$ between follow-ups 1 and 2 (Wilcoxon).

CA3c and CA1 regions of the septal and temporal hippocampus is summarized in Table 6.

Unstimulated control rats had no damage in the regions analyzed. Ten of the 14 vehicle-treated rats and 11 of the 14 LTG-treated animals had

clear hippocampal damage in thionin-stained sections. The mean severity scores of damage did not differ between the vehicle and LTG groups in the septal hilus, CA3c, or CA1 (Table 6). Also, the severity of damage in the temporal hilus and CA1 region was similar in the vehicle and LTG groups.

In the vehicle group, the severity of damage was milder contralaterally than ipsilaterally in the septal hilus ($P < 0.01$) and the septal CA1 ($P < 0.05$). In the LTG group, damage was milder contralaterally in the septal hilus ($P < 0.05$) and temporal CA1 ($P < 0.05$; Table 6).

3.4.2. Plasticity of granule cell axons (mossy fiber sprouting)

The density of mossy fiber sprouting is summarized in Table 7. Both the vehicle-treated and LTG-treated stimulated rats had an increased density of mossy fiber sprouting compared to unstimulated controls (for statistical significance, see Table 7). The density of mossy fiber sprouting in LTG-treated animals did not differ from that in the vehicle group in any of the regions analyzed (Table 7). The density of sprouting was more intense ipsilaterally than contralaterally in the vehicle

Table 6
Severity of neuronal damage in the septal and temporal ends of the hippocampus

Brain area	Sham		Vehicle		Lamotrigine	
	Ipsilateral (10)	Contralateral (10)	Ipsilateral (14)	Contralateral (14)	Ipsilateral (14)	Contralateral (14)
Septal HILUS	0	0	1.0 ± 1.1***	0.8 ± 1.1**,#	1.2 ± 1.2***	1.0 ± 1.3**,#
Septal CA3c	0	0	0.4 ± 0.6 *	0.3 ± 0.5*.#	0.3 ± 0.4*	0.3 ± 0.4*
Septal CA1	0	0	0.4 ± 0.9	0.4 ± 0.8	0.1 ± 0.2	0.1 ± 0.2
Total septal damage	0	0	1.9 ± 2.3***	1.4 ± 2.1**,#	1.7 ± 1.6**	1.4 ± 1.7**
Temporal HILUS	0	0	1.9 ± 1.4***	1.8 ± 1.3**	1.9 ± 1.4**	1.7 ± 1.4**
Temporal CA1	0	0	0.9 ± 0.9**	0.9 ± 0.9**	0.5 ± 0.4**	0.6 ± 0.4**,#
Total temporal damage	0	0	2.8 ± 2.1**	2.8 ± 1.9**	2.4 ± 1.8**	2.4 ± 1.8**
Total damage	0	0	4.7 ± 4.1***	4.1 ± 3.6***	4.1 ± 3.1***	3.8 ± 3.2***

Values are expressed as mean ± standard deviation. Number of animals in each group is shown in parenthesis. Damage was scored from thionin-stained sections as follows: 0 = no damage, 1 ≤ 20% neurons lost, 2 = 20–50% neurons lost, 3 ≥ 50% neurons lost. Total septal damage = damage in septal HILUS + septal CA3c + septal CA1. Total temporal damage = damage in temporal HILUS + temporal CA1. Total damage = total septal damage + total temporal damage. No difference was found in the severity of damage between the animals treated with vehicle or lamotrigine.

* $P < 0.05$ (compared to the sham group, Mann–Whitney's U test).

** $P < 0.01$ (compared to the sham group, Mann–Whitney's U test).

*** $P < 0.001$ (compared to the sham group, Mann–Whitney's U test).

$P < 0.05$, compared to the contralateral side (Wilcoxon).

$P < 0.01$, compared to the contralateral side (Wilcoxon).

Table 7
Density of mossy fiber sprouting in the septal and temporal ends of the hippocampus

Brain area	Sham (9)		Vehicle (13)		Lamotrigine (14)	
	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral
Septal	1.36 ± 0.40	1.41 ± 0.44	3.31 ± 1.01***	3.24 ± 0.99***	3.68 ± 1.09***	3.67 ± 1.09***
Temporal	1.24 ± 0.39	1.28 ± 0.42	3.74 ± 1.22***	3.47 ± 1.22***,#	3.93 ± 1.29***	3.88 ± 1.32***
Mean total sprouting	1.32 ± 0.39	1.36 ± 0.43	3.48 ± 1.06***	3.33 ± 1.06***,#	3.78 ± 1.15***	3.76 ± 1.16***

Values are expressed as mean ± standard deviation. Number of animals in each group is shown in parenthesis. Septal sprouting = sprouting in the (tip + mid + crest)/3. Temporal sprouting = sprouting in the (dorsal + ventral aspects of the temporal dentate gyrus)/2. Mean total sprouting = mean of sprouting scores of all areas analyzed (septal tip + septal mid + septal crest + temporal dorsal + temporal ventral)/5. Statistical significances: *** $P < 0.001$ (compared to the sham group, Mann–Whitney's U test); # $P < 0.05$ (compared to the ipsilateral side, Wilcoxon). No difference was found in the severity of sprouting between the vehicle and lamotrigine groups.

group (Table 7). In the LTG group, however, the density of sprouting was similar ipsilaterally and contralaterally (Table 7).

4. Discussion

The present study examined whether administration of a compound that is neuroprotective and delays the development of kindling will have antiepileptogenic or disease-modifying effects when administered during epileptogenic insult associated with seizure activity, and continued during the latency phase. Therefore, LTG was administered 2 h after the beginning of SE, and treatment continued for the next 11 weeks at doses that are neuroprotective and delay the development of kindling in rats. The findings indicate that LTG did not prevent the development of epilepsy. At the end of the 9-week treatment period, however, when the animals were still on medication, the duration of seizures in frequently seizing animals was shorter and their behavioral severity was attenuated compared to the vehicle group. After LTG wash-out, there was no indication of disease-modification when the seizure frequency, seizure duration, seizure severity, and hippocampal damage or mossy fiber sprouting were used as outcome measures. After wash-out of LTG behavioural seizures in frequently seizing animals were significantly more severe and were more likely to be generalised, suggesting that seizure generalization had been partially prevented during lamotrigine treatment.

4.1. Methodologic aspects

To mimic a clinical situation in which antiepileptogenic treatment would be started during or soon after the beginning of epileptogenic insult, and continued for some time thereafter, LTG was administered at the dose of 20 mg/kg (i.p.) during SE. Thereafter, treatment was continued at a dose of 20 mg/kg (p.o.) for 2 weeks and then 10 mg/kg (p.o.) for another 9 weeks.

Previous studies indicate that the ED₅₀ dose of LTG against maximal electroshock seizures in rats is 1.9 mg/kg (i.v.) (Miller et al., 1986) and against electrically evoked afterdischarges, 11.7 mg/kg (i.v.) (Wheatley and Miller, 1989). In rat amygdala or hippocampal kindling, 13.4 mg/kg (i.p.) of LTG completely abolishes the seizures and afterdischarges (Otsuki et al., 1998). A dose of 6.7 mg/kg produces a significant reduction. O'Donnell and Miller (1991) reported that LTG given per os at a dose of 12 or 18 mg/kg reduced the duration of afterdischarges in cortically kindled animals by 21 and 36%, respectively. More recently, administration of 10 or 20 mg/kg of LTG to fully kindled rats reduced the duration of afterdischarges from 50 to 28 s (by 38%) or to 22 s (by 51%), respectively (Stratton et al., 2003). Finally, administration of 20 mg/kg LTG reduced seizure duration in spontaneously seizing rats in which epileptogenesis was induced by amygdala-stimulation as in the present study (Nissinen et al., 2000). Therefore, the LTG dose used in the present study (10–20 mg/kg per day) was comparable to the dose that suppresses chemically or electrically evoked seizures as well as spontaneous seizures in rats.

Some recent studies reported inhibitory effects of LTG on the development of kindling. Pretreatment of rats with 20 mg/kg (i.p.) LTG shortens the duration of afterdischarges and reduces the severity of behavioral seizures during induction of amygdala kindling in Lister hooded rats (Stratton et al., 2003). There are less pronounced effects of LTG on the development of cortical kindling (O'Donnell and Miller, 1991). Further, it was found that a dose of LTG (15 mg/kg) enhanced the development of kindling compared to a lower dose (5 mg/kg) in Sprague–Dawley rats (Krupp et al., 2000). Here, we administered LTG at a dose of 20 mg/kg, which is comparable to the dose of LTG used in amygdala kindling. We administered LTG at a dose of 20 mg/kg per day for the first 2 weeks, which covered approximately half of the median epileptogenic period in these animals. This time period also corresponds to the duration of administration of LTG in kindling experiments (Stratton et al., 2003). The reason for the dose reduction to 10 mg/kg per day for the next 9 weeks was to minimize LTG-induced weight reduction (Halonen et al., 2001), rather than a concern regarding the facilitation of epileptogenesis.

In clinical monotherapy trials with newly diagnosed patients, LTG plasma concentrations vary between 2 and 4 $\mu\text{g/ml}$ (Brodie et al., 1995). Concentrations higher than 4 $\mu\text{g/ml}$, however, are frequently required for optimal seizure control in patients with intractable epilepsy (Brodie et al., 1995), and concentrations as high as 20 $\mu\text{g/ml}$ are not uncommon (Sander et al., 1990). In rats, administration of 12.5 mg LTG at 12-h intervals resulted in serum concentrations between 9 and 21 $\mu\text{g/ml}$ (see Halonen et al., 2001). Walton et al. (1996) measured serum concentrations of LTG in adult Sprague–Dawley rats approximately 1 h after intraperitoneal administration. According to these data, administration of 10 mg/kg of LTG results in a serum concentration of 4–5 $\mu\text{g/ml}$ and 20 mg/kg in 7–8 $\mu\text{g/ml}$. In the present study, the dose of LTG used during the first 2 weeks was 20 mg/kg and for 9 weeks thereafter, 10 mg/kg. Therefore, the serum concentrations achieved in the present study are presumably comparable to or higher than the clinically relevant concentrations in humans. It is possible that during SE the brain concentrations of LTG were higher than expected due to the leakage of the blood–brain barrier.

Peak serum LTG concentration occur within 30 min after intraperitoneal administration (Walton et al.,

1996). Therefore, we assume that the anticonvulsive concentration of LTG was achieved during the course of SE.

Finally, we administered LTG at 24-h intervals. Previous studies indicate a half-life of approximately 12.5 h in male rats. Administration of LTG at a dose of 13.4 mg/kg (i.p.), which is comparable to the chronic treatment regimen in the present study, suppressed or completely abolished both the seizure severity and afterdischarge duration, even 24 h after the injection (Otsuki et al., 1998). Therefore, we assume that our once a day administration scheme resulted in anticonvulsive LTG concentrations throughout the 10-weeks treatment period.

4.2. Effect of LTG treatment on SE

Considering the efficacy of induction of epileptogenesis by SE in different treatment groups, one critical question is: was the overall severity of SE similar in the vehicle and LTG-treated animals? LTG administration alleviated the severity of SE by reducing the number of individual electrographic seizures occurring during SE by 57% compared to the vehicle group. The seizure activity during SE, however, was not completely abolished. In fact, all animals in the LTG group had more than 38 HAFDs, which is associated with a high risk of epileptogenesis (Nissinen et al., 2000). Second, the duration of individual electrographic seizures during SE was shorter in LTG than in vehicle-treated animals. The total duration of SE was not, however, different from that in the vehicle group. Also, LTG did not eliminate the spiking activity between HAFDs (data not shown). Therefore, despite the reduction in the severity of some parameters used to assess the severity of SE, the effects of LTG were not strong enough to have a clear “initial-insult”-modifying effect comparable to that of diazepam (Nissinen et al., 2001), phenobarbital (Prasad et al., 2002), or an AMPA-antagonist, NS1209 (Pitkänen et al., 2002a), which markedly suppress the SE-associated electrographic activity and reduce the percentage of animals that develop spontaneous seizures later in life.

4.3. Effect of LTG treatment on epileptogenesis

The first period of video-EEG monitoring was started 10 weeks after SE when the animals were

still receiving vehicle or LTG treatment. A previous study demonstrated that by this time point over 90% of rats that will eventually develop epilepsy should have exhibited spontaneous seizures (Nissinen et al., 2000). Ninety-two percent of vehicle-treated rats and 100% of LTG-treated animals developed epilepsy. The second period of video-EEG monitoring was performed after a 2-weeks drug wash-out with similar results. Therefore, there was no evidence that LTG treatment prevented or delayed the development of epilepsy.

Unlike in kindling, in SE models epileptogenesis occurs in damaged brain. It is unclear whether data from the kindling model can be extrapolated to models of symptomatic focal epilepsy. As the present data indicate, an LTG-induced delay of amygdala kindling did not predict an antiepileptogenic effect in a spontaneous seizure model at a comparable dose, which is similar to data obtained previously with other antiepileptic drugs (Pitkänen, 2002a,b). Importantly, however, we did not observe any worsening of epileptogenesis by LTG treatment.

4.4. Does LTG have a disease-modifying effect?

After drug-washout 13 weeks after SE, 64% of the animals had severe epilepsy (i.e. >1 seizures per day) in both the vehicle and the LTG-treated groups. The duration of electrographic seizures, the severity of behavioral seizures, and the percentage of secondarily generalized seizures of the total seizure count in the LTG group were similar to that in the vehicle group. Therefore, LTG treatment did not have any disease-modifying effects.

Before discontinuation of the treatment, seizure duration in LTG-treated rats with severe epilepsy was only 58% of that in the vehicle group. In the LTG group, the seizure duration was prolonged after drug wash-out compared to the on-drug seizure duration. These data are consistent with those from a previous study, in which we administered LTG to previously untreated rats with severe epilepsy. The mean seizure duration was reduced from 128 to 11 s and a 50% reduction in seizure frequency in 80% of the rats. None of the eight rats tested, however, became seizure free (Nissinen and Pitkänen, 2000). In two previous studies in which the antiepileptogenic effects of valproic acid and gabapentin were investigated af-

ter kainate-induced SE, seizure frequency assessed by visual observation was lower during the end of the drug-tapering period (Bolanos et al., 1998; Cilio et al., 2001). These data together with the present observations suggest that even though an AED does not prevent epileptogenesis, the seizures emerging after the latency period are milder as long as AED administration is continued.

Postma et al. (2000) reported that the response of fully kindled seizures to LTG was reduced by prior LTG treatment. Further, administration of LTG at a dose of 15 mg/kg combined with repeated stimulations at 48-h intervals resulted in the development of tolerance (Krupp et al., 2000). As our present data demonstrate, the spontaneous seizures responded to LTG in the end of the 9-weeks treatment period despite preceding drug-administration during epileptogenesis. However, the effect of early treatment on later response to pharmacotherapy needs to be further explored.

4.5. Effect of LTG treatment on neuronal damage and axonal plasticity

Previous studies indicate that pretreatment with LTG is neuroprotective in several in vitro and in vivo models of brain damage, including SE, when the assessment of neuronal loss is performed within a few days or weeks after the insult (see references in Section 1). In the present study, in which the severity of damage was assessed 14 weeks after SE, hippocampal damage was not alleviated by LTG treatment. Also, the density of mossy fiber sprouting along the septotemporal axis of the hippocampus was similar in vehicle and LTG-treated animals, probably due to the progression of SE-induced neurodegeneration beyond the discontinuation of SE for up to 2 months after SE (Pitkänen et al., 2002b). Further, the neuroprotective effect of LTG on SE-induced cell death is most likely related to the reduction of seizure activity and consequent glutamate-induced toxicity rather than its effects on biochemical cascades that trigger delayed cell death (Halonen et al., 2001; Wang et al., 2002). Here, the lack of neuroprotective effects of LTG on SE-induced damage despite a milder SE was probably related to later degeneration, and therefore, there was no neuroprotective effect detected when assessed 3.5 months after the insult.

5. Conclusions

The present study did not provide evidence to support the idea that LTG treatment started during SE, and continued for 11 weeks thereafter, delayed or prevented SE-induced epileptogenesis. Also, there was no neuroprotective or disease-modifying efficacy of LTG treatment. In this regard, LTG did not differ from other AEDs examined in spontaneous seizure models (see, Pitkänen, 2002a,b). It remains to be tested whether AEDs have a more beneficial effect on epileptogenesis triggered by other etiologies like head trauma or stroke.

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Effect of antiepileptic drugs on spontaneous seizures
in epileptic rats

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**EFFECT OF ANTIEPILEPTIC DRUGS
ON SPONTANEOUS SEIZURES IN EPILEPTIC RATS**

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Abstract

The present study investigated whether spontaneously seizing animals are a valid model for evaluating antiepileptic compounds in the treatment of human epilepsy. We examined whether clinically effective antiepileptic drugs (AEDs), including carbamazepine (CBZ), valproic acid (VPA), ethosuximide (ESM), lamotrigine (LTG), or vigabatrin (VGB) suppress spontaneous seizures in a rat model of human temporal lobe epilepsy, in which epilepsy is triggered by status epilepticus induced by electrical stimulation of the amygdala. Eight adult male rats with newly diagnosed epilepsy and focal onset seizures were included in the study. Baseline seizure frequency was determined by continuous video-electroencephalography (EEG) monitoring during a 7 -baseline period. This was followed by a 2 to 3 -d titration period, a 7 -d treatment period, and a 2 to 3 -d wash-out period. During the 7 -d treatment period, animals were treated successively with CBZ (120 mg/kg/d), VPA (600 mg/kg/d), ESM (400 mg/kg/d), LTG (20 mg/kg/d), and VGB (250 mg/kg/d). VPA, LTG, and VGB were the most efficient of the compounds investigated, decreasing the mean seizure frequency by 83%, 84%, and 60%, respectively. In the VPA group, the percentage of rats with a greater than 50% decrease in seizure frequency was 100%, in the LTG group 88%, in the VGB group 83%, in the CBZ group 29%, and in the ETM group 38%. During the 7-d treatment period, 20% of the VPA -treated animals and 14% of the CBZ -treated animals became seizure free. These findings indicate that rats with focal onset spontaneous seizures respond to the same AEDs as patients with focal onset seizures. Like in humans, the response to AEDs can vary substantially between animals. These observations support the idea that spontaneously seizing animals are a useful tool for testing novel compounds for the treatment of human epilepsy.

Key words: amygdala, antiepileptic drug, electroencephalography, temporal lobe epilepsy, video-EEG monitoring

1. Introduction

The efficacy of anticonvulsant compounds has traditionally been investigated using maximal electroshock and pentylenetetrazol mouse and rat seizure models (Löscher, 2002; Walker and Fisher, 2004; Shorvon, 2004). The possibility of implementing new testing systems to find compounds with novel mechanisms of action and to develop study designs that better mimic clinical trials has been discussed (Stables et al., 2003). One possible approach is to use chronic animal models with spontaneous seizures instead of acutely induced seizures.

Recent developments in animal models have produced preparations in which spontaneous seizures can be triggered in either chemically or electrically induced status epilepticus (SE) (Mazarati et al., 2006), ischemic stroke (Kelly et al., 2006; Karhunen et al., 2006), or traumatic brain injury (Pitkänen et al., 2006). In all of these models, spontaneous seizures last for approximately 60 s and occur at rates that vary from 0.5 to 30 seizures per day (Nissinen et al., 2000; Karhunen et al., 2006). Relatively few studies, however, have investigated the effect of antiepileptic drugs (AEDs) on the occurrence and severity of spontaneous seizures in these models. In the pioneering study by Leite and Cavalheiro (1995), the effects of carbamazepine (CBZ), valproic acid (VPA), phenobarbital (PB), phenytoin (DPH), and ethosuximide (ETM) on spontaneous seizures were investigated in a post-SE model of epilepsy induced by pilocarpine. They reported that 0 % to 75% of rats responded (total remission of seizures) to at least one of the drugs. More recently, Brandt et al. (2004) reported that 64% of phenobarbital treated rats with post-SE epilepsy induced by amygdala stimulation had at least a 50% reduction (responder) in seizure frequency. Further, Glien et al. (2002) reported that 50% of levetiracetam (LEV) - treated rats in the same model were responders.

Video-electroencephalography (EEG) monitoring is a useful tool for assessing the effects of AEDs on seizure frequency (Mazarati et al. 1998, Halonen et al., 2001, Glien et al., 2002, Mazarati et al., 2002, Brand et al., 2004). The occurrence of partial seizures without secondary generalization can be more reliably assessed with video-EEG than with video monitoring alone. Further, the effect of AEDs on seizure duration can be measured. Here, we assessed the effects of AEDs on spontaneous seizures in video-EEG monitored rats. The aim of the present study was to provide further evidence for the validation of post-SE models in the identification of novel AEDs effective for the treatment of human epilepsy. The experiments were designed to address four questions: 1) whether AEDs used to treat patients with focal onset seizures suppress spontaneous seizures in an amygdala stimulation model of post-SE epilepsy; 2) whether ESM, an AED used to treat absence seizures, has any effect; 3) whether the responder rate (>50% decrease in seizure frequency) differs between drugs; and 4) what proportion of animals become seizure free? The data obtained support the view that post-SE models are a useful tool for investigating the antiepileptic efficacy of novel candidate AEDs.

2. Methods

The study design is summarized in figure 1.

2.1. Animals

Adult male Harlan Sprague-Dawley rats (n=49) weighing 330 ± 15 g (mean \pm standard deviation) at the time of surgery were used. The rats were singly housed in cages and maintained on a standard light-dark cycle (lights on 07:00-19:00), constant room temperature $22 \pm 1^{\circ}\text{C}$, and humidity 50 % to 60% with free access to food and tap water. Experiments were approved by the Committee for the Welfare of Laboratory Animals of the University of Kuopio and the Provincial Government of Kuopio. All procedures were conducted in accordance with the European Community Council directives 86/609/EEC.

2.2. Implantation of amygdaloid and cortical EEG electrodes

Animals were anesthetized using an intraperitoneal injection (6 ml/kg) of a mixture of sodium pentobarbital (58 mg/kg), chloral hydrate (60 mg/kg), magnesium sulfate (127.2 mg/kg), propylene glycol (40%), and absolute ethanol (10%). Thereafter, a bipolar electrode (diameter 0.127 mm, dorsoventral distance between the tips 0.4 mm; Franco Corradi, Milano, Italy) was implanted into the lateral nucleus of the left amygdala. Coordinates for the tip of the lower electrode were 3.6 mm posterior to bregma, 5.0 mm lateral to bregma, and 6.5 mm ventral to the surface of the brain [according to the rat brain atlas of Paxinos and Watson (1986)].

To record the spread of electrographic seizure activity to the contralateral cortex, a screw electrode (Plastics One Inc., Roanoke, VA) was implanted into the skull overlying the contralateral frontal cortex (3.0 mm anterior and 2.0 mm lateral to bregma). Two monopolar stainless steel screw electrodes that were fixed to the skull symmetrically over the cerebellum

with dental acrylate (Selectaplast *CN*, Dentsply DeTrey GmbH, Dreieich, Germany) served as ground and reference electrodes.

2.3. Induction and monitoring of SE

SE was induced by stimulating the lateral nucleus of the left amygdala. The stimulation consisted of a 100-ms train of 1-ms biphasic square wave pulses (400 μ A peak to peak) that were delivered at 60 Hz every 0.5 s using a A300 Pulsemaster Stimulator (World Precision Instruments, UK) connected to two A360 Constant Current Stimulus Isolators (World Precision Instruments). Rats were connected to the system via a 6-channel commutator (Plastics One Inc.) and shielded cables. The development and duration of SE in freely moving animals were monitored continuously for 18 h *via* amygdalar and cortical electrodes using the Nervus EEG Recording System (Taugagreining, Iceland), which was connected to an ISO-1032 Amplifier (Braintronics, Netherlands), or using the Stellate EEG Monitor system (Montreal, Canada), which was connected to two ISO-DAM 8 Amplifiers (World Precision Instruments). The manifestations of seizure activity were analyzed from Digital Data Storage-files.

Each rat was stimulated continuously for 20 min. Thereafter, stimulation was interrupted, and the behavioral and electrographic seizure activity of the animal was observed for 60 s. If the behavior of the animal revealed the presence of epileptic activity (head nodding/or limb clonus), the observation was continued for up to 5 min. If the animal did not meet the criterion of clonic SE (continuous electrographic epileptiform spiking and recurrent clonic seizures), stimulation was resumed and the behavior of the animal was checked again after 5 min. Once the criterion of SE was achieved, no further stimulation was given. Stimulation time never exceeded 40 min.

2.4. Selection of epileptic animals for AED monotherapy trial

Epileptic animals were selected for the AED trial 3 mo after SE. This time point was chosen based on previous findings that animals that eventually develop epilepsy express spontaneous seizures at 3 mo (Nissinen et al. 2000). Also, at 3 mo the seizure frequency in epileptic animals is stable.

Altogether, 49 animals were electrically stimulated; 3 animals did not develop SE and 10 animals died before the first monitoring at 3 mo post-SE. Thus, 36 animals were monitored (24 h/d) with the video-EEG recording system for 2 to 3 d to identify epileptic animals. The first eight animals with at least two spontaneous seizures were selected for the study.

2.5. Administration of AEDs

Carbamazepine (# C-4024, Sigma Chemical Co., St. Louis, MO) was suspended in 0.1% carboxymethyl cellulose (CM-52, Whatman, Maidstone Kent, UK) and administered intragastrically 3 times a day (40 mg/kg, 20 mg/ml, 2 ml/kg) at 8-h intervals (total daily dose 120 mg/kg). Valproic acid (# P-4543, Sigma Chemical Co.) was dissolved in 0.9% saline and given intraperitoneally twice a day (300 mg/kg, 2 ml/kg) at 12-h intervals (total daily dose 600 mg/kg). Ethosuximide (# E-7138, Sigma Chemical Co.) was dissolved in 0.9% saline and given intraperitoneally twice a day (200 mg/ml, 2 ml/kg,) at 12-h intervals (total daily dose 400 mg/kg). Lamotrigine (a generous gift from Glaxo Wellcome; at present GlaxoSmithKline, Hertfordshire, UK) was suspended in 0.1% carboxymethyl cellulose and given intragastrically twice a day (5 mg/ml, 2 ml/kg) at 12-h intervals (total daily dose 20 mg/kg). Vigabatrin (a generous gift from Hoechst Marion Roussel, Swindon, UK; at present Sanofi Aventis, Paris,

France) was dissolved in 0.9% saline and administered intraperitoneally once a day at a dose of 250 mg/kg.

Baseline seizure frequency was determined based on continuous 7 -d video-EEG monitoring, during which the animals were injected with vehicle (carboxymethyl cellulose or 0.9% NaCl). After the baseline monitoring, the dose of the AED was gradually increased to the final dose during a 2-d titration period. After a 7-d treatment period with a constant dose, the treatment was gradually discontinued during a 2-d wash-out period. This was followed by a 7-d baseline period before starting the next compound. During this time, animals were injected with the vehicle used to dissolve the next AED and were continuously monitored with video-EEG. If the animal died during the experiment (1 during CBZ treatment, 3 during VPA treatment, and 2 during VGB treatment) it was replaced by a new epileptic rat for the next drug studied.

Animals were exposed to various AEDs in the following order: CBZ → VPA → ESM → LTG → VGB. The order of drug treatments selected to avoid successively administering two compounds with a similar clinical spectrum in humans. Thus, we first administered CBZ, which is used to treat seizures with a focal onset; then VPA, which is efficacious against both focal onset and primarily generalized seizures; then ESM, which protects against primarily generalized absence -type seizures; then LTG, which has effects on both focal onset and primarily generalized seizures; and finally, VGB which is used to treat seizures with a focal onset. The doses of AEDs used in the present study resulted in plasma levels that are antiepileptic in clinical trials and that protect rats from fully kindled and/or spontaneous seizures (Cereghino, J.J. 1975; Leite and Cavalheiro, 1995; Halonen, et al., 1995; Pitkänen et al., 1996; Otsuki et al., 1998; Halonen et al., 1999; Löscher et al., 2002; Stratton et al., 2003; Schwabe et al., 2004).

2.6. Outcome measures

Five outcome measures were quantified to compare the efficacy of different treatments: (1) mean seizure frequency, which was calculated as mean seizure frequency both during the 7-d baseline or 7-d treatment periods (calculated separately for each rat), (2) mean seizure duration, which was calculated as mean duration of all seizures per 7-d baseline or treatment periods (calculated separately for each rat), (3) percentage of rats with a greater than 50% decrease in seizure frequency, (4) percentage of rats with a greater than 75% decrease in seizure frequency, and (5) percentage of rats that were seizure-free.

2.7. Statistical analysis

Data were analyzed using SPSS for Windows (Version 9.0; SPSS Inc, Chicago, IL). Non-parametric statistics were used because the distribution of values was not normal, values were on an ordinal scale, or the number of animals in treatment groups was small. Seizure frequency between baseline, treatment, and the next baseline period was analyzed using the Wilcoxon signed rank test. The change in seizure frequency over different treatment periods (baseline, treatment, next baseline) between different treatment groups was tested with a Kruskal-Wallis test followed by the Mann-Whitney *post hoc* test. A *p*-value of less than 0.05 was considered statistically significant.

3. Results

Baseline seizure frequency did not differ between the groups (Table 1, $p > 0.05$, Kruskal-Wallis). Mean baseline seizure duration, however, differed between groups (Table 1, $p < 0.01$,

Kruskall-Wallis). *Post-hoc* analysis with the Mann-Whitney-*U*-test indicated that seizure duration was longer in the CBZ ($p<0.05$), ESM ($p<0.05$), and LTG ($p<0.01$) groups than in the VGB group.

The effect of CBZ, VPA, ESM, LTG, and VGB on spontaneous seizures is summarized in Tables 1 and 2. LTG was the most effective compound for suppressing spontaneous seizures. Mean seizure frequency decreased from 18.3 to 1.6 (Table 1, $p<0.05$). The mean decrease in seizure frequency was $84 \pm 22\%$ (median 92). Seizure frequency decreased by greater than 50% or 75% in 88% (7/8) of the rats (in one rat, seizures decreased by 31%). None of the rats became seizure -free. During the post-treatment baseline period, seizure frequency increased back to the baseline level. LTG treatment did not affect seizure duration (Table 1).

Treatment with VPA reduced the mean seizure frequency from 33.5 to 6.5 seizures per day (Table 1, $p<0.05$). The mean decrease in seizure frequency was $83 \pm 14\%$ (median 84). Seizure frequency decreased by greater than 50% or 75% in 100% (5/5) and 60% (3/5) of the rats, respectively. One of the animals became seizure -free. After discontinuation of VPA, seizure frequency increased (Table 1, $p<0.05$). VPA treatment decreased the duration of seizures by 61% compared to that at baseline (Table 1).

In the VGB group, the mean daily seizure frequency decreased from 25.9 to 8.5 (Table 1, $p<0.05$). The mean decrease in seizure frequency was $60 \pm 15\%$ (median 64). Seizure frequency decreased by greater than 50% or 75% in 83% (5/6) and 17% (1/6) of the animals, respectively. Vigabatrin treatment had no effect on seizure duration.

In the ESM treatment group, seizure frequency decreased in 63% of rats as compared to baseline. The remaining animals, however, had a substantially increased seizure frequency compared to that at baseline. Consequently, the mean seizure frequency increased by $26 \pm 146\%$

(median 25) during ESM treatment. Seizure frequency decreased by more than 50% in 38% (3/8) of the animals. Actually, the same animals had a more than 75% decrease in seizure frequency. ESM treatment decreased the duration of seizures by 28% compared to that at baseline (Table 1, $p < 0.05$).

CBZ treatment did not affect mean seizure frequency as compared to baseline (Table 1). Analysis of data from individual rats, however, indicated that 29% (2/7) of rats had at least a 50% reduction in seizure frequency, and 14% (1/7) of rats had a 75% reduction in seizure frequency compared to baseline. Furthermore, one rat became seizure -free. In the remaining animals (71%), seizure frequency increased compared to baseline ($66 \pm 110\%$, median 67). CBZ treatment decreased seizure by 41% compared to baseline (Table 1, $p < 0.05$).

4. Discussion

The present study examined whether clinically efficacious AEDs suppress spontaneous seizures in an animal model of human temporal lobe epilepsy. First, the efficacy of the AEDs to suppress spontaneous seizures decreased in the order of $LTG \approx VPA > VGB > CBZ \approx ESM$. Second, 56% of rats responded to at least one of the treatments. Third, VPA, CBZ, and ESM reduced seizure duration. Fourth, only two rats (5%) became seizure-free with any of the treatments.

Methodologic considerations

Epileptogenesis was triggered in rats by electrically induced SE, and the animals were used in the study at 3 mo post-SE. Our previous data indicate that the median latency from SE to epilepsy diagnosis is 33 d, seizure frequency is stabilized by 2 mo, and all seizures have a focal onset with or without secondary generalization (Nissinen et al., 2000). Thus, we consider the animals included in the present study comparable to newly diagnosed patients with focal onset seizures. In the beginning of the study, the seizure frequency varied from 0.4 to 64.4 seizures/d between the rats, which resembles the variability in seizure frequency of newly diagnosed epilepsy patients (Brodie et al., 1995). The administration of various AEDs to the same rat in sequential order was designed to help to overcome the problems related to the contribution of the severity of epilepsy to the therapeutic response (Kwan and Sander, 2004).

Like other recent studies (Brandt et al, 2004; Volk et al., 2005), we took advantage of video-EEG monitoring to verify seizure occurrence. Although laborious, video-EEG analysis allowed us to record subclinical seizures, which might be of particular importance when

investigating AEDs that suppress the spread of seizure activity. Video-EEG monitoring also made it possible to measure the effect of AEDs on seizure duration.

Recently, Grabenstatter and coworkers (2005) proposed a study paradigm that can be used to compare the antiepileptic efficacy of compounds after a single administration and 2-d video-EEG monitoring. Here, we tried to overcome the problems related to the pharmacokinetics of various AEDs in rat by including a titration phase, treatment phase, wash-out phase, and a new baseline phase before starting the next compound, and by administering the compounds at intervals that resulted in therapeutic plasma levels of AEDs for most of the 24-h period. We did not observe a substantial increase in seizure frequency during the wash-out phase in any of the rats, suggesting that rebound seizures as a response to lowering drug plasma levels did not compromise the data interpretation.

Subpopulation of epileptic rats responded to the same AEDs that are used to treat epilepsy in humans

The percentage of rats with a more than 50% decrease in seizure frequency (responders) varied from 29% to 100% for different AEDs. That is, in the VPA group 100%, LTG group 88%, VGB group 83%, and CBZ group 29% of the rats responded to treatment. Clinical data indicate that 40% to 80% of newly diagnosed patients with focal onset seizures respond to AEDs (Kosteljanetz et al., 1979; French, 1999; Sobaniec et al., 2005). These data suggest that rats with focal epilepsy respond to the same compounds that are used to treat focal onset seizures in humans. Differences between study designs, doses of AEDs administered, duration of treatment, and species-specific pharmacokinetic differences, however, might explain some of the variability in responder rates between experimental and clinical studies. In experimental studies, the effect

of etiology on antiepileptic response can be eliminated (Brodie et al., 2005), because epileptogenesis was triggered by SE in all animals.

Unlike in previous clinical studies (*e.g.*, Kälviäinen et al., 1995), the responder rate for CBZ was substantially lower than that for LTG, VPA, or VGB. It is difficult to provide a single explanation for the lower efficacy of CBZ, for example, compared to that of LTG (responder rate 29% vs 88%). A short elimination half-life of CBZ (1-2 h; Schmutz, 1985) in rats resulting in a low plasma and brain concentration of the drug does not explain the lower efficacy in the present study. We administered CBZ at a dose of 40 mg/kg at 8-h intervals 3 times a day. As we previously demonstrated, this results in plasma concentrations of 35, 23, and 16 $\mu\text{mol/ml}$ at 2, 5, and 8 h, respectively (Halonen et al., 1999). Thus, CBZ levels were in the anticonvulsant range during the most of the treatment phase. Further, the dose used suppresses induced seizures in several animal models. For example, Schmutz (1985) reported that CBZ antagonizes electroshock-induced seizures in mice and rats with an ED_{50} of 5 to 20 mg/kg. Hönack and Löscher (1989) indicated that treatment with 30 mg/kg CBZ reduced the severity and duration of motor seizures as well as the duration of afterdischarges in fully kindled rats. Inclusion of a larger number of animals in preclinical trials as well as optimizing the AED dose and delivery will likely solve some of the problems related to the comparison of efficacy of various compounds in animals, as well as the comparison of experimental and human data.

The limited efficacy of ESM compared to that of VPA, LTG, and VGB was expected based on clinical data demonstrating the poor efficacy of ESM against focal onset seizures. In fact, 37% of animals treated with ESM had an increased seizure frequency compared to baseline. This is consistent with previous observations in spontaneously seizing rats that seizure frequency increased in 50% of ESM-treated rats (Fig.7., Leite and Cavalheiro, 1995).

Treatment with AEDs results in a small subpopulation of seizure-free epileptic rats

In the present study, 14% of rats became seizure-free with CBZ and 20% of rats became seizure free with VPA treatment. Leite and Cavalheiro (1995) reported that 38% of CBZ and 75% of VPA - treated rats became seizure -free, based on video-monitoring. The doses of CBZ and VPA used in that study were comparable to those used in the present study. In the previous study, however, authors used video monitoring for seizure detection, which might underestimate the occurrence of partial seizures without secondary generalization, and therefore, overestimate the rate of seizure freedom. None of the rats on ESM became seizure -free, which is consistent with previous observations (Leite and Cavalheiro, 1995). Recently, a video-monitoring study by Glien and coworkers (2002) in a pilocarpine model demonstrated total remission of spontaneous seizures in 25% of LEV -treated animals. Further, Brandt et al. (2004) reported that 55% of animals treated with phenobarbital achieved total remission. Taken together, the percentage of seizure-free animals varies between different studies, and can be substantially higher than that in clinical studies where seizure freedom is typically less than 20% (Kwan and Brodie, 2001). The differences between animal studies might relate to the AED dose used, the severity of epilepsy in rats included in the study, as well as to the frequency and duration of video-EEG monitoring.

Conclusions

The present study demonstrates that rats with focal epilepsy respond to the same compounds that are used to treat seizures of focal onset in humans. The use of spontaneously seizing animals makes it possible to mimic clinical study designs of pre-clinical trials. In addition, novel outcome measures like assessment of seizure duration can be included in the data

analysis, which might be useful for assessing the disease-modifying properties of various AEDs. Further developments in the models and study designs, for example, regarding the selection of animals with similar severity of epilepsy at baseline or standardization of the frequency and duration of video-EEG follow-up will facilitate comparisons between the studies.

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FIGURE LEGENDS

Figure 1. (A) A schematic representation of different phases of the study. After a 20 to 30 min electrical stimulation of the amygdala, rats developed self-sustained status epilepticus (SE). Three months later, animals with spontaneous seizures in video-EEG monitoring were randomized to successively receive carbamazepine (CBZ), valproate (VPA), ethosuximide (ESM), lamotrigine (LTG), and vigabatrin (VGB) treatment. (B) Animals were continuously video-EEG monitored during the drug administration. During the baseline phase, animals were treated with vehicle. During a 2-d titration phase, the dose of the AED was gradually increased to the level that was then administered for 7 d (treatment phase). Thereafter, the compound was washed-out during the next 2 d, and animals entered a new baseline phase, during which they were treated with the vehicle of the next compound.

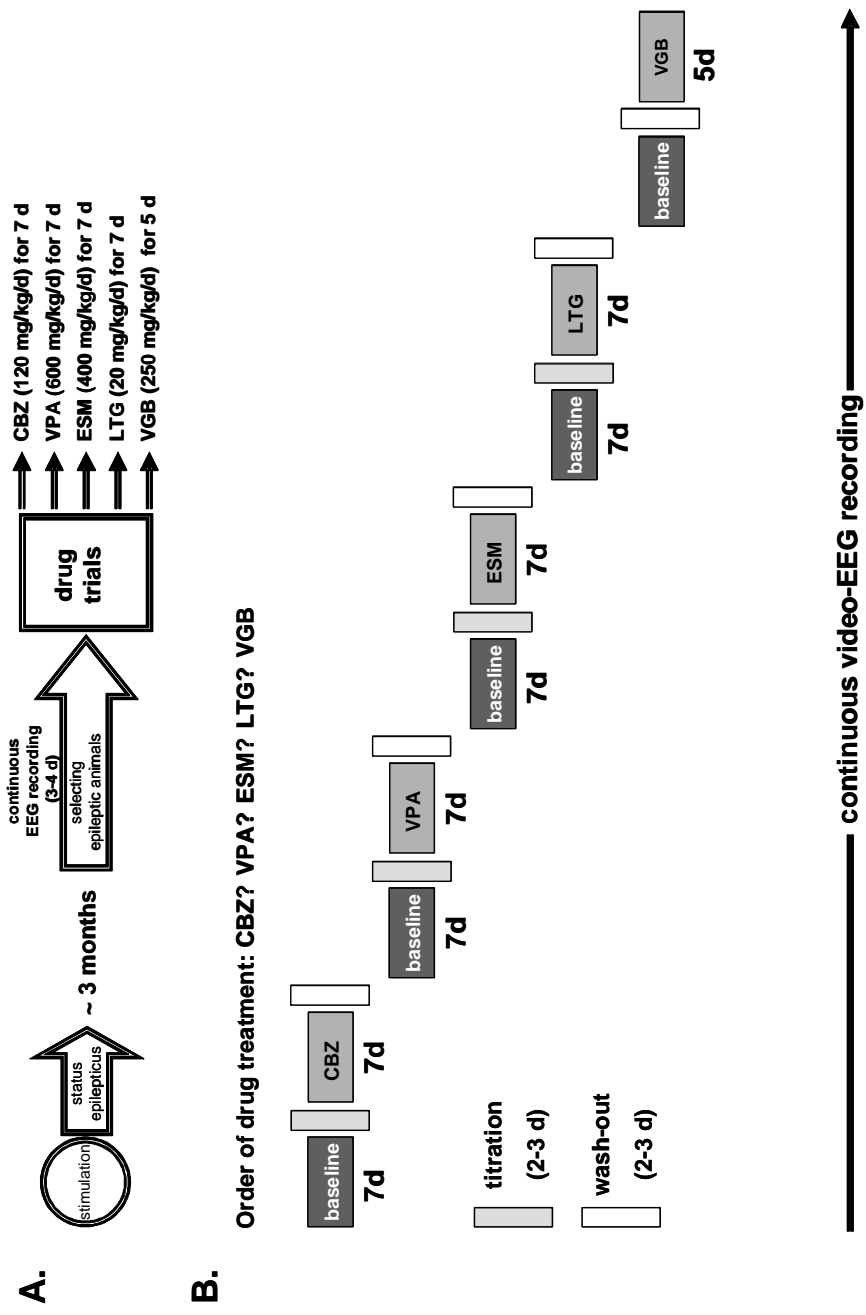


Figure 1. Nissinen and Pitkähnen
 Effect of antiepileptic drugs...

Table 1. Effect of carbamazepine (120 mg/kg/d), valproic acid (600 mg/kg/d), ethosuximide (400 mg/kg/d), lamotrigine (20 mg/kg/d) and vigabatrin (250 mg/kg/d) on spontaneous seizures in rats.

Group	Baseline period		Treatment period	
	Frequency (seizures per day)	Duration (sec)	Frequency (seizures per day)	Duration (sec)
Carbamazepine (n=7)	22.2 ± 18.1	56 ± 6	37.8 ± 33.4	33 ± 18*
Valproic acid (n=5)	33.5 ± 18.8	46 ± 15	6.5 ± 5.4*	18 ± 14*
Ethosuximide (n=8)	15.2 ± 12.9	57 ± 9	12.8 ± 11.9	41 ± 13*
Lamotrigine (n=8)	18.3 ± 12.4	58 ± 9	1.6 ± 1.0*	43 ± 25
Vigabatrin (n=6)	25.9 ± 24.8	42 ± 9	8.5 ± 7.6*	50 ± 16

Values are expressed as mean ± standard deviation. Number of animals is in parenthesis. Statistical significances: * p<0.05 compared to baseline period (Wilcoxon Signed ranks test).

Table 2. Responder rate in different treatment groups.

All animals	CBZ (7)	VPA (5)	ESM (8)	LTG (8)	VGB (6)
% of rats with decreased seizure frequency	29% (2/7)	100%* (5/5)	63% (5/8)	100%** (8/8)	100%* (6/6)
> 50% decrease	29% (2/7)	100%* (5/5)	38% (3/8)	88%** (7/8)	83% (5/6)
> 75% decrease	14% (1/7)	60% (3/5)	38% (3/8)	88%* (7/8)	17%* (1/6)
seizure-free	14% (1/7)	20% (1/5)	0% (0/8)	0% (0/8)	0% (0/6)

Abbreviations: CBZ, carbamazepine; ESM, ethosuximide; LTG, lamotrigine; VGB, vigabatrin; VPA, valproic acid. Number of animals is in parenthesis. Number of responders between treatment groups was tested with χ^2 -test (Fisher's Exact Test). Statistical significances: ** p<0.01, * p<0.01 compared to CBZ treatment; % p<0.05 compared to LTG treatment.

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