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An In Vitro Model of Status Epilepticus: Kindling and Neuroprotection

Deborah P. Nocent, B.Sc. Honours

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of Master of Science

Carleton University
Ottawa, Ontario, Canada
June, 1999

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submitted by

Deborah Nocent

in Partial fulfillment of the requirements for
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Abstract

In many kinds of *in vitro* slice preparations the removal of Mg^{2+} from the perfusing medium induces spontaneous, self-sustained, recurrent epileptiform discharges. This type of epileptiform activity has been suggested as an *in vitro* model of status epilepticus (SE). The purpose of the present experiment was to examine the effects of protracted exposure to Mg-free perfusate in nonkindled and amygdala-kindled tissue using the amygdala/piriform/perirhinal slice preparation. The first objective was to assess the viability of the *in vitro* Mg-free model of SE, by determining whether protracted exposure to a Mg-free perfusate would result in pattern of pathology similar to that associated with *in vivo* models of SE. The second objective of this experiment was to determine whether amygdala-kindled tissue exposed to protracted Mg-free perfusate would display kindling-induced neuroprotection similar to that seen against *in vivo* kainic acid SE. Degenerating/damaged cells were visualized for argyrophilic response using a modified Gallyas silver stain method. Results revealed that the classic laminar pattern of pathology seen in control tissue exposed to *in vivo* models of SE can be produced in the coronal amygdala/piriform/perirhinal slice preparation using an *in vitro* Mg-free model of SE. However, in contrast to the complete neuroprotection observed in the perirhinal and piriform cortex of kindled animals after *in vivo* kainic acid SE only a limited kindling-induced neuroprotection was seen in kindled slices in the piriform cortex layer 2 following *in vitro* Mg-free SE. The significance of these effects were discussed.

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General Introduction

Epilepsy is characterized by transient, recurrent seizures. Such seizures arise when the normal balance between excitatory and inhibitory processes, in neuronal assemblies, is disrupted (Prince, 1985). Typically the disruption appears as abnormal excitability, reflected by synchronous neuronal hyperactivity and may be either restricted to one area of the brain or widespread throughout it. This variability in the propagation of seizure activity is directly responsible for the heterogeneity seen in the clinical expression of seizures.

Epilepsy can be divided into two classes based on the site of seizure initiation. Partial seizures begin in one cerebral hemisphere (focally) and are restricted to that site. These are usually referred to as simple partial seizures. On the other hand, seizures can spread or secondarily generalize to involve both cerebral hemispheres in the epileptiform activity. In this case, they are referred to as complex partial seizures and involve impaired consciousness. In contrast, generalized seizures are typified by epileptiform activity in both cerebral hemispheres from onset.

The mechanisms that underlie seizure activity may differ (Heinemann et al., 1993). Complex partial and convulsive seizures are characterized by an evolving EEG pattern and postictal depression. Conversely, nonconvulsive seizures (i.e., absence seizures) display a non-evolving EEG pattern and no postictal depression. While absence seizures are relatively benign, partial or convulsive seizures may not be. The latter type of seizure involves GABA-ergic breakdown (Schwartzkroin & Prince, 1980) and prolonged depolarization of postsynaptic membranes. Under such conditions, voltage-

dependent Mg^{2+} inhibition is relieved and NMDA receptors become activated allowing Ca^{2+} influx into cells. A deleterious consequence of prolonged partial or convulsive seizures involves seizure-induced brain damage, which is thought to depend on the amount of Ca^{2+} entering cells during the prolonged excitation and how effective the individual neuron's capacity is to buffer free intracellular Ca^{2+} (Gloor, 1989).

Status epilepticus (SE) is a condition involving epileptiform activity distinct from epilepsy. The fundamental difference between SE and the discrete seizures of epilepsy is its duration. SE is a dynamic condition with a stereotypical physiological (Meldrum & Horton, 1973) and electrographical evolution (Treiman *et al.*, 1990). Physiological differences (i.e., GABA-ergic, systemic, metabolic) between SE and a discrete seizure are the direct result of SE's enduring nature, and make SE a serious medical emergency. Like epileptic seizures, the clinical expression of SE is heterogenous (Gastaut, 1983). SE may not necessarily be expressed in a way that is overtly obvious to the clinician. Consequently, without the use of an EEG, subtle forms may go undetected (Gastaut, 1983).

It has been somewhat of a challenge for clinicians to reach consensus on the minimum duration of time a seizure episode must occur to be defined as SE. Experimentally, SE is generally defined as a condition enduring 30 min or longer, involving either one prolonged seizure or recurrent seizures with impaired consciousness between seizures (Hauser, 1983).

Epileptiform EEG discharges recorded during SE can be divided into spike-wave (SW) and non-SW discharges (Fountain & Lothman, 1995). Absence SE, a form of

nonconvulsive SE, is characterized by 3-Hz SW EEG discharges. SW discharges are the result of cellular hyperpolarization and the activation of T-type calcium channels (Pape & McCormick, 1989). Because GABA-ergic postsynaptic inhibition remains intact in this form of SE, NMDA activation and associated Ca^{2+} entry into cells is prevented. Thus, absence SE is considered relatively benign (Gloor, 1989). Conversely, convulsive SE, in particular generalized convulsive SE, is characterized by non-SW discharges (i.e., tonic-clonic discharges). Non-SW discharges are associated with excess excitation and impaired inhibition; thus, EPSPs become prolonged and activate NMDA receptors, allowing excess Ca^{2+} entry into cells. Unlike absence SE, generalized convulsive SE is associated with high morbidity and mortality, and prompt termination of this type of SE is crucial (Towne *et al.*, 1994).

The incidence of SE in the United States has been estimated to be 102,000 to 152,000 persons per year (DeLorenzo *et al.*, 1996). SE occurs in both epileptic and non-epileptic populations. In fact, a prospective study found that the majority of cases of SE occurred in adults (in particular, the elderly: adults aged 60 years and older) and children without a prior history of epilepsy (DeLorenzo *et al.*, 1996). DeLorenzo *et al.* (1996) reported that children under 1 year old showed the highest incidence of SE; the second highest incidence of SE was observed in the elderly. The major cause of SE in children was infections with fever, whereas in adults cerebrovascular disease was the major etiological factor (DeLorenzo *et al.*, 1992; DeLorenzo *et al.*, 1996). The mortality associated with SE is highest in the elderly and lowest in children (DeLorenzo *et al.*, 1992; DeLorenzo *et al.*, 1996; Maytal *et al.*, 1989). One major controversial issue

surrounding the sustained presence of SE concerns the probability of developing brain damage, and whether such brain damage has the potential to be epileptogenic. In this case, a damaged brain would place an individual at greater risk for the development of future seizures (Lothman & Bertram, 1993).

In Vivo

Much of our knowledge about SE comes from *in vivo* animal models because studies involving the clinical human population have been predominantly retrospective and unable to determine whether the brain damage (observed at autopsy or during a surgical resection) was the cause or consequence of SE. Many *in vivo* animal models of SE exist. They can be divided into two basic types based on the method of SE induction, i.e., either electrical stimulation or administration of pharmacological agents. Common to these models is a temporal evolution of electrographic discharges analogous to those recorded from patients with SE, involving 1) discrete seizures; 2) merging seizures; 3) continuous or rhythmic ictal activity; 4) continuous ictal activity intermittently suppressed; and 5) periodic epileptiform discharges on a flat baseline background (Treiman *et al.*, 1990).

Meldrum *et al.* (1973) showed that bicuculline-induced SE for greater than 3 hours in paralyzed, ventilated adolescent baboons produces brain damage in the neocortex, thalamus, and hippocampus. This study was the first to establish that brain damage can occur with electrographic SE alone, in the absence of behavioural convulsions and relatively independent of cardiopulmonary and/or systemic factors.

Of course, brain damage is more severe when animals are not paralyzed and ventilated due to the negative contribution of systemic disturbances (Meldrum & Brierley, 1973). Numerous other *in vivo* models of SE replicate the finding that SE kills neurons in limbic structures (for reviews, see Fountain & Lothman, 1995; Wasterlain *et al.*, 1993).

Homeostatic mechanisms breakdown within the first 30 min of SE and neuronal death in limbic structures appears as early as 40-60 min after the onset of SE (Lothman, 1990); thus, the treatment of SE requires immediate neuroprotective strategies.

Kindling

The kindling model has been used extensively to study focal epilepsy with secondary generalization and is an animal model of human complex partial seizures. Kindling, a process discovered by Goddard *et al.* (1967, 1969), involves repeated electrical stimulation of a forebrain site at an intensity that will trigger a primary afterdischarge (AD) (Racine, 1972). This daily electrical stimulation results in a progressive increase in the frequency, amplitude, duration and complexity of the primary afterdischarge, and the spread of this afterdischarge to contralateral homotopic sites and other brain areas. With electrographic development, a cumulative, stereotypical pattern of convulsive behaviour evolves (i.e., stages 1 to 5; see Racine, 1972). The triggering of stage-5 generalized convulsive seizures typically marks the completion of the kindling process for most studies. However, with long-term kindling, spontaneous generalized convulsive seizures eventually occur (Pinel & Rovner, 1978).

The neural plasticity involved in kindling seems to be an enduring phenomenon, since reapplication of the kindling stimulus to previously kindled animals, that have not

been stimulated for months, can trigger stage-5 generalized convulsive seizures (Goddard *et al.*, 1969). The neuronal mechanisms responsible for the kindling effect have yet to be determined. Histological comparison of brain tissue taken from the site of the implanted electrode of control (nonkindled) and kindled animals revealed no significant differences, i.e., no damage or structural changes specific to the kindled site that could account for the kindling-induced plasticity (Goddard *et al.*, 1969; Goddard & Douglas, 1975).

Attempts to understand the remodelling that results in epileptogenesis have focused primarily on the hippocampus for several reasons, one of these being a type of pathology routinely found in hippocampal tissue obtained from temporal lobe epileptics, either at autopsy or during a temporal lobe resection. The pathology, called Ammon's horn sclerosis, is characterized by neuronal loss in areas CA1, CA3 and the hilus of the dentate, whereas relatively resistant neurons are found in CA2 and the granule cells of the dentate gyrus (Gloor, 1991). The selective loss of neurons is believed to be directly related to Ca^{2+} permeability (i.e., the cell's density of glutamate receptors) and the cell's Ca^{2+} buffering capacity [i.e. intracellular concentration(s) of Ca^{2+} buffering protein(s)]. However, Ammon's horn sclerosis is not restricted to temporal lobe epilepsy and is observed in other conditions involving excitotoxic damage, e.g., anoxia-ischemia. At present the relationship between Ammon's horn sclerosis and epileptogenesis in temporal lobe epilepsy is not fully understood and modifications in other structures like the amygdala, piriform, perirhinal cortex are certainly involved.

Another reason the hippocampus has been the primary site of investigation in the search for remodelling that can account for kindling-induced epileptogenesis is the

observed mossy fibre sprouting (i.e., granule cell axonal growth/ reorganization) and neosynaptogenesis in the dentate gyrus in response to kindling stimuli, and the high correlation of it's appearance with the development and establishment of kindling (Cavazos & Sutula, 1990; Cavazos *et al.*, 1991). Sprouting has also been observed in other temporal lobe structures like the amygdala and perirhinal cortex in response to electrically induced SE (McIntyre *et al.*, in press).

In addition to morphological changes like cell death and/or sprouting, kindling-induces diverse molecular responses. One of these responses is the upregulation of neurotrophins and their receptors, which are believed to play an important role in the development of sprouting (Rashid *et al.*, 1995; Van der Zee *et al.*, 1995; Isackson *et al.*, 1991; Bengzon *et al.*, 1993). This upregulation of neurotrophins and their receptors in response to kindling stimuli may in fact facilitate epileptogenesis.

One of the most notable kindling-induced changes in brain functions involves an unexpected neuroprotection against *in vivo* kainic acid SE (Kelly & McIntyre, 1994). Systemic administration of the glutamate analogue kainic acid at a high dose induces severe limbic SE lasting 30 min or longer in adult rats. *In vivo* kainic acid SE in control rats produces a characteristic pattern of brain damage, in particular, extensive, bilateral loss of the piriform cortex (Lothman & Collins, 1981; Ben-Ari, 1985; Kelly & McIntyre, 1994). Thus, in an experiment that was initially designed to examine whether a previously kindled dorsal hippocampus could still triggered generalized convulsive seizures after removal of the piriform cortex through kainic acid SE, Kelly and McIntyre (1994) fortuitously discovered that, in the context of brain damage, a kindled brain

responds very differently to kainic acid SE than a control (nonkindled) brain. The most significant difference was the complete neuroprotection observed in the piriform cortex and substantia nigra reticulata of dorsal hippocampal kindled animals, despite that fact that the kindled rats experienced SE as severe, behaviourally and electrographically, as that of controls. Neuroprotection against *in vivo* kainic acid SE was also observed in amygdala kindled rats; however, the protection was found only in the amygdala-piriform region ipsilateral to the kindled focus and not in contralateral structures (Kelly & McIntyre, 1991).

Three of the many factors that are associated with the degree of brain damage resulting from *in vivo* kainic acid SE are the behavioural and electrographic form of SE and the duration of severe limbic SE. McIntyre *et al.* (in press) have identified four distinct forms of SE that vary in behavioural and electrographic severity. These forms, as listed from mildest to most severe and include 1) immobile SE with EEG spiking at < 0.5 Hz; 2) ambulatory SE at 1-2 Hz; 3) masticatory SE at 3-5 Hz; and 4) generalized convulsive SE at 5-8 Hz. Each form produces a characteristic pattern of brain damage that is additive with the increasing severity of SE. The kindling-induced neuroprotection discussed above is found against all forms of SE.

The brain damage observed after many hours of SE is progressive with the duration of SE (Kelly & McIntyre, 1994). Examination of its time course reveals an interesting laminar pattern of damage in the perirhinal and piriform cortex. In control (nonkindled) animals at 3 hours after the onset of kainic acid SE, damage was found in the perirhinal cortex layers 6B and 5A, and in layer 3 of the piriform cortex (McIntyre *et*

al., 1995). Later at 6 hours, damage appears in the perirhinal cortex layer 3A and in the piriform cortex layer 2B. Kindling of the dorsal hippocampus largely protects against the majority of this damage, indicated by the normal appearance of cells days or weeks after SE: although the cells might be distressed during the SE experience. Amygdala kindling provides a similar protection in piriform and perirhinal cortices.

In Vitro

In vitro slice preparations have allowed researchers to investigate the cellular mechanisms involved in epileptogenesis. The *in vitro* model has provided information about the origin and propagation of epileptiform activity in local circuits. Manipulations of ion concentrations and/or the addition of pharmacological agents to the bathing medium will result in spontaneous epileptiform activity *in vitro*; however, the characteristics of this abnormal activity (i.e., frequency, duration and/or morphology of the discharge) will vary depending on the region of the brain slice (Heinemann *et al.*, 1993) and the modification(s) to the perfusing medium.

Alterations in the perfusing medium can produce effects at three cellular levels: intrinsic, synaptic, and nonsynaptic. These alterations can a) effect the intrinsic properties of neurons by disrupting the balance between the ionic (Na^+ , Ca^{2+} , K^+ and Cl^-) currents, which maintain a stable neuronal resting membrane potential; b) enhance excitatory or block inhibitory synaptic transmission (Heinemann *et al.*, 1991); and/ or c) effect the size of the extracellular space, influencing ephaptic interactions, as well as, electrical synapses via gap junctions (Lux *et al.*, 1986; Jefferys & Haas, 1982).

The two basic types of epileptiform activity observed *in vitro* are interictal-like and ictal-like discharges. These discharges are differentiated primarily by their duration. Interictal-like discharges are brief events, usually consisting of a single population burst about 50-100 ms in duration (Johnston & Brown, 1981). The paroxysmal depolarization shift (PDS) that underlies the interictal-like discharge is a burst of action potentials riding on a large depolarization shift (Dichter & Ayala, 1987). The PDS results from a giant EPSP (Johnston & Brown, 1981) and represents the synchronous activity of many neurons connected by recurrent excitatory circuits. However, ictal-like discharges are longer lasting events (15-45 sec.) (Valenzuela & Benardo, 1995), consisting of a rapid series of population bursts (i.e., PDS's). An *in vitro* model of SE requires that prolonged, self-sustained ictal-like discharges occur for 30 min or longer, and be similar to the EEG spike and wave discharges recorded *in vivo* during SE.

Few *in vitro* models of SE exist, despite the fact that there are numerous ways of producing epileptiform activity *in vitro*. This is because the majority of the epileptiform activity induced is interictal-like. However, in one *in vitro* model of limbic SE, tetanic stimulation of the Schaeffer collaterals of the CA3 pyramidal neurons in a hippocampal slice bathed in a low Mg^{2+} medium results in two types of SE-like activity (Rafiq *et al.*, 1995). This SE-like activity is either spontaneous, recurrent ictal-like discharges for 30 min or longer (occurring 60-70% of slices), or self-sustained continuous epileptiform discharges for 30 min or longer (occurring in 5-10% of slices) (Rafiq *et al.*, 1995). Once established, the activity of both types of SE-like discharges appears to be independent of NMDA receptor activation, because the application of the NMDA receptor antagonist

APV to the perfusing medium results in enhanced rather than suppressed activity (Rafiq *et al.*, 1995). However, these two types of SE-like activity can be reduced or blocked with the addition of benzodiazepines to the perfusing medium (Rafiq *et al.*, 1995), similar to *in vivo* models of SE.

Another *in vitro* model of SE, using the combined hippocampal-parahippocampal slice preparation developed by Heinemann and colleagues, in the presence of Mg^{2+} -free perfusing medium, produces SE-like tonic clonic discharges in the entorhinal cortex (Jones & Heinemann, 1988; Drier & Heinemann, 1990, 1991). These discharges evolve over time into either continuous, clonic discharges or recurrent, tonic activity (Drier & Heinemann, 1990). The initial tonic clonic discharges are suppressed by application of the clinical anticonvulsant, valproic acid (VPA), to the bathing medium (Drier & Heinemann, 1990). However, the later appearing recurrent, tonic discharges are insensitive to VPA; thus, it has been suggested that these discharges represent a model of drug resistant SE (Drier & Heinemann, 1990). The removal of Mg^{2+} from the perfusing medium also induces spontaneous, self-sustained, ictal-like activity in neocortical (Valenzuela & Benardo, 1995), amygdala-piriform (Gean & Shinnick-Gallagher, 1988; McIntyre & Plant, 1989), and perirhinal-piriform slices (Plant & McIntyre, 1988).

In a normal perfusing medium [approximating physiological concentrations (2mM)], NMDA receptors are blocked by Mg^{2+} in a voltage-dependent manner (Nowak *et al.*, 1984). Switching the medium to one that is Mg^{2+} -free relieves this Mg^{2+} blockage from the NMDA receptors, thus, making them voltage-independent (Nowak *et al.*, 1984). Removal of the Mg^{2+} blockage from the NMDA receptors is believed to be mainly

responsible for the generation of epileptiform activity induced by a Mg^{2+} -free perfusate because the application of NMDA receptor antagonists to the bathing medium, i.e., 2-AP5 (Jones & Heinemann, 1988), 2-APV (Walther *et al.*, 1986; McIntyre & Popham, 1992), CCP (Valenzuela & Benardo, 1995), reversibly blocks this activity. Another effect caused by a Mg^{2+} -free perfusate, that may contribute to the epileptiform activity, is the reduction of membrane surface charge screening, which can lower activation thresholds for inward Na^+ and Ca^{2+} currents and lead to increased levels of transmitter release from presynaptic terminals and enhanced postsynaptic activation (Heinemann, 1987).

Slice preparations have principally focused on the hippocampus as an epileptogenic area because this region has the lowest threshold for AD's, although it kindles very slowly. However, faster rates of kindling epileptogenesis are obtained from other limbic structures, including the perirhinal cortex (McIntyre *et al.*, 1993; Felstead *et al.*, 1995), piriform cortex (McIntyre & Plant, 1989), and amygdala (Goddard *et al.*, 1969; for review, see Gloor, 1992), respectively. In addition, the hallmark of epilepsy, the interictal spike, almost always begins in the piriform region, independent of the kindling site (Kairiss *et al.*, 1984; Racine *et al.*, 1988).

To further investigate the properties of the amygdala-piriform region in epilepsy, McIntyre and Wong (1985) developed the coronal amygdala-piriform slice preparation. Subsequently, they demonstrated that the enhanced excitability attained through *in vivo* amygdala kindling is retained in the *in vitro* amygdala-piriform slice preparation (McIntyre & Wong, 1986). Since this kindling-induced change is retained *in vitro*, the

question of whether other kindling-induced changes might also be preserved *in vitro* naturally arose, i.e., could kindling-induced neuroprotection be observed against an *in vitro* model of SE.

As discussed above, the removal of Mg^{2+} from the perfusing medium has been suggested as an *in vitro* model of SE. Many differences exist between an *in vitro* model of SE induced by Mg^{2+} -free perfusate and an *in vivo* model of SE induced by kainic acid. Some of these being a) in the slice preparation only local circuits are preserved; b) systemic factors such as: O₂, energy supply and temperature are held constant *in vitro*; and c) the two mechanisms of SE induction are different despite the fact that both produce spontaneous, self-sustained, ictal-like activity. Nevertheless, common to these two models is the belief that seizure-induced brain damage involves excessive Ca^{2+} influx into cells during prolonged excitation.

This *in vitro* model of SE, using the coronal amygdala-piriform slice preparation (McIntyre & Wong, 1985) and 0 Mg^{2+} allowed us to evaluate 1) if we could produce a laminar pattern of cell damage in nonkindled tissue similar to that seen in nonkindled animals after *in vivo* kainic acid induced SE; and 2) if previously kindled tissue in 0 Mg^{2+} *in vitro* would display a pattern of kindling-induced neuroprotection similar to that seen in kindled animals against *in vivo* kainic acid induced SE. Future investigations of the pathophysiological mechanisms involved in SE-induced cell damage and testing of possibly effective treatments to prevent such damage would benefit from such an *in vitro* SE model.

Purpose

The purpose of this thesis was to investigate the effects of protracted exposure to 0 Mg^{2+} perfusate on coronal slices from the amygdala-piriform cortex. The first experiment assessed the viability of the 0 Mg^{2+} preparation as an *in vitro* model of SE by determining whether protracted exposure to a 0 Mg^{2+} perfusate would result in a pattern of cell damage similar to that induced by *in vivo* models of SE. Normal tissue was exposed to either 10 hr. of normal perfusate or 10 hr. of 0 Mg^{2+} perfusate. It was predicted that tissue taken from normal rats and exposed to normal Mg^{2+} containing perfusate would appear undamaged after 10 hr., while normal tissue exposed to 0 Mg^{2+} would display a pattern of cell damage similar to *in vivo* models of SE. The second experiment determined whether amygdala-kindled tissue exposed to 10 hr. of 0 Mg^{2+} would display kindling-induced neuroprotection similar to that seen against *in vivo* kainic acid SE. It was expected that amygdala-kindled tissue would suffer less cell damage, specifically within the piriform cortex, than normal tissue under the same 0 Mg^{2+} condition.

Method and Procedures

Treatment of subjects was in accordance with the Animals for Research Act, the Guidelines of the Canadian Council on Animal Care, and was approved by the Carleton University Animal Care Committee.

Subjects

Fifty-two male Long-Evans Hooded rats (Charles River, Canada) weighing from 240–400g were shoe-box caged, 2 rats per cage, prior to preparation for surgery and/or

the slice chamber. Animals were maintained in a temperature-controlled colony room with a 12 hour light-dark cycle. Animals had free access to food and water in their home cages throughout the experiment. Rats that underwent surgery subsequently were individually housed in hanging wire cages.

Surgery

Rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). Two electrodes (constructed of two twisted strands of 127 μ m diameter Nichrome wire, Diamel-insulated and attached to male Amphenol pins) were stereotaxically implanted in the basal amygdala and secured to the skull with 6 stainless steel jewellers screws and dental acrylic, and fitted into a headplug assembly (Molino & McIntyre, 1972). The coordinates used for the amygdala were 0.2 mm posterior to bregma, 4.5 mm lateral to the midline, and 8.3 mm ventral to the surface of the skull (Pellegrino *et al.*, 1979).

Kindling began 7 days after surgery.

Kindling

Seven days after surgery, the afterdischarge (AD) threshold [defined as the minimum stimulus intensity to provoke an AD outlasting the stimulus by 2 seconds or more] at one electrode site was determined in all rats. A 2 s, 60 Hz sine wave stimulus was administered at 1 minute intervals with increasing intensity (15, 25, 35, 50, 75, 100, 150 and 200 μ A peak-to-peak) until an AD was triggered. Animals were stimulated at their AD threshold intensity once a day until 6 generalized stage-5 convulsions (Racine, 1972) were recorded. Animals were decapitated for use in the slice preparation at least 7 days after their sixth generalized stage-5 convulsion.

Slice Preparation

Following decapitation, the whole brain was rapidly extracted (~ 2 min) and placed in freezing, oxygenated (95% O₂- 5% CO₂), sucrose-based artificial cerebral spinal fluid (SaCSF) containing (in mM): 262 sucrose, 5 KCl, 1.25 Na₂PO₄, 26 NaHCO₃, 9.4 D-glucose, 2 MgSO₄, 2 CaCl₂ and 0.68 kynurenic acid. After 60-120 s of incubation in partially frozen SaCSF, the brain was removed and placed on an ice chilled inverted petri dish and a block of tissue containing the amygdala, piriform, and perirhinal cortex was removed; in the case involving kindled animals, the selected area was taken from the kindled hemisphere, and followed the procedure of McIntyre & Wong (1985, 1986). This block of tissue was fixed to a cutting block with cyanoacrylate glue and was placed in the oxygenated SaCSF filled slicing chamber of a Vibratome (Lancer 1000, St. Louis, MO): from this block, a series of coronal sections (400 μ m) were cut. Slices were then transferred to an interface chamber, placed on lens paper (to enable later removal from the chamber) and perfused (1.8 ml/min) with warmed (32°C) oxygenated normal artificial cerebral spinal fluid (aCSF) containing (in mM): 5 KCl, 1.25 Na₂PO₄, 26 NaHCO₃, 9.4 D-glucose, 2 MgSO₄, 124 NaCl and 2 CaCl₂ for a period of 1 hr. After 1 hr of normal aCSF perfusion, the tissue was either perfused for an additional 10 hrs with the same solution or switched to an aCSF solution in which the MgSO₄ was omitted for 10 hrs (0 Mg²⁺ condition). All tissue was perfused at 2.6 ml/min. All solutions were prepared daily with fresh double distilled water.

Extracellular Field Recordings

The microelectrodes for extracellular field recordings were pulled from borosilicate glass pipettes (WPI, O.D. 1.0 mm, I.D. 0.58 mm) on a Flaming Brown Micropipette puller (Sutter Instruments, Model P-87) to a resistance of 80- 120 M Ω , bevelled back to a resistance of 4-20 M Ω and backfilled with 2 M NaCl. Extracellular field recordings of spontaneous events were made using a A-M Systems Model 1600 Neuroprobe amplifier and stored on a Sony Video FM Recorder (model SLV-555 UC) with Hi-Fi stereo FM tapes using an A-D System from Medical Systems Corp. (model PCM 4/8). Extracellular field recordings were taken from the piriform cortex layer 2 at 30 min, 1 hr, 2 hrs, 3 hrs, 4 hrs and ~ 10 hrs of perfusion with Mg-free aCSF of one slice; when possible this recording electrode was not displaced and stayed on the slice for the full duration of the experiment. In addition, all other slices in the chamber were checked for ongoing activity at the end of ~ 10 hrs of perfusion with Mg-free aCSF.

Data Analysis

The latency to onset of epileptic events during perfusion with Mg-free aCSF in control, operated and kindled tissue was compared using analysis of variance (ANOVA). In addition, a general activity score [frequency (mean number of epileptiform events counted in 5 min for each recording interval) x duration (mean duration of 10 representative events for each recording interval)] was created to account for the tendency that the frequency and duration of the epileptiform events had toward an inverse relationship, as previously described by McIntyre and Plant (1993). General activity scores were compared between treatments at 1 hr, 2 hrs, 3 hrs, 4 hrs and ~ 10 hrs of

perfusion with Mg-free aCSF using ANOVA. When appropriate individual group comparisons were made using Tukey's test.

Histological Analysis

Following 10 hrs of perfusion in the interface chamber, slices were immediately removed and fixed in 4% paraformaldehyde solution for at least 2 days. Each 400 μm slice was resectioned on the sliding microtome at 40 μm . Degenerating/damaged cells were visualized for argyrophilic response using a modified Gallyas silver stain method (Nadler & Evenson, 1983) (see Appendix A).

For a slice to be included in histological and statistical analysis, the following criteria had to be met: the slice had to have a) an intact piriform cortex layer 1; b) epileptic activity at the end of ~ 10 hrs of perfusion with Mg-free aCSF in either the piriform cortex or perirhinal cortex; c) no excessive mechanical distortion on the tissue block during Vibratome cutting; d) well filtered water for solution preparation; e) no damage incurred through resectioning of the tissue on the sliding microtome; and f) good silver impregnation. Additional analysis of an overall qualitative description of the laminar pathology of nonkindled and kindled tissue was made. Cell counts were taken in selected areas of the piriform cortex layers 2 and 3 and across a horizontal strip encompassing all layers of the perirhinal cortex in nonkindled and kindled tissue. These areas are indicated schematically in Figure 1. Differences in cell numbers were assessed parametrically with multivariate analysis of variance (MANOVA) and ANOVA and, when appropriate individual group comparisons were made using Fisher's LSD test or the Tukey test.

Figure 1. Schematic diagram of the amygdala/piriform/perirhinal slice preparation. The boxed areas indicate where cell counts will be taken. PRC = perirhinal cortex; RF = rhinal fissure; PC = piriform cortex; EX = external capsule; ST = stria terminalis; L = lateral amygdala; ABL = basolateral amygdala; CE = central amygdala; M = medial amygdala; and CO = cortical nuclei of the amygdala (from McIntyre & Plant, 1989).

Results

The results described below were obtained on a sample of slices from 16 control rats (N=18 slices), 5 operated rats (N=5 slices) and 10 kindled rats (N= 12 slices). Slices that did not satisfy the minimum criteria (refer to method section) were excluded from the statistical analyses. For example, Figure 2 shows a photograph of the perirhinal cortex from a coronal amygdala/piriform/perirhinal slice excluded from statistical analyses. No electrical activity was detected in this slice at 10 hrs. of perfusion with *Mg-free* (0 Mg^{2+}) aCSF. The random/widespread argyrophilic response in the neurons reflects this lack of activity (i.e. the extent to which the cells were damaged/degenerating/) and this massive but random pattern of argyrophilic response is unlike the normal pattern seen in slices that displayed activity at 10 hrs. of perfusion with *Mg-free* aCSF.

Extracellular Field Recordings

As expected, switching the perfusing medium from *normal* aCSF to *Mg-free* aCSF resulted in the generation of spontaneous epileptiform activity in the amygdala/piriform/perirhinal slice. Extracellular recordings from the piriform cortex revealed that the mean latency to onset of epileptiform events was 43.8 ± 5.6 min (range 29-66 min) in control slices, 55.0 ± 3.1 min (range 45-64 min) in operated slices and 37.3 ± 3.1 min (range 26-49 min) in kindled slices. A one-factor analysis of variance (ANOVA) was conducted to compare the mean latency to onset of epileptiform events for control, operated and kindled slices. The analysis revealed a significant main effect of treatment, $F(2, 16) = 4.65$, $p < 0.05$. *Post hoc* Tukey tests comparing the mean

Figure 2. A photograph of the perirhinal cortex from a coronal amygdala/piriform/perirhinal slice in which no electrical activity was detected at 10 hr. of perfusion with Mg-free aCSF. Note the massive but random pattern of argyrophilic response.

latency to onset of epileptiform events for each of the three treatments with the remaining two revealed a significant difference in mean latency to onset between only the kindled and operated slices ($p < 0.01$).

As previously stated (refer to method section), consideration for the inverse relationship between the frequency and duration of epileptiform events was achieved by combining the two measures to create a general activity score. The mean general activity (frequency x duration) as recorded at 1, 2, 3, 4 and 10 hrs. of perfusion with *Mg-free* aCSF for control, operated and kindled slices is shown in Figure 3. As can be seen, activity was greater in control slices than in either operated or kindled slices. A two-factor ANOVA (treatment by time) with one factor repeated (time) was conducted on the mean activity across time. The analysis resulted in a significant main effect of treatment, $F(2, 17) = 13.88$, $p < 0.001$, a significant main effect of time, $F(4, 68) = 15.77$, $p < 0.001$, but did not result in a significant treatment by time interaction, $F(8, 68) = 1.76$, $p > 0.05$.

Post hoc Tukey tests comparing each of the three treatments (averaged over time) with the remaining two were conducted to further isolate the significant effects. Results indicated that the control tissue showed greater activity when compared to each of the other two treatments ($p < 0.05$). As can be seen in Fig. 3, no significant difference in activity was observed between the operated slices and kindled slices ($p > 0.05$).

Post hoc Tukey tests comparing each of the five intervals when activity was recorded (averaged over treatment) with the remaining four resulted in a significant difference when the 1 hour interval was compared with either the 2, 3, 4, or 10 hour

Figure 3. Activity [the mean number of epileptiform events (in 5 min) times the mean duration of 10 events (in sec.) $\pm$ s.e.m.] recorded in control, operated and kindled slices at 1, 2, 3, 4 and 10 hrs. of perfusion with Mg-free aCSF.

interval ($p < 0.05$); thus, activity increased after the first hour. None of the other comparisons were found to be significant ($p > 0.05$).

Histological Analysis

Table 1 shows the results of the silver impregnation argyrophilic technique conducted on control and kindled slices that underwent 10 hours of perfusion with *normal* aCSF. As can be seen, the mean percentage of cells showing an argyrophilic response in both control and kindled slices was very low. A multivariate analysis of variance (MANOVA) was conducted on the mean percentage of cells showing an argyrophilic response in control and kindled slices after 10 hours of perfusion with *normal* aCSF across the nine brain regions/layers examined (i.e., piriform cortex layers 2 and 3, and perirhinal cortex layers 2, 3a, 3b, 5a, 5b, 6a, and 6b). The analysis did not produce a significant main effect of brain region, Pillai (8, 6) = 0.796, $p > 0.05$, or a significant brain region by treatment interaction Pillai (8, 6) = 0.671, $p > 0.05$.

The results of the silver impregnation argyrophilic technique conducted on control, operated and kindled slices that underwent 10 hours of perfusion with *Mg-free* aCSF are shown in Table 2. As can be seen in this table, Mg-free conditions resulted in a great deal of argyrophilic activity compared to normal medium. A MANOVA was conducted on the mean percentage of cells showing an argyrophilic response in control, operated and kindled slices after 10 hours of perfusion with *Mg-free* aCSF across the nine brain regions examined. The analysis resulted in a significant main effect of brain region, Pillai (8, 10) = 29.325, $p < 0.001$, however, the brain region by treatment interaction was not significant Pillai (16, 22) = 1.475, $p > 0.05$. In addition, individual univariate

TABLE 1

Percentage of cells showing an argyrophilic response in amygdala/piriform/perirhinal slices from control and kindled tissue after 10 hours perfusion with normal aCSF.

	Control	Kindled
Piriform Cortex		
Layer 2	8.80 $\pm$ 4.72	0 $\pm$ 0
Layer 3	8.51 $\pm$ 3.72	0 $\pm$ 0
Perirhinal Cortex		
Layer 2	5.63 $\pm$ 4.16	0 $\pm$ 0
Layer 3a	2.22 $\pm$ 2.22	0 $\pm$ 0
Layer 3b	3.21 $\pm$ 1.36	0 $\pm$ 0
Layer 5a	12.84 $\pm$ 6.36	0 $\pm$ 0
Layer 5b	2.51 $\pm$ 1.45	0 $\pm$ 0
Layer 6a	3.78 $\pm$ 2.76	0 $\pm$ 0
Layer 6b	5.16 $\pm$ 2.00	11.43 $\pm$ 5.72

Argyrophilic response is expressed as a mean percentage $\pm$ S. E. M.

TABLE 2

Percentage of cells showing an argyrophilic response in amygdala/piriform/perirhinal slices from control, operated and kindled tissue after 10 hours perfusion with Mg-free aCSF.

	Control	Operated	Kindled
Piriform Cortex			
Layer 2	19.12 $\pm$ 8.67	18.78 $\pm$ 10.71	2.28 $\pm$ 1.29
Layer 3	39.98 $\pm$ 3.87	34.42 $\pm$ 9.90	31.68 $\pm$ 8.83
Perirhinal Cortex			
Layer 2	31.76 $\pm$ 7.79	36.66 $\pm$ 5.92	13.65 $\pm$ 5.46
Layer 3a	33.41 $\pm$ 10.62	32.12 $\pm$ 7.03	25.80 $\pm$ 2.83
Layer 3b	20.78 $\pm$ 4.40	17.54 $\pm$ 4.55	15.13 $\pm$ 4.79
Layer 5a	39.30 $\pm$ 5.82	57.66 $\pm$ 3.03	62.23 $\pm$ 3.97
Layer 5b	12.78 $\pm$ 3.25	12.06 $\pm$ 3.38	8.67 $\pm$ 2.45
Layer 6a	14.19 $\pm$ 3.77	17.56 $\pm$ 4.57	10.18 $\pm$ 4.21
Layer 6b	34.80 $\pm$ 3.45	33.16 $\pm$ 4.11	33.75 $\pm$ 4.85

Argyrophilic response is expressed as a mean percentage $\pm$ S. E. M.

ANOVAs comparing control, operated, and kindled slices on each of the nine brain regions/layers were not significant ($p > 0.05$), with the exception of the perirhinal cortex layer 5a, $F(2, 19) = 6.076$, $p < 0.05$. Further, *post hoc* Fisher LSD comparing each of the three treatments with the remaining two resulted in a significant difference when control slices were compared with either operated or kindled slices ($p < 0.05$). None of the other comparisons was found to be significant ($p > 0.05$). Hence, both operated and kindled tissue showed a greater argyrophilic response in layer 5a of the perirhinal cortex than control tissue after 10 hours perfusion with *Mg-free* aCSF.

Due to the small number of slices in the operated group and the large S.E.M's around the mean percentages of argyrophilic response (particularly relevant with regard to layer 2 of the piriform cortex; see Figure 4), an alternative analysis was run that excluded slices from the operated group. This MANOVA was conducted on the mean percentage of cells showing an argyrophilic response in control versus kindled slices after 10 hours of perfusion with either *normal* or *Mg-free* aCSF across the nine brain regions/layers examined. The analysis resulted in a significant main effect of brain region, Pillai (8, 19) = 25.391, $p < 0.001$ and a significant interaction between the brain region, treatment and medium, Pillai (8, 19) = 3.78, $p < 0.05$. To further analyze the nine brain regions/layers examined in control and kindled slices after 10 hours perfusion with either *normal* or *Mg-free* aCSF, nine two-factor ANOVAs (treatment by medium) were conducted on the mean percentage of argyrophilic response. The analysis of the piriform cortex layer 2 resulted in a significant main effect of treatment, $F(1, 26) = 4.36$, $p < 0.05$, but did not result in a significant main effect of medium, $F(1, 26) = 1.05$, $p > 0.05$, or

Figure 4. Percentage of piriform cortex layer 2 and 3 cells that exhibited an argyrophilic response in brain slices perfused with either normal aCSF or 0 Mg²⁺ aCSF. Shown are means $\pm$ s.e.m. (Dots represent individual data points).

treatment by medium interaction, $F(1, 26) = 0.43$, $p > 0.05$. The nonsignificant main effect of medium in the piriform cortex layer 2 is evident in the fact that the mean percentage of argyrophilic response in control slices in *normal* aCSF was 8.80 ± 4.72 and 19.12 ± 8.67 in *Mg-free* aCSF, whereas kindled slices had a mean percentage of argyrophilic response of 0 ± 0 in *normal* aCSF but a mean of only 2.28 ± 1.29 in *Mg-free* aCSF (see Figure 4), an average increase of approximately 200% in argyrophilic response (over baseline) in both treatment groups.

The other eight ANOVAs conducted on piriform cortex layer 3; perirhinal cortex layer 2; 3a; 3b; 5a; 5b; 6a, and 6b all resulted in a significant main effect of medium, $F(1, 26) = 42.29$, $p < 0.01$; $F(1, 26) = 11.37$, $p < 0.01$; $F(1, 26) = 17.42$, $p < 0.01$; $F(1, 26) = 22.62$, $p < 0.01$; $F(1, 26) = 63.24$, $p < 0.01$; $F(1, 26) = 15.38$, $p < 0.01$; $F(1, 26) = 9.44$, $p < 0.01$ and $F(1, 26) = 44.65$, $p < 0.01$, respectively. However, none of these eight analyses resulted in a significant main effect of treatment ($p > 0.05$) or a significant treatment by medium interaction ($p > 0.05$), with the exception of the perirhinal cortex layer 5a. The analysis of the perirhinal cortex layer 5a resulted in a significant treatment by medium interaction, $F(1, 26) = 10.29$, $p < 0.01$. A test of simple main effects for the perirhinal cortex layer 5a showed a significant effect of treatment only in *Mg-free* aCSF, $F(1, 26) = 8.457$, $p < 0.05$, and a significant effect of medium in both treatments, i.e., control, $F(1, 26) = 14.068$, $p < 0.01$ and kindled, $F(1, 26) = 51.899$, $p < 0.001$. Thus, control and kindled slices showed significantly more damage in the perirhinal cortex layer 5a in *Mg-free* aCSF than *normal* aCSF, but kindled slices showed significantly

more damage in the perirhinal cortex layer 5a in *Mg-free* aCSF than controls (consistent with results from previous analysis, see above).

These *in vitro* results compare favourably with the argyrophilic response of these neuroanatomical areas to *in vivo* SE. For example, Figure 5A and 5B are representative photographs of coronal brain sections of the perirhinal cortex and piriform cortex, respectively, showing the classic pattern of pathology seen in the *in vivo* kainic acid model of SE. These photographs were taken from control animals after 6 hours of ambulatory (see Fig. 5A) and 6 hours of masticatory (see Fig. 5B) SE induced by systemic injection of kainic acid (McIntyre, Kelly & Staines, in press). Despite the inherent variability from one animal to the next, a stereotypical pattern of argyrophilic response can be seen in the perirhinal cortex layers 6B, 5A and portions of layer 3 (see Fig. 5A) and in the piriform cortex in layer 3 and layer 2B.

In summary, after 10 hours of perfusion with *normal* aCSF little damage (i.e., argyrophilic response) was observed in the perirhinal and piriform cortices of control and kindled slices (see Figure 6A and 6B, and 7A and 7B; respectively). However, after 10 hours of perfusion with *Mg-free* aCSF, the pattern of argyrophilic response in the perirhinal cortex layers 6B, 5A and 3 (see Fig. 8a) and in the piriform cortex layers 3 and 2B (see Fig 8B) of control tissue bears a striking resemblance to that seen following *in vivo* kainic acid SE. Kindled tissue after 10 hours perfusion with *Mg-free* aCSF showed, in general, less damage in the piriform cortex layer 2 (see Fig 9A) but retained the same overall pattern of argyrophilic response (see Fig. 9B) as seen in control tissue following *in vivo* kainic acid SE, as well as control slices from the *in vitro* Mg-free model of SE.

Figure 5. Representative photographs of coronal brain sections of the perirhinal cortex (A) and the piriform cortex (B) showing the classic pattern of pathology seen in the *in vivo* kainic acid model of SE (McIntyre, Kelly & Staines, in press). Note the argyrophilic response in the perirhinal cortex layers 6B, 5A and portions of layer 3 (A) and in the piriform cortex in layer 3 and layer 2B (B).

A **B**

Figure 6. Representative photographs of the perirhinal cortex (A) and piriform cortex (B) from a coronal amygdala/piriform/perirhinal control slice after 10 hr. of perfusion with normal aCSF.

A **B**

Figure 7. Representative photographs of the perirhinal cortex (A) and piriform cortex (B) from a coronal amygdala/piriform/perirhinal kindled slice after 10 hr. of perfusion with normal aCSF.

A **B**

Figure 8. Representative photographs of the perirhinal cortex (A) and piriform cortex (B) from a coronal amygdala/piriform/perirhinal control slice after 10 hr. of perfusion with Mg-free aCSF. Note the argyrophilic response in the perirhinal cortex layers 6B, 5A and portions of layer 3 (A) and in the piriform cortex in layer 3 and layer 2B (B) and the striking resemblance of these photographs to Figure 5A and 5B.

A **B**

Figure 9. Representative photographs of the perirhinal cortex (A) and piriform cortex (B) from a coronal amygdala/piriform/perirhinal kindled slice after 10 hr. of perfusion with Mg-free aCSF. In particular, less argyrophilic response was observed in kindled slices in the piriform cortex layer 2 (B) when compared with control slices (see Figure 8B).

A **B**

Discussion

There were two major findings from this thesis. One, this work is the first to demonstrate that the classic laminar pattern of pathology seen in control tissue after *in vivo* kainic acid SE (McIntyre, Kelly & Staines, in press) can be produced in the coronal amygdala/piriform/perirhinal slice preparation using an *in vitro* Mg-free model of SE. And, two, using this *in vitro* model of SE we were unable to duplicate the type/extent of kindling-induced neuroprotection seen in kindled animals against *in vivo* kainic acid induced SE. Only a minimal kindling-induced neuroprotection was seen in a sub-area (piriform cortex layer 2) of the amygdala-piriform-perirhinal kindled slice against *in vitro* Mg-free SE. A discussion of the results obtained from the extracellular field recordings and the silver impregnation of the tissue in this study follows; as well as, some remarks on the value and implications of an *in vitro* Mg-free model of SE for future research.

Electrophysiological Observations

The present experiment replicates the findings of McIntyre and Plant (1988; 1989), that the local neural circuits present in the coronal amygdala/piriform/perirhinal slice preparation are sufficient to support the genesis of epileptiform activity using a Mg-free perfusate. This result supports the suggestion of Heinemann and colleagues that removal of Mg^{2+} from the perfusing medium might serve as an *in vitro* model of SE in their combined hippocampal-parahippocampal slice preparation (Jones & Heinemann, 1988; Dreier & Heinemann 1990, 1991). The results of the current investigation extend that suggestion to the amygdala/piriform/perirhinal slice preparation, since we were able

to maintain spontaneous, recurrent, epileptiform, SE-like discharges with little or no break in activity for 10 hours when tissue was perfused with a Mg-free aCSF.

When examining the genesis of the SE *in vitro* we compared the latency to onset of epileptiform activity in control, operated and kindled slices to establish whether the slices from these three treatments seized for approximately the same amount of time. The results indicated that there was no significant difference between control and kindled tissue, however, operated slices required a significantly longer perfusion time with Mg-free aCSF than kindled slices before the development of epileptiform activity. Thus importantly, we can state that the differences in the argyrophilic response of control and kindled tissue, after perfusion with Mg-free aCSF, were not the result of different durations of epileptiform activity.

However, these latency to SE onset results are unlike those previously found in our laboratory, in which amygdala kindled slices were observed to require a significantly longer perfusion time with Mg-free aCSF than control slices before the onset of epileptiform activity (note: operated, nonstimulated tissue was not included in that study) (McIntyre & Plant, 1993). It should be noted, however, that other investigators have reported significant variability in the onset of epileptiform activity between slices in different slice preparations that is consistent with the present results (Walther *et al.*, 1986; Drier & Heinemann, 1991). One possible methodological difference that may account for the slightly longer latencies to onset of epileptiform activity after Mg-free perfusion observed in the present study than those reported by McIntyre & Plant (1993) is that our brain tissue was placed (immediately after extraction) into SaCSF containing kynurenic

acid, and although tissue was later perfused for 1 hour with normal aCSF to allow for washout/equilibration before switching over to Mg-free aCSF, this addition of kynurenic acid to the SaCSF may have contributed to the slightly longer latencies to onset of epileptiform activity seen in the tissue from the present study and eliminated seizure onset differences between kindled and control slices.

Observation of the recorded activity from control, operated and kindled tissue at 1, 2, 3, 4, and 10 hours of perfusion with Mg-free aCSF revealed a tendency toward the same type of relationship between the frequency and duration of epileptiform discharges described by McIntyre and Plant (1993). In accordance with McIntyre and Plant (1993), initial discharges generally tended to be shorter but more frequent than the less frequent but longer duration discharges observed at later time intervals. Such temporal changes appear to be part of the dynamic alterations of SE development in the amygdala/piriform area.

Tissue from each of the three treatments was found to have greater general activity (frequency x duration) after the first hour of perfusion with Mg-free aCSF. This result is consistent with previous findings that spontaneously, recurring discharges became stable after about 60-90 min of perfusion with Mg-free aCSF (Walther *et al.*, 1986). However, at all the time intervals recorded, the general activity was found to be greater in control tissue than in either operated or kindled tissue. This result is unlike the previous findings of McIntyre and Plant (1993) in which the frequency and duration of spontaneous discharges in control tissue were not obviously different from those of kindled tissue. The overall profile of the general activity (across time) in operated and

kindled slices appeared very similar. This result suggests that the implantation of an electrode in the basal amygdala was sufficient to produce a change in the general activity (across time) of operated tissue, which resulted in its resemblance to the general activity of kindled tissue, but not control tissue. In this context, it is noteworthy that Loscher *et al.* (1995) reported a pro-kindling influence following prolonged electrode implantation, i.e., that animals kindled faster when the kindling stimulations began after prolonged period since implantation of a bipolar electrode. Because electrode implantation is known to produce local microhemorrhages, which result in the deposition of iron (Boast *et al.*, 1976), and iron deposits have been found to produce epileptogenesis in cortical and limbic regions (Willmore *et al.*, 1978; Pico *et al.*, 1994), Losher *et al.* (1995) propose that this pro-kindling effect, induced by prolonged electrode implantation, was most likely due to the epileptogenic effect of iron. Collectively, these findings support the need for nonimplanted control tissue (i.e., normal control slices), since some of the local intrinsic electrophysiological characteristics of the implanted, nonstimulated tissue (i.e., operated control slices) were altered by the process of electrode implantation and its prolonged presence in the tissue.

Histological Observations

The present study demonstrated a remarkable viability of the *in vitro* amygdala/piriform/perirhinal model during normal aCSF in the interface chamber. The fact that we were able to maintain such healthy tissue, as evident in the lack of argyrophilic response, in both control and kindled slices after 10 hours of perfusion with normal aCSF serves to validate the baseline methods of normalcy used in this study.

Against this background of normal cellular appearance in normal aCSF, the results of the current investigation dramatically support the removal of Mg^{2+} from the perfusing medium as an *in vitro* model of SE. In particular, we were able to produced a pattern of laminar pathology in the amygdala/piriform/perirhinal slice preparation that was virtually identical to that seen in normal tissue after exposure to either *in vivo* kainic acid induced SE or *in vivo* electrically induced SE.

In the present experiment, histological examination of control tissue after 10 hours of Mg-free aCSF revealed an argyrophilic response pattern that always included layers 6B, 5A and portions of layer 3 in the perirhinal cortex, and layers 3 and 2B in the piriform cortex, similar to *in vivo* SE models. In the same vein, kindled tissue, after 10 hours of perfusion with Mg-free aCSF, also presented the same overall pattern of argyrophilic response as seen in control animals. In addition, we did find significantly more damage in layer 5a of the perirhinal cortex in the kindled tissue. However, some specific neuroprotection was seen in the kindled tissue. A significant effect of treatment between control and kindled slices in layer 2 of the piriform cortex suggested that kindled slices experienced neuroprotection in this sub-area of the piriform cortex. Furthermore, the neuroprotection we observed in layer 2 of the piriform cortex in kindled tissue is unlikely due to its lower general activity (across time), since this type of minimal neuroprotection was not observed in operated slices, which displayed a general activity profile that clearly resembled kindled and not control tissue.

Histological Digression

Appendix B shows a photograph of the perirhinal cortex from a coronal section of the amygdala/piriform/perirhinal slice of control tissue after 10 hours of Mg-free perfusion. It should be noted that healthy/intact cells stain a light yellow-brown, whereas degenerating cells/structures stain black (Nadler & Evenson, 1983). Degenerating cells appear as either small black spots (or a small black spot appearing inside a cell body), or a blacked outlined cell body and/or its processes (these cells often appeared to still have a relatively normal shape). The modified Gallyas silver stain method used in the present study visualizes degenerating terminals and lysosomes (Nadler & Evenson, 1983); however, we do not know the temporal nature of this stain for marking degenerating cells, e.g., did the two different types of cellular staining we observed identify injured cells at different time points in their degeneration. Unpublished findings from our laboratory (M. E. Kelly, personal communication) support the notion that many cells that display an argyrophilic response might later recover. In the future, other staining techniques will be necessary to resolve the full meaning of an argyrophilic cell outcome (i.e., death versus recovery).

Mechanisms of Excitotoxicity

Heinemann *et al.* (1990) suggested that in the Mg-free *in vitro* preparation, the later appearing recurrent, epileptiform discharges that were insensitive to VPA [and believed to be associated with cellular energetic failure (Schuchmann *et al.*, 1999)] may represent a model of drug resistant SE. But, the question still remains as to which *in vivo* model of SE the *in vitro* Mg-free model of SE most directly corresponds. Despite the

fact that both the *in vivo* kainic acid and *in vitro* Mg-free models of SE produce spontaneous, self-sustained, ictal-like activity, the mechanisms of SE induction in these two models are different. In the *in vitro* model of SE, removal of Mg^{2+} from the perfusing medium relieves the Mg^{2+} voltage-dependent block from the NMDA receptors (Nowak *et al.*, 1984), and this is believed to be mainly responsible for the development of epileptiform activity. As already stated, NMDA receptor antagonists reversibly block this activity (Walther *et al.*, 1986; Jones & Heinemann, 1988). Conversely, in the *in vivo* kainic acid model of SE, kainic acid is thought to activate kainate receptors, which in turn increase glutamate release, and through giant secondary depolarizations, induce NMDA receptor activation.

Evidence suggests that seizure-induced brain damage is associated with the accumulation of abnormally high levels of intracellular calcium (Choi, 1988). The NMDA receptor-activated channel is thought to be the major route of Ca^{2+} influx into a neuron, although research now suggests that the type of subunit composition determines the calcium permeability of the AMPA and kainate receptors (Lerma, 1997). Further, Arias *et al.* (1999) have demonstrated, using an *in vitro* model of ischemia, that excitotoxic and ischemic loss of neuronal viability is dependent on extracellular calcium in both NMDA-induced excitotoxicity and AMPA-induced excitotoxicity. The coapplication of the noncompetitive NMDA receptor antagonist (MK-801) and the noncompetitive AMPA receptor antagonist (GYKI-52466) was found to result in almost complete neuroprotection of the hippocampal slice, and was comparable to the neuroprotection observed by withholding extracellular calcium (Arias *et al.*, 1999).

As previously stated, many differences exist between an *in vivo* kainic acid model of SE and an *in vitro* Mg-free model of SE. In the slice preparation, the fact that only local circuits are maintained means that this preparation has a limited glial matrix for support and likely less buffering capacity (i.e., glutamate uptake). Also, any potential neuroprotective factors that may be released into the extracellular space of the slice, e.g., free radical scavengers, would be washed out by the constant perfusion of the slice in the chamber. Systemic factors such as O₂, energy supply, and temperature represent other sources of difference when comparing the *in vivo* and *in vitro* models of SE. For example, the temperature in the slice chamber was held constant at 32°C, whereas the body temperature of animals during kainic acid-induced SE vary considerably. In addition, there is evidence that kainic acid-induced seizures and seizure-related brain damage are temperature dependent, i.e., hypothermia (23°C - 28°C) reduces seizures and seizure-related brain damage, whereas hyperthermia (42°C) increases the severity of seizures and seizure-related brain damage (Liu *et al.*, 1993). Furthermore, the effects of temperature on *in vitro* potassium-induced epileptiform activity were such that the activity could be reversibly stopped by decreasing the temperature from 35 – 37°C to 28 – 30°C (Traynelis & Dingledine, 1988). Thus, the impact of constant temperature in the slice chamber against varying temperatures in *in vivo* SE are further unknowns when comparing the brain damage versus neuroprotection developed in the two different models.

Other neuroprotective and/or death mechanisms associated with kindling (Ben-Ari *et al.*, 1990; Cavazos *et al.*, 1990; 1991; Pretel *et al.*, 1997) involve diverse molecular

responses, such as, the modification of neurotransmitter receptor expression and/or functional properties (Kohr *et al.*, 1993; Kohr & Mody, 1994; Gall *et al.*, 1990; Kamphuis *et al.*, 1992) and the upregulation of neurotrophins and their receptors (Isackson *et al.*, 1991; Bengzon *et al.*, 1993). Tremblay *et al.* (1999) demonstrated that BDNF pretreatment protects cortical neurons from excitotoxic death. They propose that this neuroprotection resulted from a BDNF-mediated blockage of protein kinase C inactivation in the NMDA receptor, and not from an alteration in the amount of Ca^{2+} entry through the NMDA receptor. But if NGF is not present at the time of BDNF upregulation, then the latter signals a cascade leading to cell death. All of these various possibilities need to be examined to help explain the differences in our *in vivo* and *in vitro* models of SE to more adequately assess the various mechanisms of cell loss or preservation as a consequence of protracted seizure and kindling-based neuroprotection.

In summary, the present investigation suggests that, regardless of the differences between the *in vivo* kainic acid model of SE and the *in vitro* Mg-free model of SE, the latter model induced a pattern of pathology that was similar to that seen in control tissue exposed to either *in vivo* electrical or kainic acid SE. In doing so, the histological outcome identified most of the same cell populations as either relatively vulnerable or resistant to excess excitatory stimulation. The major difference in kindled tissue from the *in vitro* Mg-free model of SE and the *in vivo* kainic acid model of SE was the extent of the kindling-induced neuroprotection. Kindled slices after *in vitro* Mg-free SE displayed no kindling-induced neuroprotection in the perirhinal cortex and only restricted protection in the piriform cortex layer 2. This is compared with the complete

neuroprotection observed in the perirhinal and piriform cortex of kindled animals after *in vivo* kainic acid SE. The goal of future research will be to understand the mechanism(s) involved in kindling-induced neuroprotection against *in vivo* kainic acid SE that result in the change(s) that dramatically reduce the vulnerability of specific cell populations to kainic acid SE. The ease with which manipulations can be made to the perfusing medium make this *in vitro* Mg-free model of SE extremely useful in such an assessment. Future studies using this model will likely evaluate therapeutic neuroprotective strategies and their impact on electrophysiology and neuronal survival.

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Science, 200, 1501-1502.

Appendix A

Silver Stain Method

Reagents

Stock Solutions

Protect solution C from light. Store all solutions at room temperature. Stable for several weeks. Use distilled H₂O.

Solution:

- A 9% (w/v) sodium hydroxide (NaOH)
 90 g into 1 liter H₂O
- B 16% (w/v) ammonium nitrate (NH₄NO₃)
 80 g into 500 ml H₂O
- C 50% (w/v) silver nitrate (AgNO₃)
 3 g into 6 ml H₂O
- D 1.2% (w/v) ammonium nitrate (NH₄NO₃)
 12 g into 1 liter H₂O

E Add 5 g anhydrous sodium carbonate (Na₂CO₃) to 300 ml of 95% ethanol and 600 ml of H₂O. Q.S. to 1 liter.

F Add 0.5 g anhydrous citric acid to 15 ml of 37% formalin, 100 ml of 95% ethanol and 700 ml H₂O. Adjust pH to 5.8-6.1 with solution A. Q.S. to 1 liter.

- G 0.5% (v/v) acetic acid
 5 ml into 1 liter H₂O

Working Solutions

Prepare no more than 1 hour before beginning the staining procedure.

Pretreating Solution: Mix equal volumes of solutions A and D.
 100 ml A + 100 ml D

Impregnating Solution: Add 1.5 volumes of solution A to each volume of solution B. Then add 0.5-0.6 ml of solution C for each 100 ml of total volume.

$$60 \text{ ml A} + 40 \text{ ml B} + 0.6 \text{ ml C} = 100 \text{ ml}$$

Washing Solution: Mix 1 ml of solution D with each 100 ml of solution E.

300 ml E + 3 ml D

Developing Solution: Mix 1 ml of solution D with each 100 ml of solution F.
100 ml F + 1 ml D

Silver Staining Procedure

Keep dishes covered. Do not use metal. Drain quickly. Agitate x1 min except when in developer. When possible, keep tissue submerged and away from sides. With each step the tissue is transferred into a new dish.

- Steps:
1. H₂O --- 5 min
 2. H₂O --- 5 min
 3. H₂O --- 5 min
 4. Pretreatment Solution --- 5 min
 5. Pretreatment Solution --- 5 min
 6. Impregnating Solution --- 10 min
 7. Washing Solution --- 2 min
 8. Washing Solution --- 2 min
 9. Washing Solution --- 2 min
 10. Developing Solution --- at least 1 min

Cover Slipping Procedure

When sections have been mounted on slides and are dry, they go through a post-silver stain process prior to cover slipping.

1. Solution G --- 10 min
2. Solution G --- 10 min
3. Solution G --- 10 min
4. H₂O --- 2 min
5. 50% ethanol --- 2 min
6. 70% ethanol --- 2 min
7. 80% ethanol --- 2 min
8. 90% ethanol --- 2 min
9. 95% ethanol --- 2 min
10. 100% ethanol --- 2 min
11. Butanol --- 2 min
12. Xylene --- 2 min
13. Xylene --- 2 min
14. Xylene --- 2min
15. cover slipping (from xylene)

Appendix B

A photograph of the perirhinal cortex from a coronal section of the amygdala/piriform/perirhinal slice of control tissue after 10 hours of Mg-free perfusion. Note the two different types of cellular staining (indicated by the arrows) that we observed.