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**EPILEPTOGENESIS CAUSES LONG-TERM PLASTICITY CHANGES IN
EXPRESSION OF CALBINDIN D-28K IN THE RAT PILOCARPINE MODEL
OF ACQUIRED EPILEPSY**

A thesis submitted in partial fulfillment of the requirements for the degree of Master of
Science at Virginia Commonwealth University

by

Anne Elizabeth Johnston Harrison

Bachelor of Science in Pharmacy
Virginia Commonwealth University
May 1996

Bachelor of Science in Psychology
The College of William and Mary
May 1993

Director: Robert J. DeLorenzo, M.D., Ph.D., M.P.H.
Professor
Department of Neurology

Virginia Commonwealth University
Richmond, Virginia
December 2005

DEDICATION

To my husband, Steve, for all of his encouragement, love, and support. Te amo mejo.

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank a number of people without whom I could not have completed this thesis. Each of you has made immeasurable contributions to my education and personal growth. First, I must credit my family for their constant support, love, and encouragement in all of my endeavors, and for emphasizing the significance of continued learning throughout life.

I owe special thanks to my graduate advisor, Dr. Robert J. DeLorenzo, for his guidance and tremendous support. His enthusiasm for scientific research is an inspiration. I must also thank Dr. Robert Blair for his help with dissections, western blots, data analysis, and figure preparation. Bob, thank you for your tireless assistance with my project. Thank you to the other students in the lab--Laxmikant Deshpande and especially to Katherine Falenski--who were a great source of knowledge and assistance. Katie, I owe you a special thanks for taking me under your wing and teaching me so much. To my co-workers in the DeLorenzo laboratory: Dr. Dawn Carter, Dr. Nageeb Haider, Elisa Atkisson, and Pat Blanton. Your support and encouragement mean more than you know.

I am also grateful to Drs. Som Sombati and Sandra Welch, for serving on my graduate advisory committee. Finally, I'd like to thank my co-workers and students in the Churn and Miles laboratories whose support and enthusiasm make the sixth floor of the Medical Sciences Building such a wonderful place to be.

TABLE OF CONTENTS

	Page
List of Figures.....	vi
List of Abbreviations.....	vii
Abstract.....	viii
I. Introduction.....	1
A. Epilepsy.....	1
B. Molecular Mechanisms of Epilepsy.....	4
C. Glutamate and Glutamate Receptors.....	5
Ionotropic glutamate receptors.....	6
Metabotropic glutamate receptors.....	7
D. Calcium Ion Homeostasis.....	8
E. Intracellular Calcium Regulation.....	9
Influx of extracellular calcium across the plasma membrane.....	9
Calcium extrusion across the plasma membrane.....	9
Calcium buffering, sequestration, and storage.....	10
F. Calcium Binding Proteins.....	10
G. Central Hypothesis and Rationale.....	11
II. Materials and Methods.....	13
A. Pilocarpine-Induced Status Epilepticus.....	13
B. Epileptic Seizure Monitoring.....	14
C. Tissue Preparation and Immunohistochemistry.....	14
D. Western Blot Protocol.....	15
III. Results.....	21
A. Immunohistochemistry.....	21
B. Western Blot.....	23

IV.	Discussion.....	47
	A. Rationale and Summary of the Central Hypothesis.....	47
	B. Protein Synthesis.....	47
	C. Calcium as a Major Signaling Molecule.....	48
	D. The Calcium Hypothesis of Epileptogenesis.....	49
	E. Conclusions.....	52
V.	List of References.....	54
VI.	Vita.....	62

LIST OF FIGURES

Figure	Page
1. A silver stain was performed to assess correct protein loading into lanes of western blot.....	20
2. Naive controls and pilocarpine-treated animals have similar calbindin expression four days after treatment	26
3. Overall calbindin expression is not significantly decreased in epileptic animals one week after status epilepticus.....	28
4. Two weeks post status epilepticus, overall calbindin protein levels are not significantly decreased	30
5. Calbindin expression is significantly decreased in epileptic animals one month post status epilepticus.....	32
6. Four months post status epilepticus, calbindin still appears to be decreased in epileptic rats compared to controls.....	34
7. Calbindin expression remains significantly decreased in epileptic animals six months following pilocarpine treatment.....	36
8. Immunohistochemistry of hippocampal CA3 dendritic field reveals decreases in calbindin immunoreactivity at 6 months, but not at 4 days post pilocarpine treatment.....	38
9. Four days post-SE, high magnification photos reveal similar calbindin immunoreactivity in the dentate gyrus of epileptic and control rats.....	40

10. The dentate gyrus of epileptic rats six months post-SE show significantly decreased calbindin immunoreactivity.....	42
11. The CA1 dendritic field shows a loss of calbindin-positive neurons in epilepsy.....	44
12. Western blot analysis shows significant decreases in calbindin expression in the supernatant (cytosolic fraction) of epileptic hippocampi when compared to naive controls.....	46

ABSTRACT

EPILEPTOGENESIS CAUSES LONG-TERM PLASTICITY CHANGES IN CALBINDIN D-28K IN THE RAT PILOCARPINE MODEL OF ACQUIRED EPILEPSY

Anne Johnston Harrison

A Thesis submitted in partial fulfillment of the requirement of the degree of Master of Science at Virginia Commonwealth University

Virginia Commonwealth University, 2005

Director: Robert J. DeLorenzo, M.D., Ph.D., M.P.H.
Professor
Department of Neurology and Pharmacology/Toxicology

Epilepsy is one of the most common neurological disorders, affecting more than 2% of children and 1% of adults in the U.S. Emerging research has demonstrated that calcium, as a major second messenger system, underlies many of these injury-induced plasticity changes associated with the development of epilepsy. Recent evidence has suggested that long term elevations in neuronal resting calcium levels play a role in

initiating and maintaining epileptogenesis (the development of epilepsy). Collaborations between our lab and others have produced microarray data that suggests that a major calcium-binding protein, calbindin D-28k, mRNA levels are decreased in epileptic rats even up to one year following pilocarpine treatment. The goal of this research effort was to determine if epileptogenesis alters basal calcium levels by producing a long-term change in the expression of the major calcium binding protein in neurons, calbindin D-28k. Immunohistochemistry (IHC) and western blot experiments have been conducted to test the hypothesis that epileptogenesis produces a long lasting decrease in the expression of calbindin in the hippocampus in the rat pilocarpine model of acquired epilepsy. IHC experiments indicated that changes in calbindin expression occur gradually over a 2-4 week interval after the initial injury. Significant decreases in calbindin immunoreactivity are seen in the hippocampus of epileptic animals, at one month, four months, and six months post-pilocarpine treatment. However, these changes were not seen as early as 4 days post-status epilepticus. Western blots quantitated differences between epileptic animals and naive controls. Long lasting decreases in calbindin may play an important role in the altered calcium homeostatic mechanisms observed in epileptic neurons. These findings will help to elucidate one of many changes that occurs in epilepsy.

INTRODUCTION

Epilepsy

Epilepsy is a neurological disorder characterized by recurrent, unprovoked seizures. A seizure is defined as the synchronous discharge and uncontrollable firing of a population of neurons (Lothman et al., 1991). Seizure discharges have a distinct onset and termination. The occurrence of seizure activity does not necessarily indicate a permanent alteration in neuronal function. An isolated episode can be precipitated by a variety of insults to the brain, including trauma, toxins, drug withdrawal, and metabolic disturbances. Status epilepticus (SE) is defined as continuous seizure activity that lasts for 30 minutes or longer, or intermittent seizures without regaining consciousness between seizures lasting 30 minutes or longer. It can damage brain tissue and lead to the generation of spontaneous recurrent seizures (SRSs).

Epilepsy can vary in age of onset, cause, seizure type, and pattern of the electroencephalogram (DeLorenzo, 1989, 1991). This diversity of expression has led to the standard classification of epilepsy and the numerous different epilepsy syndromes (Frazen, 2000). A classification system has been developed that incorporates data from animal models and clinical evaluations (Lothman et al., 1991). *Partial* seizures involve specific brain foci and exhibit a variety of behavioral and motor characteristics. This

class of seizures is subdivided into *simple* and *complex*, the latter involving loss of consciousness. Partial seizures have the ability to propagate throughout the brain and become generalized. *Generalized* seizures involve large areas of the brain and are bilateral in nature. Although epilepsy can manifest itself in a number of different ways, each type of epilepsy shares the common feature of persistently increased neuronal excitability that manifests sporadically as seizure generation (Lothman et al., 1991; McNamara, 1994, 1999). Behavioral manifestations that ensue with generalized seizures range from loss of consciousness to major motor convulsions (tonic, clonic, or tonic-clonic). It has been estimated that approximately 10% of the population will experience a seizure at some time during their lifetime (Lothman et al., 1991).

Epilepsy impacts society on many different levels. From the economic standpoint, the total annual cost of epilepsy is estimated at nearly four billion dollars in direct medical expenses combined with indirect expenses such as lost wages, cost of home care, and premature death (Murray et al., 1996). Although advances have been made in the development of anticonvulsant drugs and the surgical treatment of epilepsy, approximately 50% of epilepsy cases remain refractory to medical interventions. This condition greatly burdens the quality of life of 1-2 million Americans (Hauser, 1990). In daily life, epileptics must deal with disease treatment issues and can suffer from limitations in mental and physical function which may affect the employment status of both the individual and family caregivers, as well as interpersonal relationships at home, work, and school (DeLorenzo et al., 1996; Cramer et al., 1999; Buelow, 2001). Thus, the

stigma associated with epilepsy as well as functional disabilities of the disease can greatly diminish the quality of life of persons with epilepsy.

Unlike a single seizure, epilepsy is associated with spontaneous recurrent seizures (SRS) and is the result of a long-lasting alteration in neuronal function at the molecular level. In roughly 50% of cases there is no known cause for the disorder (Hauser, 1983; DeLorenzo, 1991). These epilepsies are called idiopathic, in that they occur in the absence of other brain abnormalities (Frazee, 2000.) Ongoing research in the field of medical genetics has led to the elucidation of an underlying cause for some of these idiopathic cases with the identification of cell migration abnormalities (Copp and Harding, 1999; Rakic, 2000) and numerous gene mutations in humans (Bertrand et al., 1998; Wallace et al., 1998) and mouse models of epilepsy (Puranam and McNamara, 1999) that may underlie some of these idiopathic epilepsies. However, in the majority of idiopathic cases, the underlying cause of the epileptic phenotype is still unknown.

In the remaining estimated 50% of cases, a known cause or injury produces permanent plasticity changes that lead to the development of acquired epilepsy (AE) (Hauser, 1983; Lothman et al., 1991; McNamara, 1999). The transformation of healthy CNS tissue with a normal balance between excitation and inhibition to a brain with hyperexcitable populations of neurons is called epileptogenesis. It involves a permanent plasticity change from a known cause or injury that leads to the development of AE (Lothman et al., 1991; McNamara, 1999).

Molecular mechanisms of epilepsy

Epileptogenesis has been attributed to alterations in both excitatory and inhibitory synaptic function. Changes in these two systems, either independently or in concert with each other, play an important role in seizure induction. At the functional level, the multitude of underlying regulatory pathways involved in sustaining the epileptogenic phenotype is very complex. Many investigations employing both in vitro and in vivo models of epilepsy have shed light on some of these underlying mechanisms.

In many seizure models, a significant and well-documented phenomenon is the observation of selective cell loss in the hippocampus, accompanied by sprouting of mossy fibers onto targets that did not receive those inputs previously (Parent et al., 1997). This is also seen in brain tissue taken from temporal lobe epilepsy (TLE) patients (Sutula, 1990). The re-wiring of neuronal circuitry has been shown to predominantly occur in the hilar cells of the dentate gyrus (DG), a region usually resistant to seizure induction (Parent et al., 1997).

As part of the hippocampal formation, the dentate gyrus participates in the processes of learning, memory, motivation, integration of cognitive functions, alerting responses (Block, 1993). Via the entorhinal cortex, the dentate gyrus integrates inputs from a variety of cortical regions (Amaral and Witter, 1995). The loss of inhibition in the dentate gyrus may cause hyperexcitability of the CA3 and CA1 subregions; and this changed excitability state in the whole hippocampal formation could be one of the underlying mechanisms in the generation of spontaneous recurrent seizures. This

neurogenesis and aberrant synaptic reorganization leads to an increased number of excitatory synapses, culminating in the induction of a hyperexcitable state. The potentiation of neuronal excitability is thought to contribute to the pathophysiological manifestations evident in these models (Parent et al., 1997).

Glutamate and glutamate receptors

L-Glutamate is the most widespread amino acid in the brain and serves a number of functions in the CNS (Nicholls and Attwell, 1990). For instance, this dicarboxylic amino acid is a precursor to the inhibitory amino acid neurotransmitter γ -aminobutyric acid (GABA), for the Krebs cycle intermediate α -ketoglutarate, and for the amino acid glutamine. Glutamate also functions as a detoxification agent for ammonia products in the brain. In addition to the many metabolic roles of glutamate, the most significant function of glutamate in the brain is its function as the primary excitatory neurotransmitter (Mayer and Westbrook, 1987).

As a neurotransmitter, extracellular glutamate levels must be maintained at controlled levels. Under physiological conditions, extracellular glutamate has been measured in the range of 1-5 μ M (Wahl et al., 1994). Although transporters exist to move glutamate into the brain across the blood-brain-barrier, the majority of glutamate is synthesized *de novo* from either glucose, glutamine, or aspartate (Lattera et al., 1999). Glutamate is stored in synaptic vesicles at concentrations in excess of 20 mM via a magnesium (Mg^{2+})/ATP dependent transporter (Dingledine et al., 1999). The primary

mechanism for uptake of extracellular glutamate is a class of high affinity Na^+ dependent glutamate transporters found on neurons and astrocytes (Gegelashvili and Schousboe, 1997).

The signaling actions of glutamate are mediated at the neuronal membrane through specialized receptor macromolecules. The binding of glutamate to specific sites on its receptor molecule causes a conformational change that initiates signal transduction cascades in the neuron. Glutamate receptors are broadly categorized based on the signaling cascade they trigger. Ionotropic glutamate receptors are coupled to ion permeable channels which, under physiological conditions, depolarize neurons. In contrast, metabotropic receptors are coupled are coupled to guanosine triphosphate binding proteins (G proteins) and second messenger systems that modulate synaptic transmission (Dingledine et al., 1999).

Ionotropic Glutamate Receptors

The ionotropic glutamate receptors are post-synaptic, ligand-gated ion channels (Dingledine et al., 1999). Three types of ionotropic glutamate receptors have been categorized and named according to selective ability of N-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), or kainate (KA) to activate them (Dingledine et al., 1999).

The AMPA receptor contributes to the early, fast component of the excitatory post-synaptic potential (EPSP). As a low affinity glutamate receptor, the AMPA receptor

is typically permeable to the monovalent cations, sodium (Na^+) and potassium (K^+). However, AMPA receptors that lack a GluR2 subunit are also permeable to the divalent cation, Ca^{2+} (Wisden and Seeburg, 1993). This ligand-gated channel demonstrates little voltage dependence, and currents are very brief (a few milliseconds) due to the low glutamate affinity and a high rate of desensitization (Boulter et al., 1990; Dingledine et al., 1999).

KA receptors are very similar in function to AMPA receptors. Like AMPA receptors, KA receptors are voltage-independent, monovalent cation permeable channels with low affinity and fast kinetics (Michaelis, 1998). KA receptor-mediated EPSPs have smaller peak amplitudes and slower decay kinetics than those derived from AMPA receptors (Frerking and Nicoll, 2000).

The NMDA receptor is quite different from the AMPA and KA subtypes of glutamate receptor. First, in addition to their permeability to Na^+ and K^+ , NMDA receptors have high permeability to Ca^{2+} (Dingledine et al., 1999). NMDA receptors also have slower kinetics attributed to a much higher affinity for glutamate (Conti and Weinberg, 1999). The conductance through NMDA receptors can last several hundred milliseconds and constitutes a slower, later phase of the EPSP (Conti and Weinberg, 1999).

Metabotropic Glutamate Receptors

As previously mentioned, G-protein coupled metabotropic receptors are the other major category of glutamate receptors. There are eight types of metabotropic glutamate

receptors (mGluRs) that are further classified according to the second messenger systems to which they are linked (Conn and Pin, 1997). These receptors are found both on the pre-synaptic and post-synaptic membranes. Pre-synaptic mGluRs decrease neurotransmitter release, while mGluRs on the post-synaptic membrane regulate the function of ligand-gated ion channels including all three subtypes of ionotropic glutamate receptors (Anwyl, 1999). Thus, metabotropic glutamate receptors can act to modulate synaptic transmission in the CNS.

Calcium ion homeostasis

Calcium plays a fundamental role in the cell as a second messenger governing cellular functions such as differentiation and growth, membrane excitability, exocytosis, and synaptic activity. Neurons possess specialized homeostatic mechanisms to ensure tight command of cytosolic Ca^{2+} levels so that multiple independent Ca^{2+} -mediated signaling pathways can exist in the normal cell (Arundine and Tymianski, 2003). In excitotoxicity, excessive stimulation of glutamate receptors and an increase in extracellular glutamate concentration can lead to the dysregulation of Ca^{2+} homeostasis (Arundine and Tymianski, 2003). An overwhelming increase in free intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) can activate a self-destructive cellular cascade involving many calcium-dependent enzymes, such as phosphatases (eg, calcineurin), proteases (the calpains), and lipases. Lipid peroxidation can also cause production of free radicals which damage vital cellular proteins and lead to neuronal death (Choi, 1988; Michaels and Rothman, 1990; Tymianski and Tator, 1996; Delorenzo et al., 2005).

Intracellular calcium regulation

Influx of extracellular Ca^{2+} across the plasma membrane

The neuronal plasma membrane is relatively impermeable to Ca^{2+} with exclusion of three fundamental mechanisms of Ca^{2+} entry: ligand-gated cation channels, voltage-gated Ca^{2+} channels (VGCCs), and store-operated Ca^{2+} channels (SOCs). The NMDA receptor, a ligand-gated cation channel, mediates the vast majority of Ca^{2+} influx during excitatory neurotransmission (Ozawa, 1993). In addition, AMPA and KA receptors of certain subunit composition are permeable to Ca^{2+} (Jonas and Burnashev, 1995).

Calcium extrusion across the plasma membrane

Two transport systems exist to pump free intracellular Ca^{2+} out of the neuron into the extracellular space. Because Ca^{2+} extrusion acts against a large Ca^{2+} concentration gradient, these systems are energy-dependent and are, therefore highly susceptible to ischemic injury (Tymianski and Tator, 1996). The ATP-driven Ca^{2+} pump (Ca^{2+} - ATPase) expends one molecule of ATP for each Ca^{2+} ion extruded and is modulated by calmodulin, fatty acids, and protein kinases (Carafoli, 1992). The second transport system, the Na^{+} - Ca^{2+} exchanger, is indirectly coupled to ATP utilization in that it utilizes the Na^{+} concentration gradient maintained by the ATP driven Na^{+} - K^{+} exchanger. This electrogenic exchange system is triggered by increases in $[\text{Ca}^{2+}]_i$ and extrudes one Ca^{2+} for every two or three Na^{+} that enter the neuron (Tymianski and Tator, 1996).

Calcium buffering, sequestration, and storage

Calcium buffering and sequestration can also reduce free intracellular Ca^{2+} levels. The endoplasmic reticulum (ER) functions as a Ca^{2+} store. The ER accumulates Ca^{2+} via the sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase (SERCA). This enzyme is similar to the Ca^{2+} -ATPase of the plasma membrane in that it requires ATP. However, unlike the plasma membrane Ca^{2+} -ATPase, SERCA function is independent of calmodulin and it moves two Ca^{2+} ions into the ER for each ATP molecule utilized (Tymianski and Tator, 1996).

Due to their buffering properties, EF hand Ca^{2+} -binding proteins (CaBPs) can affect intracellular Ca^{2+} homeostasis. They play an important role as calcium transporters and represent one of the most important calcium compartments in the brain (Krsek et al., 2004). Calbindin D-28k, one of the major CaBPs, is present at high cytosolic concentrations in neurons such as purkinje cells and hippocampal granule cells. Together with its high cytosolic concentration, the ability of calbindin to bind up to four Ca^{2+} ions at a time suggests that it plays an important role in Ca^{2+} buffering (Mattson et al., 1995).

The hippocampal formation is a locus of epileptic seizure activity (Lothman et al., 1991). Recent research suggests that the absence of calcium buffer proteins results in marked abnormalities in cell firing (Bastianelli, 2003). The calcium-binding proteins are present mainly in GABAergic interneurons, thus their disturbance could result in an alteration of inhibitory mechanisms (Krsek et al., 2004). Hippocampal neurons rich in the main Ca^{2+} -binding protein, calbindin D-28k, appear to be relatively resistant to

degeneration in a variety of acute and chronic disorders (Sloviter, 1989; Hauser and Annegers, 1991; Magloczky et al., 1997).

Calbindin-like immunoreactivity is present in all dentate granule cells and some, but not all, CA1 and CA2 pyramidal cells in rat hippocampi. In area dentata, calbindin immunoreactivity is normally present in a small number of interneurons of the molecular and granule cell layers and in a small population of presumed basket cells in or below the granule cell layer. Calbindin immunoreactivity has typically been found in interneurons of the strata radiatum and lacunosum-moleculare layers of the CA1 field (Sloviter, 1989). Recent studies suggest that there is a loss of calbindin from granule cells of the dentate gyrus and select CA1 neuron populations in mouse models (Kohr et al., 1991; Gary et al., 2000) and in rat kindling models of epilepsy (Kohr et al., 1991). Thus, the possible role of Ca^{2+} as a second messenger mediating some of these changes in hippocampal CA neurons, dentate granule neurons, and interneurons is an important area of investigation.

Central Hypothesis

The rationale for this thesis comes from collaborations between our lab and others that have produced microarray studies showing a decrease in calbindin mRNA levels in rats six months and one year following pilocarpine-induced status epilepticus (DeLorenzo, Miles, in press). The central hypothesis to be tested in this thesis is that epileptogenesis causes long term plasticity changes in the expression of calbindin D-28k. Using the rat pilocarpine model of acquired epilepsy, this hypothesis will be tested by systematically addressing the following specific aims:

1. Determine the effect of epileptogenesis on the distribution of calbindin using immunohistochemistry.
2. Quantitate the effect of epileptogenesis on calbindin level using western blot analysis.
3. Evaluate the time course of the change in expression of calbindin employing immunohistochemistry.

MATERIALS AND METHODS

Pilocarpine-Induced Status Epilepticus

Male Sprague-Dawley rats weighing 200 to 250 g were used in accordance with university animal care and use protocols. Animals were housed in single cages on a 12-h/12-h light/dark cycle (lights on at 7:00 AM) and were provided food and water ad libitum. Animals were made epileptic using a modified protocol of (Mello et al., 1993) that is well established in our laboratory (Rice and DeLorenzo, 1998). Before pilocarpine injections, animals were administered methylscopolamine nitrate (1 mg/kg i.p.) to minimize peripheral, parasympathetic effects of pilocarpine treatment. Pilocarpine nitrate (375 mg/kg i.p.) was then administered 30 min later. Onset of status epilepticus (SE) typically occurred within 20 to 40 min after pilocarpine injection and was determined when the animal displayed continuous moderate to severe behavioral seizures characterized by forelimb clonus, rearing, and falling.

SE was defined as continuous seizure activity that lasted 30 min or longer or intermittent seizures without regaining consciousness between seizures that lasted 30 min or longer. The severity of convulsions was evaluated, and only those animals that displayed behaviors consistent with ongoing SE were used in the study (Rice and

DeLorenzo, 1998). Seizure activity was terminated by consecutive diazepam injections (5 mg/kg i.p., solubilized in 10% ethanol, 45% propylene glycol, and 45% H₂O) at 1, 3, and 5 h after the onset of SE. Animals continuing to display seizure activity beyond 6 h post onset of SE were euthanized. Control groups were composed of both naive and sham control animals that received methylscopolamine nitrate and diazepam injections only. Approximately 75% of the SE animals developed epilepsy under these conditions, and the mortality rate from SE was approximately 10%. SE animals that did not stop seizing with diazepam treatment were uncommon and represented less than 2% of the animals injected with pilocarpine.

Epileptic Seizure Monitoring

Seizures were monitored in freely moving animals via 24-hour video monitoring starting two weeks after pilocarpine treatment (Rice and DeLorenzo, 1998). Seizures were then evaluated using established techniques (Rice and DeLorenzo, 1998) and confirmed by an observer blind to experimental treatment. Only seizures of grade 3 or greater on the Racine (1972) scale were scored (i.e. forelimb clonus ± rearing ± falling). The onset of spontaneous seizure occurrence was typically 4-6 weeks after drug injection.

Tissue Preparation and Immunohistochemistry

Five epileptic and five control rats from each time point (except the 6-month group where only 4 epileptic and 4 controls were available) were transcardially perfused with isotonic saline, and brains were quick frozen and stored at –80°C in embedding

compound (Sakura Inc., Japan). Cryostat sections (10 μ M) were prepared for immunostaining using established techniques (Scharfman et al., 2002; Krsek et al., 2004). Adjacent tissue sections were evaluated morphologically using Nissl stain in order to determine general histological characteristics. There is approximately only a 10% cell loss in the hippocampus associated with pilocarpine treatment.

Adjacent tissue sections from each rat were processed for calbindin immunoreactivity. Briefly, sections were blocked in bovine serum for 1 h and then incubated with calbindin antiserum (Sigma-Aldrich, St. Louis, MO) at a 1:5000 dilution for 48 hours at 4°C. Tissue slices were then washed in PBS (three washes, each for 5 min), followed by biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) secondary antibody at 1:200 dilution for 1 h at room temperature. After again washing in PBS for 15 min, calbindin immunoreactivity was visualized by exposure to avidin-biotin complex and 3-3'-diaminobenzidine (Vector Laboratories, Burlingame, CA). Stained tissue sections were evaluated using a binocular microscope (Olympus America Inc., Melville, NY) and were photographed using a digital camera (Olympus America Inc.). Images were analyzed using pixel values from Scion Image analysis software (Scion Corporation, Frederick, MD).

Western Blot Protocol

Gel electrophoresis was carried out on rat hippocampal homogenate and cytosolic preparations from epileptic animals 4 months after SE and age-matched, naive controls.

After monitoring of epileptic animals to verify seizure activity, the rats were sacrificed, and hippocampal tissue was harvested on ice. Hippocampi were homogenized in 50 mM Tris, pH 7.5, 6 mM EGTA, 6mM EDTA, 320 mM sucrose, 1 mM dithiothreitol, and 0.3 mM phenylmethylsulfonyl fluoride. Samples were supplemented with the following protease inhibitors: aprotinin (10 μ g/ml), PMSF (1 mmol/L) and leupeptin, (20 μ g/ml) (Sigma-Aldrich, St. Louis, MO). Cytosolic fractions were isolated from neuronal membranes by centrifugation (Morris et al., 2000). Before electrophoresis, samples were thawed on ice and protein concentration per sample was calculated using the Micro Bradford reagent system (Bio-Rad, Hercules, CA) quantified using a UV-2101PC ultraviolet spectrophotometer (Shimadzu, Kyoto, Japan).

Samples were balanced to 8 μ g protein/gel lane and denatured in beta-mercaptoethanol and loading dye buffer. Samples were then heated to 90°C for 5 min in a programmable thermal controller PTC100 (MJ Research, Watertown, MA) and allowed to cool to room temperature before loading onto a 4-12% Bis-Tris-HCl Ready Gel (Invitrogen Life Technologies, Carlsbad, CA). A colorimetric molecular mass marker including standards ranging from 10 to 182 kDa (ProSieve; Cambrex Bio Science Rockland, Inc., Rockland, ME) was loaded onto the last lane of the gel to aid in determining protein size. Gels were assembled into a MiniProtean II Electrophoresis System (Bio-Rad, Hercules, CA) and resolved for 90 min at 220 V constant in Tris buffer (Bio-Rad). After electrophoresis, gels were Western blot transferred to Immobilon nylon membrane (Millipore Corp., Bedford, MA) overnight at 4°C using a Genie transfer

apparatus (IDEA Scientific, Minneapolis, MN) at a constant 15 V. Transfer buffer consisted of Tris-glycine buffer containing 10% methanol. After transfer, the Western blot was stored in phosphate-buffered saline at 4°C overnight; and gels were processed with a silver stain kit (Biorad) to assess correct protein loading (Figure 1).

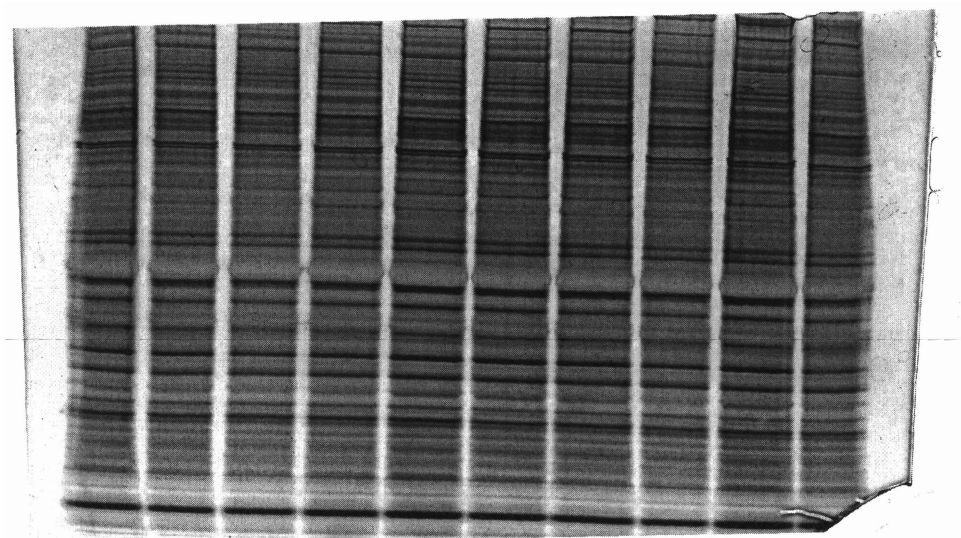
Immunostaining of the Western blot was performed by first blocking the membrane in buffer composed of 5% blotting grade blocker (Bio-Rad, Hercules, CA) and 0.05% Tween 20 in phosphate-buffered saline for 45 min at room temperature. Mouse (polyclonal) anti-calbindin primary antibody (Sigma-Aldrich, St. Louis, MO) was added to the blocking solution at a dilution of 1:3000, and the membrane was incubated for 90 min at room temperature. After primary antibody incubation, the membrane was washed for a total of 25 min (five times for 5 min each) in phosphate-buffered saline with Tween (PBS-T). The membrane was then reblocked in fresh blocking buffer for 30 min. Anti-mouse IgG-horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was then added to the blocking solution in a 1:1000 dilution, and the membrane was incubated for a final 45 minutes. Western blots were washed (five times for 5 min each) in PBS-T and incubated for 5 min in SuperSignal (Pierce Chemical, Rockford, IL) for enhanced chemiluminescent analysis. Chemiluminescent images were visualized using Kodak X-Omat Blue XB-1 X-ray film (Eastman Kodak, Rochester, NY) and developed using a Kodak M35A X-Omat Processor (Eastman Kodak). Film images were digitized using a gel scanner and analyzed by computer-assisted densitometry (Amersham Biosciences Inc., Piscataway, NJ).

Membranes were stripped and re-blotted with anti- β -actin antibody (Sigma-Aldrich, St. Louis, MO) (1:5000 in PBS) as a second means to assess correct protein loading. Using the molecular mass marker as reference, the only visible protein band was determined to correspond to a mass of 28 kDa.

Statistical Analyses

Results are given as means \pm standard error of the mean (SEM). Statistical comparisons were made using SigmaStat (SPSS Science, Chicago, IL). The student's t-test was utilized to compare control and pilocarpine-treated groups. Graphs were generated using Origin 6.1 software (Microcal Software, Inc., Northampton, MA).

Figure 1. Gels were processed with Silver Stain Plus kit (Biorad) immediately following the transfer step to assess correct protein loading.



RESULTS

This study is based on 68 animals that had been either treated with pilocarpine and subsequently had status epilepticus (SE) ($n = 34$) or age-matched naive controls ($n = 34$). Of the 34 pilocarpine-treated rats, animals were perfused at varying times after pilocarpine-induced status epilepticus (4 days, 1 week, 2 weeks, 1 month, 4 months and 6 months post-SE). The pilocarpine-treated animals in the 1 month, 4 month, and 6 month post-SE groups all had been observed having recurrent, spontaneous motor seizures after SE. Motor seizures were noticed as early as two weeks after pilocarpine treatment and continued for months until animals were sacrificed. Animals had at least one seizure when observed during at least two separate 24-hour video monitoring periods, and manifested an average of 3.0 seizures per 24-hour period. Control rats never exhibited behavioral seizures. They were perfused at the same time points as age-matched post-SE epileptic animals.

Immunohistochemistry

To evaluate the anatomical distribution of the decrease in calbindin expression in epileptic brains, we conducted immunohistochemical staining of calbindin protein on coronal hippocampal sections using established techniques (Wittner et al., 2002; Dinocourt et al., 2003; Krsek et al., 2004). In addition to measuring right and left whole

hippocampi, pixel values from a non-brain region of the slide were measured. In an effort to minimize the influence of any background staining, background values were subtracted before averaging the right and left hippocampi together. Epileptics were compared with naive controls using Scion Image computer software. All measurements were in the linear range between 0 (lightest) and 250 (darkest).

Animals that experienced SE that were sacrificed four days ($n = 10$) and 1-week ($n = 10$) after pilocarpine showed calbindin staining patterns that were indistinguishable from naive controls in most instances. Pixel value means and standard errors were 43.7 ± 3.3 and 48.3 ± 6.4 for naive and SE rats, respectively, in the 4-day post-SE group as shown in Figure 2. For animals in the 1-week group, these values were 9.9 ± 4.2 and 9.4 ± 3.2 for naives and post-SE animals (Figure 3).

Two weeks post-pilocarpine treatment there also did not appear to be a significant overall decrease in calbindin immunoreactivity (IR). Control animals ($n = 5$) had a mean pixel value of 22.7 ± 2.7 , while epileptic rats ($n = 5$) had a mean of 17.9 ± 2.8 (Figure 4). Interestingly, slides from two of the five rats that had experienced status in the two-week group had noticeably less calbindin present than naive animals indicating that they were sacrificed as changes and possible synaptic reorganization were occurring.

In control animals of all groups, calbindin immunoreactive neurons predominated in stratum pyramidale of the hippocampus, and in stratum granulosum and hilus of the dentate gyrus. Average pixel value analysis of 1 month, 4 month, and 6 month post-SE

epileptic animals showed a significant overall decrease in calbindin immunoreactivity ($p < 0.05$) using the student's t-test for statistical analysis (Figures 5, 6, and 7). A significant decline in the number of calbindin immunoreactive neurons was demonstrated in the CA3 region (Figure 8), and in both blades of the dentate gyrus (Figures 9 and 10) in pilocarpine-treated rats sacrificed at one month, four months, and six months post-SE. A majority of epileptic animals also showed a loss of calbindin in the CA1 dendritic field (Fig. 11). These differences were especially striking when compared to data from the 4-day post-SE animals.

Western blot analysis

Using Western blot analysis, we compared naive control with epileptic hippocampal cytosolic fractions (supernatant) and membrane fractions (P2 pellets). The data show a significant decrease in average optical densities in the five epileptic lanes when compared to control loaded with 8 μ g of protein from the cytosolic fraction, the compartment of the cell where calbindin is found (Figure 12B). Samples consisted of five naive and five epileptic animals sacrificed 4 months post-SE as described previously using anti-calbindin primary antibody at a dilution of 1:3000 (Sigma-Aldrich, St. Louis, MO) and goat anti-mouse IgG HRP secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a dilution of 1:1000. Quantification of the bands shown in Figure 11A revealed that expression of this protein was decreased 63% in the cytosolic fraction of hippocampi from epileptic rats ($n = 5$) compared with naive control animals ($n = 5$; $p <$

0.006, student's t-test), suggesting that a long-term plasticity change in the expression of calbindin occurs with epilepsy. After correcting calbindin protein levels to internal protein standards in a silver stain, we still observed a significant decrease in the calbindin expression in epileptic animals.

Figure 2. Calbindin expression was not significantly decreased in pilocarpine-treated rats sacrificed four days after status epilepticus (SE). **A:** Mean pixel value for the control group 43.7 ± 3.3 ($n = 5$). Pilocarpine treated rats ($n = 5$) had a mean pixel value of 48.3 ± 6.4 . **B:** Representative control and pilocarpine-treated hippocampi of animals sacrificed four days post pilocarpine treatment. In this and all other figures, images were acquired with an Olympus DP 10 digital camera and photos were assembled using Adobe Photoshop (version 5.0); images were digitally sharpened, contrast was changed, and color was adjusted to reflect the original tissue specimens. (Data are reported as mean \pm SEM.)

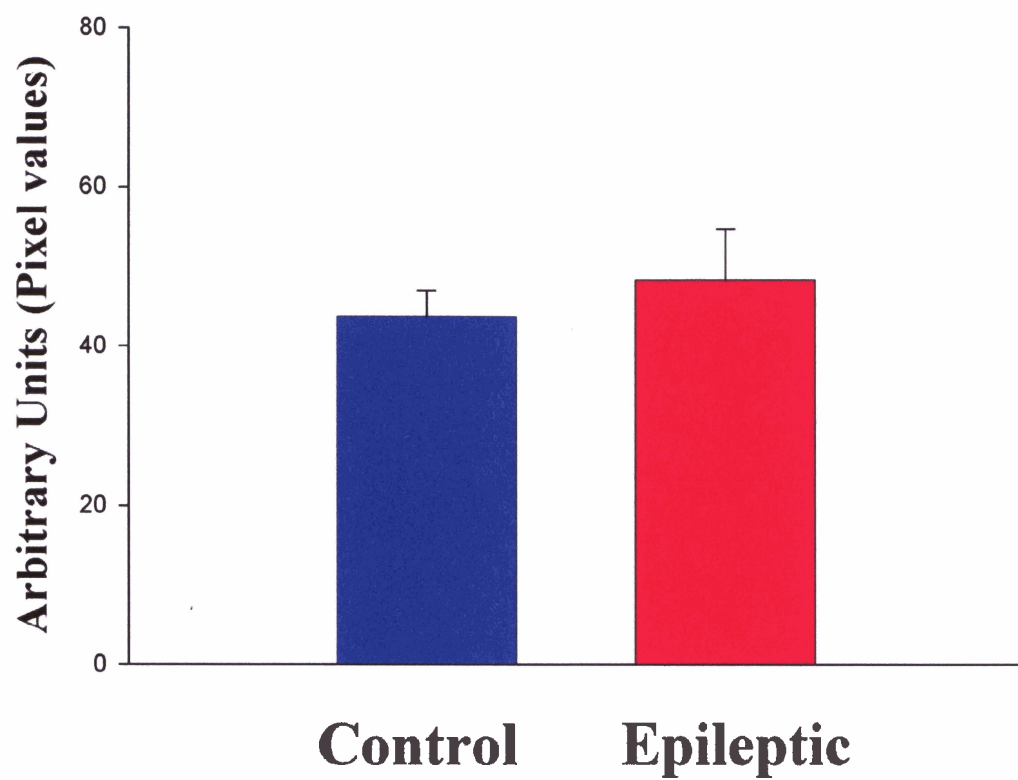
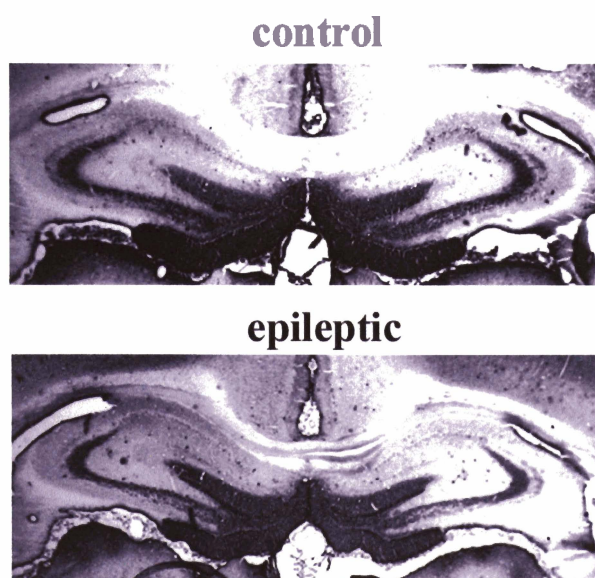
A**B**

Figure 3. Calbindin expression was not significantly decreased in pilocarpine-treated rats sacrificed one week after status epilepticus (SE). **A:** Mean pixel value for the control group was 9.9 ± 2.1 ($n = 5$). Pilocarpine treated rats ($n = 5$) had mean pixel value of 9.4 ± 3.2 . **B:** Representative control and pilocarpine-treated hippocampi of animals sacrificed one week post pilocarpine treatment are shown.

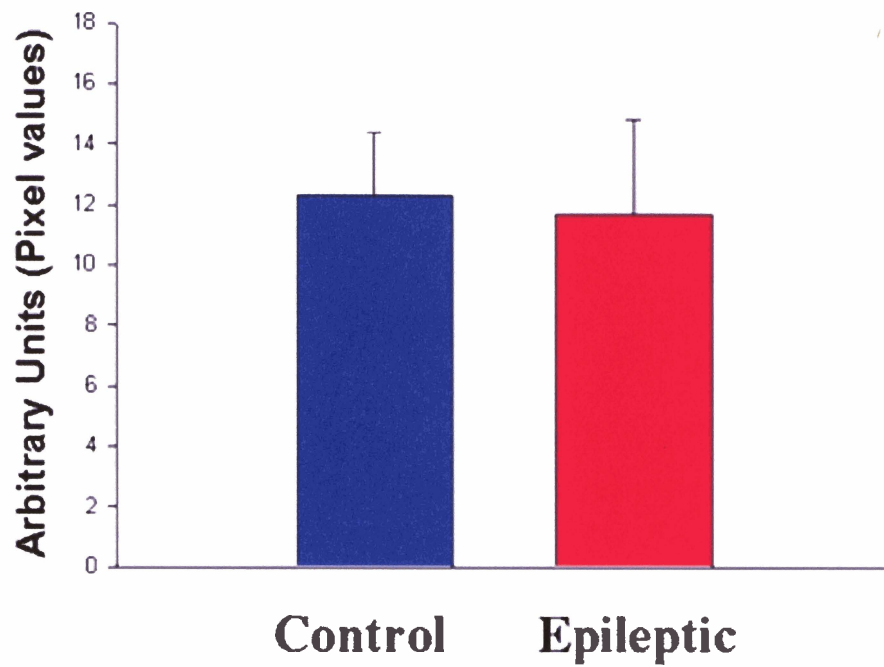
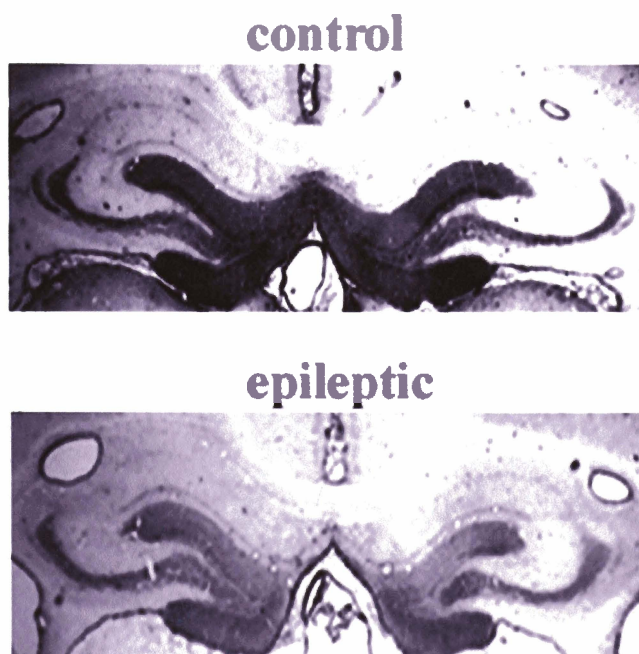
A**B**

Figure 4. Two weeks after pilocarpine treatment, there was not a significant overall decrease in calbindin protein levels. **A:** Mean pixel value for control group was 22.7 ± 2.7 ($n = 5$). Pilocarpine treated rats ($n = 5$) had mean pixel value of 17.9 ± 2.8 . **B:** Representative control and pilocarpine-treated hippocampi of animals sacrificed two weeks post pilocarpine treatment.

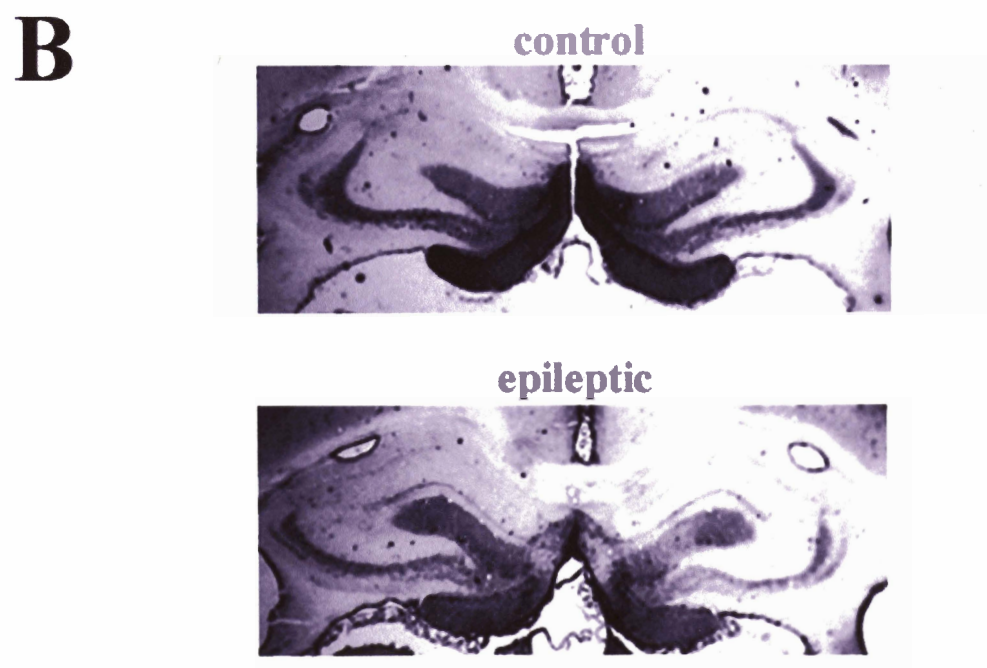
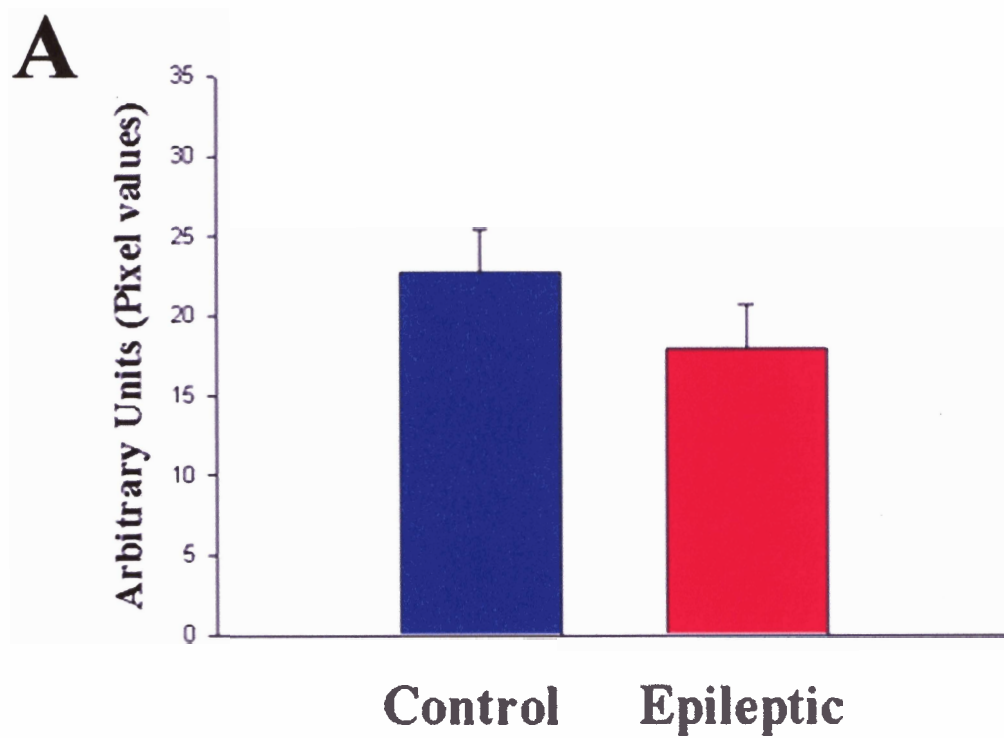


Figure 5. Calbindin expression is significantly decreased one month after status epilepticus (*, $p < 0.05$, student's t-test). **A.** Mean pixel value for control group was 43.9 ± 4.3 ($n = 5$). Pilocarpine treated rats ($n = 5$) had mean pixel value of 26.9 ± 4.3 . **B.** Representative control and pilocarpine-treated hippocampi of animals sacrificed one month post pilocarpine treatment.

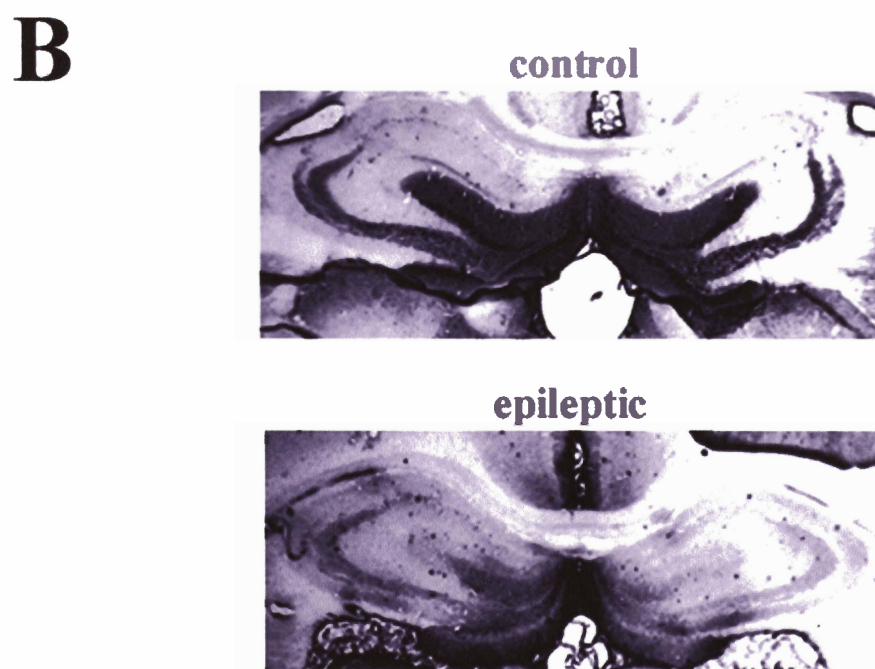
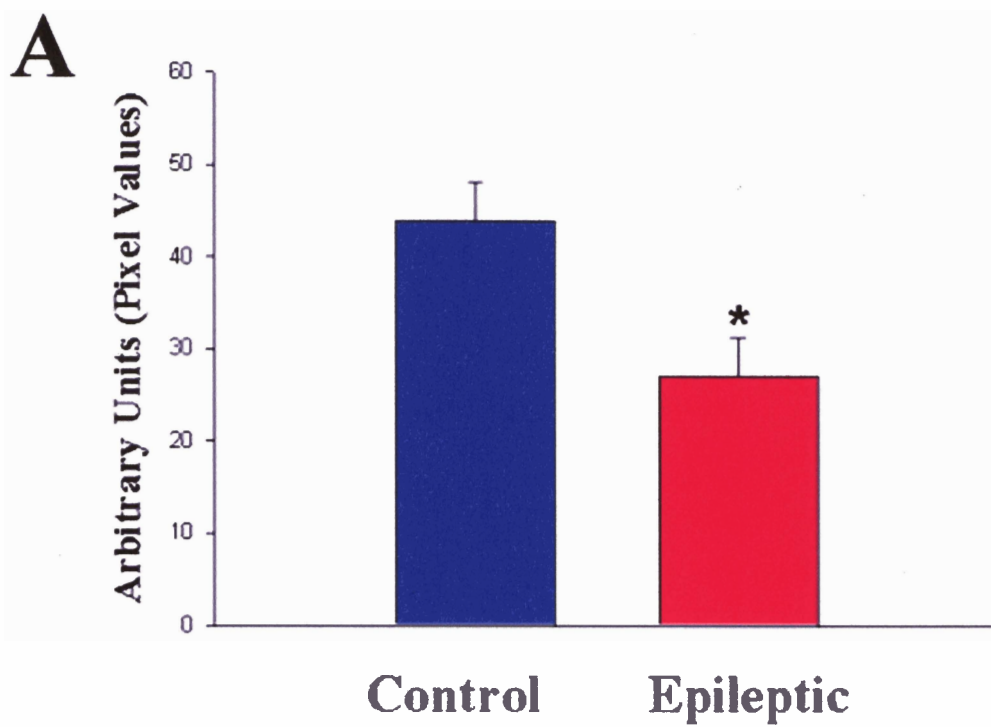


Figure 6. Four months after status epilepticus, calbindin protein levels remain significantly decreased ($p < 0.05$, student's t-test). **A.** Mean pixel value for control group was 49.7 ± 3.5 ($n = 5$). Pilocarpine treated rats ($n = 5$) had mean pixel value of 35.1 ± 2.4 . **B.** Representative control and pilocarpine-treated hippocampi of animals sacrificed four months post pilocarpine treatment.

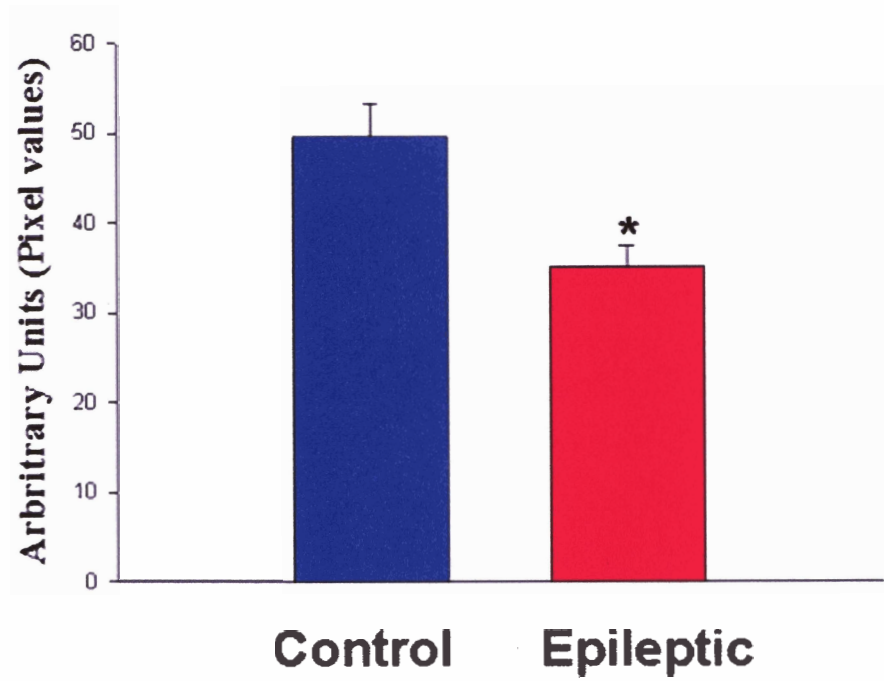
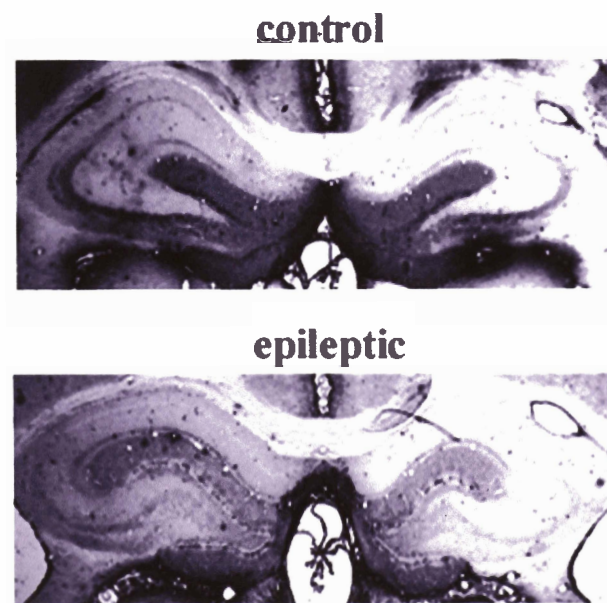
A**B**

Figure 7. Six months post pilocarpine treatment, overall calbindin protein levels remain significantly decreased ($p < 0.05$, student's t-test). **A.** Mean pixel value for control group was 27.2 ± 4.3 ($n = 4$). Pilocarpine treated rats ($n = 4$) had a mean pixel value of 12.1 ± 2.4 . **B.** One representative control and epileptic hippocampi of animals sacrificed six months post status epilepticus are shown.

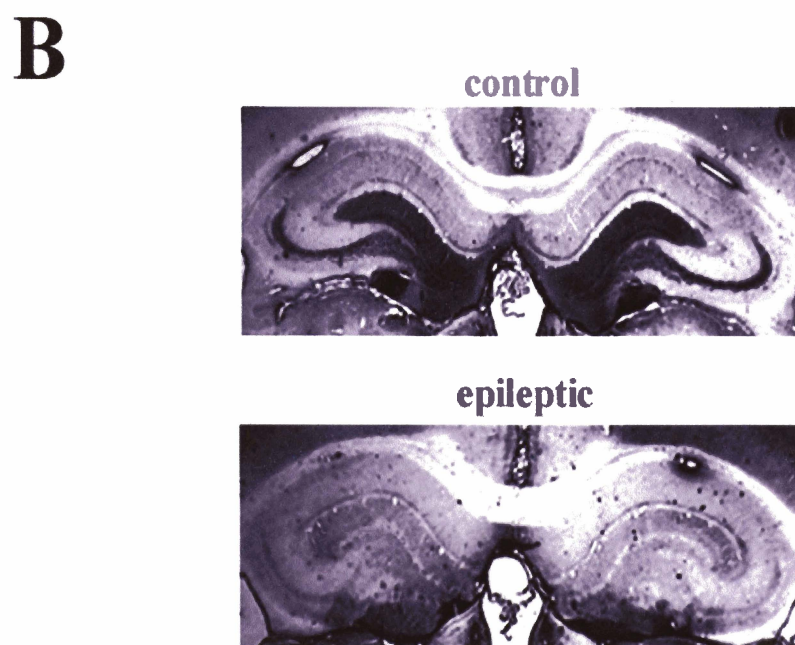
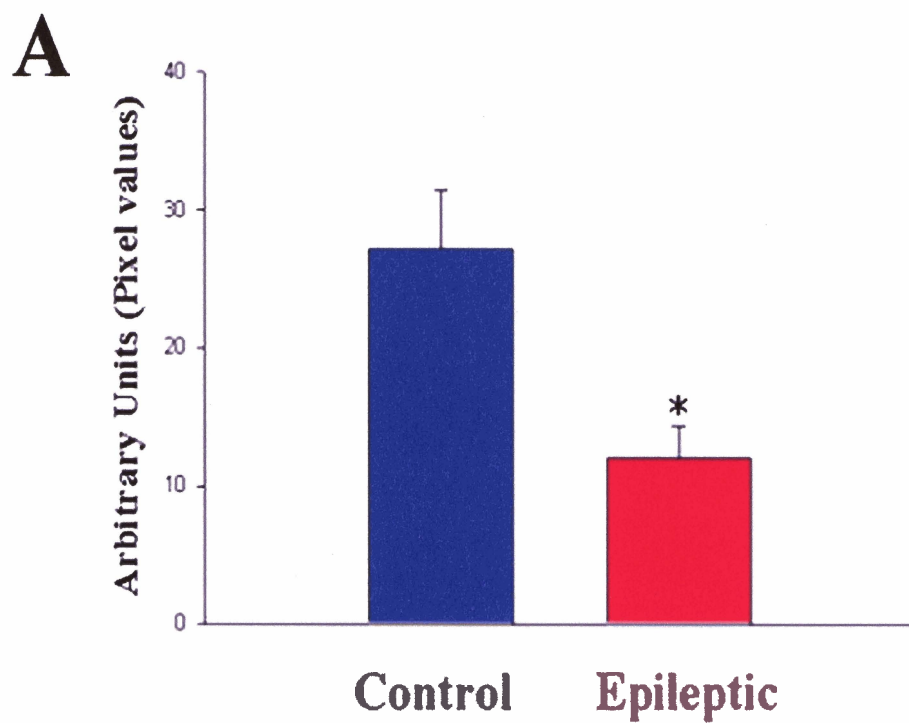


Figure 8. Immunohistochemical analysis of control and pilocarpine treated rats reveals no significant decrease in calbindin in CA3 dendritic field in animals that were sacrificed four days post treatment (A). In contrast, epileptic rats show a significant loss of calbindin protein in the CA3 field at the one month post-SE time point (B) as well as at the six month post-SE time point (C).

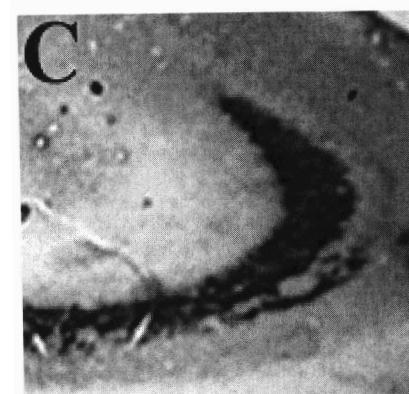
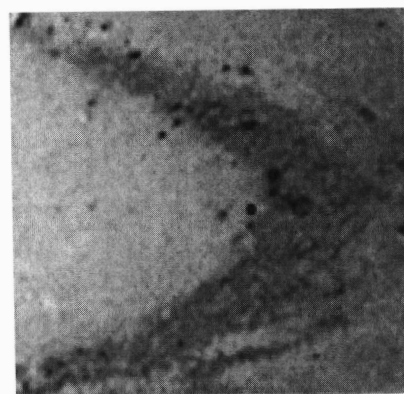
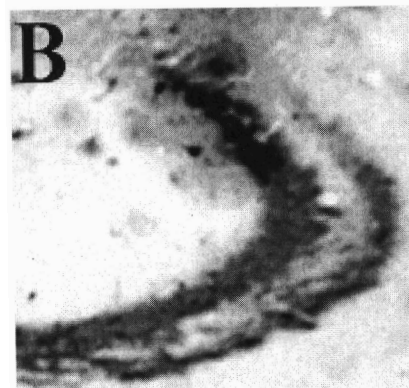
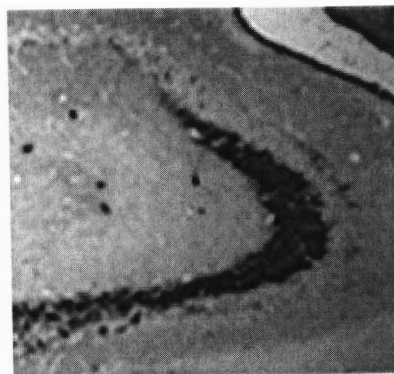
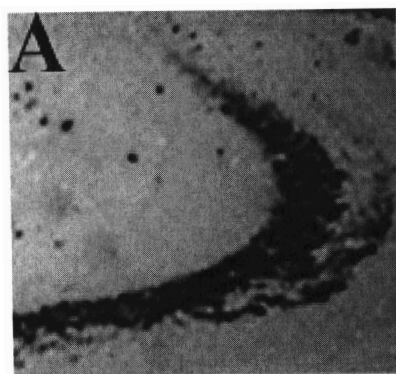
control**epileptic**

Figure 9. Calbindin expression in the granule cell layer of the dentate gyrus in the hippocampi of naïve rats (A) is similar to pilocarpine treated animals (B) at 4 days post-SE. Higher magnification photos of the dentate gyrus of control (C) and epileptic (D) animals reveal almost identical calbindin staining patterns.

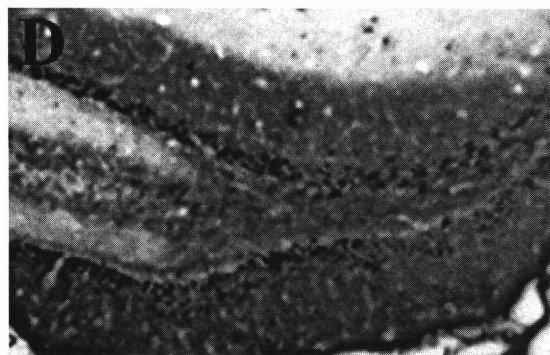
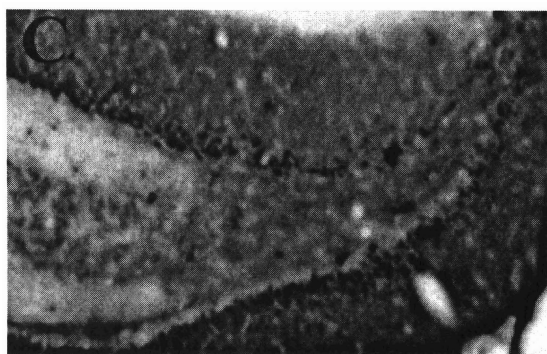
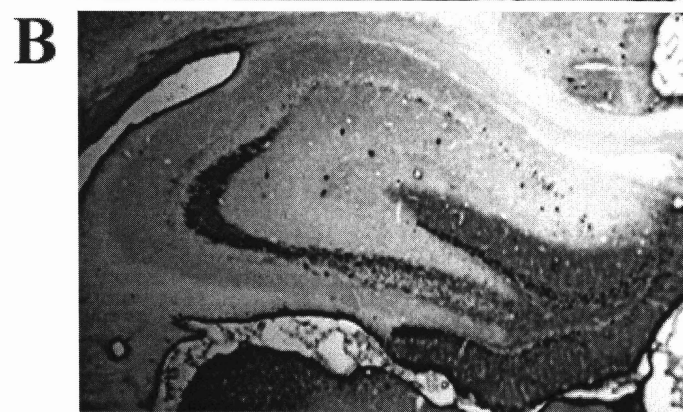
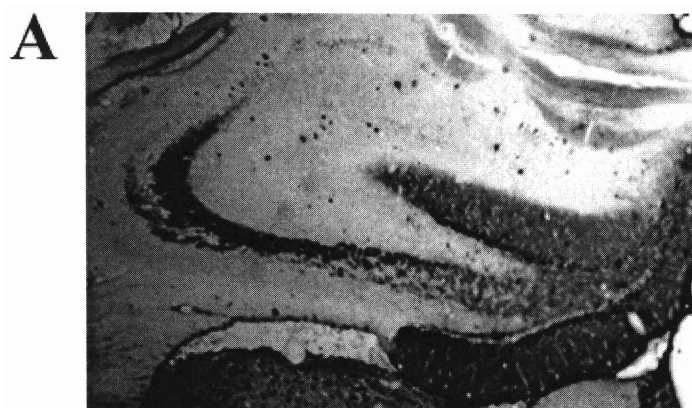


Figure 10. The dentate gyrus of control rat (**A**) exhibits greater calbindin immunoreactivity than an epileptic rat six months post-SE (**B**). Higher magnification photos reveal the significant loss of calbindin in the blades of the dentate gyrus in epileptic rats (**D**) when compared to naive controls (**C**).

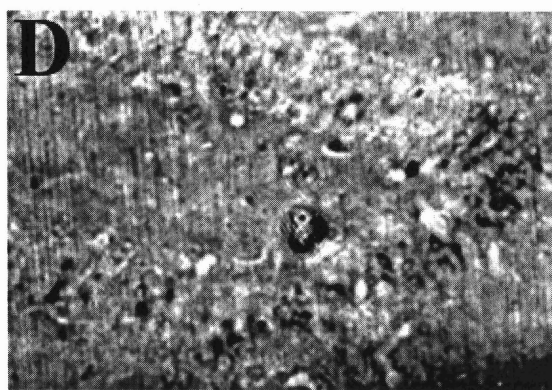
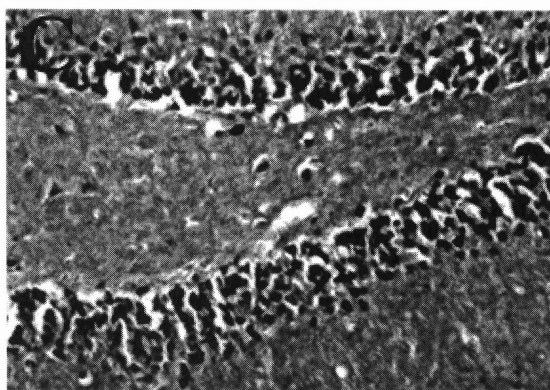
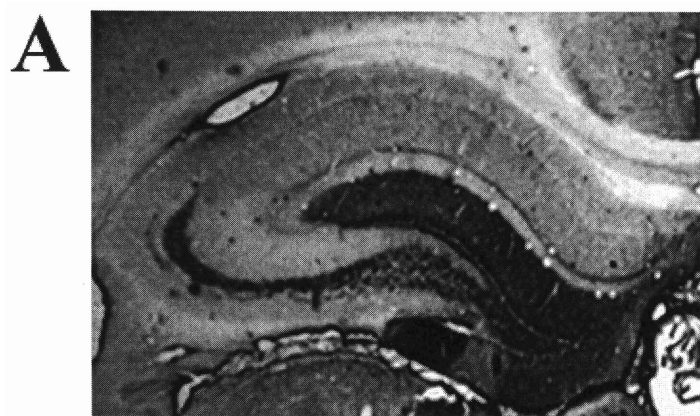


Figure 11. High magnification (200 x) photographs show a loss of calbindin-positive neurons in the CA1 dendritic field in epileptic rats (**B**) when compared to naive controls (**A**). o = stratum oriens; p = stratum pyramidale; r = stratum radiatum.

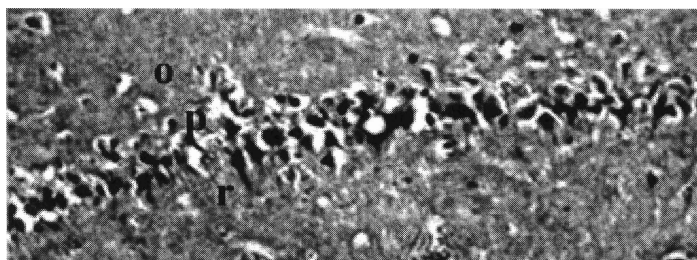
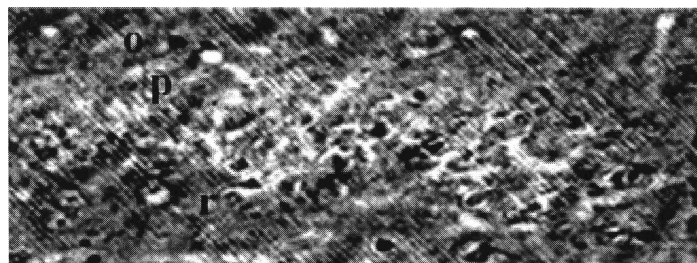
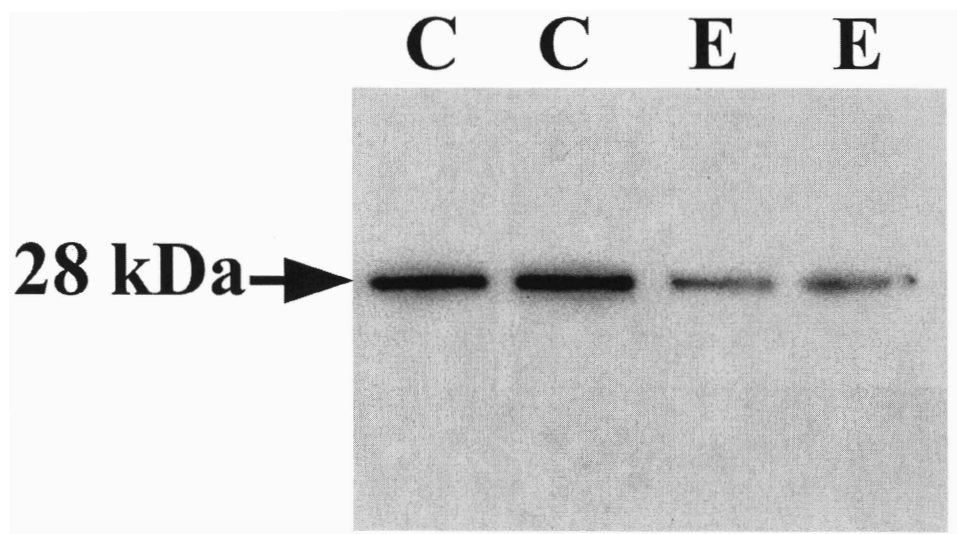
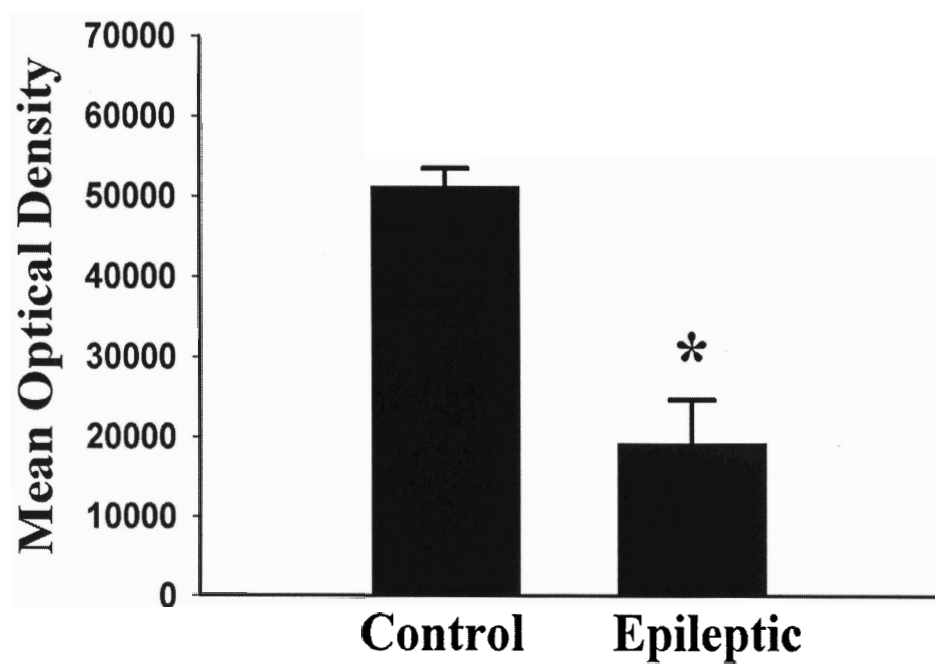
A**B**

Figure 12. Quantification of western blots revealed a significant decrease in calbindin levels in the supernatant (cytosolic fraction) of epileptic hippocampi. **A.** A blot with four representative lanes, two control (C) and two epileptic (E) samples. **B.** Mean optical densities of all ten lanes averaged together is significantly less in epileptic tissue when compared to naive animals (*, $p < 0.006$, student's t-test).

A**B**

DISCUSSION

Rationale and Summary of the Central Hypothesis

The calcium-binding protein, calbindin, plays an important role as a calcium transporter and as a buffering system for intracellular calcium ions, and represents one of the most important calcium compartments in the brain (Newman et al., 2002). Neuronal calbindin mRNA decreases don't necessarily correspond to a lower amount of protein translation. Because of the possibility of post-transcriptional modifications that can occur and the number of steps involved in translation of mRNA into protein, decreased mRNA levels do not always lead to a decrease in protein synthesis. Microarray studies suggest a decrease in calbindin mRNA levels as long as one year post-pilocarpine treatment. This thesis study found decreases in calbindin in epilepsy and looked at protein levels to determine when changes in expression were occurring. Previous studies have reported the vulnerability of calbindin-positive neurons in the dentate granule cell layer of the hippocampus in epilepsy (Scharfman et al., 2002; Krsek et al., 2004). The present findings confirm these observations and further demonstrate that this loss in protein corresponds to a decrease in calbindin mRNA expression.

Protein Synthesis

Protein synthesis requires highly specific molecular signals and occurs in three

carefully orchestrated phases--initiation, elongation, and termination. Messenger RNA (mRNA) carries the genetic information copied from DNA in the form of a series of three-base code "words," each of which specifies a particular amino acid. Protein synthesis is a multi-step process where amino acids are added one at a time. Each polypeptide chain has a specific starting point, and growth proceeds in one direction to a fixed terminus. Often, the primary synthetic product is then modified. Translation is the whole process by which the base sequence of an mRNA is used to order and to join the amino acids in a protein. The three types of RNA (mRNA, transfer RNA, and ribosomal RNA) participate in this essential protein-synthesizing pathway in all cells (Lodish et al., 2000).

Calcium as a Major Signaling Molecule

Calcium is a major signaling molecule in neurons; therefore, neuronal free $[Ca^{2+}]_i$ is highly regulated. Normal neuronal $[Ca^{2+}]_i$ is maintained around 100 nM (Mody et al., 1995). This concentration is less than one ten-thousandth of the free extracellular Ca^{2+} concentration (Putney and McKay, 1999). Calcium plays a pivotal role in normal neuronal function (Berridge, 1998; DeCoster et al., 1992; Tymianski and Tator, 1996). Calcium signaling in neurons is involved in processes as diverse as cell growth and differentiation (Spitzer, 1994), synaptic activity (Brose et al., 1992; Llinas et al., 1992), maintenance of the cytoskeleton (Trifaro and Vitale, 1993), and gene expression (Carafoli et al., 1997). Brief, controlled elevations in Ca^{2+} occur during physiological

processes such as neurotransmitter release and the plasticity changes of long-term potentiation in learning and memory (Malenka and Nicoll, 1999; Gnegy, 2000; West et al., 2001; Tzounopoulos and Stackman, 2003; Delorenzo et al., 2005).

The Calcium Hypothesis of Epileptogenesis

It is important to emphasize that epileptogenesis is a complex process, and there may be many other second messenger systems interacting with Ca^{2+} or acting independently in producing and maintaining AE. However, the evidence for the role of Ca^{2+} in this process and the close relationship between this second messenger to injury make it a likely important regulator of epileptogenesis.

A major theory in developing the role of Ca^{2+} in the development of AE is that there is a continuum of severity in the effects of Ca^{2+} on neuronal tissue. Olney (1969) initially developed this concept in the discovery of excitotoxicity. Small changes in Ca^{2+} levels produced by glutamate receptor stimulation results in activities related to synaptic transmission and normal physiological activity. However, excessive activation of the glutamate receptors can actually excite the neuron to death, leading to the term, excitotoxicity. In between normal physiologic functions and cell death, there are other effects of prolonged activation of glutamate receptors, especially the NMDA receptors. Prolonged or increased activation of glutamate receptors have been implicated in the neuronal plasticity changes of memory and long-term potentiation (Davies et al., 2002; Lynch, 2004).

The development of AE is one of the most dramatic examples of long-term plasticity changes in neurons. Following an initial neuronal injury, permanent plasticity changes develop, which lead to the induction and maintenance of AE. Since dead neurons do not seize, it is reasonable to assume that the injury phase of AE produces prolonged activation of glutamate receptors that exceed the effects developed in normal physiological function and memory, yet stop short of producing excitotoxicity and cell death. Thus, the severity of glutamate stimulation and its duration are essential in the development of AE (DeLorenzo et al., 2005).

Overwhelming, irreversible elevations in $[Ca^{2+}]_i$, as observed in glutamate excitotoxicity, have been implicated in mechanisms of delayed neuronal death secondary to SE and other conditions such as stroke and traumatic brain injuries (TBIs) (Golding et al., 1999; Gopinath et al., 2000; Lenzlinger et al., 2001). The Ca^{2+} hypothesis of epileptogenesis postulates that the pathophysiological effects of Ca^{2+} on neuronal function may lie on a continuum with one extreme characterized by brief, controlled Ca^{2+} loads of normal function, another extreme characterized by irreversible Ca^{2+} loads and neuronal death, and a middle ground that is characterized by sublethal, prolonged, but reversible, elevations in $[Ca^{2+}]_i$ that trigger pathological plasticity changes, leading to the development of epilepsy and the persistent elevations in $[Ca^{2+}]_i$ that play a role in maintaining chronic epilepsy (DeLorenzo et al., 2005). In other words, both excitotoxicity and epileptogenesis require NMDA receptor activation and the presence of extracellular

Ca^{2+} during initiation. With both excitotoxicity and epileptogenesis neurons endure large elevations of $[\text{Ca}^{2+}]_i$. In excitotoxicity, these elevations progress to an irreversible loss of Ca^{2+} homeostasis and neuronal death. In epileptogenesis, though, these elevations, though prolonged, are buffered over time and lead to permanent plasticity changes and neuronal hyperexcitability.

The findings presented in this thesis study demonstrate that a significant change in calbindin expression occurs with the epileptic phenotype. The data suggest that calbindin levels in the hippocampus, specifically in the dentate granule cells, CA3 region, and CA1 dendritic field, decrease significantly between 2 weeks and 1 month after CNS insult/injury, during the time that corresponds to the latency phase of epileptogenesis. Preliminary immunohistochemical analysis of specific regions was performed. Results indicated no significant differences between controls and epileptics in the 4 day post-SE group when comparing dentate granule cells, CA1, and CA3 regions. However, one month post-SE, epileptic animals had even more significantly decreased calbindin protein immunoreactivity ($p < 0.005$) in the molecular layer of the dentate gyrus and in the CA3 region than controls when compared to pixel values for whole hippocampus.

Because calcium-binding proteins are present mainly in GABAergic interneurons, their disturbance could therefore refer to an alteration of inhibitory mechanisms (Krsek et al., 2004). This type of alteration has been observed in the dentate gyrus of rats previously exposed to pilocarpine-induced SE. Dentate granule cells demonstrated

prolonged EPSPs and discharged more action potentials in comparison with controls. In addition, IPSP conductances as well as frequency of GABA-A spontaneous and miniature IPSCs were decreased, thus confirming a loss of inhibition of granule cells (Kobayashi and Buckmaster, 2003; Krsek et al., 2004). According to microarray studies, calbindin mRNA is still decreased in epileptic rats six months and even one year after pilocarpine treatment, indicating that this change in calbindin expression is prolonged and probably permanent.

Conclusions

The major finding of this thesis was that calbindin expression significantly decreases between two weeks and one month after status epilepticus in the rat pilocarpine model of epilepsy. The decreased calbindin expression displayed in the hippocampi of epileptic animals was regionally specific, occurring primarily in the dentate gyrus, CA3 dendritic field, and in some animals, in the CA1 field. This decrease in calbindin expression was seen up to six months after the induction of epilepsy and thus demonstrates a long-lasting or permanent plasticity change in the brain that may play a role in the pathophysiology of epilepsy. The observation that morphological changes occur over the course of two to four weeks after initial insult suggests that interventions during this time period or sooner could possibly be beneficial. Although it is unlikely that restoring calcium homeostatic mechanisms to normal in epileptic brain tissue will completely reverse all of the complex changes associated with AE, it is possible that it

may restore enough normal physiological function to the epileptic neuron to decrease or even terminate seizure discharge. A better understanding of the mechanisms that underlie the pathophysiological changes occurring after a debilitating, but not lethal, CNS insult will aid in the elucidation of the pathogenesis of epilepsy.

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VITA

Anne Elizabeth Johnston Harrison was born on July 25, 1971 in Richmond, Virginia and is an American citizen. She graduated from Highland Springs High School in 1989, and earned a Bachelor of Science degree from the College of William and Mary in 1993. Anne graduated from Virginia Commonwealth University's School of Pharmacy in 1996, and is a registered pharmacist practicing in retail and hospital settings.

During graduate school, Anne has presented abstracts and posters at numerous meetings and symposiums including Daniel T. Watts Poster Symposium, Central Virginia Chapter Society for Neuroscience, and the Society for Neuroscience. In addition to her graduate studies, Anne served on the Medical College of Virginia Honor Council. She is also a member of the American Pharmacist Association and the Virginia Pharmacy Association.

ABSTRACTS

Harrison, A. J., Blair, R. E., Miles, M. F., and DeLorenzo, R. J. Vimentin Expression in the Rat Pilocarpine Model of Acquired Epilepsy. Twenty first Annual Daniel T. Watts Poster Symposium, Abstract and poster, 2004.

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Falenski, K. W., Blair, R. E., Harrison, A. J., Martin, B. R., and DeLorenzo, R. J. Immunohistochemical Time-Course of Hippocampal Cannabinoid CB₁ Receptor Redistribution in the Rat Pilocarpine Model of Acquired Epilepsy. Society for Neuroscience Annual Meeting, Abstract and Poster, 2005.

MANUSCRIPTS

Harrison, A. J., Blair, R. E., Falenski, K. W., Miles, M. F., and DeLorenzo, R. J.
Epileptogenesis Causes Long-Term Plasticity Changes in the Expression of
Calbindin D-28k in the Rat Pilocarpine Model of Acquired Epilepsy. In
preparation, The Journal of Pharmacology and Therapeutics.