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A thesis submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements of the Degree of Doctor of Medicine

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ABSTRACT

Neurodegeneration (cell death) is a feature of many neurological diseases including human temporal lobe epilepsy (TLE). Recently, it has been suggested that *excitotoxicity*, a process by which neurons are killed by prolonged or excessive exposure to glutamate or its analogs, may contribute to neuronal injury observed in many diseases characterized by neurodegeneration. Thus, in a model of TLE, the potent glutamate analog kainate has been shown to induce seizures and subsequent cell death in rat hippocampus. Although the pathology produced with this model resembles that observed in human TLE, it does not completely replicate it. Accumulating evidence now suggest that activity of the Na^+/K^+-ATPase enzyme (Na^+/K^+ pump), the ion pump responsible for neuronal ionic homeostasis, may be compromised or insufficient in many neurodegenerative diseases, particularly in TLE. In this thesis project, the hypothesis that a reduction of Na^+/K^+ pump capacity will reduce neuronal survival of excitotoxicity and produce a pathology in young adult rats similar to that observed in human TLE was investigated.

To test this hypothesis, brain Na^+/K^+pump capacity was partially inhibited in young adult rats treated with subtoxic dose of kainate. Seizure activity in these animals was verified by electroencephalography (EEG) and damage to the hippocampus was documented by a silver staining method for dead/dying neurons. Control animals were treated with either kainate or the Na^+/K^+ pump inhibitor, ouabain, alone.

In further studies, the chronic pathological and behavioral consequences of these treatments were determined. The following features of human TLE were studied in these animals: 1) a pattern of cell death in the hippocampus described as Ammon's horn sclerosis; 2) spontaneous, recurrent seizures; and 3) evidence of hippocampal remodeling.

Results of these experiments revealed that partial inhibition of the Na^+/K^+pump in young adult rats with ouabain was not itself neurotoxic. This treatment, however, markedly potentiated ordinarily subtoxic doses of the glutamate analog, kainate. Thus, rats treated with intraperitoneal (i.p.) injections of kainate (5 mg/kg; i.p.) followed by intraventricular (i.c.v) ouabain (3 nmoles) experienced behavioral and EEG recorded seizures. Subsequent silver staining of the brain of these animals showed hippocampal damage. In contrast, ouabain alone or kainate alone did not produce seizures or hippocampal damage ($p < 0.001$; chi-square analysis). These results were replicated with intraperitoneal injections of both kainate (7 mg/kg; i.p.) and ouabain (1 mg/kg; i.p.). Furthermore, Na^+/K^+ pump impairment was found to be associated with kainate treatment in producing seizure activity and subsequent neuronal death. Thus, when ouabain (3 nmoles; i.c.v) was delayed (90 min instead of 30 min) after kainate (5 mg/kg; i.p.), seizure onset was also delayed and seizure duration was reduced. Finally, treatment with the combination of ouabain and kainate produced features commonly observed in human TLE: i) acute limbic type seizures; ii) a pattern of cell death in the hippocampus replicating that observed in the human disease; iii) a chronic epileptic state characterized by spontaneous, recurrent seizures;

iv) evidence of plasticity and hippocampal remodeling.

The results of this study demonstrated a critical role for the Na^+/K^+ pump in neuronal survival of excitatory stimuli. The hypothesis that reduction of Na^+/K^+ pump capacity decreases neuronal threshold for hyperexcitability and injury was supported, producing a seizure syndrome and pathology in adult rats closely replicating human TLE.

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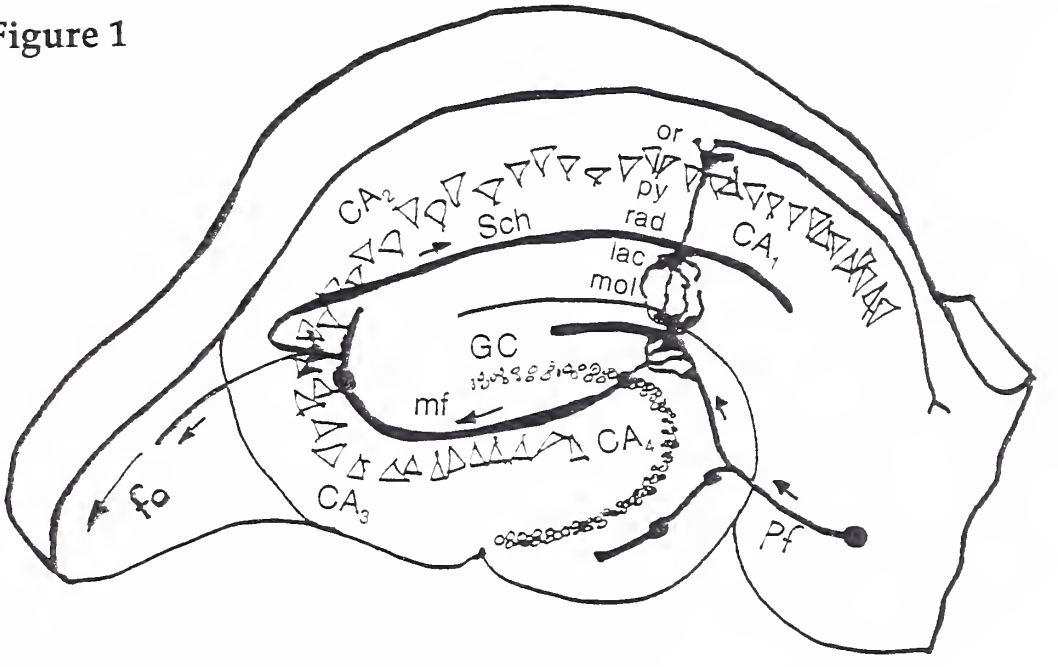
INTRODUCTION

Neurodegeneration and Temporal Lobe Epilepsy

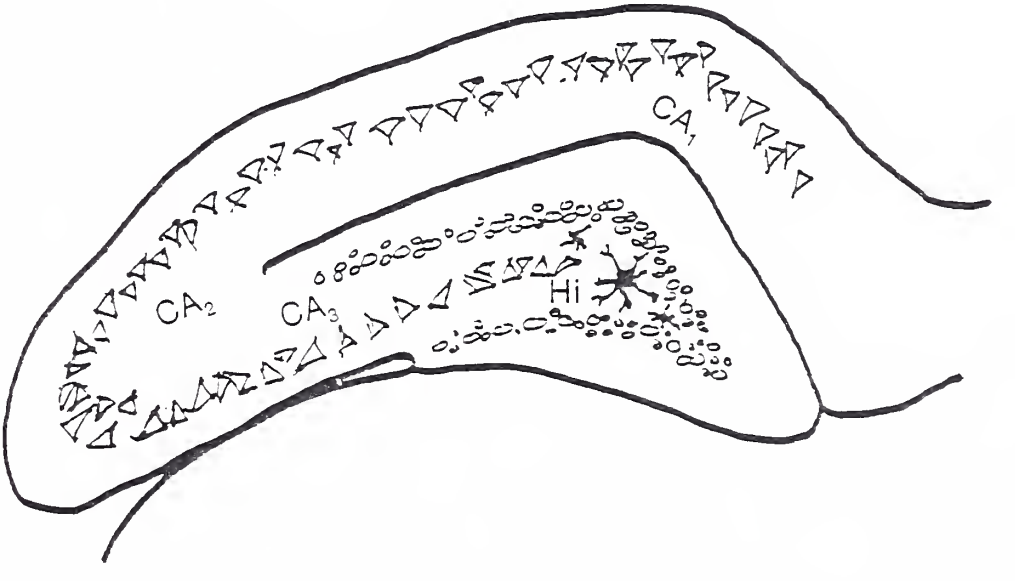
Human Epilepsy is a significant public health problem affecting approximately 2/1000 persons (Booss, 1995). This illness is particularly frequent in children where convulsions may lead to brain damage and subsequent seizure activity in adulthood. In the adult population, temporal lobe epilepsy (TLE) is the most common and devastating form of epilepsy. Synonyms of this type of epilepsy include limbic seizures, psychomotor epilepsy and complex partial seizures, deriving from the fact that it originates from the limbic structures of the brain, i.e., hippocampus, amygdala, and other limbic structures. Clinical manifestations of TLE include sensory symptoms, visual or auditory hallucinations, visceral symptoms such as chewing and salivation, and somatomotor symptoms including tonic-clonic movements. The disorder is often extremely resistant to currently available anticonvulsant drugs. When uncontrolled, attacks may predispose to bodily injuries and sometimes life-threatening situations (DeLorenzo, 1991).

In 1880, Sommer (Sommer, 1880) published a landmark paper entitled "Disease of Ammon's Horn as an Aetiological Factor in Epilepsy." This paper carried the description of the pattern of histological damage now known as Ammon's horn sclerosis or hippocampal sclerosis. In the brain of these patients, principal pyramidal neurons of hippocamapus CA1 - CA4 subfields as well as dentate hilar cells (Figure 1; next page) were variably destroyed. More recent studies of surgically obtained brain specimens have

Figure 1



A. Human Hippocampus



B. Rat Hippocampus

Figure 1: **A. Schematic drawing of transverse section of human hippocampal formation.**

Pyramidal cells of area CA1 - 4 are depicted in the pyramidal layer (py). Dendrites of the pyramidal cells radiate into the *stratum oriens* (or), *stratum radiatum* (rad), *stratum lacunosum* (lac), and *stratum moleculare* (mol). The dentate hilus is the area of cells circumscribed by the granule cells in the dentate gyrus. The mossy fibers (mf; axons of granule cells) making synaptic connection with pyramidal cells of area CA3 are shown. Synaptic connections also exist between pyramidal cells via the Schaffer collaterals (Sch). The major *afferent* pathway to the hippocampus is the perforant pathway (pf). This pathway is excitatory and synapses with granule cells. *Efferent* fibers leaving the hippocampus in the fornix (fo) are axons of pyramidal cells.

B. Transverse section through rat hippocampus.

The overall organization of the rat hippocampus is very similar to that of human. Areas CA1 - 3 are shown. The hilar region corresponds to area CA4 in human hippocampus. Afferent and efferent pathways (not shown) are also similar. (Other abbreviations: Dentate hilus (Hi). (Brodl, 1980; Paxinos and Watson, 1986).

confirmed Sommer's findings (Margerison and Corsellis 1966; Corsellis and Bruton, 1983; Babb et al., 1984; de Lanerolle et al., 1992). This type of irreversible neurodegeneration is not a feature of TLE alone; it has been reported for many other chronic neurological conditions such as Huntington's chorea, Alzheimer's disease, and Parkinson's disease (Margerison and Corsellis, 1966; Babb et al., 1984; Meldrum, 1985; Rothman and Olney, 1987; Choi, 1988b; Lees, 1991; de Lanerolle et al., 1992). Whether the neurodegeneration observed in the brain of patients suffering from TLE is the cause or effect of this disease is a century-old debate. More recently, however, there has been considerable effort to understand the pathogenesis of cell death in TLE. It is hoped that elucidation of such mechanisms would provide means to prevent or ameliorate this potentially debilitating disease.

In 1980, the work of Wyllie and his colleagues suggested that most cell death could be separated on the basis of morphology into "apoptosis" and "necrosis" (Wyllie et al., 1980). Apoptosis is characterized by an initial condensation of cellular and nuclear elements. This is followed by a process of cytoplasmic vacuolization. Membrane permeability and organelle integrity are preserved, but cells become detached from adjacent cells and form apoptotic bodies. Examples of apoptosis include normal programmed cell death, glucocorticoid-induced death of immature thymocytes, and radiation cytotoxicity. Wyllie and his co-workers suggested that the pathophysiology of apoptosis might involve endonuclease digestion of nuclear chromatin. On the other hand, necrosis describes cell death that is marked in the early stages by chromatin clumping, progressing with swelling of mitochondrial matrix, and terminating with eventual rupture of nuclear, organelle, and plasma membranes. Examples of necrosis include cell death induced by hypoxia-ischemia, toxins, or complement. It has been suggested that the

pathophysiology of necrosis involves loss of cellular volume homeostasis secondary to altered membrane permeability, as evidenced by abnormal permeability to dyes or chromium (Wyllie et al., 1980).

EXCITOTOXICITY

An interesting discovery of modern neuroscience research is that excitatory amino acids (EAA) such as glutamate and its analogs (e.g. kainate) can kill neurons after brief, intense exposure (Rothman, 1985; Choi et al., 1987). This phenomenon has been termed *excitotoxicity* (Olney, 1986). The exact morphological features of the cell death induced by this mechanism remains to be classified either as necrotic, apoptotic, or a combination of both; however, there is increasing evidence to suggest that this mechanism participates in the pathogenesis of many neurodegenerative conditions including stroke, epilepsy, and Parkinson's disease (Choi, 1992).

The first *in vivo* evidence of excitotoxicity were noted more than 20 years ago. In these studies, characteristic pathological changes were noted in the circumventricular regions of young monkeys and rodent brains treated with high systemic doses of glutamate (Olney, 1969; Olney et al., 1972). Within 30 min of glutamate administration, acute neuronal swelling was noted by electron microscopy. Subsequently, neurons became necrotic and underwent phagocytosis by macrophages over the next several hours.

Similarly, kainic acid, literally meaning the ghost of the sea in Japanese (Takemoto, 1978), is a potent analog of glutamate (Shinozaki and Konishi, 1980; Shinozaki, 1981) that has been shown to produce "axon-sparing lesions" in rat brain (Coyle et al., 1978). The use of kainate to circumvent the destruction of *fibres de passage* (axons which were destroyed by conventional electrolytic lesions) led to studies that have provided several experimental

models of human diseases such as hemiballism (Hammond et al., 1979), Huntington's chorea (Coyle et al., 1981), and human TLE (Ben-Ari et al., 1981).

The association between excitotoxicity and neuronal death in human neurodegenerative diseases has come mainly from indirect evidence (Meldrum, 1985; Rothman and Olney, 1987; Choi, 1988). In one particular study, brain extracellular concentrations of excitatory amino acids were sampled by microdialysis in conscious epileptic patients. This study demonstrated that extracellular glutamate levels increased significantly prior to seizure episodes and remained elevated during seizure activity, reaching potentially neurotoxic concentrations in the hippocampus (During and Spencer, 1993).

Transmembrane ion imbalances created by excitotoxicity kills neurons

In vitro experiments using cultured cells and patch-clamp methodology have helped to elucidate the interaction between EAA's and cortical cells during excitotoxicity. When neurons are exposed to glutamate or its agonists, there is an immediate depolarization accompanied by Na^+ influx and K^+ efflux. Additionally, a Ca^{++} influx is detected on prolonged neuronal exposure to glutamate. These and other studies have led to the identification of two components of excitotoxicity. During the first phase, there is an influx of Na^+ that is accompanied passively by influx of Cl^- and water, leading to cell swelling (Rothman, 1985; Choi et al., 1987). This phase need not be toxic to the cell; volume homeostasis may be regained if the excitatory insult is removed (Choi, 1992). If cells continue to be exposed to glutamate, a second set of events occur that is marked by delayed neuronal disintegration beginning several hours after initial exposure. This phase is

most likely triggered by excessive Ca^{++} influx into the cell, since it is dependent on the presence of extracellular Ca^{++} (Choi, 1985).

In summary, profound and potentially toxic ionic changes take place both inside and outside the cell milieu after brief or prolonged intense exposure to glutamate and other excitatory amino acids. Excessive or repetitive stimulation may lead to cell death (Lowe, 1971; Choi, 1985; Rothman, 1985; Mayer and Westbrook, 1987; Rothman and Olney, 1987). **These observations have suggested that neuronal mechanisms for ionic homeostasis may be overwhelmed during excitotoxicity.**

THE Na^+/K^+ PUMP

The sodium/potassium ATPase (Na^+/K^+-ATPase; Na^+/K^+pump) is a transmembrane protein of ubiquitous distribution in mammals. Both human and rat brains are especially enriched with this pump (Brines et al., 1991; Mata et al., 1991; Watts et al., 1991; Brines and Robbins, 1993). Even under resting conditions, at least 60% of the energy released by respiration is consumed by brain ATPase, compared with about 5% in liver and striated muscle (Lowe, 1971; Ritchie and Straub, 1980; Astrup et al., 1981; Choi, 1985; Hansen, 1985; Rothman, 1985; Mayer and Westbrook, 1987; Rothman and Olney, 1987).

The Na^+/K^+-ATPase is a heterodimeric enzyme (Figure 2; next page) consisting of a large catalytic (α) subunit (~115 kD) and a smaller glycoprotein (β) subunit (~45 kD). Currently three catalytic isoforms ($\alpha1$, $\alpha2$ and $\alpha3$) with two glycoprotein isoforms ($\beta1$, $\beta2$) are recognized (Shull, 1986; Shull, 1987; Young, 1987). Tissue-specific isoform distribution exists for some isoforms of this pump. Thus, the $\alpha3$ isoform is virtually exclusive to the nervous system

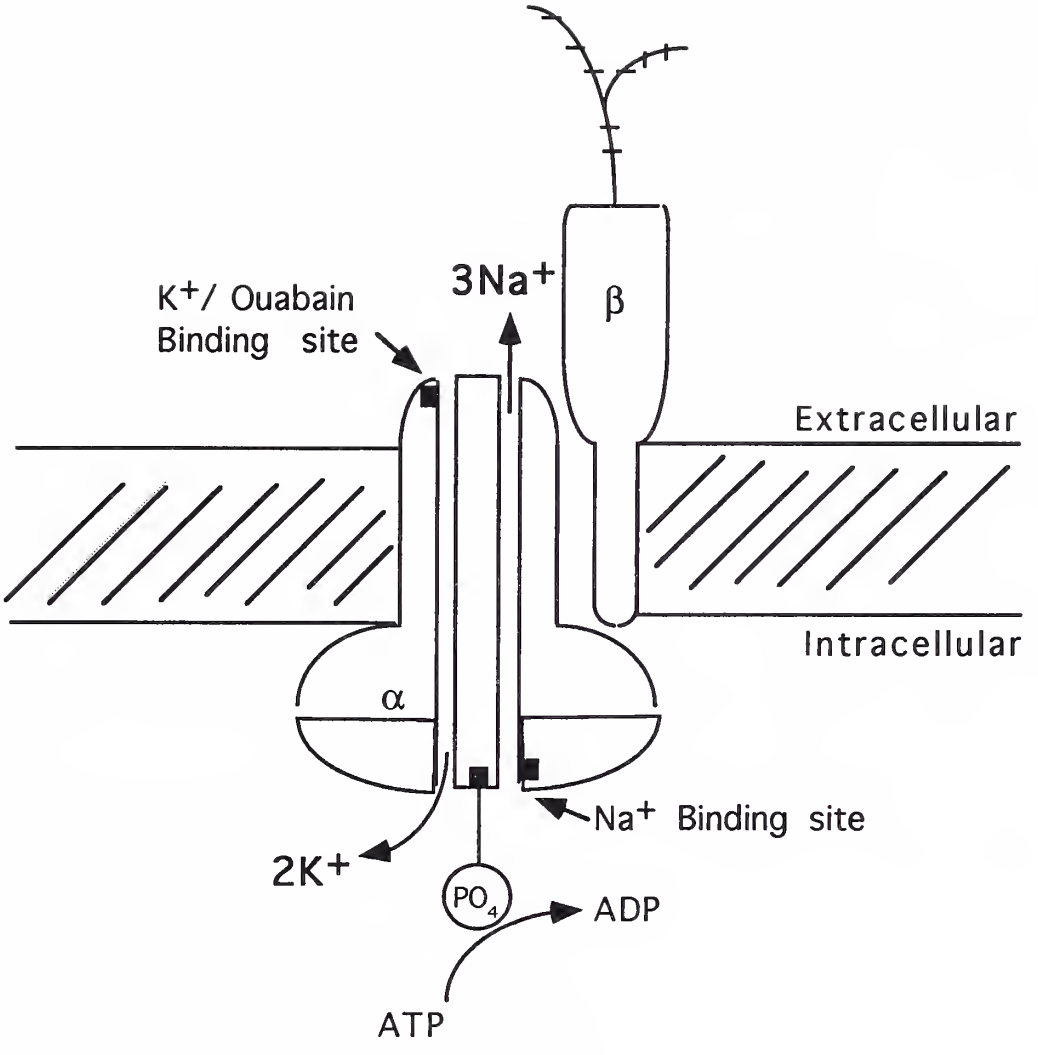


FIGURE 2: The Na^+/K^+-ATPase enzyme

This enzyme consists of a large catalytic (α) subunit (~ 115 kD) and a smaller glycoprotein (β) subunit (~45 kD). Currently three catalytic isoforms and two glycoprotein isoforms are recognized (Young et al., 1987; Shull et al., 1986). Ouabain binds reversibly to the K^+ binding site.

and is expressed at very high levels by neurons (Schneider et al., 1988; Filuk et al., 1989; Brines et al., 1991; Watts et al., 1991). Regional as well as cell type-specific distribution also exist for pump isoforms (Caspers et al., 1987; Brines et al., 1991). The olfactory nuclei, dentate gyrus, hippocampus are among brain regions expressing the highest levels of the Na^+/K^+ATPase enzyme. The large neurons of the olfactory bulb and pyriform cortex express high levels of $\alpha 3$ and lesser levels of $\alpha 1$ mRNA. Principal cells of the hippocampus express all three isoforms; however, the $\alpha 3$ mRNA predominates. Finally, the distribution of functional Na^+/K^+ATPase enzyme, as localized by [^{3}H] ouabain binding, is highest in the neuropil of hippocampus and cerebral cortex and lower over perikarya and white matter, reflecting higher expression of pump activity by neurons.

The Na^+/K^+ pump provides several critical functions for the cell (Figure 3; next page), (reviewed by Skou, 1988). This pump is responsible for restoring transmembrane gradients of Na^+ and K^+ after neuronal excitation. In experiments on vagus nerve energy metabolism, the increased glucose metabolism resulting from stimulation of the nerve is largely used for restoring the ionic gradient across the cell membrane. This increase in glucose utilization is blocked by the Na^+/K^+-ATPase inhibitor ouabain (Yarowsky and Ingvar, 1981). The pump also provides the energy required for driving other transport mechanisms including Na^+/Ca^{++} exchange and uptake of many small molecules such as glucose and excitatory amino acids (e.g. glutamate). In a recent study with glial cultures and rat brain synaptosomes, uptake of glutamate was sodium-dependent, driven by the transmembrane gradient established by the Na^+/K^+pump. (Barbour, 1988; Erecinska, 1989). Further evidence also supports a role for both glia and

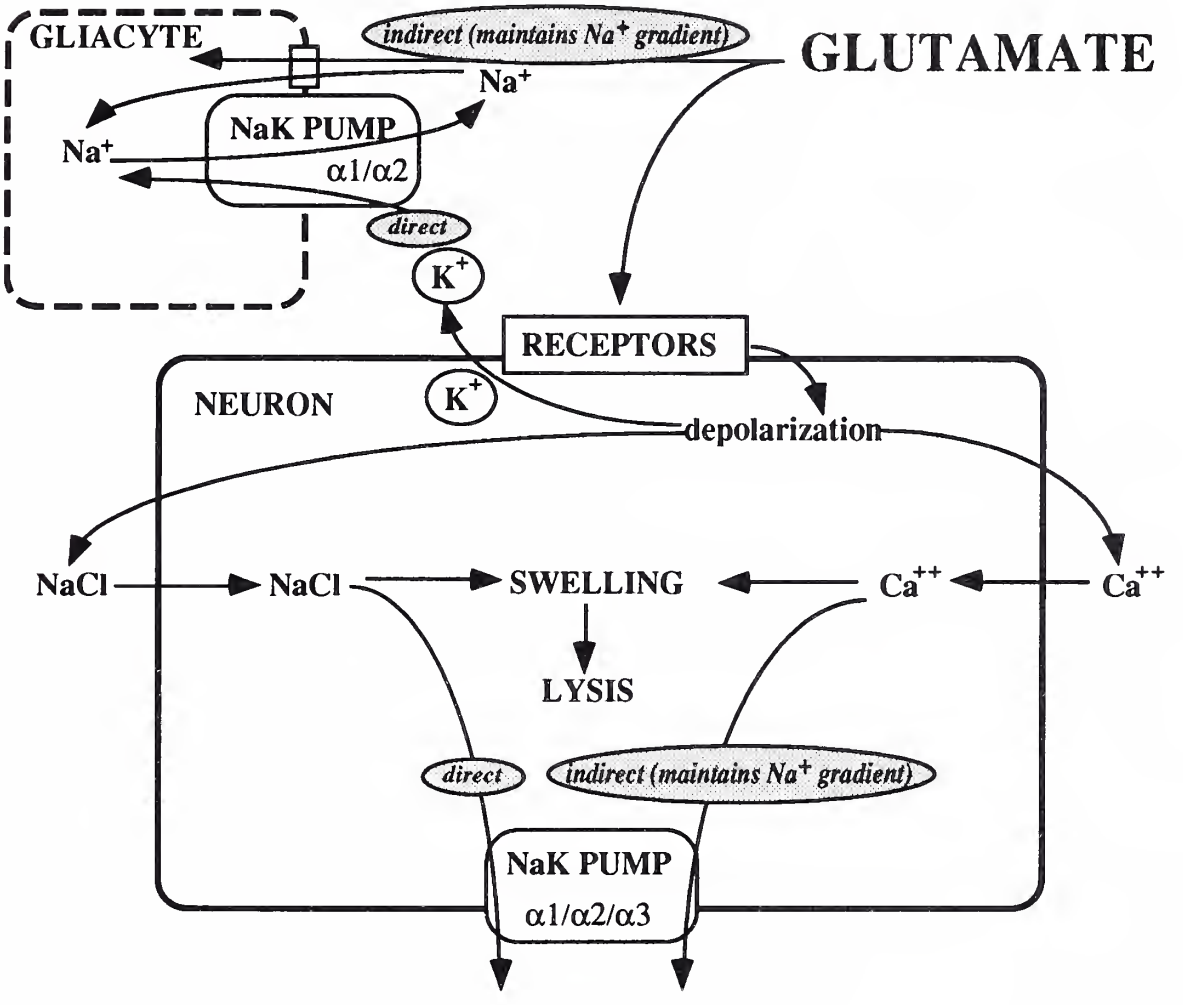


FIGURE 3: Multiple ionic homeostatic mechanisms of the Na^+/K^+-ATPase enzyme family: The Na^+/K^+-pump buffers effects of stimulation (e.g., by glutamate) via direct (uptake of K^+ and extrusion of Na^+) and indirect (maintainance of sodium gradient for glutamate uptake and extrusion of Ca^{++}) mechanisms.

neuronal Na^+/K^+ATPase in buffering potential glutamate toxicity in rodent cultures (Rosenberg, 1991; Rosenberg et al., 1992). Lastly, but not least, this pump plays the important role of maintaining cellular integerity by regulating cell volume (Lowe, 1971; Ullrich, 1979; Stahl, 1986; Skou, 1988; Horisberger et al., 1991).

An interesting feature of the Na^+/K^+-ATPase is that it holds tremendous reserve capacity such that it is able to compensate for increased requirement for Na^+ transport by increased enzymatic activity. The exact mechanisms of this upregulation are still being investigated. However, unlike the sodium-impermeable glia, neurons possess a significant sodium permeability at rest (Kuffler et al., 1984) and, not surprisingly, express the majority of brain Na^+/K^+ pump (Brines et al., 1991; Mata et al., 1991). At resting metabolic activity the extracellular K^+ site of the pump is saturated and intracellular Na^+ is present at non-saturating concentrations (Jorgensen and Skou, 1969; Skou, 1988). Under these conditions, the pump operates at ~20 - 25% of its maximum capacity (**Vmax**) (Skou, 1988). Experiments in synaptoneurosomes have demonstrated that maximum Na^+/K^+ capacity is only half achieved when maximum demand for ion transport is imposed. Thus, when the sodium ionophore, monensin, was used to increase intracellular Na^+ to levels equivalent to that in the extracellular milieu, a less than 50% increase in ouabain-inhibitable rubidium pumping (a measure of ATP-ase-regulated K^+ influx) was observed (Swann, 1991). Further evidence from membrane experiments suggest that Na^+/K^+-ATPase response to small increases in intracellular Na^+ concentrations may occur on the order of seconds (Skou, 1988). **Despite the tremendous ionic homeostatic mechanisms subserved by the Na^+/K^+ ATPase, neuronal injury is a constant consequence**

of excitotoxicity. One possibility is that Na^+/K^+ pump capacity may be insufficient or compromised during excitotoxicity.

Evidence for reduced Na^+/K^+ ATPase capacity in epilepsy and other energy-deficient states.

In the past, it has been argued that the regional increase in blood flow accompanying seizure activity precludes ischemia that would significantly decrease cellular ATP concentrations (Meldrum, 1983; Ben-Ari, 1985; Auer and Siesjo, 1988). More recently, studies by Brines and co-workers have revealed that reduced levels of cytochrome c oxidase activity may be present in epileptic hippocampi (Brines et al., 1995) . This enzyme is the main source of energy substrate for the Na^+/K^+-ATPase (Dagani and Erecinska, 1987). It is estimated that ATPase activity is significantly reduced when ATP concentration declines below 0.4 mM (ibid). Thus, intrinsic defects in cellular energy metabolism, rather than external factors such as ischemia alone, may adversely impact Na^+/K^+ ATPase activity in epileptic hippocampi.

Arachidonic acid, other long-chain fatty acids, lysophospholipids and prostaglandins accumulate during seizure activity (Siesjo et al., 1982). All these compounds are known potent inhibitors of the Na^+/K^+pump (Baran et al., 1987); arachidonate being the most potent. At concentrations of 0.1 - 0.4 mM this fatty acid is sufficient to cause over 90% inhibition of the Na^+/K^+-ATPase in the cortex (Chan et al., 1983; Swann, 1984). During seizure activity free fatty acids including arachidonate increase to a level enough to inhibit ~40% of total brain Na^+/K^+ pump capacity (Siesjo et al., 1982; Yoshida et al., 1987).

An integral component of the Na^+/K^+-ATPase is a phospholipid moiety that binds with high affinity to calcium and is required for pump activity (Charnock et al., 1975; Abeywardena and Charnock, 1983). It is reported that up to 10% of cellular phospholipids are degraded during seizure activity. In particular, phosphotidylinositol and polyphosphoinositide, the major phospholipids essential for pump activity, are most vulnerable (Eiselet et al., 1984; Yoshida et al., 1986; Abe et al., 1987). **Arguably then, several mechanisms including those described above may contribute to the neuronal death associated with seizure activity by directly or indirectly impairing Na^+/K^+-ATPase activity.**

Hypothesis

It has been suggested that the Na^+/K^+pump is probably the single most important ion pump in the cell (Lees, 1991), playing the critical homeostatic role of maintaining the transmembrane ion gradient responsible for excitability and integrity of the neuron. Yet, the role of the Na^+/K^+ pump in modulating nervous system activity and, particularly, in seizure generation and maintenance is only poorly characterized.

A model of how the Na^+/K^+ pump may interact with the process of excitotoxicity is summarized in Figure 4 (next page). At resting states, endogenous levels of glutamate and its analogs (e.g. kainate) act via specific receptors to depolarize neurons. Such depolarization leads to Na^+ influx (followed passively by Cl^-) and K^+ efflux. Ordinarily, the Na^+/K^+ pump functions to maintain transmembrane ionic equilibrium by rectifying these disturbances. When chronic hyperstimulation occurs due to chronic or intense exposure to EAA, Na^+/K^+ pump capacity may be overwhelmed. Sodium may accumulate in the cell, cell swelling results, and irreversible cell

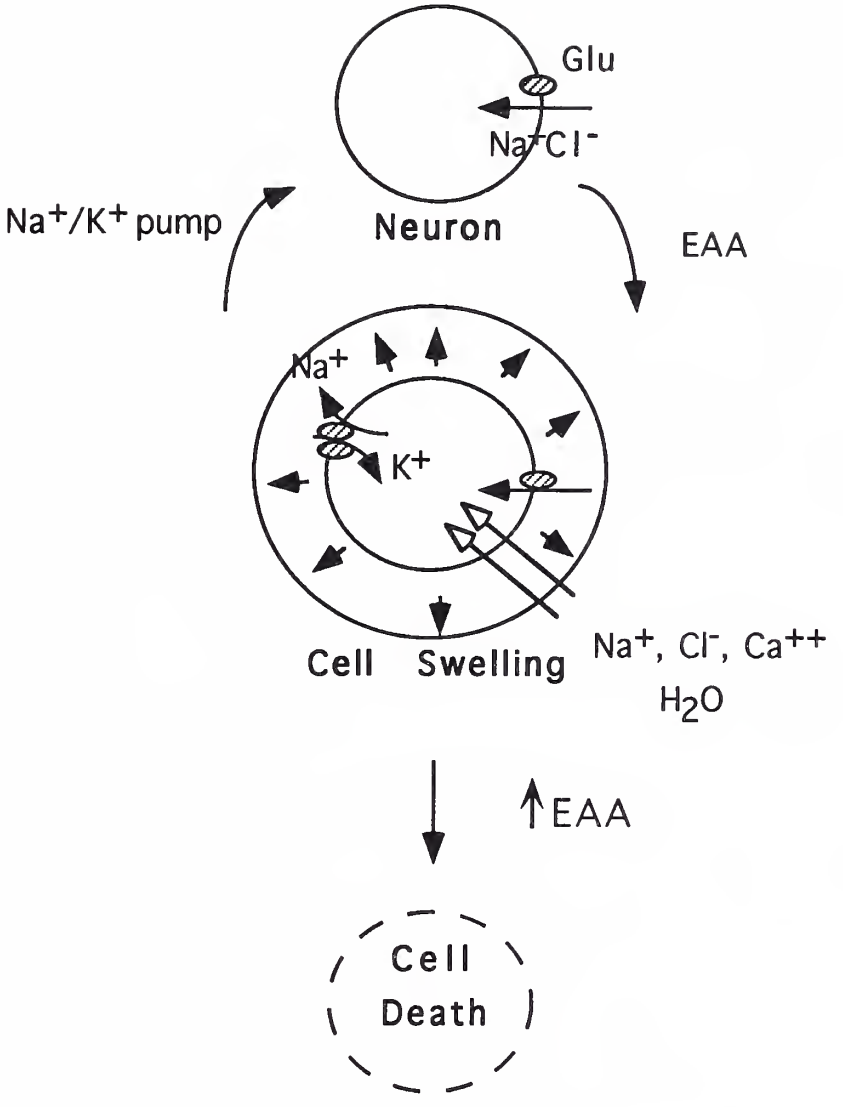


FIGURE 4: EXCITOTOXICITY

Ordinarily, the Na^+/K^+ pump rectifies ion imbalance due to innocuous levels of excitatory amino acids (EAA). However, prolonged or intense stimulation may overwhelm the Na^+/K^+ pump and cell swelling may ensue with eventual cell death.

injury ensues. Based on this model, it was hypothesized that:

Impairment of brain Na^+/K^+ pump capacity will decrease neuronal survival of excitatory stimuli.

Some experimental evidence exists in support of this hypothesis. Novelli and co-workers (Novelli et al., 1988) examined glutamate neurotoxicity using cultured neonatal cerebellar granule cells. Specifically, they examined the ATP dependence of glutamate toxicity and observed that a variety of treatments, including 1 μM ouabain, potentiated subtoxic concentrations of glutamate. However, this experiment has limited application to excitotoxicity for several reasons. First, their experimental model consisted of a single neuronal type which is relatively insensitive to glutamate. Also, Na^+/K^+ pump mRNA mapping studies suggest that cerebellar cells may possess only the α1 isoform of the pump which is relatively insensitive to ouabain at 1 μM concentrations.

Brines and co-workers have also tested this hypothesis *in vitro* . Using fetal telencephalic cultures, α2/α3 isoforms of the Na^+/K^+ pump were inhibited with low concentrations of ouabain (1 μM). Under these conditions, total sodium pump activity was reduced by 65% as accessed by rubidium-uptake (Brines and Robbins, 1992). This treatment was not neurotoxic under basal conditions. However, when cultures were subsequently subjected to the additional stress of normally non-toxic levels of glutamate (100 μM), neurons were preferentially destroyed.

THESIS PROPOSAL

Although evidence from *in vitro* studies lend support to the concept that the Na^+/K^+-ATPase is critical to neuronal survival of excitotoxicity, several obvious limitations exist in trying to understand human pathological processes on the basis of *in vitro* findings. First, cultured cells derived from fetal tissue may behave differently from adult cells. Furthermore, experiments *in vitro* are conducted in isolated systems and therefore do not account for the complex interactions that may take place with other systems *in vivo*.

The principal goal of this project was to test the hypothesis that reduction of Na^+/K^+ pump capacity will limit neuronal survival of excitatory activity *in vivo* and produce a pathology in young adult rats similar to that observed in human TLE. The kainate rat model was conveniently adopted for this purpose. The seizure syndrome and subsequent pathology induced by the potent glutamate analog, kainate, in rat has been extensively studied and shown to possess some features of human TLE: 1) The hippocampus, amygdala and other limbic structures play a central role in the seizure syndrome induced by kainate acid (Schwob et al., 1980; Ben-Ari et al., 1981; Lothman and Collins, 1981). 2) Spontaneous, recurrent limbic type seizures are reproducibly observed after parenteral (Tremblay and Ben-Ari, 1985) or intracerebral administration of kainic acid (Cepeda et al., 1982; Cavalheiro et al., 1983). 3) Anticonvulsants are weak against the seizures generated by kainic acid (Stone and Javid, 1980; Clifford et al., 1982). However a number of important differences exist between the kainate model and the human pathology. Pyramidal cells of hippocampus subfield CA1 are destroyed in the human pathology (Margerison and Corsellis, 1966; Corsellis and Bruton, 1983;

de Lanerolle et al., 1992 and 1994). These are not routinely destroyed in the kainate model. Damage to these cells is achieved only with very high doses of kainate, leading to high animal mortality. Moreover, unlike the human pathology in which neuronal damage is mostly confined to the hippocampus, kainate induces extrahippocampal damage in the amygdala, pyriform cortex, entorhinal cortex, and thalamus.

Thus, several potential benefits existed for testing the above stated hypothesis in the kainate rat model. First, it allowed verification of results of previous *in vitro* investigations of this hypothesis (Novelli et al., 1988; Brines and Robbins, 1992). Second, successful reproduction of the human pathology by impairing Na^+/K^+pump capacity in the kainate model would 1) provide experimental, *in vivo* support for Na^+/K^+-ATPase insufficiency or dysfunction in the neuropathology of human TLE, and 2) provide an animal model which may be used as a tool for further investigation of human TLE.

METHODS AND MATERIALS

EXPERIMENTAL DESIGN

This study consisted of two parts: A *direct* test of the experimental hypothesis was carried out in three steps in part one. First, a subtoxic dose of kainate (a dose of kainate not producing seizures and/or neurotoxicity in young adult rats) was determined. Subsequently, conditions for partial impairment of brain total Na^+/K^+pump capacity were established. In a final step to test the hypothesis, Na^+/K^+ capacity was impaired after treating rats with a subtoxic dose of kainate. In part two, the behavioral and pathological consequences of Na^+/K^+pump inhibition in kainate-treated animals were characterized.

PART I

1) DETERMINATION OF A SUB-TOXIC DOSE OF KAINATE

Response to the glutamate analog, kainate, is very variable in different animals and even within animals of the same species (Ben-Ari, 1985; Sperk, 1994). Factors such as species of animal, commercial breeder, inter-species variations (e.g. sex, age and weight) and route of drug administration may influence response to kainate. Therefore, to establish a subtoxic dose of kainate it was necessary to derive this from a dose-response curve. This curve was achieved by administering a wide range of experimentally reported doses of kainate (3 - 12 mg/kg body weight) intraperitoneally (IP; i.p.) and observing animals for overt behavioral seizures. Twenty-four hours after the start of experiments, animal brains were sacrificed and their brains were

removed and examined for cell death in the hippocamapus, using a technique of silver staining that stains for dead/dying neurons.

2) PARTIAL IMPAIRMENT OF TOTAL BRAIN Na^+/K^+ PUMP CAPACITY

The Na^+/K^+ ATPase is essential for the maintenance of cellular integrity of neurons and glia. Therefore, complete inhibition of the Na^+/K^+ pump with high concentrations of ouabain is toxic to neurons (Lowe, 1978; Garthwaite et al., 1986; Lees et al., 1990) . However, unlike most mammalian Na^+/K^+ATP-ase which possess high affinity (10^{-9} - 10^{-8}M) for glycosides, rodents α1 isoform has a low affinity (10^{-4} - 10^{-3}M) for ouabain, and thus is less sensitive to ouabain inhibition (Sweadner 1989; Brines and Robbins 1992). Consequently, it is possible to titrate glycoside concentrations to achieve partial inhibition of the Na^+/K^+ pump.

Previous experiments in cultured telencephalic cells by Brines and co-workers have shown that ~65% reduction of total Na^+/K^+ pump activity (as assessed by rubidium up-take studies) is achieved with ouabain (1 µM) (Brines and Robbins, 1992). Assuming a uniform distribution throughout the volume of brain extracellular fluid (Kuncz et al., 1990), it was estimated that ouabain (3 nmoles) attains ~0.5 - 1 µM ambient brain concentrations. However, ouabain crosses the blood-brain barrier (BBB) very poorly (Dutta and Marks, 1966; Dutta et al., 1977). Therefore, to ensure that ouabain reaches the brain at desired concentrations it was administered intracerebrovascularly (ICV; i.c.v) through surgically implanted infusion ports (cannula). The level of Na^+/K^+ pump inhibition achieved with ouabain was assessed by [^{3}H] ouabain binding studies. Potential epileptogenic effects of ouabain were monitored by intrahippocampal electroencephalographic (EEG) electrodes implanted at time of surgery for cannula placement. Animals were sacrificed

24 hours after the start of experiments and potential neurotoxic effects of ouabain were studies using the silver stain technique. In control experiments, animals were treated with artificial cerebrospinal fluid (CSF).

3) DOES PARTIAL IMPAIRMENT OF Na^+/K^+-ATPase AMPLIFY KAINATE NEUROTOXICITY?

After establishing steps 1 & 2 above, these conditions were combined to test the proposed hypothesis. Animals were treated with a sub-toxic dose of kainate (i.p.) followed later by ouabain (i.c.v.). Since ouabain was administered directly into the brain, it was delayed by 1/2 h to allow adequate CNS penetration by kainate. Intrahippocampal EEG was recorded to document seizure activity. Subsequently, animal brains were examined for neuronal damage. Control animals received either kainate or ouabain alone. In additional experiments, the time of ouabain administration after kainate injections was varied from 1/2 h to $1^1/_2$ h to further investigate the association between ouabain and kainate in producing seizures.

Experiments were repeated using intraperitoneal injections of both kainate and ouabain. According to estimates of volume of body distributions in young rats, ouabain (~ 1 mg/kg; i.p.) produces plasma concentrations in the range of 1 μM (Watkins et al, 1987; Tatsuji et al 1982). In experiments in which systemic and CSF levels of [^{3}H]ouabain were monitored over a 60 min period after intravenous (IV; i.v.) administration of this drug, plasma/CSF ratio for ouabain remained at ~ 1: 40, although CSF appearance of the drug was delayed by ~ 10 min (Dutta et al., 1977). Thus in intraperitoneal experiments, ouabain was administered first and followed by kainate at 15 min interval due to the less effective CNS penetration of ouabain. Even with

a delay in kainate administration, it was expected that kainate would attain appreciable levels in the brain prior to ouabain.

PART II

CHARACTERIZATION OF THE SEIZURE SYNDROME AND NEUROPATHOLOGY IN Na^+/K^+ PUMP-IMPAIRED, KAINATE-TREATED RATS

Three features of human TLE were investigated in Na^+/K^+ pump impaired, kainate-treated animals : 1) The pattern of hippocampal cell death was characterized by the silver staining method for dead/dying neurons. 2) The presence of a chronic epileptic state was documented by observing animals for spontaneous, recurrent seizures over a period of 30 - 90 days. 3) Histological evidence of plasticity and remodeling in the hippocampus was determined by the Timm stain technique at 30 and 90 days after initial experiments.

METHODOLOGICAL DETAILS

Animals and Materials

Adult male Sprague-Dawley rats weighing 250 - 300g were used in these experiments (Charles River Breeders). Animals were housed at the Boyer Animal Center (Yale University, School of Medicine) under 12 hr light/ dark cycle and provided with food and water *ad lib.* Animal surgery was performed using Kopf stereotactic frames (Kopf Instruments) and the coordinates of Paxinos and Watson (1986). Animals were anesthetized with a mixture of xylazine (20mg/ml; Butler Co.) and ketamine (100 mg/ml; Henny Schein). Ouabain (Sigma, Chemical Co.) was administered into to right lateral cerebral ventricle using a slow-infusion pump (Harvard apparatus) connected by tubing to a cannula system implanted into the right lateral ventricle of the animal. A standard infusion rate (1 µl/min) was used in all experiments. A stock concentration of ouabain (0.333 nmol/µl) was prepared in artificial CSF (NaCl: 135 mM; KCl: 3 mM; $MgCl_2$: 1 mM; $CaCl_2$: 1.2 mM; ascorbate: 200µM; and sodium mono- and dibasic phosphate: 2 mM to pH 7.4) stored at -20 °C, and thawed freshly prior to use. Kainic acid was dissolved in normal saline (0.9%, w/v, NaCl in distilled H_20) and administered intraperitoneally.

Animal Surgery

(i) Cannula Placement

Five to seven days prior to experiments, animals underwent stereotactic cannula placement into the right, lateral ventricle (AP = -0.3, L= +1.4, V= - 3.0). Animals were anesthetized with a mixture of ketamine/ xyalzine (8/80 mg/kg; i.p.). After onset of anesthesia, animals were placed in

a kopf stereotactic frame, hair over the cranium was shaved, and the scalp was sterilized with alcohol and betadine. A subcutaneous injection of epinephrine/lidocaine was administered to provide local anesthesia and hemostasis. A longitudinal incision was then made in the scalp approximately 2 cm long from the coronal suture to the lambda. The cranium was exposed after blunt dissection of the subcutaneous tissue. After marking the desired stereotactic coordinates using bregma as reference, the skull was drilled with a 0.80 screw on a hand-held drill (Plastics One, Inc.). A pre-cut guide cannula was stereotactically placed approximately 2 mm above the right ventricle and secured to cranial screws using Loctite glue (Bearing Distributors). The exposed cranium was then covered with a hygenic repair resin (Dental Lab. Distributors) to provide additional support for the cannula system. The skin incision was closed with a 3-0 prolene stitch. A "dummy" cannula was advanced through the guide cannula into the right ventricle and left in place for the animals to recover from the operation (Figure 5; next page). Correct cannula placement was verified by serial brain sectioning.

(ii) Electrode placement

In experiments in which EEG monitoring of seizure activity was required, bilateral, intra-hippocampal electrodes were placed at the time of surgery for cannula implantation. Routinely, four thin (1mm) platinum-coated, stainless steel electrodes were placed: an active electrode in the right hippocampus with its tip aiming at area CA3 of the hippocampus (AP = -4.2; ML = +2.0; DV= -3.0), a second active electrode with a tip aiming at area CA3 of the left hippocampus (AP = -4.2; ML = -2.0 DV= -3.0), a reference electrode in the left frontal area (AP = +3.0; ML = -2.0 DV= -2.0), and a ground electrode attached to a cranial screw. All electrodes were secured to the skull using

Figure 5

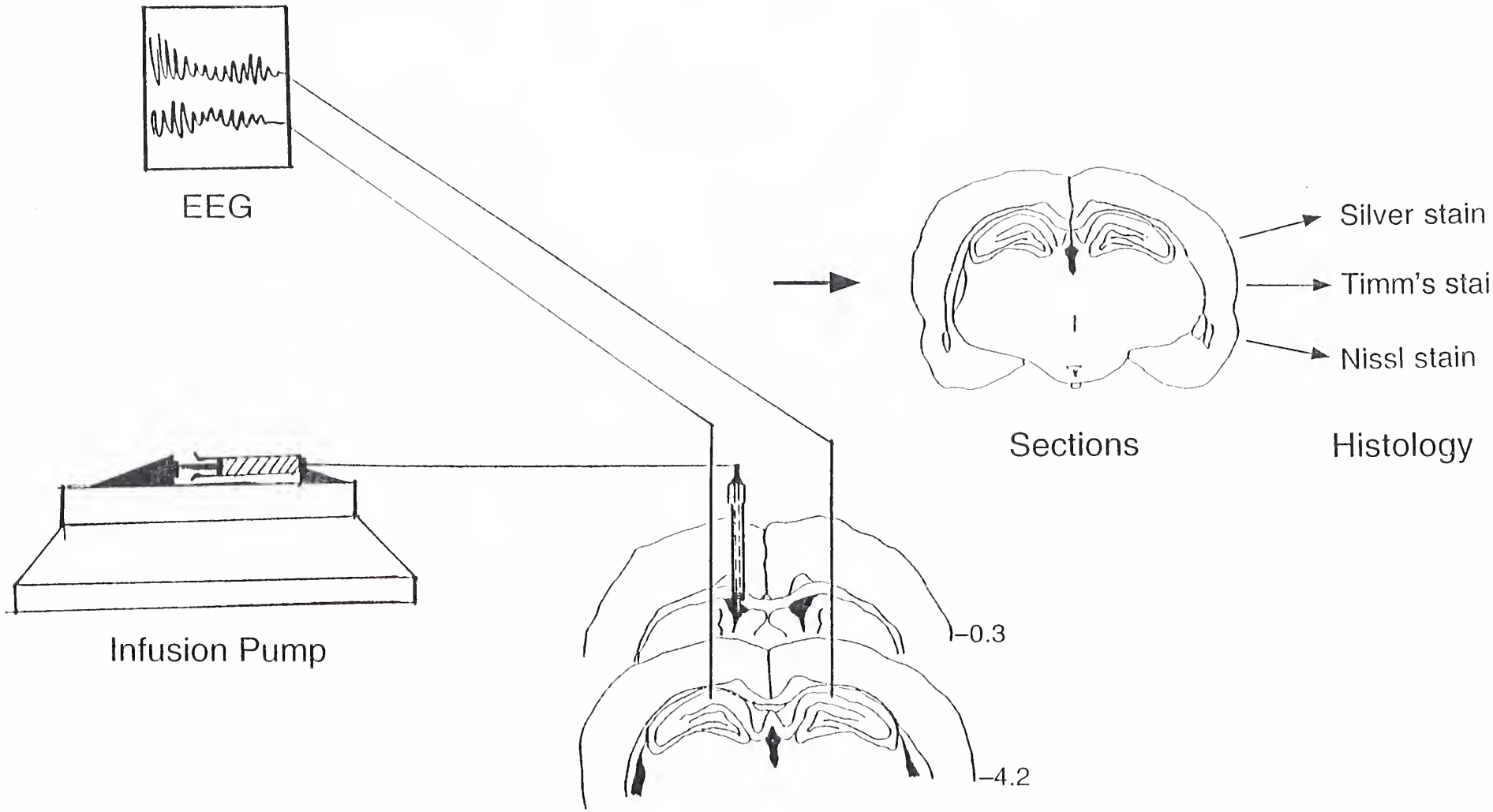

EEG
Infusion Pump
Silver stain
Timm's stai
Nissl stain
Sections
Histology
–0.3
–4.2

Figure 5: **EXPERIMENTAL SET-UP:**

Five to seven days prior to experiments, animals underwent surgery and were implanted with guide cannula (right lateral ventricle) and electroencephalographic electrodes (intra-hippocampal). On the day of experiments, kainic acid was injected intraperitoneally (IP; i.p.). Ouabain was either injected IP or intraventricularly (ICV; i.c.v.) through the implanted cannula. Animals were observed behaviorally for seizures and hippocampal activity was monitored by electroencephalography (EEG). Twenty-four hours after experiments, animals were sacrified and their brains were examined for cell death using the silver staining method. Dead cells were identified by silver staining. Cell loss was assessed by Nissl staining. At 30 and 90 days after experiments, animals were sacrified and their brains were observed for hippocampal remodeling using the Timm stain (refer to text for detail).

Loctite glue and subsequently with hygenic repair resin as described above. All electrodes were brought into a "female nut" and left in place during animal recovery (Figure 5). Correct electrode placement was verified by serial brain sectioning.

Tissue Preparation

SILVER STAIN

The silver staining method is a staining technique used to reveal dead and degenerating neuronal cell bodies beginning at least 8 hrs after brain injury. Complete characterization of brain damage is best achieved with this technique when animals are perfused 24 hrs after the initial brain injury (Nadler and Evenson, 1983). This stain was selected so as to visualize cell bodies primarily and not their processes.

Animals were anesthetized with chloral hydrate (50mg/kg i.p.) and perfused trans-cardially with normal saline for 30 sec and then with 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 15 - 20 min (200ml). The brains were then removed and fixed further in cold, buffered paraformaldehyde for 3 days at room temperature. Brain specimens were embedded in 5% agarose (w/v) in 0.1 M phosphate buffer, cut into 40 μM thick sections on a Lancer vibrotome in phosphate buffer, pH 7.4.

Details of procedure:

Reagents:

A. 9% (w/v) NaOH

B. 16% (w/v) $NH_4 NO_3$

C. 50% (w/v) $Ag NO_3$

D. 1.2% (w/v) $NH^4 NO_3$

E. Dissolve 5 g of anhydrous Na_2CO_3 in 300 ml of 95% ethanol and 600 ml of water. Dilute to 1 litre with water.

F. Dissolve 0.5 g of anhydrous citric acid in 15 ml of 35% formalin, 100 ml of 95% ethanol, and 700 ml of water. Add solution A with stirring until the pH reaches 5.8 - 6.1 then dilute to 1 litre with water.

G. 0.5% (v/v) acetic acid

Working Solutions:

(These solutions were prepared not more than 1 hr before staining).

1. Pretreating solution: Equal amounts of solutions A & D.
2. Impregnating Solution: Add 1.5 volumes of solution A to each volume of solution B. Then add 0.5 - 0.6 ml solution C (1.5 ml) for each 100 ml of total volume.
3. Washing Solution: Mix 1 ml of solution D with each 100 ml of solutionE.
4. Developing Soliution: Mix 1 ml of solution D with each volume of solution F.

Staining Procedure:

Sections were washed free of fixatives by immersion in three changes of water for 5 min each. Sections were then transferred sequentially into two changes of pretreating solution for 5 min each, impregnating solution for 10 min, and finally developing solution for at least 1 min. Sections were mounted from developing solution onto acid-cleaned glass slides that have been precoated with 0.5% (w/v) chromium potassium sulfate, 0.5% (w/v) gelatin. When the mounted sections have dried, they were immersed in three changes of solution G for 10 min each, washed with water, dehydrated

with a graded series of ethanol solutions, cleared with butanol and three changes of xylene, and covered with coverslips.

TIMM STAIN

The heavy metal, zinc, is released from mossy fibres (MF) of the dentate granule cells in the hippocampus and is also found to accumulate in injured neurons in the hippocampus (Sloviter, 1982). Following kainate-induced lesions, the MF of the granule cells send out collaterals which grow across the granule cell layer and form a plexus in the inner molecular layer of the granule cell dendrites (Laurberg and Zimmer, 1981). This type of hippocampal remodeling has been termed 'sprouting' and is also observed in human epileptic hippocampus (Babb et al., 1991; de Lanerolle et al., 1994). The Timm technique stains for zinc and therefore is used to identify sprouting in the injured hippocampus.

To prepare rat brains for this technique, animals were anesthetized with chloral hydrate and perfused trans-cardially using 0.37% sodium sulphide in phosphate-buffered solution, pH 7.2, for 5 min and with 10% neutral buffered formalin (NBF) for 5 min. (Sulphide in the perfusate impregnates neurons, and it is later oxidized to sulphite in lysed cells, staining them black). Rat brains were removed and further fixed in NBF solution for at least 2 hrs, but not more than 3 days. Coronal sections (40 μM) are cut on a Lancer vibrotome into phosphate buffer pH 7.4, collected onto subbed-slides, and allowed to dry at room temperature.

Details of Procedure:

Reagents:

A. Gum Arabic Solution; 50% (w/v) gum arabic in water.

B. Citrate Solution: 9.4 g sodium citrate in water 10.2 citric acid monohydrate in water.

C. Hydroquinone Solution: hydroquinone in water.

D. Silver Nitrate Solution: (w/v) $AgNO_3$ in water.

E. Developer: Mix 250 ml of solution A, 40 ml of solution B, and 120 ml of solution C and warm the mixture to 26°C. 2 ml aliquot of solution D is added just before staining.

Staining Procedure:

Slides are rinsed serially in 70% ethanol, 50% alcohol, and then in four changes of water for 3 min each. The slides are covered with developer and placed in a water bath maintained at 26 °C in a dark room for 45 min. Slides are washed in tap water in the dark for 5 -10 min to terminate development. The sections are then dehydrated with graded ethanol (70%, 95%, 100%, 100%) and xylene for 3 min each and coverslipped for viewing under the microscope.

NISSL STAIN

The Nissl stain is a basic neurological staining techniques that stains for nissl granules in neuron. It was used to counter stain Timm stain to help delineate nerve cells and neuronal architecture.

Details of Procedure:

Reagents:

Toluidine blue (stock) : 0.1% (w/v) toluidine blue in distilled water.

Working solution:

A: 10 ml of stock toluidine blue in 10 ml of 0.1M phosphate buffer.

Staining procedure:

Slides were immersed in xylene to remove coverslips from Timm stain procedure. Sections were then rehydrated by serial changes through graded ethanol (100%, 95%, 70%) for 1 min each and then washed by dipping in water 5 - 10 times. Slides were then covered with toluidine blue for 30 seconds and washed again with distilled water. After a one-minute wash in 70% acid alcohol, sections were dehydrated with graded alcohol changes (70%, 95%, 100%, 100%) for 1 min each and then through two changes of xylene, 5 min each. Finally, slides were coverslipped for viewing under the light microscope.

In situ [^{3}H] ouabain binding studies

Ouabain specifically binds only to sodium pump in an active conformation, therefore it has been used widely to assess Na^+/K^+ pump function/capacity (Hegyvary, 1976; Hootman and Ernst, 1981; Kjeldsen and Norgaard, 1987). *In situ* [^{3}H]ouabain binding studies were carried out to quantitate high-affinity Na^+/K^+ pump active sites in hippocampal sections. Animals were sacrificed as above, and their brains were quickly removed, blocked, and flash-frozen in embedding medium on dry ice. Brain specimens were then stored at -70°C until use within 5 - 7 days.

On the day of binding studies, coronal sections (20 µM) of the dorsal hippocampus were cut on a Reichert cryostat (2800E) at -15 °C and thaw-mounted onto gelatin-potassium dichromate-subbed slides. Sections were dried for 2 min at 37 °C and then frozen at -70 °C. Subsequently, sections were

brought to room temperature and preincubated in TM buffer (tris-HCl 50 m, $MgCl_2$ 10 mM, pH 7.4) for 15 mins at 27 °C to dissociate any unbound ouabain-like substances. Excess solution was blotted from each slide, and 1 ml of TM buffer containing 10 nM ^{3}H-ouabain (Dupont, NAN), 100 mM NaCl, 5 mM ATP, with or without 1 μM cold ouabain (Sigma Chemical Co.) was layered over each section. Tissue was incubated at room temperature for 45 min. Previous studies by Brines and co-workers have shown that ouabain binding attains equilibrium in 30 min at 27 °C and remains constant for at least 60 min (Brines et al., 1991). To terminate ouabain binding, slides were washed twice in ice-cold buffer for 5 min each, rinsed in deionized water to remove buffer salts, and rapidly dried within 1 min with cool air. Dried slides were then exposed to Ultrofilm (Reichart, Inc.) along with appropriate tritium plastic standards (American Radiolabeled Chemicals) and exposed for two weeks.

EEG Studies

Brain electrical activity was recorded on a Grass Model 6 EEG (Grass Instrument Co.) from platinum electrodes stereotactically implanted into hippocampi as described above. The EEG acquisition montage consisted of a combination of monopolar and bipolar techniques. A left frontal lobe electrode served as reference for both monopolar hippocampi electrodes, and differential activity between both hippocampi was obtained by a bipolar connection (Kooi et al., 1978) A frequency range of (0.3-70 Hz) was used at a sensitivity of 50. All electrodes were grounded from a skull screw. Routinely, a baseline EEG recording was obtained with artificial CSF infusion (1 μl/min) for 30 min prior to start of experiments. EEG monitoring were continued throughout experiments.

Data analysis

According to a grading scale advanced by Sperk and co-workers (Sperk et al., 1983), grade I - II seizures are mild limbic seizures manifesting as immobility, staring, facial grimaces, and occasional episodes of characteristic torso-and-rear shakes termed wed-dog-shakes (WDS). Grade III seizures are moderate seizures characterized by more frequent WDS, automatism including head-bobbing, forelimb clonus, and some foaming at the mouth. Grade IV seizures comprise all the above described features of limbic features and include standing on rear limbs, rearing and eventual loss of balance due tonic-clonic convulsions. Grade V seizures are severe seizures leading to death of the animal. In this study, grade III - V seizures were considered overt, behavioral seizures.

Cell death in hippocampus subfields CA1 - 3 and the dentate hilus was determined by examining high-power fields (3 - 4) under the light microscope. For each animal, average cell death was obtained from 6 - 10 successive sections through the dorsal hippocampus. In pilot experiments, all dead/dying cells were clearly identifiable by the silver stain technique and no correction for cell loss was required. Also, the silver stain allowed visualization of both dead/dying neurons as well as background neuronal architecture, so that counter-staining was not routinely required. Statistical significance of the association between treatments (experimental and controls) and a particular outcome (cell death or seizures) was determined by the chi-square analysis. In these calculations, cell death was treated as a variable.

[^{3}H]-ouabain studies produced film images of exposed sections (autoradiographs). After development, autoradiographs were analyzed by the MCID densitometric System (Imaging Research, St. Catharines, Canada).

Densities were determined as the average of multiple small windows sampled from 6 - 10 enlarged images taken randomly from cortical and hippocampal regions. Statistical difference between groups was assessed by chi-square analysis.

All EEG tracings were compared to baseline recording on the basis of amplitude, frequency and pattern of waveforms.

RESULTS

The kainate dose-response curve

The dose-response curve for young adult, male, Sprague-Dawley rats obtained in these experiments is illustrated in Figure 6. Kainate was given in the range 3 - 12 mg/kg (i.p.). The dose-response curve was steep, reflecting the drastically different behavioral responses observed for animals receiving low doses of kainate compared to animals receiving moderate to high doses. At low kainate doses of 3, 5, and 7 mg/kg (i.p.), i.e., kainate doses $\leq$ 7 mg/kg, only 2 of 19 animals experienced seizures. For a moderate dose of kainate (8 mg/kg i.p.), 7/8 animals experienced seizures. High-dose kainate (10 and 12 mg/kg; i.p.) produced severe and prolonged seizures in all animals (n= 6).

Behavioral observations

The behavioral response to varying doses of kainate has already been extensively reviewed (Ben-Ari, 1985; Sperk, 1994) and will be presented only briefly here. Most aninimals (17/19) treated with low-dose kainate exhibited Sperk grade I - II seizures (Sperk, 1983). In general, animals became restless, moving incessantly around the cage few minutes after kainate injection. Between 20 to 30 minutes after kainate injection, animals displayed repetitive scratching behavior interrupted by episodes of immobility and staring. Thereafter, between 30 - 40 min after kainate injections, animals exhibited WDS. WDS were the most severe behavioral changes observed in most (17/19) animals receiving $\leq$ 7 mg/kg (i.p.) of kainate. Typically, these WDS increased in frequency and abated subsequently within 60 - 90 min.

Animals treated with moderate or high-dose kainate behaved similarly to animals treated with low-dose kainate up until onset of WDS. In moderate and high-dose kainate-treated animals experiencing seizures, WDS increased in frequency until generalized, clonic-tonic convulsions appeared (Sperk grade III - IV). These episodes consisted of up to 3 min of sustained seizure activity manifested as head bobbing, facial grimaces and foaming from the mouth. In severe cases, rearing and eventual standing on rear limbs with bilateral fore-limb clonus (pedaling) occurred, ending sometimes in loss of balance and falling. There was a trend to suggest that the seizure severity and rate of progression to ictal behavior increased with increasing dose of kainate. In animals receiving moderate dose kainate (8 mg/kg; i.p.; n= 8), the latency to onset of WDS measured 39 $\pm$ 5 min (SEM; measured for 4/8 animals). Latency to onset of overt seizures was on average 112 $\pm$ 20 min (SEM; measured for 2/8 animals) and lasted on average 130 $\pm$ 12 min (SEM; measured for 3/4 animals). At high-dose kainate (10 mg/kg i.p.), WDS occurred later 57 $\pm$ 7 min (SEM; n=5), but seizures occurred earlier (91 min $\pm$ 5 min, SEM; n=5) and lasted longer 300 $\pm$ 50 min (SEM; measured for 3/5 animals). The mortality rate was 20% (1/5) at kainate concentration of 10 mg/kg, death occurring within the hour (Sperk grade V). At 12 mg/kg kainate, the mortality was 100% (1/1).

Histology

Silver staining of brains from all surviving animals (31/33) used for the dose-response curve revealed neuronal death only in animals experiencing behavioral seizures. Behavioral changes progressing to and ending with WDS did not lead to brain damage. In the brain of animals experiencing seizures with moderate-dose kainate treatment (7/8), cell death

was observed in subfields CA2 and CA3, the dentate hilus, and the granule cell layer. Pyramidal cells of area CA1 were not routinely damaged by this dose of kainate. Although not routinely assessed, extra-hippocampal damage was observed in hippocampal sections containing parts of the thalamus and amygdala complex. The pattern of damage in the hippocampus of animals experiencing seizures with high-dose kainate (10 mg/kg; i.p.; 3/5 animals) was similar to that of animals treated with moderate-dose kainate. However, at high-dose kainate cells in hippocampus area CA1 were routinely damaged.

Partial inhibition of total brain $Na^{\pm}/K^{\pm}$-ATPase Capacity with ICV ouabain

Four animals implanted with cannula as well as intra-hippocampal EEG electrodes were used for these experiments (Figure 5).

Behavioral observations

In the first few minutes after beginning ouabain infusion (3 nmoles, i.c.v. over 9 min), animals generally showed irritability by walking around the cage in circles. Five minutes into ouabain infusion, animals became more restless, and circling behavior was interrupted only by brief periods of immobility, staring, and occasional WDS. In subsequent minutes, animals demonstrated increased overall level of agitation and respiration. At the end of injections (10 min) animals routinely exhibited vigorous running activity, lasting on the average ~ 60 sec. Subsequently, animals showed a variable period of hypotonia (up to 30 min), sporadic head or body clonus, decreasing respiratory rates, and eventual return to normal within an hour after cessation of ouabain infusion.

EEG Correlates

EEG recordings during ouabain infusion revealed decrease in brain activity starting by the end of the first minute (Figure 7). Reduced wave frequency and spike activity compared to baseline was first recorded in the right hippocampus (the site of cannula placement) with subsequent progression to the left hemisphere (Figure 7 B & C). Vigorous running at the end of ouabain infusion was recorded as artifact on the EEG (data not shown). Reduced brain activity persisted for 30 - 45 min after ouabain infusion, reverting to baseline patterns thereafter. No epileptiform discharges were recorded in any animals (n = 4).

Histology

Silver staining revealed no evidence of brain damage in all four animals treated with ouabain (3 nmoles; i.c.v) (Figure 8, panel A) or in one control animal treated with artificial CSF (data not shown).

Results of [^{3}H]Ouabain Binding studies

Densitometric analysis of autoradiographs produced in [^{3}H]-ouabain binding studies revealed that the number of active pump sites in ouabain-treated animals (n = 3) was approximately twice that of control animals (n = 2) (Figure 9; $p < 0.001$, chi-square analysis). The concentrations of [^{3}H]-ouabain (1 μM) used in this detection method allowed assessement of only high-affinity isoforms ($\alpha 2/\alpha 3$). Also, the limited spatial resolution of Ultrafilm autoradiography allowed assessement of only gross regional variation in Na^+/K^+pump density. Thus, in the brain of ouabain-treated animals and in control animals treated with artificial CSF, Na^+/K^+ density in the hippocampus was twice that of surrounding cortical regions ($p < 0.001$; chi-square analysis; n = 3 and 2, respectively).

Partial impairment of total brain Na+/K+-ATPase capacity amplifies kainate neurotoxicity

From the dose-response curve, a subtoxic dose of kainate (5 mg/kg i.p.) was determined. At this dose of kainate, 1/10 animals were observed to exhibit seizures (Figure 6), and the brain of 9/10 animals not experiencing seizures revealed no neuronal damage (Figure 8, panel B). In preliminary experiments, EEG activity increased above baseline within 10 min after kainate (5 mg/kg i.p.), suggesting CNS access after IP administration of kainate within this interval. Thus, six surgically-prepared animals were treated with 5 mg/kg (i.p.), followed 1/2 h later by ouabain (3 nmoles; i.c.v.). Four control animals received ouabain alone 1/2 h after a sham saline IP injection. Also, six other control animals received kainate (5 mg/kg; i.p.) followed by a sham ICV infusion with artificial CSF.

In control animals treated with ouabain alone, no seizure activity was detected either behaviorally or by EEG, in agreement with earlier experiments. Also, subsequent histological evaluations revealed no evidence of brain damage in these animals (Figure 8, panel A). Similarly, kainate (5 mg/kg, i.c.v.) alone failed to elicit behavioral seizures, epileptiform activity on EEG, or cell death upon silver staining (Figure 8, panel B).

On the other hand, all 6 animals treated with kainic acid (5 mg/kg; i.p.) followed by ouabain (3 nmoles, i.c.v.) exhibited behavioral seizures as well as epileptiform activity on EEG (Figure 10). Histological examinations of brain sections from these animals also revealed brain damage in all six animals (Figure 8, Panels C -F).

Behavioral observations

Typically, animals treated with kainate (5 mg/kg i.p.) and ouabain (3 nmoles; i.c.v) became mildly agitated and showed occasional episodes of immobility and staring after kainate treatment and prior to ouabain treatment. Behavioral changes during subsequent ouabain administration were initially very similar to those observed with ICV ouabain-only treatments. Typically, animals expressed increased levels of restlessness, scratching, and occasional WDS. These culminated in vigorous running activity at the end of injections (9 - 10 min). WDS appeared on the average 4 min $\pm$ 2 min (SEM; n= 4) after initiating ouabain infusion. The hypotonia observed after this running episode in ouabain-only experiments was not routinely observed in these animals. WDS returned promptly after the episode of running and progressed in frequency, culminating in overt seizure episodes. The average latency to onset of the first seizure episode was 116 $\pm$ 13 min after ouabain injection (SEM; n= 4). These convulsions were similar to those of high kainic acid treatment alone, were not associated with obvious apneic spells, and continued sporadically for 345 $\pm$ 9 min (SEM; n = 4). In spite of the severity of seizures occurring after ouabain and kainate, no animal mortality was observed.

EEG Correlates

EEG recordings were obtained with four animals treated with kainate (5 mg/kg i.p.) followed by ouabain (3 nmoles, i.c.v.) (Figure 10). After kainate injections and prior to ouabain treatment, an increase in the frequency and amplitude of brain waves above baseline was observed (Figure 10, panels A, B, C & D). The increase in brain activity correlated with increased restlessness during this period. Upon ouabain administration, characteristic ouabain

suppression of brain activity was observed beginning within the first minute (Figure 10, panel E). Attenuated brain waveforms were first observed in the right hemisphere (cannula site) and then in the left hemisphere 5 - 10 seconds later. However, unlike ouabain-only experiments, brain activity returned promptly (Figure 10, panel F). After the vigorous running activity observed at 9 - 10 min, the first set of epileptiform discharges occurred with an average latency of 17.5 $\pm$ 2.5 min after the start of ouabain infusion (SEM; n = 4). Again, seizure activity was first observed in the right hemisphere (site of ouabain infusion) before appearing in the left hemisphere, separated by 2 - 5 seconds. Subsequently, a variable period of 15 - 60 min intervened during which epileptiform discharges were observed on EEG in the absence of any overt behavioral manifestations of seizure activity. Some of these discharges corresponded with immobility and staring or facial grimaces. In general, these discharges became more frequent just prior to the first observed behavioral seizure. Epileptiform discharges were subsequently recorded on EEG beginning on the average 116 $\pm$ 13 min (SEM; n=4), and sporadically for the 6 h for which animals were routinely monitored.

Histology

Analysis of the hippocampus of animals treated with kainate (5 mg/kg; i.p.) and ouabain (3 nmoles; i.c.v.) revealed widespread damage to hippocampal neurons. Neurons of the dorsal hippocampus were heavily injured, particularly neurons in CA1 and CA3 subfields (Figure 8, panel C.). Hilar interneurons were consistently damaged (Figure 8, panels C & D). Damage was not routinely observed in extra-hippocampal regions. Cell death was observed in the amygdala, but virtually no cell death was observed in the

thalamus, neocortex, or frontal lobe (data not shown). Cell death was also observed in hippocampal area CA2 and in the granule cell layer (Figure 8 C).

Statistical analysis revealed that pairing of ouabain and kainate reliably produced seizures and neuronal degeneration (6/6 animals; $p < 0.001$; chi-square analysis). In contrast, kainate or ouabain alone did not (0 of 6 and 0 of 4 animals, respectively; Figure 11).

Onset and severity of seizures depend upon time of ouabain administration

In 8 animals, ouabain was delayed by 90 min after kainate administrations. Of these, 3 animals were monitored by EEG. All 8 animals experienced seizures, albeit at different latencies compared to animals treated with ouabain 30 min after kainate injection. Latency of onset of seizures was recorded for 5/8 animals and was observed on average to be 282 $\pm$ 30 min (SEM) after kainate treatment. This reflected an increase in latency of seizure onset after kainate treatment of ~70 $\pm$ 15 min (SEM), compared to animals treated at 30 min interval. However, latency of seizure onset from ouabain injection did not differ significantly for both groups (Figure 12). The duration of overt, successive seizure episodes also shortened notably (~ 150 min) as determined for 6/8 animals. Notable differences between EEG recorded for animals treated with ouabain at 90 min versus 30 min after kainate injection included: i) an almost complete return to baseline of brain activity 60 - 80 min after kainate injection and prior to ouabain infusion, and ii) a reduction in the number of, and duration in which, epileptiform discharges occurred. When ouabain was administered at 90 min, the first epileptic discharge appeared on EEG 22 $\pm$ 4 min (SEM; n = 3) after ouabain injection and was not significantly different in latency of onset compared to animals administered ouabain 30 min after kainate. Histological study of neuronal death in these

animals revealed a pattern of brain damage similar to those described above for animals receiving kainate and ouabain at 30 min interval.

Intraperitoneal injections of both ouabain and kainate replicate results of experiments with kainate (i.p.) and ouabain (i.c.v)

In preliminary studies, ouabain (1 mg/kg; i.p.) in combination with kainate (5 mg/kg; i.p.) failed to elicit any observable seizure activity (data not shown). Subsequently, an experimental group was treated with ouabain (1 mg/kg; i.p.) followed by kainate (7 mg/kg; i.p.; 8 animals). Kainate control animals received saline followed by kainate (7 mg/kg; i.p.; n = 8). Four ouabain control animals received ouabain (1 mg/kg; i.p.) followed with saline (i.p.). Animals were then observed for behavioral manifestations of seizures for up to 8 hrs after kainate injection.

Table 1 (next page) illustrates results from this experiment. Within 5 min of injections, all 4 control animals treated with ouabain (1 mg/kg; i.p.) demonstrated mild agitation and increased respiration rate with return to normal within 1 hr. Seizures or hippocampal cell death were not observed in these animals. Seizures were observed in only 1 of 8 control animals treated with kainate (7 mg/kg; i.p.). The rest of kainate control animals not seizing (7/8) typically experienced WDS which appeared between 20 - 40 minutes after kainate injections and tapered off within an hour of onset. Six of eight animals receiving ouabain (1 mg/kg; i.p.) followed by kainate (7 mg/kg; i.p.) experienced overt limbic type seizures.

Histological studies of neurodegeneration revealed no brain damage in all control animals, except in the one control animal that experienced seizures with 7 mg/kg (i.p.) kainate. Silver staining of animals experiencing seizures from treatments with ouabain followed by kainate (7 mg/kg; i.p.)

TABLE 1

CONTROLS	Seizures	Neuronal Death	Mortality	TOTAL
Ouabain (1 mg/kg; i.p.)	–	–	–	4
kainate (7 mg/kg; i.p.)	1	1	–	8
EXPERIMENTAL				
Ouabain (1mg/kg; i.p.) + kainate (7mg/kg; i.p.)	6	6	–	8

revealed neuronal injury in the hippocampus similar to that already described for animals treated with ouabain (i.c.v.) and kainate (i.p.) (Figure 13A & B).

Statistical analysis of these results showed that pairing of ouabain and kainate reliably produced seizures and neuronal degeneration (6/8; $p < 0.001$; chi-square analysis), compared to control animals treated with ouabain (0/6) and kainate (1/8).

Pattern of hippocampal damage and chronic features of kainate and ouabain treated animals

i) Pattern of hippocampal cell death

The pattern of hippocampal cell damage observed for animals treated with kainate and either ouabain (i.c.v) or ouabain (i.p.) were essentially the same. All hippocampal cells were affected, including cells in subfields CA1 - CA3. Cell death was observed in the dentate hilus and in the granule cell layer. Granule and CA2 pyramidal cells were relatively spared (Figures 8, panels C, D, E, &F and Figure 13).

ii) Spontaneous, recurrent seizures

Three animals treated with ouabain (1 mg/kg; i.p.) followed by kainate (7 mg/kg; i.p.) were allowed to recover and observed for spontaneous behavioral manifestations of seizures. Two animals were observed for 30 days and one for 90 days. Handling elicited spontaneous limbic type seizures in all three animals, beginning day 14, 15, and 30, respectively. These episodes were characterized by head clonus, facial grimaces and automatism. No overt tonic-clonic seizures were observed. In a control animal treated with sham surgery, no spontaneous recurrent seizures were observed.

iii) Hippocampal remodeling

At 30 and 90 days after experiments, animals observed for spontaneous recurrent seizures were sacrificed and their brains were examined for evidence of hippocampal remodeling by Timm stain.

Examination of the dorsal hippocampus of an animal that seized from treatment with ouabain (1 mg/kg; i.p.) and kainate (7 mg/kg; i.p.) showed sprouting of granule cells into the inner molecular layer (Figure 14 B). Also notable was a thinning of the heavily stained outer molecular layer of area CA1 compared to control (Figure 14B; arrow heads). Timm staining of brain sections from control animals three months after the experiment showed no remodeling of the hippocampus (Figure 14A).

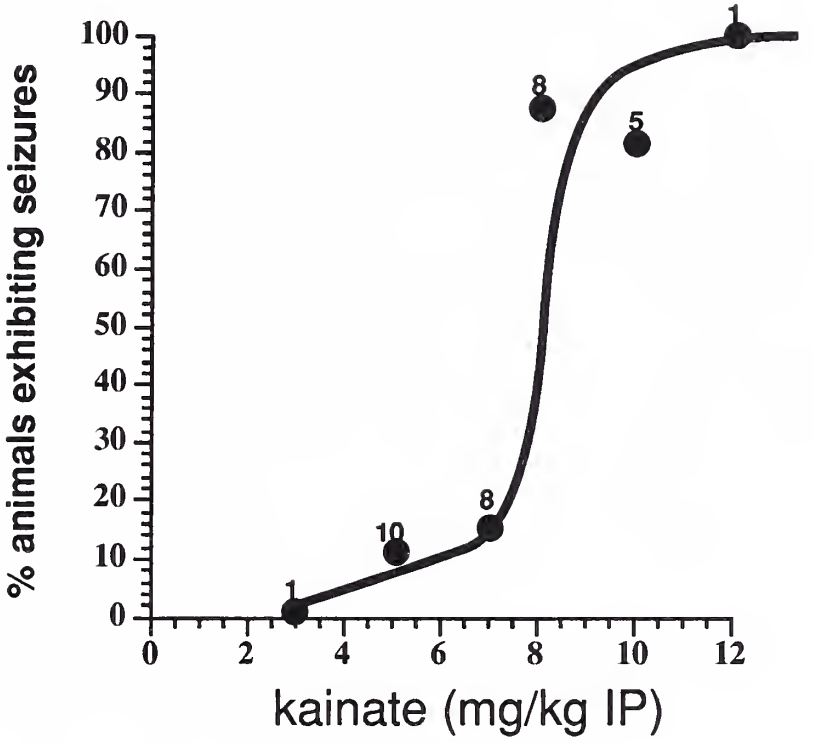


FIGURE 6: The kainate dose-response curve

This graph illustrates the percentage of animals exhibiting overt behavioral seizures. (Numbers of animals at the dose are over black dots).

Figure 7

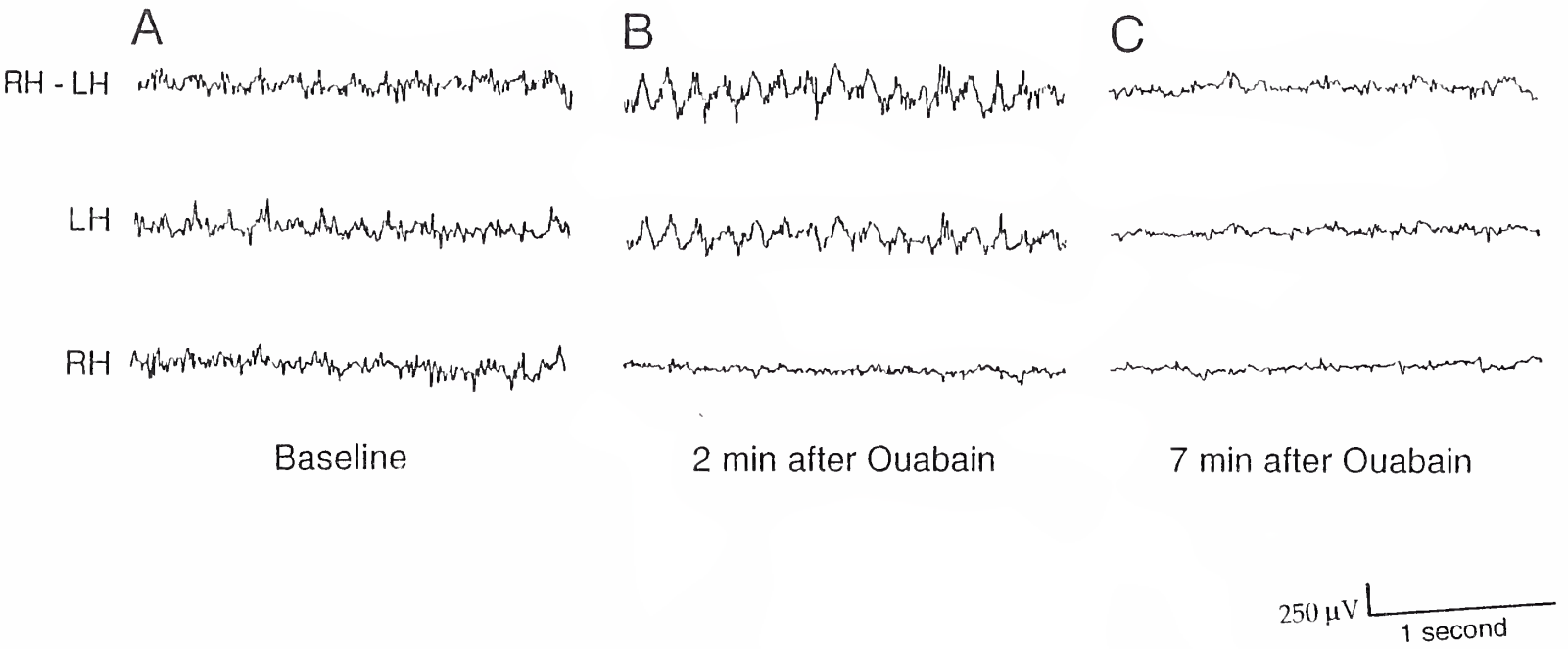

A
B
C
RH - LH
LH
RH
Baseline
2 min after Ouabain
7 min after Ouabain
250 µV
1 second

Figure 7: **Electroencephalographic recording during ouabain (3 nmoles; i.c.v).**

Panel A shows typical, baseline, resting activity prior to injection. Panel B shows hippocampal activity 2 min after start of ouabain infusion. There is decreased EEG amplitude in the right hippocampus (RH) - the hemisphere containing the infusion port - with persistent baseline activity in the left hippocampus (LH). The effects of ouabain are seen ~ 5 min later in the left hippocampus with persistent suppression of activity in the RH (Panel C). (RH - LH: potential difference between RH and LH in reference to frontal lobe electrode).

Figure 8

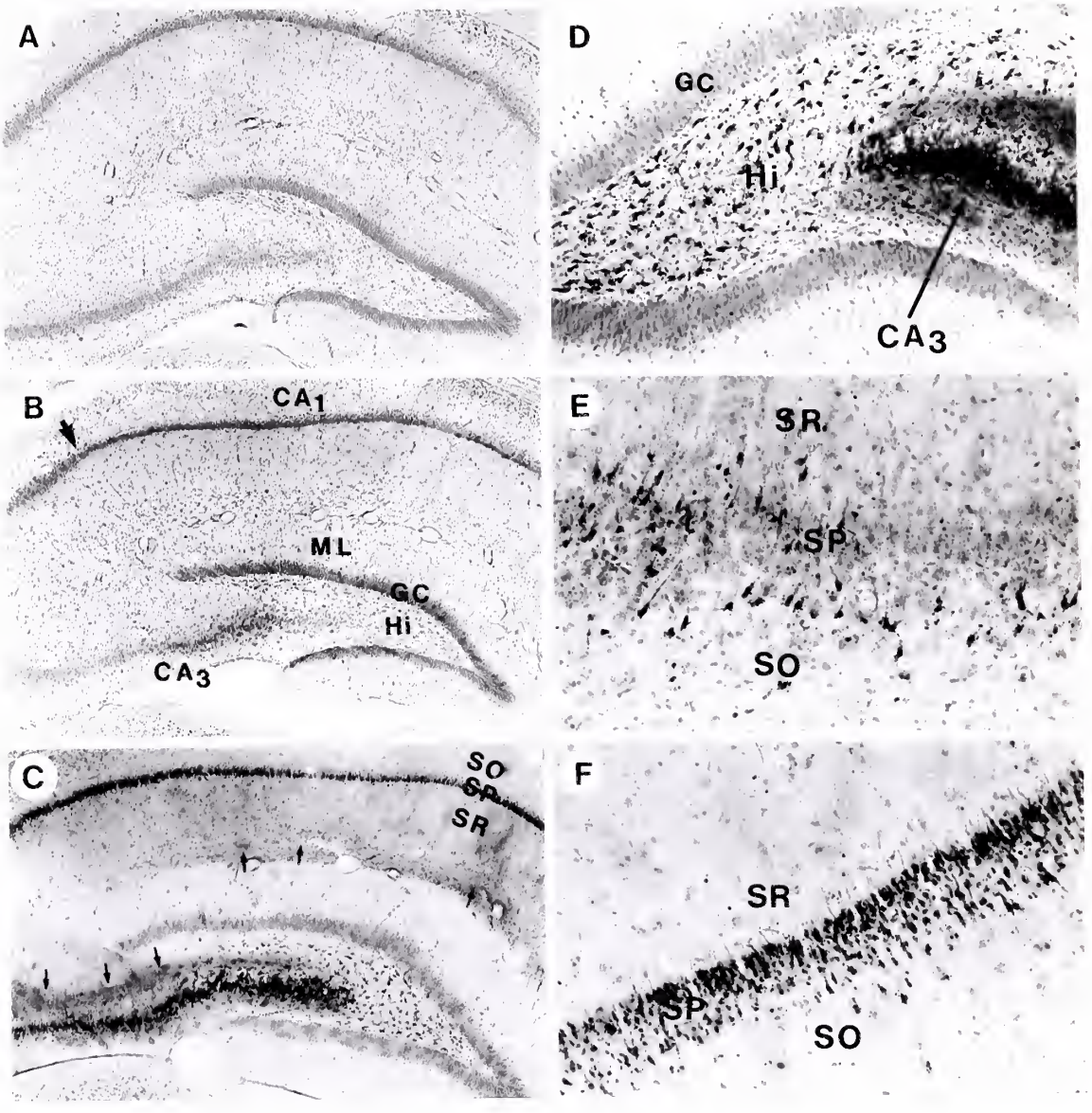


Figure 8: **Neuronal death in the dorsal hippocampus associated with various treatments.**

Ouabain (3nmoles; i.c.v.), although producing an extreme pattern of motor behavior did not produce neuronal death (panel A). Non-seizure producing dosages of kainate (e.g. 5 mg/kg; panel B) do not cause neuronal death as assessed by silver degeneration stain which shows dead/dying cells and their processes as black (arrow in panel B seperates CA2 and CA1 regions). Pairing of ouabain and kainate produced widespread death throughout the hilus (Hi) and CA1/CA3 subfields (panel C). Note fiber staining of apical dendrites of pyramidal cells in *stratum radiatum* (SR) in panel C (small arrows). Similar to human TLE, pyramidal neurons of the CA2 subfield were predominantly spared. A variety of hilar neurons are affected (Panel D: Higher magnification and orientation reversed) including those of the mossy cell morphology. Panels E and F correspond to high power views of degenerating CA3 and CA1 pyramidal neurons. (ML : molecular layer of the dentate gyrus; GC: granule cell layer; SO, SP, and SR: *stratum oriens, pyramidale, and radiatum* , respectively).

Figure 9

A B

Figure 9: **[^{3}H]Ouabain Binding Studies**

A. CSF controls

B. Ouabain-treated aninals.

Autoradiography to assess Na^+/K^+pump density was achieved by pre-incubating sections to allow bound ouabain (administered *in vivo*) to dissociate and then incubating with [^{3}H]-ouabain to equilibrium. Densitometric analysis revealed that on average, binding density approximately doubled with ouabain pre-treatment compared to controls ($p < 0.001$; chi-square analysis; $n = 3$).

Figure 10

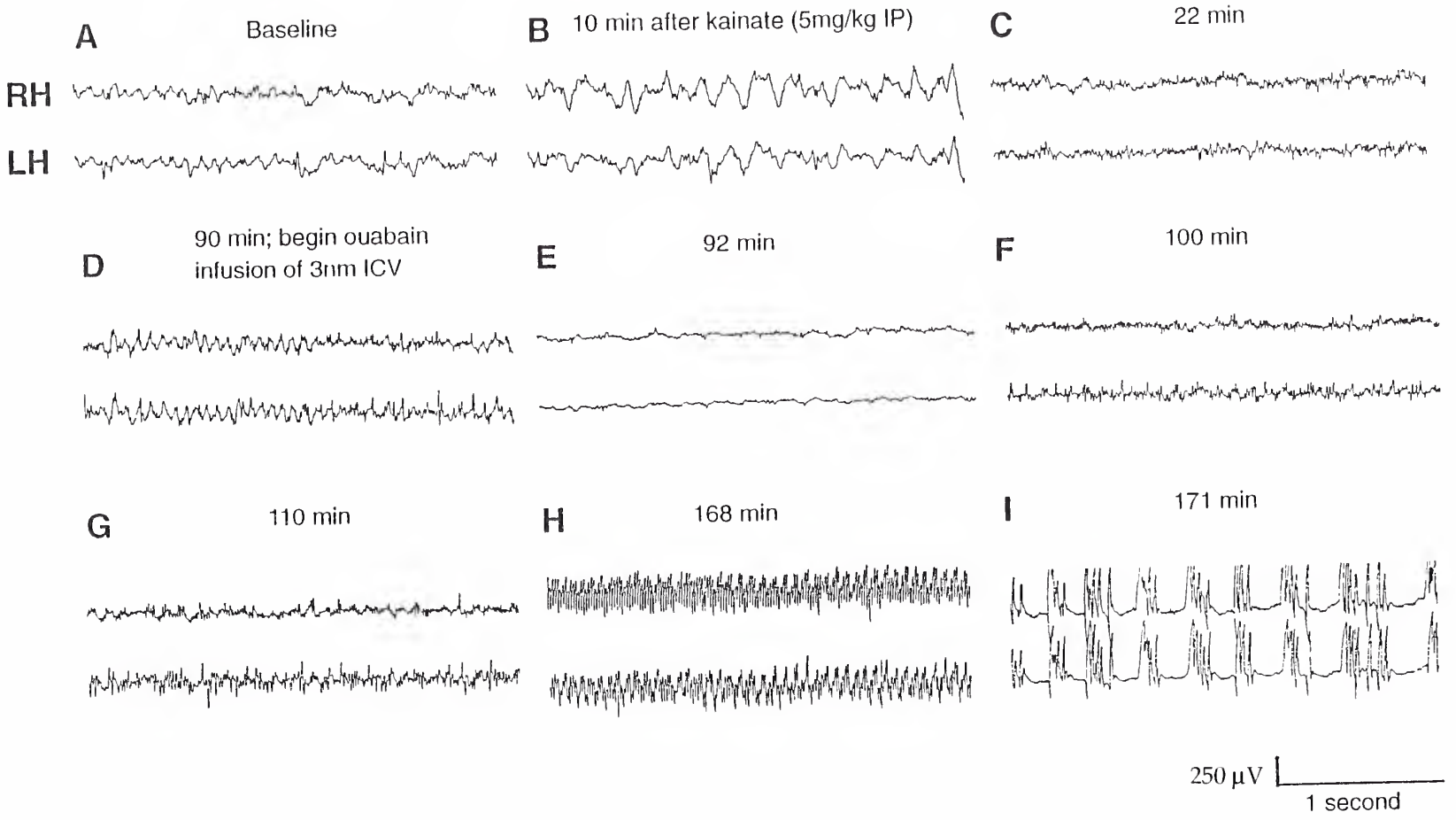

A
Baseline
B
10 min after kainate (5mg/kg IP)
C
22 min
RH
LH
D
90 min; begin ouabain infusion of 3nm ICV
E
92 min
F
100 min
G
110 min
H
168 min
I
171 min
250 µV
1 second

Figure 10: **Representative electroencephalographic recording of ouabain and kainate-treated animal.**

A. Shows typical, baseline resting activity prior to drug injection.

B. Shows hippocampal activity 10 min after a subtoxic dose of kainate (5 mg/kg; i.p.) was injection (note prominent spikes). Later recordings, panels (C & D), show progression of activity back towards baseline. Panel E shows ouabain (3 nmoles) injection into the right ventricle; note drastic suppression of bilateral hippocampal activity. Activity resumes promptly, displaying progressively higher frequencies and amplitude (panels F, G). Panel H and I shows two patterns of EEG activity during seizures. (RH: right hippocampus; LH: left hippocampus).

Figure 11

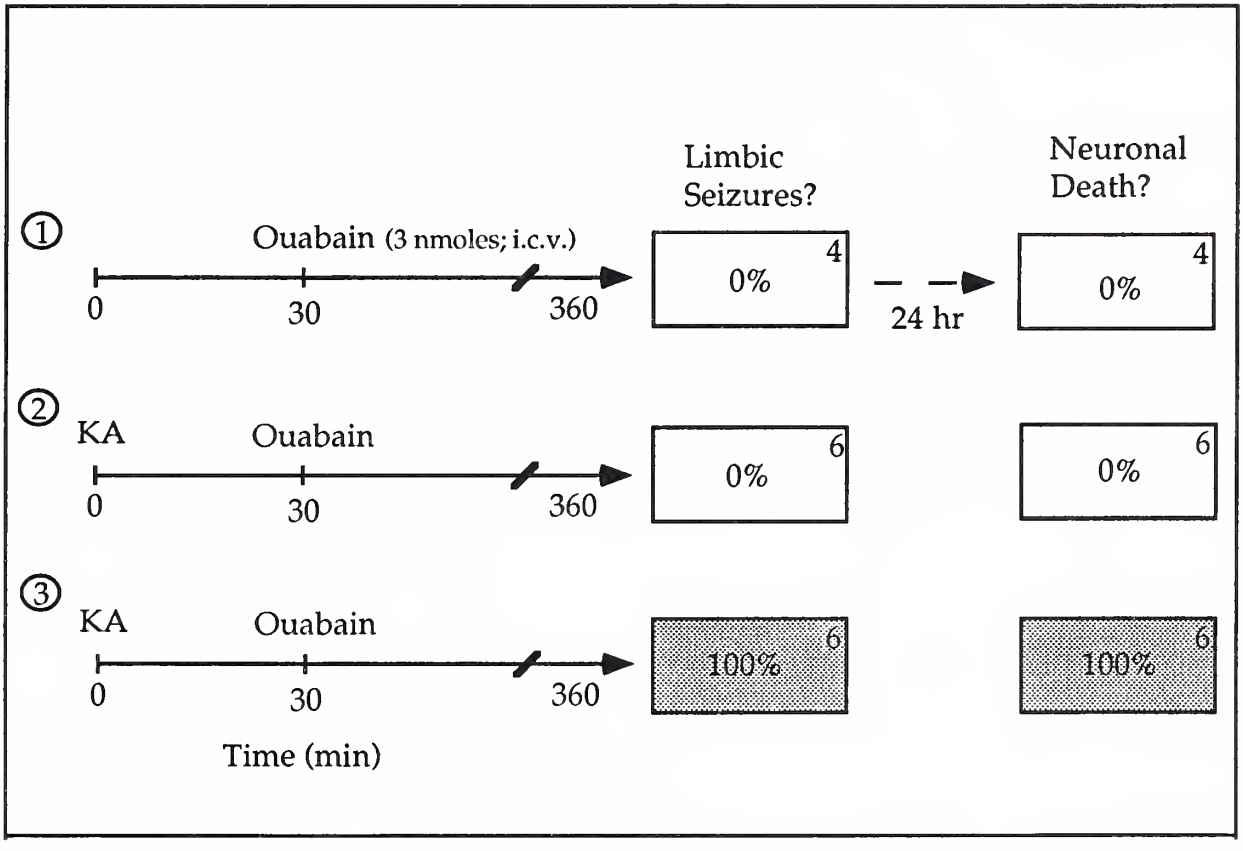

Limbic Seizures?
Neuronal Death?
①
Ouabain (3 nmoles; i.c.v.)
0
30
360
0%
4
24 hr
0%
4
②
KA
Ouabain
0
30
360
0%
6
0%
6
③
KA
Ouabain
0
30
360
100%
6
100%
6
Time (min)

Figure 11: **Ouabain potentiates sub-toxic kainic acid.**

This figure shows results of experiments to test the hypothesis that partial inhibiton of Na^+/K^+ pump capacity with ouabain increases kainate neurotoxicity. 1) Four control animals were treated with ouabain alone and none of these animals (0%) experienced seizures or neuronal death. 2) Six control animals were treated with kainic acid alone and no animals (0%) experienced seizures or neuronal death. 3) Six animals were treated with a combination of ouabain and kainate and all six (100%) experienced seizures and neuronal death. (Number of animals and percentage of positive outcomes appear in boxes below questions investigated; KA: Kainic acid).

Figure 12

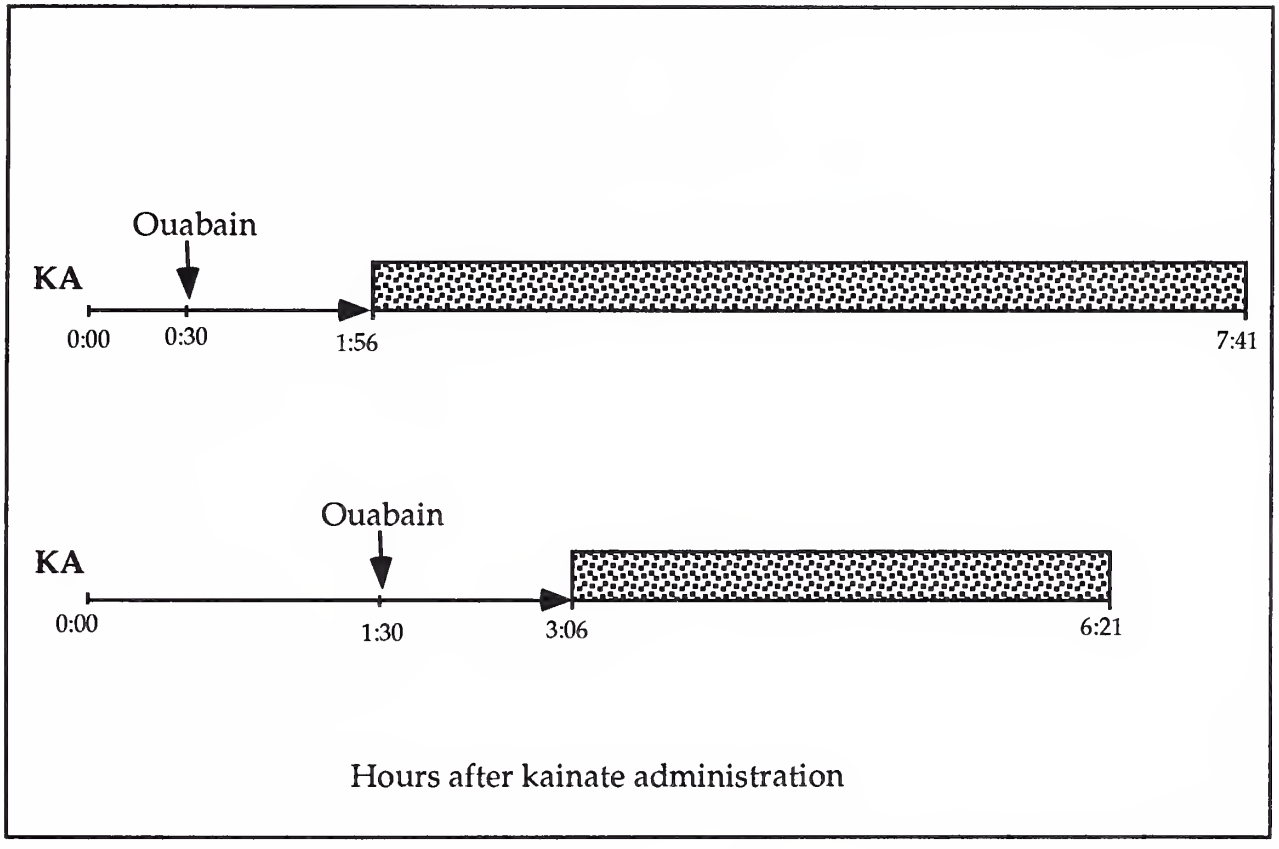

Ouabain
KA
0:00
0:30
1:56
7:41
Ouabain
KA
0:00
1:30
3:06
6:21
Hours after kainate administration

Figure 12: **A delay in ouabain administration increases the latency of seizure onset after low-dose kainate but shortens seizure duration.**

When subtoxic dosages of kainate (5mg/kg; i.p) were followed 30 min later by ouabain (3 nmoles; i.c.v.), severe seizures routinely developed with an average latency of 116 $\pm$ 13 min (SEM) hrs after kainate. These convulsions continued for a duration of 345 $\pm$ 9 min (SEM). Delaying ouabain administration by 90 min increased the latency of the onset of seizures (by 70 $\pm$ 15 min), and the duration of epileptic discharges under this paradigm shortened notably (~150 min). (KA: Kainic acid).

Figure 13

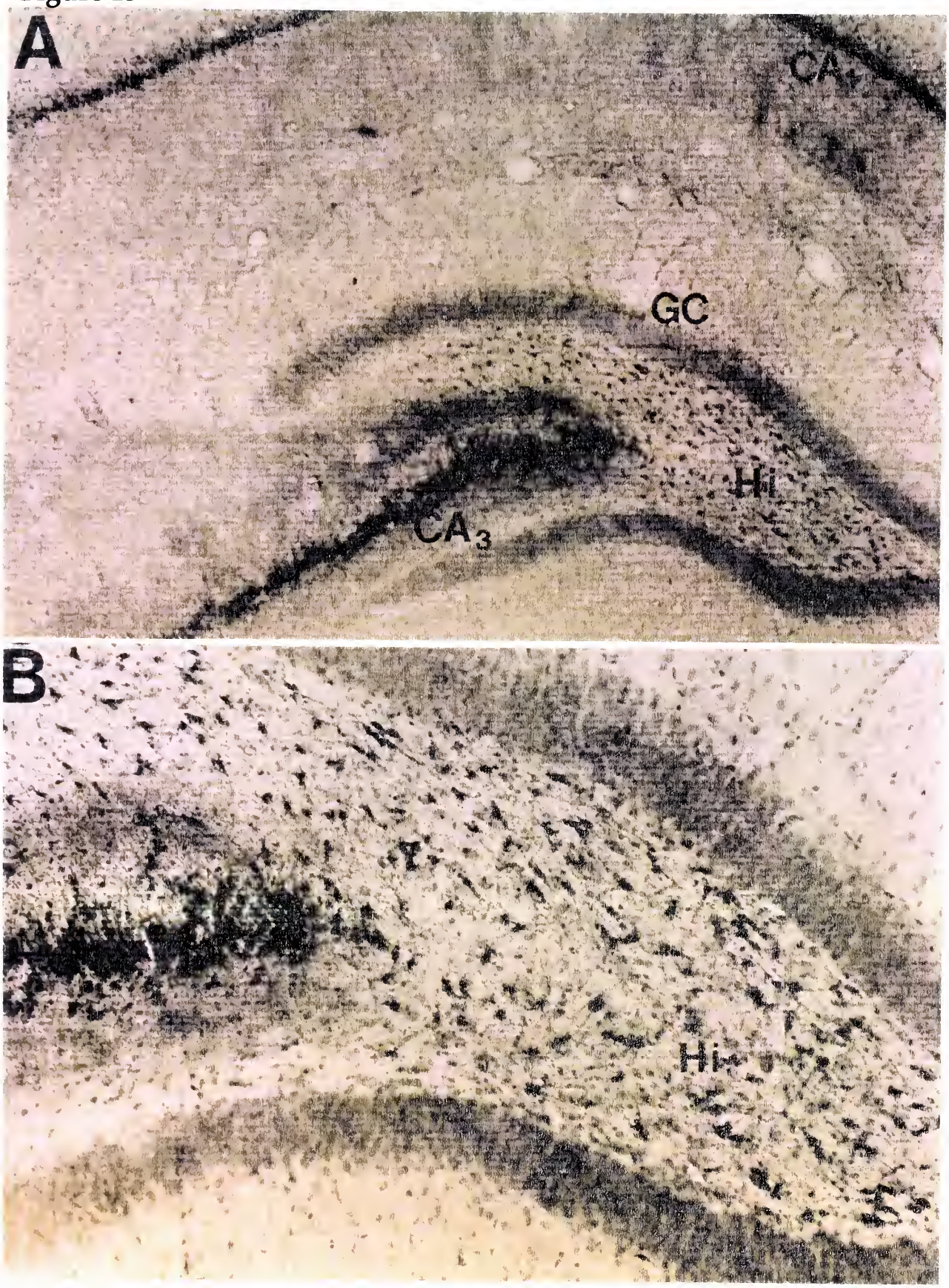


Figure 13: **Silver stain of hippocampus of animal treated with ouabain (1 mg/kg; i.p.) and kainate (7 mg/kg; i.p).**

A. Pyramidal cells of areas CA3 and area CA1 are heavily damaged (dark silver granules). Neuronal death is also present in the hilus (Hi). Cells of the granule cell layer (GC) and area CA2 (not shown) are relatively spared.

B. Higher magnification of hilus in A, showing "selective" hilar damage.

Figure 14

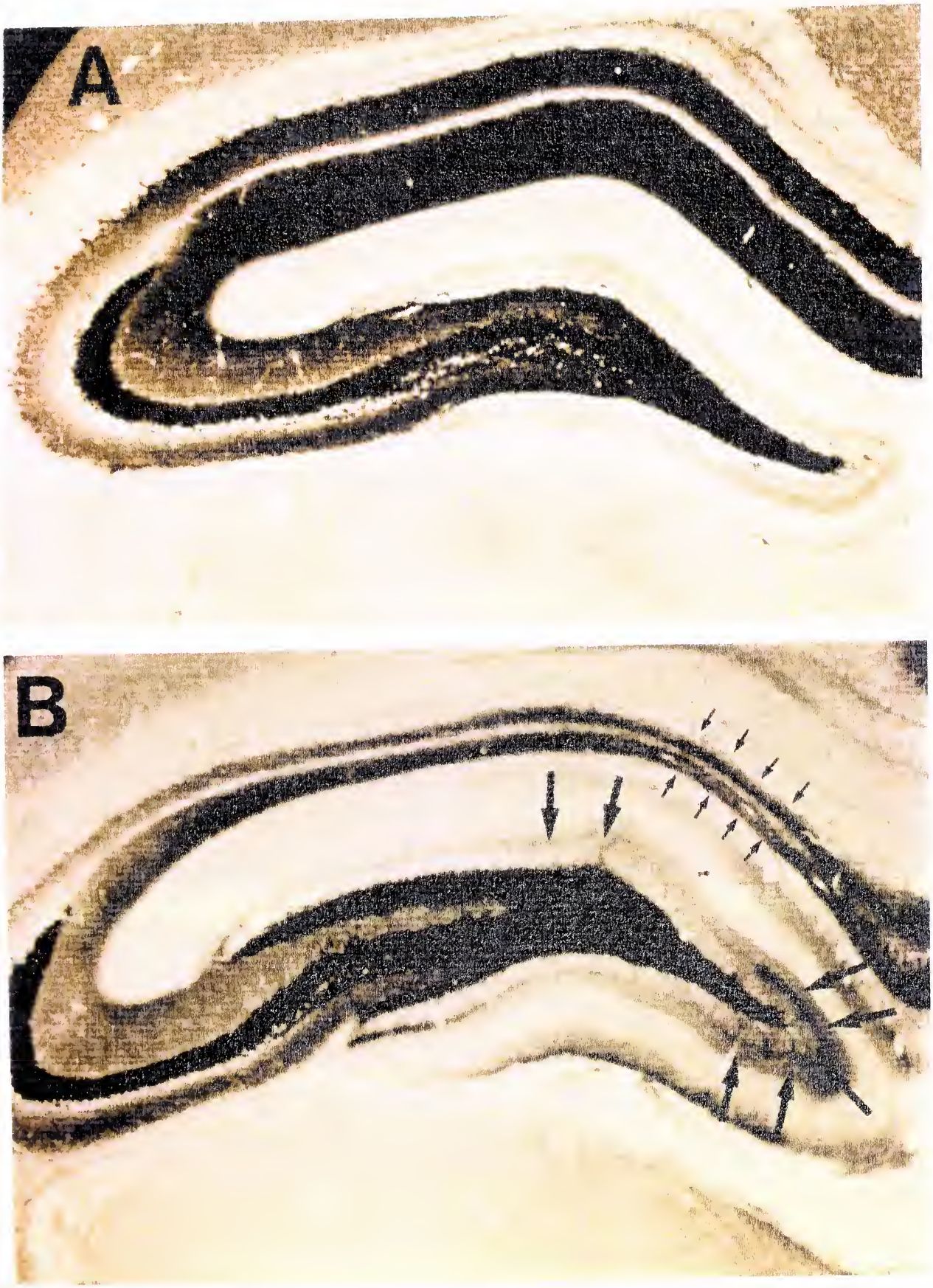


Figure 14: **Timm stain of rat hippocampus**

A. Control

B. 30 days after treatment with kainate (7mg/kg; i.p.) and ouabain (1 mg/kg; i.p). Sprouting of mossy fibers into the inner molecular layer of the dentate girus is present (large arrows). A prominent loss of staining is observed in area CA1 (small arrows), reflecting significant damage to cells in this area by initial treatment with kainate/ouabain.

DISCUSSION

THE KAINATE DOSE RESPONSE CURVE

As previously stated, the response to kainate in many animals including rats is not only dose dependent, but it is also influenced by age, sex, route of administration, and strain variations in the same species of animal (Sperk, 1994). Steep dose-response curves similar to the one observed in this study have previously been reported, albeit under different conditions. Lothman and co-workers (Lothman and Collins, 1981) administered increasing amounts of kainate (0.3 - 12 mg/kg) intravenously to albino rats weighing 280 - 350 g and identified three dose ranges: with a low dose range (0.3 and 1 mg/kg) animals exhibited staring only, without signs of seizure activity; animals treated with an intermediate dose of kainate (4 mg/kg) exhibited staring, WDS, automatisms and mild limbic convulsions, but never experienced severe limbic convulsions; and animals treated with high-dose kainate exhibited a range of seizure manifestations including severe limbic convulsions.

The characteristic steepness of the dose-response curve observed may be partly explained by changes in BBB permeability after systemically administered kainic acid. Kainate penetrates the BBB poorly. Less than one percent of the systemically injected kainic acid reach the brain (Berger et al., 1986). Access to the brain after systemic administration is also gradual, reaching plateau levels in perfused brain of 10-day old Winstar rats in approximately the same time as the onset of seizures (~ 20 min). Studies

have shown that this kinetics of CNS penetration is partly dependent on BBB disruption by increasing doses of kainic acid reaching the brain (Saija et al., 1992). Thus, at sufficient doses of kainate, CNS penetration by the neurotoxin become synergistic, gradually enhanced by disruption of BBB, leading to the sigmoid shape of the dose response curve observed (Figure 6).

OUABAIN INDUCES 2-FOLD INCREASE IN NA^+/K^+PUMP DENSITY

In this study, the concentrations of ouabain used allowed inhibition of the high-affinity ($\alpha 2/\alpha 3$) Na^+/K^+ pump isoforms. This preferential inhibition, as already explained, establishes partial impairment of total Na^+/K^+ pump capacity (Brines and Robbins, 1992). Total inhibition of brain Na^+/K^+ pump would kill animals, since neurons require some Na^+/K^+ pump for cellular integrity (Garthwaite et al., 1986; Lees et al., 1990).

In assessing Na^+/K^+ pump density in ouabain-treated animals, conditions of incubation were selected to activate the Na^+/K^+ pump uniformly by providing the required cations and sufficient ATP (Caspers et al., 1987; Brines et al., 1995). The paradoxical increase in the Na^+/K^+ pump density in ouabain-pretreated animals is most likely in response to impairment with ouabain. It is likely that complete inhibition of the high affinity ($\alpha 2/\alpha 3$) isoforms induced a near total replacement of these pump units. Consequently, a doubling in the population of high-affinity isoforms, was detected. Although the exact mechanisms of upregulation are not clear (via production of new protein or cell surface translocation from inactive pools) this type of recruitment of additional pump units may result in a *net* reduction of the total (active + dormant) Na^+/K^+ pump capacity. However Na^+/K^+pump inhibition could not be quantitated by the technique used here.

OUABAIN POTENTIATES KAINATE NEUROTOXICITY

Our experimental results demonstrated that partial inhibition of Na^+/K^+ pump capacity decreases neuronal ability to survive otherwise innocuous excitatory stimuli. All experimental animals treated with kainate (5 mg/kg; i.c.v.) followed with ouabain (3 nmoles; i.c.v.) experienced seizures and subsequent brain damage, however no control animals experienced seizures or neuronal injury. Further experimental evidence suggested that neurotoxicity due to kainate (5 mg/kg; i.p.) was intimately associated with Na^+/K^+ pump inhibition. Thus time of seizure onset after kainate treatment was dependent on time of ouabain administration (Figure 12). The shortened duration of epileptiform activity observed when ouabain is administered at 90 min, instead of 30 min after kainate, could be explained by decreasing brain levels of kainate with time. Such that, when ouabain is administered at 90 min, lower brain levels of kainate are present. Indeed, the behavioral manifestations as well as neurotoxicity of systemically administered kainate is dose-dependent (Sperk et al., 1983; Ben-Ari, 1985), as supported by our dose-response curve. These findings were replicated by intraperitoneal injections of both kainate and ouabain. Thus, experimental groups treated with a combination of kainate and ouabain were significantly different from controls, producing seizures and subsequent neuronal death ($p< 0.001$; chi-square analysis).

EEG recording after administration of kainate (5 mg/kg i.p.) revealed increased neuronal activity above baseline. This can be explained by the excitatory action of kainate, since neuronal exposure to low levels of glutamate or its analogs (including kainate) leads to immediate depolarization, influx of Na^+ and efflux of K^+ (Rothman, 1985). Under these conditions, the Na^+/K^+ pump is secondarily activated to rectify the resultant

ionic disturbance (Rossier et al., 1987; Skou, 1988). Indeed, increased metabolic activity correlating with systemic treatment with kainate has been observed with 2-deoxyglucose uptake studies. Thus, when male Wistar rats were treated with kainate (i.v.), increased glucose uptake was detected in the brain, albeit only in the hippocampus and lateral septum at low-dose kainate (6 mg/kg). At higher doses (12 mg/kg), increased metabolic activity was detected in other brain regions as well (Saija et al., 1992). However, as evidenced by the absence of brain damage in control animals, homeostatic mechanisms, most likely dependent on Na^+/K^+ pump activity, prevented neuronal injury from sub-toxic kainate (5 mg/kg; i.p.).

The scenario, however, changes with injection of ouabain (3nmoles; i.c.v.) 30 min after kainate (5 mg/kg; i.p.) treatment. Impairment of Na^+/K^+ -ATPase in these circumstances may interfere with neuronal mechanisms to cope with an otherwise innocuous brain level of kainate. Precedence for this concept exists *in vitro*. Stimulation of CA1 pyramidal cells with impairment of Na^+/K^+pump activity markedly reduced the capacity of these cells to restore transmembrane gradients (Novelli et al., 1988). Also, inhibition of Na^+/K^+ -ATPase function in these neurons markedly potentiated and prolonged glutamate depolarization (Thompson and Prince, 1986; Fukuda and Prince, 1992).

MECHANISMS BY WHICH OUABAIN MAY POTENTIATE KAINATE

There are several potential mechanisms by which ouabain may potentiate kainate neurotoxicity.

Increased endogenous levels of glutamate or its agonists

In addition to an already higher than normal brain level of kainate imposed by our experimental design, endogenous extracellular levels of

glutamate may rise due to Na^+/K^+ pump inhibition. Sampling of rat brain extracellular milieu by microdialysis after Na^+/K^+ pump inhibition with ouabain have shown 2-3 fold increase in the release of glutamate (Jacobson et al., 1986; Westerink et al., 1989). Furthermore, inhibition of Na^+/K^+ ATPase may lead to a block of glutamate re-uptake mechanisms, causing an increase in intracellular levels of endogenous glutamate (Kanner and Sharon, 1978; Dagani and Erecinska, 1987). Since the Na^+/K^+ pump is required for repolarizing the cell membrane after depolarization, chronic neuronal depolarization (by glutamate and kainate) in the face of reduced Na^+/K^+pump capacity may lead to inadequate neuronal repolarization (Skou, 1988). This situation would lead to increased excitability of the neuron and subsequent seizure generation.

Release of other potentiating compounds

Infusion of ouabain into rat striatum has been demonstrated to produce a massive release of dopamine (Westerink et al., 1989; Fairbrother et al., 1990). On the other hand, since dopamine may inhibit Na^+/K^+-ATPase (Bertorello et al., 1990), a vicious cycle may be created to perpetuate a spiral decrease in Na^+/K^+ ATPase activity. Other compounds released after Na^+/K^+ pump release include gamma-amino butyric acid (GABA) and acetylcholine (O'Fallon et al., 1981; Jacobson et al., 1986; Westerink et al., 1989). Indeed, GABA has been shown to accelerate cortical neuronal death in vitro (Erdo et al., 1991). Also, acetylcholine can potentiate glutamate toxicity of hippocampal cell cultures *in vitro* (Mattson et al., 1989).

Limited Na^+/K^+ pump reserve capacity

The paradoxic doubling of Na^+/K^+ pump binding sites in the brain of animals treated with ouabain (i.c.v.) as observed by tritiated ouabain studies has already been discussed. Although the concept of neuronal recruitment of Na^+/K^+ ATPase reserve-capacity to meet increasing demand is not clearly understood, it is likely that the cell is unable to recruit reserve pump units *fast* enough to prevent irreversible injury. Conversely, a scenario may be engendered in which all recruited pumps (whether by translocation to the cell surface from existing stores or by *de novo* synthesis) are readily inhibited by ambient ouabain. Since ouabain was used in the micromolar range, the high affinity $\alpha 2/\alpha 3$ isoform may be chronically inhibited, and $\alpha 1$ isoforms may in fact become the only recruitable Na^+/K^+ pump pool. Indeed it has been suggested that the significance of differential affinities and Km's observed for rodent Na^+/K^+ pump isoforms may be a teleological one: to allow the neuron to establish reserve Na^+/K^+ pump capacity for recruitment as needed (McGrail et al., 1991; Brines and Robbins, 1993). In any case, with unmatched demand for pump activity, the stage may be created for irreversible events of excitotoxicity (Choi, 1992).

Synergistic disturbance in the neuronal ionic milieu

As already introduced, neuronal exposure to EAA leads to disturbance in ionic balance resulting primarily from Na^+ and Ca^{++} influx and K^+ efflux. Extensive evidence has shown that the ionic changes in Na^+, K^+, and Ca^{++} occurring with direct or indirect inhibition of the Na^+/K^+ pump (Baker et al., 1969; Goddard and Robinson, 1976; Reeves and Sutko, 1979; Ullrich et al., 1982) are exactly those observed by excitotoxicity, albeit by different mechanisms (Choi, 1988; Kohr and Heinemann, 1988; Stable et al., 1990).

Consequently, ouabain may potentiate kainate neurotoxicity by a synergistic ionic disturbance.

Excitotoxicity as a final common pathway

Any of the above described mechanisms may eventually lead to a net increase in intracellular Na^+ due to compromised or insufficient pump activity. Water may follow passively into the cell and cell swelling may result. This would mark the first phase of excitotoxicity. Subsequently, the second phase of excitotoxicity may ensue if pump capacity is continuously impaired. This would be marked by Ca^{++} influx and inevitable neuronal injury. (Choi, 1992). Neuronal injury resulting from Na^+/K^+ pump inhibition during excitatory stimulation has been previously observed *in vitro* by Brines and co-workers. Up to a 6-fold increase in neuronal injury (as assessed by lactic dehydrogenase release) was observed when ouabain treated cultures were stimulated with glutamate (Brines and Robbins, 1992). As suggested by Choi, "[neuronal] insults are likely to induce cell death by several simultaneous injury processes (Choi, 1992)." Occasionally, some of these processes may act synergistically to induce cell death when the summation of their individual effects overwhelms the cell's primary ionic homeostatic mechanism, the Na^+/K^+ pump.

Evidence from human epileptic hippocampi

Results of studies from surgical specimen of epileptic hippocampi provide some impetus for the hypothesis tested here. Mapping of Na^+/K^+ pump density per unit of protein for various regions of epileptic and non-epileptic hippocampi revealed that hippocampal areas usually destroyed in epilepsy (e.g. area CA1) possessed the lowest total density of Na^+/K^+pump units, whereas those more resistant areas (area CA2, the dentate molecular

layer, and the subiculum) possessed the highest Na^+/K^+ pump density (Brines et al., 1995). In this same study, activity of cytochrome c oxidase, the enzyme producing ATP required for Na^+/K^+ pump function was generally reduced in epileptic hippocampi compared to hippocampi from autopsy controls. Consequently, although pump density was determined to be increased in vulnerable areas of the brain in epileptic patients, the capacity to power them was reduced. These findings suggested two things: 1) in the epileptic hippocampi, cells expressing lower densities of Na^+/K^+ pump are more susceptible to excitotoxicity as may occurs in seizures and 2) a defect in energy production may be part of the pathoetiology of epilepsy.

NEUROPATHOLOGY

The pattern of cell death observed after kainate/ouabain treatments, like that observed with high-dose kainate, is one of selective cell damage. A notable difference, however, include death to pyramidal cells in area CA1 with kainate/ouabain treatments. These cells express only low levels of kainate receptors (Monaghan and Cotman, 1982) and this may explain their lower vulnerability to kainate-only treatment. However, like other cells in the hippocampus, CA1 cells express high levels of glutamate receptors (Sperk, 1994). Consequently, increase in endogenous glutamate levels either from inhibition of re-uptake mechanism (Kanner and Sharon, 1978; Dagani and Erecinska, 1987) or by neuronal depolarization (Jacobson et al., 1986; Westerink et al., 1989) may lead to excitotoxicity of CA1 cells. Conversely, these cells may have been damaged by hypoxia, since they are exquisitely sensitive to this insult (Wasterlain et al., 1993). Although arterial oxygen saturation were not measured during seizures, apneic episodes were not observed to suggest cell death by hypoxia.

On the other hand, the destruction of pyramidal cells of hippocampus area CA3 observed with the combination of kainate and ouabain have been observed in several other situations. These cells are vulnerable to several treatment including kainate alone (Ben-Ari et al., 1981), ischemia (Johansen et al., 1987), and electrical stimulation of the perforant pathway into the hippocampus (Sloviter, 1987). The exceptional vulnerability of these cells may be explained by one or a combination of several factors. Cells in area CA3 express the highest density of kainate receptors in the hippocampus (Monaghan and Cotman, 1982; Berger and Ben-Ari, 1983; de Lanerolle et al., 1992) making them more vulnerable to direct kainate excitation. Secondly, area CA3 is the region of the termination of a major excitatory pathway to the hippocampus, the perforant pathway (Figure 1). Consequently, these neurons may be chronically stimulated during seizures. Thirdly, the hippocampus, the olfactory cortex, the retina, and cerebellum are among some of brain regions expressing the highest level of Na^+/K^+ATPase. Consequently, Na^+/K^+ pump inhibition may be particularly severe in the hippocampus making these cells more vulnerable. It is unlikely that ouabain acts by other mechanisms such as modification of cerebrospinal fluid to produce neuronal injury, since the choroid plexus appears to express only the $\alpha 1$ (low affinity) isoform (Brines et al., 1991).

Although it is believed that impairment of Na^+/K^+ pump capacity led first to seizure generation and subsequently to neuronal injury, the possibility that seizure activity itself contributes to the pathology observed in kainate/ouabain treated animals cannot be ruled-out. Parenteral administrations of kainic acid may not allow toxic levels of kainic acid to be reached in the vicinity of all neurons. Initiation of seizure activity in areas of low seizure threshold such as area CA3 may exert strong excitatory actions

upon certain neuronal pathways (Figure 1), which in turn may cause seizures and distant, albeit excitotoxic, brain damage in certain target neurons. The most direct support for this mechanism comes from the work of Sloviter and co-workers. These investigators demonstrated that sustained stimulation of the perforant path (Figure 1) in the rat results in similar damage of CA3 and hilar neurons of the hippocampus as observed in kainic acid induced seizures (Sloviter, 1983). Further support for this mechanism stems from the effect of anticonvulsants on kainate-induced neurotoxicity. The mode of action of these drugs is such that they do not interfere with kainate binding to neuronal cells (hence the direct interaction of kainate with neuronal cells persists), instead their neuroprotective action is achieved by increasing the seizure threshold (Bernard et al., 1980; Frandsen et al., 1990; Stone and Javid, 1980). Thus, these agents reduce neurotoxicity by mitigating a contribution from seizure activity. In this respect, seizure activity itself, by establishing a chronic, recurrent neuronal excitation may contribute to neuronal cell death.

A second seizure-related mechanism of kainate neurotoxicity suggests that brain damage may be related to hypoxia, hypoglycemia or edema. Kainic acid induced damage to the *frontobasal* as well as the *temporobasal* portions of the brain have been attributed to this mechanism. A characteristic incomplete parenchymal necrosis is observed in these areas (Spielmeyer, 1927; Levine, 1960), although the pattern of cell death is still quite different from that observed by hypoxia or ischemia alone (Sperk et al., 1983). It has also been suggested that the early edema resulting from severe seizures may compromise venous drainage and blood supply to affected brain areas (Spielmeyer, 1927; Lassmann et al., 1984; Lassmann, 1984; Sperk et al., 1983). This mechanism may play a role in damage to extra-hippocampal brain areas; however, there is less support for its role in the damage to Ammon's horn

during seizures. Since, substantial decrease (35%) in partial oxygen pressures in the hippocampus during the initial phases of seizure activity is compensated for by at least a 3-fold increase in local blood flow in this region (Pinard et al., 1984).

TEMPORAL LOBE EPILEPSY AND ANIMAL MODELS

The human pathology

In the period since Sommer's qualitative description of Ammon's horn sclerosis (Sommer, 1880), several more elaborate and quantitative descriptions of the neuropathology of TLE have been presented (Margerison and Corsellis, 1966; Corsellis and Bruton, 1983; Babb et al., 1984; Gloor, 1991; de Lanerolle et al., 1992 and 1994; Kim et al., 1990). The present consensus is that neuropathology of human TLE is mostly confined to the hippocampus and involves other brain regions only minimally. Also, a highly variable pattern of cell loss has been described (de Lanerolle et al., 1994). Cell loss may be minimal, involving only hilar interneurons (Margerison and Corsellis, 1966) or it may be pervasive throughout Ammon's horn with substantial (>50%) loss of CA1 - CA4 pyramidal cells and dentate granule cells (Babb et al., 1984; Kim et al., 1990). Granule cells and CA2 pyramidal cells are generally less vulnerable, although all neuronal populations can be affected. Furthermore, damage to cells of the dentate gyrus is selective, with preferential loss of interneurons expressing somatostatin (SOM), neuropeptide Y (NPY) and substance P (SP) (de Lanerolle et al., 1989). In fact, the selective loss of these interneurons is a reliable diagnostic feature of mesial TLE patient (de Lanerolle et al., 1994). Another histological feature of epileptic hippocampi is neuronal plasticity and remodeling. Sprouting describes a process of axonal growth and establishment of new fibre systems in the epileptic hippocampi. Although several types of sprouting have been observed, mossy fiber sprouting into the dentate inner molecular layer has been more extensively studied. The functional consequence(s) of sprouting remains to be established, although it is thought that sprouting may participate in the pathology of human TLE (Sloviter, 1992).

Animal models

There has been considerable effort to create an experimental animal model to study the etiology of seizures and neuronal damage in human TLE. To bear some relevance to the human disease, a useful animal model must fulfill the following criteria at least: (a) the focus of seizure generation must be in the hippocampus; (b) it must be chronic, reproducing spontaneously and repetitively; (c) the pathology must resemble that observed in the human disease, showing damage to pyramidal cells, granule cells, and hilar interneurons as described above; (d) damage to hilar interneurons must be selective for NPY, SP, and SOM expressing neurons; and (e) it must show evidence of hippocampal plasticity (sprouting).

The kainate, rat model

Kainic acid has been administered intraperitoneally (Ben-Ari et al., 1981), intravenously (Lothman and Collins, 1981) intraventricularly (Nadler et al., 1987), and directly into the hippocampus (Ben-Ari et al., 1979; Schwob et al., 1980). Limbic structures, primarily the hippocampus, are the focus of seizure activity for kainate treated animals exhibiting seizure activity (Sperk, 1994). Spontaneous seizure recurrence has been observed with this model (Pisa et al., 1980; Cronin and Dudek, 1988). Kainate-lesioned hippocampi also show long term remodeling (Tauck and Nadler, 1985; Cronin and Dudek, 1988). However, as already introduced, there are several differences between this model and the human pathology. To adequately replicate the human pathology, high doses of kainate are used. This not only leads to a high animal mortality (Ben-Ari et al., 1981, Lothman and Collins, 1981; Sperk, 1994), but also it leads to widespread, extra-hippocampal brain damage not commonly observed in the human pathology (Ben-Ari et al., 1981).

Moreover, cells of area CA1 which are destroyed in the human disease are not routinely damaged in the kainate rat model. From the data available, it appears that the kainate rat model does not replicate the selective hilar cell loss observed in the human disease. In contrast to the human situation, hilar SOM mRNA content was observed to decrease between 10 - 30 days after kainate treatment, where as NPY mRNA increased and SP content remained unchanged (Sperk, 1994).

The pilocarpine model

This model uses intraperitoneal injections of pilocarpine to induce severe motor limbic seizures and status epilepticus in rats (Turski et al., 1983; Turski et al., 1989). Following the acute seizure episode, the animals show relatively normal behavior for several weeks, and then a high percentage develop spontaneous recurrent seizures (Leite et al., 1990; Cavalheiro et al., 1991). Evidence for substantial mossy fiber recurrent sprouting has also been shown to occur in the hippocampus of these animals (Mello et al., 1990). However, the pathology described for this model considerably falls short of the human scenario. Widespread, extra-limbic damage not observed in the human pathology, including damage to the mediodorsal thalamic nuclei, substantia nigra and neocortex is commonly observed (ibid). Damage of CA3 and CA1 neurons is achieved at high levels of pilocarpine, resulting in high animal mortality (Turski et al., 1986). Although, the pattern of hilar cell loss is not completely characterized in this model, preliminary studies suggest a loss of GABAergic interneurons (Obenaus et al., 1993). This is in sharp contrast to the human pathology in which GABAergic interneurons are quite resistant to injury (Babb et al., 1989). The status of SP, NPY, and SOM containing hilar interneurons remains to be established for this model.

The afferent excitation model

Sloviter and coworkers (Sloviter, 1983; Sloviter, 1987) advanced the afferent excitation model produced by 8 - 24 hrs of intermittent stimulation of the perforant pathway in anesthetized rats. This method reliably results in selective damage of specific neurons in the dentate hilus, including SOM and NPY containing interneurons (Sloviter, 1991). Damage to pyramidal cells by this technique is more variable. Prolonged stimulation (>12 hrs) results in selective, but often inconsistent, damage to neurons of hippocampus area CA3 and CA1; however, as in the human pathology, pyramidal cells of area CA2 and granule cells are relatively undamaged (ibid). An increased excitability of granule and CA1 pyramidal cells is observed in this model after the period of intermittent stimulation. Although this represents 'hippocampal remodeling' it is dissimilar to 'sprouting' as it does not involve the growth of new fiber systems. Moreover, convulsions and chronic, recurrent seizures do not occur with this model (Sloviter, 1987). Consequently, the afferent excitation model not only fails to adequately replicate the human pathology, but it also fails to reproduce the seizure syndrome observed in the human disease.

The kainate/ischemia rat model

More recently, intraventricular kainate and ischemia have been combined to produce a 'mesial temporal sclerosis' in rats (Franck and Roberts, 1990). The pattern of brain damage observed here is a combination of ischemia-induced and kainate-induced damage. Reportedly, there is a selective loss of dentate hilar neurons and CA3/CA1 pyramidal cells of the ventral hippocampus. Like the damage observed in the human disease, dentate granule cells and an intermediate portion of the pyramidal cell layer

(presumably area CA2) are spared. This model suggests that the pathology observed in human TLE is partly a result of ischemia. Many authors, however, do not believe that the damage to hippocampal pyramidal cells is due to seizure-related ischemia. As discussed previously, direct evidence suggests that there is no mismatch between blood supply to, and metabolic activity in, Ammon's horn during seizure activity (Meldrum, 1983; Auer and Siesjo, 1988). This model remains to be adequately characterized. A seizure syndrome has not been described. Spontaneous, recurrent seizures as well as long term remodeling and immunohistochemical changes have also not been reported. Hilar cells containing GABA are mostly spared in the human disease (Babb et al., 1989). However, these cells are relatively vulnerable to ischemia (Ribak et al., 1985; Obenaus et al., 1993). It remains to be determined whether these cells are spared in the kainate/ischemia model.

The Amygdala kindled rat model

Kindling is a very old technique that has been used traditionally to induce the epileptic state in animal models of epilepsy. This technique involves repeated, daily use of low-voltage electrical stimulation of the amygdala region until a state of enhanced sensitivity is attained (McNamara et al., 1985). Seizure activity can then be elicited as desired with lower voltage electrical stimulation. Initial electrical stimulus as well as subsequent stimulation in amygdala-kindled rats often elicits limbic type seizures (Racine, 1972). Although inter-ictal spikes are observed on EEG, spontaneous, recurrent seizures do not occur ordinarily without stimulation in kindled animals. In this sense, the kindling model fails to completely replicate the clinical picture of the human disease (Klass, 1975). However, in more recent studies, extended electrical kindling has been shown to lead to the

development of spontaneous seizures (Milgram et al., 1995). These results remain to be replicated. The pattern of neuronal cell death observed in kindled animals is highly variable and appears to be dependent on the number of seizure episodes. Brain damage may be limited to interneurons in the dentate hilus or may evolve to include CA1 and eventually CA3 pyramidal cells depending on the number of repeated stimulation (McNamara et al., 1985). Long-term hippocampal remodeling has been demonstrated for amygdala-kindled rats in the form of changes in dendritic synapses in the medial amygdaloid nucleus (Okada et al., 1993); however, this type of remodeling is dissimilar to the growth of new axonal systems observed in the human pathology. Several authors have reported increase in somatostatin levels in amygdala and various cortical regions in amygdala kindled rats (Kato et al., 1983). This is in contrast to the decrease observed in the human pathology. The status of NPY and SP hilar interneurons is unclear in either amygdala or hippocampus kindled rats.

The ouabain-potentiated, kainate rat model

Clearly, the limbic type behavioral seizures observed as well as evidence from EEG point to the hippocampus and other limbic structures as the focus of seizure activity in kainate/ouabain treated animals. An epileptic state characterized by spontaneous, recurrent seizures supervenes in these animals. The pattern of cell damage produced with this model closely replicates what is commonly observed in the human disease. As in the human pathology, all cells in Ammon's horn are damaged, but CA3 and CA1 pyramidal cells are significantly more affected than the granule and CA2 pyramidal cells. Hippocampal remodeling in the form of mossy fiber sprouting into the inner molecular layer is also present in this model.

Finally, there is significant damage to cells in the dentate hilus, although it remains to be determined whether this damage is selective for SOM, NPY, and SP interneurons.

Unique features of this model compared to current animal models of TLE are: 1. With intraperitoneal injections of kainate and ouabain, this model is very easy to replicate. 2. This technique routinely produces damage to cells of hippocampus area CA1; a lesion not always observed with other animal models without a high animal mortality. 3. Treatment with ouabain and kainate yields no animal mortality, as both drugs are administered well below their toxicity levels. 4. Unlike many of the models described above, brain damage in this model is confined to the hippocampus with minimal or no extrahippocampal damage 5. Although not completely characterized, this model more closely replicates the human pathology than many existing models of TLE. 6. Finally, evidence from epileptic hippocampi for a role of the Na^+/K^+ pump in the neuropathology of human TLE has been presented (Grisar et al., 1992; Brines et al., 1995). Since this model is based on this very concept, it may serve as a useful tool for investigating the role of the Na^+/K^+ pump in human TLE.

Although not extensively investigated in this model, recurrent, spontaneous seizure episodes after the acute phase of kainate-induced seizures has been well documented for most kainate rat models (Ben-Ari et al., 1981; Sperk, 1994). These studies have shown that spontaneous seizures do not always recur after initial seizures with kainate. The reported rate is 50 - 60% (Cronin and Dudek, 1988). Optimum monitoring for chronic epilepsy in these animals is best achieved by 24 hr video monitoring. This technique was not used in this study; a less optimum technique (handling of animals to induce seizures at time of observation) was used instead. This may explain

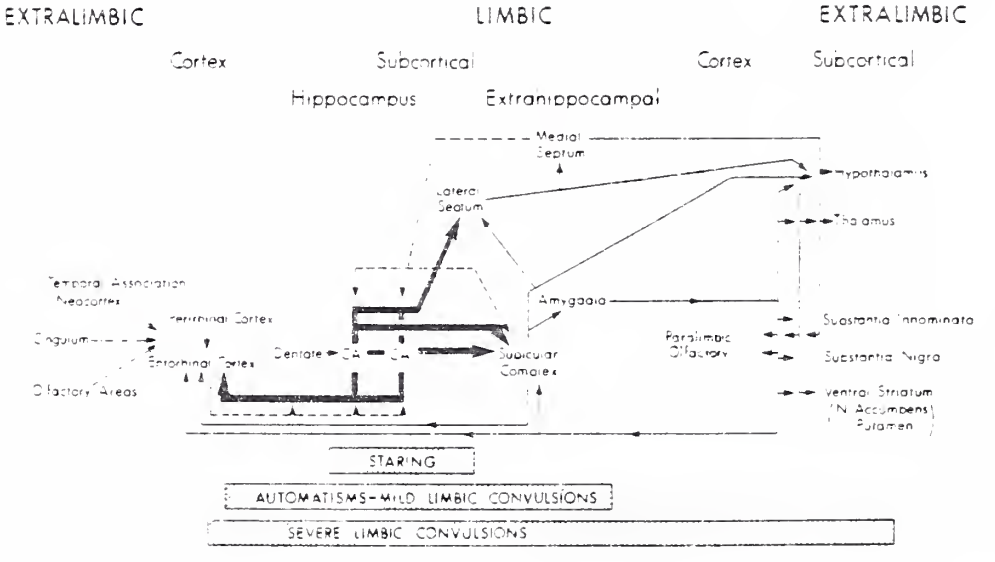
the rather low number of seizure episodes witnessed in the three animals followed. The latency to spontaneous seizure recurrence after kainate treatment is at least a weak and may be delayed for up to several weeks (Pisa et al., 1980; Cavalheiro et al., 1982; Tanaka et al., 1988). The first recurrence of epileptic attacks after kainate/ouabain treatment in three animals occurred 14, 15, 30 days respectively, in agreement with previously published reports. Finally, immunohistochemistry to characterize the subset of destroyed hilar interneurons in this model was not successful, mainly due to technical problems. However, with the overwhelming damage to this region, some damage of interneurons expressing NPY, SP, and SOM is expected. Confirmation of this criteria awaits further characterization of this model.

Anatomic basis and Pathway of Limbic seizures in rat hippocampus

Lothman and co-workers (1981) as well as others (Ben-Ari, 1985) have postulated a pathway for generation and propagation of limbic seizures (Figure 15; p. 84). It is believed that area CA3 of the hippocampus is the "pacemaker" for initiation and propagation of limbic seizures. This is supported by the finding that cells of area CA3 possess a low seizure threshold (Schwartzkronin and Prince, 1978) . Cells in this area of the brain are also the first to show an increase in metabolic activity in the early stages of seizure activity (Nadler et al., 1981). It is believed that heavy projections from the hippocampus to the lateral septum are the first efferent projections activated (Figure 15; bold lines). This correlates with behavioral manifestations of staring in the initial stages of seizure generation (Swanson and Cowan, 1977). The second stage of limbic seizures is thought to involve non-hippocampal limbic centers including the entorhinal cortex, the amygdala, subiculum, and medial/lateral septum. Increase in glucose utilization and detection of

electrical activity in these areas correlate with automatisms and mild limbic convulsions. The third stage of limbic seizures is characterized by head-bobbing followed by bilateral fore-limb clonus and complete tonic-clonic convulsions. During this stage, electrical activity and increased deoxyglucose utilization have been recorded both in limbic and extralimbic areas as well as on surface EEG leads. It is believed that propagation of seizure activity to these areas is along higher-order axons from secondary centers. The substantia nigra, midline thalamic nuclei and some paralimbic (olfactory, perirhinal, periamygdaloid and cingular) cortical sites become activated.

Figure 15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Medial Septum
Lateral Septum
Hypothalamus
Thalamus
Temporal Association Neocortex
Amygdala
Cingulum
Entorhinal Cortex
Dentate
CA
Subicular Complex
Parolfactory
Substantia Innominata
Substantia Nigra
Ventral Striatum (N. Accumbens) Putamen
Olfactory Areas
STARING
AUTOMATISMS-MILD LIMBIC CONVULSIONS
SEVERE LIMBIC CONVULSIONS

Figure 15: **Anatomic basis and pathway of kainic acid induced limbic seizures**

The hippocampus is considered the principal brain area and its afferent (dashed lines) and efferent (bold lines) are shown. Input to the hippocampus is shown on the left and output is shown on the right. During the first stage of limbic seizures, the hippocampus and lateral septum are selectively activated and the behavioral response (lower part) is staring. when epileptic activity extendes to non-hippocampal limbic areas (entorhinal cortex, amygdala, subiculum and medial septum) mild limbic convulsions develop. With extension along synaptic pathways from these secondary limbic centers, extra-limbic centers are activated and severe limbic convulsions appear. (Adapted from Lothman and Collins, 1981).

CONCLUSION

Summary of findings:

1) Impairment of Na^+/K^+ pump capacity reduces neuronal survival of excitatory stimuli *in vivo.* Thus kainate (5 mg/kg; ip) or ouabain (1 mg/kg; i.p) do not typically cause seizures or neuronal injury in animals. However, kainate (5 mg/kg; i.p.) combined with ouabain (3 nmoles; i.c.v) reliably induces seizure activity and neuronal injury in rat hippocampus. Similarly, ouabain (1 mg/kg; i.p.) or kainate (7 mg/kg; i.p.) do not ordinarily induce seizures or neuronal injury in animals; however, a combination of these treatments induces seizures and hippocampal damage. A strong association was observed between Na^+/K^+ pump inhibition and kainate treatment in producing seizures and subsequent neuronal damage. Thus, when ouabain (3nmoles; i.c.v) is delayed (90 min instead of 30 min) after kainate (5 mg/kg; i.p.) seizure onset is also delayed and the duration of seizures is reduced, although latency to seizure onset after ouabain injection remains unchanged.

2) Treatment with the combination of kainate and ouabain induces a seizure syndrome and neuropathology similar to human TLE. The following features are produced:

A. Acute limbic seizures originating from the hippocampus.

B. Spontaneously recurring chronic seizures beginning 14 - 30 days (n =3) after initial treatments.

C. Damage to hippocampal cells in a pattern reminiscent of the human pathology.

D. Hippocampal remodeling in the form of mossy fiber sprouting into the dentate gyrus.

In conclusion, impairment of Na^+/K^+ pump capacity decreases neuronal ability to survive excitatory activity and a pathology is produced that closely replicates human TLE. To the best of our knowledge, this is the first report of an animal pathology of human TLE produced by the method described herein. This model has been termed the ouabain-potentiated, kainate (OPK) model of TLE.

Clinical relevance:

Our observation with direct pharmacological reduction of rat total Na^+/K^+ pump capacity has implications for human disease, since cardiac glycosides are widely used to treat congestive heart failure and cardiac arrhythmia. Although these drugs penetrate the CNS poorly, neurological manifestations are nonetheless side-effects of treatment (Cooke, 1993). In contrast to rodent isoforms, all three human isoforms of the Na^+/K^+pump possess high affinity binding for cardiac glycosides (Sweadner, 1989). Thus, neurotoxic threshold for glycosides may be much lower for human than for rodents. Epileptic patients in treatment for cardiac disease with glycosides may be particularly vulnerable to this potential complication, as Na^+/K^+ pump activity may already be reduced in these patients (Grisar and Delgado-Escueta, 1986; Brines et al., 1995)

Finally, results of the present study are consistent with existing evidence suggesting that Na^+/K^+ pump capacity may be insufficient or comprised in the epileptic hippocampi (Grisar, 1984; Lees, 1991; Brines et al., 1995). Thus, it can be hypothesized that seizure generation and the sequelae of neuronal injury may be prevented or ameliorated by upregulating Na^+/K^+

pump capacity in the hippocampus. Some evidence exist to suggest that Na^+/K^+ pump expression can be modulated both *in vitro* and *in vivo*. Mata and coworkers have demonstrated that prolonged stimulation of hypothalamic neurons leads to isoform specific up-regulation of Na^+/K^+ pump mRNA (Mata et al., 1980). Such isoform-specific upregulation of Na^+/K^+ pump capacity have also been reported for CA1 and CA4 pyramidal cells exposed to the mineralocorticoid, aldosterone (Farman et al., 1994). Also, up-regulation of neuronal Na^+/K^+ pump activity by treatment with insulin and insulin like growth factors (IGF) has recently been reported. This upregulation was associated with increased survival of glutamate toxicity *in vitro* (Swanson and Choi, 1993). This last finding suggest that further study of Na^+/K^+ pump regulation may lead to methods of ameliorating or preventing the hyperexcitability and neuronal damage intrinsic to the pathology of human epilepsy.

REFRENCES

Abe, K., Kogure, K., Yamamoto, H., Imazawa, M. and Miyamoto, K. Mechanism of arachidonic acid liberation during ischemia in gerbil cerebral cortex. *J. Neurochem.* 48 (1987) 503-509.

Abeywardena, M. Y. and Charnock, J. S. Modulation of cardiac glycoside inhibition of Na,K-ATPase by membrane lipids. *Biochim. Biophys. Acta* 729 (1983) 75-84.

Antonelli, M. C., Baskin, D. G., Garland, M. and Stahl, W. L. Localization and Characterization of Binding Sites with High Affinity for [^{3}H]Ouabain in Cerebral Cortex of Rabbit Brain Using Quantitative Autoradiography. *J. Neurochem.* 52 (1989) 193-200.

Astrup, J., Sorensen, P. M. and Sorensen, H. R. Oxygen and glucose consumption related to Na+/K+ transport in canine brain. *Stroke* 12 (1981) 726-720.

Auer, R. N. and Siesjo, B. K. Biological differences between ischemia, hypoglycemia, and epilepsy. *Ann. Neurol.* 24 (1988) 699-707.

Babb, T. L., Kupfer, W. R., Pretorius, J. K., Crandall, P. H. and Levesque, M. F. Synaptic reorganization by mossy fibers in human epileptic fascia dentata. *Neuroscience* 42 (1991) 351-363.

Babb, T. L., Lieb, J. P., Brown, W. J., Pretorius, J. and Crandall, P. H. Distribution of cell density and hyperexcitability in the epileptic human hippocampal formation. *Epilepsia* 25 (1984) 721-728.

Babb, T. L., Pretorius, J., Kupfer, W. and Crandall, P. H. Glutamate decarboxylate immunoreactive neurons are preserved in human epileptic hippocampus. *J. Neurosc.* 9 (1989) 2562-2578.

Baker, P. F., Blaustein, M. P., Keynes, R. D., Manil, J., Shaw, T. I. and Steinhardt, R. A. The ouabain-sensitive fluxes of sodium and potassium in squid giant axons. *J. Physiol.* 200 (1969) 459-496.

Baran, H., Heldt, R. and Hertting, G. Increased prostaglandin formation in rat brain following systemic application of kainic acid. *Brain Res.* 404 (1987) 107-112.

Barbour, B., Brew, H. and Attwell, D. Electrogenic glutamate uptake in glial cells is activated by intracellular potassium. *Nature* 335 (1988) 433-436.

Ben-Ari, Y. Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. *Neurosci.* 14 (1985) 375-403.

Ben-Ari, Y., Tremblay, E., Otterson, O. P. and Meldrum, B. S. The role of epileptic activity in hippocampal and "remote" cerebral lesions induced by kainic acid. *Brain Res.* 191 (1980) 79-97.

Ben-Ari, Y., Trembley, E., Riche, D., Ghilini, G. and Naquet, R. Electrographic, clinical and pathological alterations following systemic administration of kainic acid, bicuculline and pentetrazole: metabolic mapping using the deoxyglucose method with special reference to the pathology of epilepsy. *Neuroscience* 6 (1981) 1361-1391.

Berger, M. and Ben-Ari, Y. Autoradiographic visualization of ^{3}H kainic acid receptor subtypes in the rat hippocampus. *Neurosci. Lett.* 39 (1983) 237-242.

Berger, M. L., Lefauconnier, J. M., Tremblay, E. and Ben-Ari, Y. Limbic seizures induced by systemically applied kainic acid: how much kainic acid reaches the brain? *Adv. Exptl. Med. Biol.* 203 (1986) 199-203.

Bernard, P. S., Sobiski, R. E. and Dawson, K. M. Antagonism of a kainic acid syndrome by baclofen and other putative GABAmimetics. *Brain Res. Bull.* 5 (1980) 519-523.

Bertorello, A. M., Hopfield, J. F., Aperia, A. and Greengard, P. Inhibition by dopamine of (Na/K) ATPase activity in neostriatal neurons through D1 and D2 dopamine receptor synergism. *Nature* 347 (1990) 386-388.

Booss, J. Management of Epilepsy for Primary Care. *Federal Practitioner, Suppl* (1995) 5-6.

Brines, M. L., Gulanski, B. I., Gilmore-Hebert, M., Greene, A. L., Benz, E. J. J. and Robbins, R. J. Cytoarchitectural relationships between [^{3}H]ouabain

binding and mRNA for isoforms of the sodium pump catalytic subunit in rat brain. *Mol. Brain Res.* 10 (1991) 139-150.

Brines, M. L. and Robbins, R. J. Inhibition of α2/α3 sodium pump isoforms potentiates glutamate neurotoxicity. *Brain Res.* 591 (1992) 94-102.

Brines, M. L. and Robbins, R. J. Cell-type specific expression of Na,K-ATPase catalytic subunits in cultured neurons and glia: evidence for polarized distribution in neurons. *Brain Res.* 631 (1993) 1-11.

Brines, M. L., Tabuteau, H., Sundaresan, S., Kim, J., Spencer, D. D. and de Lanerolle, N. C. Regional distributions of hippocampal Na^+,K^+-ATPase, cytochrome oxidase, and total protein in temporal lobe epilepsy. *Epilepsia* 36 (1995) 371-383.

Brodsky, J. L. Insulin activation of brain Na,K-ATPase is mediated by α2-form of enzyme. *Am. J. Physiol.* 258 (1990) C812-C817.

Caspers, M. L., Schwartz, R. D., Labarca, R. and Paul, S. M. Autoradiographic visualization and characterization of [^{3}H]ouabain binding to the Na^+, K^+-ATPase of rat brain and pineal. *Brain Research* 409 (1987) 335-342.

Cavalheiro, E. A., DeFeo, M. R., Mecarelli, O. and Ricci, G. F. Intracortical and Intrahippocampal Injections of Kainic Acid in Developing Rats: An Electographic Study. *Electroenceph. Clin. Neurophys.* 56 (1983) 480-486.

Cavalheiro, E. A., Leite, J. P., Bortolotto, Z. A., Turski, W. A., Ikonomidou, C. and Turski, L. long-term effects of pilocarpine in rats: structural damage of the brain triggers kindling and spontaneous recurrent seizures. *Epilepsia* 32 (1991) 778-782.

Cavalheiro, E. A., Riche, D. and Le Gal La Salle, G. Long term effects of intrahippocampal kainic acid in rats: a method for inducing spontaneous recurrent seizures. *Electronceph. Clin. Neurophysiol.* 53 (1982) 581-582.

Cepeda, C., Tenaka, T., Riche, D. and Naquet, R. Limbic status epilepticus: behavior and sleep alterations after intra-amygdaloid kainic acid microinjections in papio baboons. *Electroencephal. Clin. Neurophysiol.* 54 (1982) 603-613.

Chan, P. H., Kerlan, R. and Fishman, R. A. Reductions in y-aminobutyric acid and glutamate uptake and Na^+,K^+-ATPase activity in brain slices and synaptososomes by arachidonic acid. *J. Neurochem.* 40 (1983) 309-316.

Charnock, J. S., Almeida, A. F. and To, R. Temperature activity relationships of cation activation and ouabain inhibition of Na.K ATPase. *Arch. Biochem. Biophys.* 167 (1975) 480-487.

Choi, D. W. Glutamate neurotoxicity in cortical cell culture is calcium dependent. *Neurosci. Lett.* 58 (1985) 293-297.

Choi, D. W. Glutamate neurotoxicity and diseases of the nervous system. *Neuron* 1 (1988) 623-634.

Choi, D. W. Excitotoxic cell death. *J. Neurobiol.* 23 (1992) 1261-1276.

Choi, D. W., Maulucci-Gedde, M. and Kriegstein, A. R. Glutamate Neurotoxicity in Cortical Cell Culture. *J. Neuroscience* 7(2) (1987) 357-368.

Clifford, D. B., Lothman, E. W., Dodson, W. E. and Ferendelli, J. E. Effect of anticonvulsant drugs on kainic acid-induced epileptiform activity. *Expl. Neurol.* 76 (1982) 156-167.

Cooke, D. M. The use of central nervous system manifestations in the early detection of digitalis toxicity. *Heart and Lung* 22 (1993) 477-81.

Corsellis, J. A. N. and Bruton, C. J. Neuropathology of status epilepticus in humans. *Advances in Neurology* 34 (1983) 129-139.

Coyle, J. T., Bird, S. J., Evans, R. H., Gulley, R. L., Nadler, J. V., Nicklas, W. J. and Olney, J. W. Excitatory amino acid neurotoxins: selectivity and mechanisms of action. *Neurosci. Res. Prog. Bulletin* 19 (1981) 331-427.

Coyle, J. T., Molliver, M. R. and Kuhar, M. J. In situ injection of kainic acid: a new method for selectively lesioning neuronal cell bodies while sparing axons of passage. *J. comp. Neurol.* 180 (1978)

Cronin, J. and Dudek, F. E. Chronic seizures and collateral sprouting of dentate mossy fibers after kainic acid treatment in rats. *Brain Res.* 474 (1988) 181-184.

Dagani, F. and Erecinska, M. Relationships among ATP synthesis, K gradients, and neurotransmitter amino acid levels in isolated rat brain synaptosomes. *J. Neurochem.* 49 (1987) 1229-1240.

de Lanerolle, N. C., Brines, M. L., Kim, J. H., Williamson, A., Philips, M. F. and Spencer, D. D. (1992). Neurochemical remodelling of the hippocampus in human temporal lobe epilepsy. Molecular Neurobiology of Epilepsy (Epilepsy Res. Suppl. 9). New York, Elsevier. 205-220.

de Lanerolle, N. C., Brines, M. L., Williamson, A., Kim, J. H. and Spencer, D. D. (1992). Neurotransmitters and their receptors in human temporal lobe epilepsy. The Dentate Gyrus and its Role in Seizures (Epilepsy Res. Suppl. 7). New York, Elsevier. 235-250.

de Lanerolle, N. C., Kim, J. H. and Brines, M. L. Cellular and Molecular Alterations in Partial Epilepsy. *Clin. Neurosci.* 2 (1994) 64-81.

de Lanerolle, N. C., Kim, J. H., Robbins, R. J. and Spencer, D. D. Hippocampal interneuron loss and plasticity in human temporal lobe epilepsy. *Brain Res.* 495 (1989) 387-395.

DeLorenzo, R. J. (1991). The Epilepsies. Neurology in clinical practice. Boston, Butterworth-Heineman. 1443-1445.

During, M. J. and Spencer, D. D. Extracellular hippocampal glutamate and spontaneous seizure in the conscious human brain. *Lancet* 341 (1993) 1607-1610.

Dutta, S. and Marks, B. H. Distribution of ouabain and digoxin in the rat and guinea pig. *Life Sci.* 5 (1966) 915-920.

Dutta, S., Marks, B. H. and Schoener, E. P. Accumulation of radioactive cardiac glycosides by various brain regions in relation to the dysrhythmogenic effect. *Br. J. Pharm.* 59 (1977) 101-106.

Eiselet, W. H., Domer, F. R., Jarrott, D. M. and Baricos, W. H. Cerebral phospholipid content and Na,K ATPase activity during ischemia and post-ischemic reperfusion in the mongolian derbil. *J. Neurochem.* 43 (1984) 320-327.

Erdo, S. L., Michler, A. and Wolff, J. R. GABA accelerates excitotoxic cell death in cortical cultures: protection by blockers of GABA-gated chloride channels. *Brain Res.*, 542 (1991) 254-258.

Erecinska, M. Stimulation of the Na+/K+ Pump Activity During Electrogenic Uptake of Acidic Amino Acid Transmitters by Rat Brain Synaptosomes. *J. Neurochem.* 52 (1989) 135-139.

Fairbrother, I. S., Arbuthnott, W., Kelly, J. S. and Butcher, S. P. In vivo mechanisms underlying dopamine release from rat nigrostriatal terminals: Studies using veratrine and ouabain. *J. Neurochem.* 54 (1990) 1834-1843.

Farman, N., Bonvalet, J. and Seckl, J. Aldosterone selectively increases Na,K-ATPase α3 subunit mRNA expression in rat hippocampus. *Am. J. Physiol.* 266 (1994) C423-C428.

Franck, J. E. and Roberts, D. L. Combined kainate and ischemia produces "mesial temporal sclerosis". *Neurosci. Lett.* 118 (1990) 159-163.

Frandsen, A., Quistorff, B. and Schousboe, A. Phenobarbitol protects cerebral cortex neurons against toxicity induced by kainate but not other excitatory amino acids. *Neurosci. Lett.* 111 (1990) 233-238.

Fukuda, A. and Prince, D. A. Excessive intracellular Ca^{2+} inhibits glutamate-induced Na^{+}-K^{+} pump activation in rat hippocampal neurons. *J. Neurophys.* 68 (1992) 28-35.

Garthwaite, J., Garthwaite, G. and Hajos, F. Amino acid neurotoxicity: relationship to neuronal depolarization in rat cerebellar slices. *Neuroscience* 18 (1986) 449-460.

Gloor, P. (1991). Mesial temporal sclerosis: Historical background and an overview from a modern perspective. Epilepsy Surgery. New York, Raven Press. 689-703.

Goddard, G. A. and Robinson, J. D. Uptake and release of Ca by rat brain synaptosomes. *Brain Res.* 110 (1976) 331-350.

Grisar, T. Glial and Neuronal Na^+-K^+ Pump in Epilepsy. *Ann. Neurol.* 16 (suppl) (1984) S128-S134.

Grisar, T. and Delgado-Escueta, A. V. Astroglial Contribution in Human Temporal Lobe Epilepsy: K^+ Activation of Na^+,K^+-ATPase in Bulk Isolated Glial Cells and Synaptosomes. *Brain Res.* 364 (1986) 1-11.

Grisar, T., Guillaume, D. and Delgado-Escueta, A. V. Contribution of Na,K-ATPase to focal epilepsy: a brief review. *Epilepsy Res.* 12 (1992) 141-149.

Hammond, C., Feger, J., Bioulac, B. and Souteyrand, J. P. Experimental hemibalism in the monkey produced by unilateral kainic acid lesion in corpus luysii. *Brain Res.* 171 (1979) 577-580.

Hansen, A. J. Effect of anoxia on ion distribution in the brain. *Physiol. Rev.* 65 (1985) 101-141.

Hegyvary, C. Ouabain-binding and phosphorylation of (Na^++K^+)-ATPase treated with N-ethylmaleimide or oligomycin. *Biochim. Biophys. Acta* 422 (1976) 365-379.

Hootman, S. A. and Ernst, S. A. Effect of methacholine on Na^+ pump activity and ion content of dispersed avian salt gland cells. *Amer. J. Physiol.* 241 (1981) R77-86.

Horisberger, J. D., Lemas, V., Kraehenbuhl, J. P. and Rossier, B. C. Structure-function relationship of Na^+,K^+ATPase. *Ann. Rev. Physiol.* 53 (1991) 565-584.

Jacobson, I., Hagberg, H., Sandberg, M. and Hamberger, A. Ouabain-induced changes in extracellular aspartate, glutamate, and GABA levelsin the rabbit olfactory bulb in vivo. *Neurosc. Lett.* 64 (1986) 211-215.

Johansen, F. F., Simmer, J. and Diemer, N. H. Early loss of somatostatin neurons in dentate hilus after cerebral ischemia in the rat precedes CA1 pyramidal cell loss. *Acta Neuropath.* 73 (1987) 110-114.

Jorgensen, P. L. and Skou, J. C. Preparation of highly active Na,K-ATPase from the outer medulla of rabbit kidney. *Biochem. Biophys. Res. Commun.* 37 (1969) 39-46.

Kanner, B. I. and Sharon, I. Active transport of L-glutamate by membrane vesicles isolated from rat brain. *Biochemistry* 17 (1978) 3949-3953.

Kato, N., Higuchi, T., Friesen, H. G. and Wada, J. A. Changes in immunoreactive somatostatin and b-endorphin content in rat brain after amygdaloid kindling. *Life Sci.* 32 (1983) 2415-2422.

Kim, J., Guimaraes, P., Shen, M., Masukawa, L. and Spencer, D. Hippocampal neuronal density in temporal lobe epilepsy with and without gliomas. *Acta Neuropath.* 80 (1990) 41-45.

Kjeldsen, K. and Norgaard, A. Quantification of rat sciatic nerve Na^+,K^+-ATPase by measurements of ^3H-ouabain binding in intact nerve samples. *J. Neurol. Sci.* 79 (1987) 205-219.

Kohr, G. and Heinemann, U. Differences in Mg and Ca effect on NMDA and quisqualate-induced decreases in extracellular sodium concentration in rat hippocampal slices. *Exp. Brain Res.* 71 (1988) 425-430.

Kuffler, S. W., Nichols, J. G. and Martin, A. R. From Neuron to Brain. Sunderland, Mass, Sinauer Associates, Inc. (1984).

Kuhlmann, J., Erdmann, E. and Rietbrock, N. Distribution of cardiac glycosides in heart and brain of dogs and their affinity to the Na,K-ATPase. *Naunyn-Schmied. Arch. Pharm.* 307 (1979) 65-71.

Kuncz, A., Doczi, T. and Bodosi, M. The effect of skull and dura on brain volume regulation after hypo-and hyperosmolar fluid treatment. *Neurosurgery* 27 (1990) 509-515.

Lassmann, H., Petsche, U., Kitz, K., Baran, H., Sperk, G., Seitelberger, F. and Hornykiewicz, O. The role of brain edema in epileptic brain damage induced by systemic kainic acid injection. *1984* 13 (1984) 691-704.

Laurberg, S. and Zimmer, J. Lesion-induced sprouting of hippocampal mossy fiber collaterals to the fascia dentata in developing and adult rats. *J Comp. Neurol.* 200 (1981) 433-459.

Lees, G. J. Inhibition of sodium-potassium-ATPase: a potentially ubiquitous mechanism contributing to central nervous system pathology. *Brain Res. Rev.* 16 (1991) 283-300.

Lees, G. J., Lehmann, A., Sandberg, M. and Hamberger, A. The neurotoxicity of ouabain, a sodium-potassium ATPase inhibitor, in the rat hippocampus. *Neurosci. Lett.* 120 (1990) 159-162.

Leite, J. P., Bortolotto, Z. A. and Cavalheiro, E. A. Spontaneous recurrent seizures in rats: an experimental model of partial epilepsy. *Neurosci. Biobehav. Rev.* 14 (1990) 511-517.

Levine, S. Anoxic ischemic encephalopathy in rats. *Am. J. Path.* 36 (1960) 1-17.

Lothman, E. W. and Collins, R. C. Kainic Acid Induced Limbic Seizures: Metabolic, Behavioral, Electroencephalographic and Neuropathological Correlates. *Brain Res* 218 (1981) 299-318.

Lowe, A. G., Ed. Functional aspects of active cation transport. Membranes and Ion Transport. London, Wiley-International (1971).

Lowe, D. A. Morphological Changes in the Cat Cerebral Cortex Produced by Superfusion of Ouabain. *Brain Res.* 148 (1978) 347-363.

Margerison, J. H. and Corsellis, J. A. N. Epilepsy and the temporal lobes: A clinical and encephalographic and neurologic study of the brain in epilepsy with particular reference to the temporal lobes. *Brain* 89 (1966) 499-563.

Mata, M., Siegel, G. J., Hieber, V., Beaty, M. W. and Fink, D. J. Differential distribution of (Na,K)-ATPase a isoform mRNAs in the peripheral nervous system. *Brain Res.* 546 (1991) 47-54.

Mattson, M. P., Guthrie, P. B., Hayes, B. C. and Kater, S. B. Roles for mitotic history in the generation and degeneration of hippocampal neuroarchitecture. *J. Neurosci.* 9 (1989) 1223-1232.

Mayer, M. L. and Westbrook, G. L. Cellular mechanisms underlying excitotoxicity. *Trends Neurosci.* 10 (1987) 59-61.

McGrail, K. M., Phillips, J. M. and Sweadner, K. J. Immunofluorescent Localization of Three Na,K-ATPase Isozymes in the Rat Central Nervous System: Both Neurons and Glia Can Express More Than One Na^+,K^+-ATPase. *J. Neuroscience* 11 (1991) 381-391.

McNamara, J. O., Bonhaus, D. W., Shin, C., Crain, B. J., Gellman, R. L. and Giacchino, J. L. The kindling model of epilepsy: A critical review. *CRC Crit. Rev Neurobio.* 1 (1985) 341-391.

Meldrum, B. Possible therapeutic applications of antagonists of excitatory amino acid neurotransmitters. *Clin. Sci.* 68 (1985) 113-122.

Meldrum, B. S. Metabolic factors during prolonged seizures and their relation to nerve cell death. *Adv. Neurol.* 34 (1983) 261-275.

Mello, L. E. A. M., Cavalheiro, E. A., Babb, T. L., Kupfer, W. R., Pretorius, J. K., Tan, A. M. and Finch, D. M. Pilocarpine induced status epilepticus leads to chronic seizures and mossy fiber sprouting in the hippocampus. *Epilepsia* 31 (1990) 675.

Monaghan, D. T. and Cotman, C. W. The distribution of ^{3}H-kainic acid binding sites in rat CNS as determined by autoradiography. *Brain Res.* 252 (1982) 91-100.

Nadler, J. and Cuthbertson, G. Kainic acid neurotoxicity toward the hippocampal formation: dependence on specific excitatory pathways. *Brain Res.* 195 (1980) 47-56.

Nadler, J. V. and Evenson, D. A. Use of excitatory amino acids to make axon-sparing lesions of hypothalamus. *Meth. Enzym.* 103 (1983) 393-400.

Novelli, A., Reilly, J. A., Lysko, P. G. and Henneberry, R. C. Glutamate becomes neurotoxic via the N-methyl-D-aspartate receptor when intracellular energy levels are reduced. *Brain Res.* 451 (1988) 205-212.

O'Fallon, J. V., Brosemer, R. W. and Harding, J. W. The Na,K ATPase a plausible trigger for voltage-independent release of cytoplasmic neurotransmitters. *J. Neurochem* 36 (1981) 369-378.

Obenaus, A., Esclapez, M. and Houser, C. R. Loss of glutamate decarboxylase mRNA-containing neurons in the rat dentate gyrus following pilocarpine-induced seizures. *J. Neurosci.* 13 (1993) 4470-4485.

Okada, R., Nishizuka, M., iizuka, R. and Arai, Y. Persistence of reorganized synaptic connectivity in the amygdala of kindled rats. *Brain Res. Bull.* 31 (1993) 631-5.

Olney, J. W. Brain lesion, obesity and other disturbances in mice treated with monosodium glutamate. *Science* 164 (1969) 719-721.

Olney, J. W. Inciting excitotoxic cytocide among central neurons. *Adv. Exp. Med. Biol.* 203 (1986) 631-645.

Olney, J. W., Sharpe, L. G. and Feigin, R. D. Glutamate-induced brain damage in infant primates. *J. Neuropathol. Exper. Neurol.* 31 (1972) 464-488.

Pinard, E., Tremblay, E., Ben-Ari, Y. and Seylaz, J. Blood flow compensates oxygen demand in the vulnerable CA3 region of the hippocampus during kainate-induced seizures. *Neuroscience* 13 (1984) 1039-1049.

Pisa, M., Sanberg, P. R., Corcoran, M. E. and Fibiger, H. C. Spontaneously recurrent seizures after intracerbral injections of kainic acid in rat: a possible model of temporal lobe epilepsy. *Brain Res.* 200 (1980) 481-487.

Racine, R. J. Modification of seizure activity by electrical stimulationI. Afterdischarge threshold. *Electroencephalogr. Clin. Neurophysiol.* 32 (1972) 281.

Reeves, J. P. and Sutko, J. L. Sodium-calcium ion exchange in cardiac membrane vesicles. *Proc. Natl. Acad. Sci. USA* 76 (1979) 590-594.

Ribak, C. E., seress, L. and Amaral, D. G. The development, ultrastructure and synaptic connections of the mossy cells of the dentate gyrus. *J. Neurocytol.*, 14 (1985) 835-857.

Ritchie, J. M. and Straub, R. W. Oxygen composition and phosphate efflux in mammalian non-myelinated nerve fibers. *J. Physiol.* 304 (1980) 109-121.

Rosenberg, P. A. Accumulation of Extracellular Glutamate and Neuronal Death in Astrocyte-Poor Cortical Cultures Exposed to Glutamine. *GLIA* 4 (1991) 91-100.

Rosenberg, P. A., Amin, S. and Leitner, M. Glutamate uptake disguises neurotoxic potency of glutamate agonists in cerebral cortex in dissociated cell culture. *J. Neurosci.* 12 (1992) 56-61.

Rossier, B. C., Geering, K. and Kraehenbuhl, J. P. Regulation of the sodium pump: how and why? *Trends Biochem. Sci.* 12 (1987) 483-487.

Rothman, S. M. The Neurotoxicity of Excitatory Amino Acids is Produced by Passive Chloride Influx. *J. Neurosci.* 5 (1985) 1483-1489.

Rothman, S. M. and Olney, J. W. Excitotoxicity and the NMDA receptor. *Trends Neurosci.* 10 (1987) 299-302.

Saija, A., Princi, P., Pisani, A., Santoro, G., De pasqualae, R., Massi, M. and Costa, G. Blood-brain barrier dysfunction following injection of kainic acid in the rat. *Life Sciences* 51 (1992) 467-477.

Schwartz, W. J. a. S., Frank R. Autoradiographic Maps of Regional Brain Glucose Consumption in Resting, Awake Rats Using [^{14}C] 2-Deoxyglucose. *J. Comp. Neur.* 177 335-360.

Schwartzkronin, P. A. and Prince, D. A. Cellular and field potential properties of epileptogenic hippocampal slices. *Brain Research* 147 (1978) 117-130.

Schwob, J. E., Fuller, T., Price, J. L. and Olney, J. W. Widespread patterns of neuronal damage following systemic or intracerebral injections of kainic acid: A histological study. *Neurosci.* 5 (1980) 991-1014.

Shinozaki, H., Ed. Discovery of novel actions of kainic acid and related compounds. In Kainic Acid as a Tool in Neurobiology. New York, Raven Press (1981).

Shinozaki, H. and Konishi, S. Actions of several antihelminthics and insecticides on rat cortical neurons. *Brain Res.* 24 (1980) 368-371.

Siesjo, B. K., Ingvar, M. and Westerberg, E. The influence of bicucilline-induced seizures on free fatty acid concentrations in cerebral cortex, hippocampus, and cerebellum. *J. Neurochem.* 39 (1982) 796-802.

Skou, J. C. Overview: The Na,K-Pump. *Meth. Enzymol.* 156 (1988) 1.

Sloviter, R. S. A simplified Timm stain procedure compatible with formaldehyde fixation adn routine paraffin embedding of rat brain. *Brain Res. Bull.* 8 (1982) 771-774.

Sloviter, R. S. Epileptic brain damage in rats induced by sustained electrical stimulation of the perforant path: Acute electrophysiological and light microscopic studies. *Brain Res. Bull.* 10 (1983) 675-698.

Sloviter, R. S. Decreased hippocampal inhibition and a selective loss of interneurons in experimental epilepsy. *Science* 235 (1987) 73-76.

Sloviter, R. S. and Lowenstein, D. H. Heat shock protein expression in vulnerable cells of the rat hippocampus as an indicator of excitation-induced neuronal stress. *J. Neurosci.* 12 (1992) 3004-3009.

Sommer, W. Erkrankung des Ammonshorns als aetiologisches Moment der Epilepsie. *Arch. Psychiatr. Nervenkr.* 10 (1880) 631-675.

Sperk, G. Kainic acid seizures in the rat. *Prog. Neurobiol.* 42 (1994) 1-32.

Sperk, G., Lassmann, H., Baran, S. J., Kish, S. J., Seitelberger, F. and Hornykiewicz, O. Kianic acid induced seizures: Neurochemical and histopathological changes. *Neuroscience* 10 (1983) 1301-1315.

Spielmeyer, W. Die pathogenesis des epileptischen krampfes. *Z. Neurol. Psychiat.* 109 (1927) 501-520.

Stable, J., Arens, J., Lambert, J. D. C. and Heineman, U. Effects of lowering Na^+ and K^+ and of ouabain in quisqualate-induced ionic changes in area CA1 of rat hippocampal slices. *Neurosc. Lett.* 110 (1990) 60-65.

Stahl, W. L. The Na,K-ATPase of nervous tissue. *Neurochem. Int.* 8 (1986) 449-476.

Stone, W. E. and Javid, M. J. Effects of anti-convulsants and glutamate antagonists on the convulsive actions of kainic acid. *Archs Int. Pharmacodyn. Ther.* 243 (1980) 56-65.

Stone, W. E. and Javid, M. J. Effects of anticonvulsants and glutamate antagonists on the convulsive actions of kainic acid. *Archs int. Pharmacodyn. Ther.* 243 (1980) 56-65.

Swann, A. C. Free fatty acid and Na,K ATPase: effects on cation regulation, enzyme conformation, and interactions with ethanol. *Arch. Biochem. Biophys.* 233 (1984) 354-361.

Swann, A. C. Brain Na^+,K^+-ATPase regulation in vivo: reduction in activity and response to sodium by intracerebroventricular tetrodotoxin. *Brain Res.* 543 (1991) 251-255.

Swanson, L. W. and Cowan, W. M. An autoradiographic study of the organization of the afferent connections of the hippocampal formation in the rat. *J. comp. Neurol.* 172 (1977) 49-84.

Swanson, R. A. and Choi, D. W. Glial glycogen stores affect neuronal survival during glucose deprivation in vitro. *J. Cereb. Blood Flow Metab.* 13 (1993) 162-169.

Sweadner, K. J. Isozymes of the Na^+/K^+ATPase. *Biochem. Biophys. Acta* 988 (1989) 185-220.

Takemoto, T., Ed. Isolation and structural identification of naturally occurring excitatory amino acids. In Kainic Acid as a Tool in Neurobiology. New York, Raven Press (1978).

Tanaka, S., Kondo, S., Tanaka, T. and Yonemasu, Y. Long-term observation of rats after unilateral intra-amygdaloid injection of kainic acid. *Brain Res.* 463 (1988) 163-167.

Thompson, S. M. and Prince, D. A. Activation of electrogenic sodium pump in hippocampal CA1 neurons following glutamate-induced depolarization. *J. Neurophys.* 56 (1986) 507-522.

Tremblay, E. and Ben-Ari, Y. Usefulness of parenteral kainic acid as amodel of temporal lobe epilepsy. *Rev. Electroenceph. Neurophysiol. Clin.* (1984) 241-6.

Turski, L., Cavalheiro, E. A., Schwarcz, M., Turski, W. A., Mello, L. E. A. M., Bortolotto, Z. A., klockghether, T. and Sontag, K.-H. Susceptibility to seizures by pilocarpine in rats after microinjection of isoniasid of γ-vinyl-GABA into the substantia nigra. *Brain Res.* 370 (1986) 294-309.

Turski, L., Ikonomidou, C., Turski, W. A., Bortolotto, Z. A. and Cavalheiro, E. A. Cholinergic mechanisms and epileptogenesis. The seizures induced by pilocarpine: a novel experimental model of intractable epilepsy. *Synapse* 3 (1989) 154-171.

Turski, W. A., Cavalheiro, E. A., Schwarcz, M., Czuczwar, S. J., Kleinrok, Z. and Turski, L. Limbic seizures produced by pilocarpine in rats: a behavioral, electroencephalographic and neuropathological study. *Behav. Brain Res.* 9 (1983) 315-335.

Ullrich, A., Steinberg, R., Baierl, P. and Ten Bruggencate, G. Changes in extracellualar potassium and calcium in rat cerebellar cortex related to local inhibitionof the sodium pump. *Pflugers Arch.* 395 (1982) 108-114.

Ullrich, K. J. Sugar, amino acid, and Na^+ cotransport in the proximal tubules. *Ann. Rev. Physiol.* 41 (1979) 181-195.

Wasterlain, C. G., Fujikawa, D. G., Penix, L. and Sankar, R. Pathophysiological mechanisms of brain damage from status epilepticus. *Epilepsia* 34 (1993) S37-S35.

Watts, A. G., Sanchez-Watts, G., Emanuel, J. R. and Levenson, R. Cell-specific expression of mRNAs encoding Na, K-ATPase α- and β-subunit isoforms within the rat central nervous system. *Proc. Natl. Acad. Sci. USA* 88 (1991) 7425-7429.

Westerink, B. H. C., Damsma, G. and De Vries, J. B. Effect of ouabain applied by intrastriatal microdialysis on the in vivo release of dopamine, acetylcholine, and amino acids in the brain of conscious rats. *J. Neurochem.* 52 (1989) 705-712.

Wyllie, A. H., Kerr, J. F. R. and Currie, A. R. Cell death: the significance of apoptosis. *International Review of Cytology* 68 (1980) 251-306.

Yarowsky, P. J. and Ingvar, D. H. Neuronal activity and energy metabolism. *Fed. Proc.* 40 (1981) 2353-2362.

Yoshida, S., Ikeda, M., Busto, R., Santiso, M., Martinez, E. and Ginsberg, M. D. Cerebral phosphoinositide, triacylglycerol, and energy metabolism in reversible ischemia: origin and fate of free fatty acids. *J. Neurochem.* 47 (1987) 774-757.

Yoshida, S., Ikeda, M., Busto, R., Santiso, M., Martinez, E. and Ginsberg, R. Cerebral phosphoinositide, triacylglycerol and energy metabolism in reversible ischemia; origin and fate of free fatty acids. *J. Neurochem.* 47 (1986) 774-757.

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