Published in final edited form as:
Epilepsy Res. 2008 May ; 79(2-3): 213–223. doi:10.1016/j.eplepsyres.2008.02.006.

Long-term decrease in calbindin-D28K expression in the hippocampus of epileptic rats following pilocarpine-induced status epilepticus

Dawn S. Carter[a], **Anne J. Harrison**[a], **Katherine W. Falenski**[a], **Robert E. Blair**[a], and **Robert J. DeLorenzo**[a,b,c,*]

[a] Department of Neurology, Virginia Commonwealth University School of Medicine, Richmond, VA 23298, United States

[b] Department of Pharmacology and Toxicology, Virginia Commonwealth University School of Medicine, Richmond, VA 23298, United States

[c] Department of Molecular Biophysics and Biochemistry, Virginia Commonwealth University School of Medicine, Richmond, VA 23298, United States

Summary

Acquired epilepsy (AE) is characterized by spontaneous recurrent seizures and long-term changes that occur in surviving neurons following an injury such as status epilepticus (SE). Long-lasting alterations in hippocampal Ca^{2+} homeostasis have been observed in both in vivo and in vitro models of AE. One major regulator of Ca^{2+} homeostasis is the neuronal calcium binding protein, calbindin-D28k that serves to buffer and transport Ca^{2+} ions. This study evaluated the expression of hippocampal calbindin levels in the rat pilocarpine model of AE. Calbindin protein expression was reduced over 50% in the hippocampus in epileptic animals. This decrease was observed in the pyramidal layer of CA1, stratum lucidum of CA3, hilus, and stratum granulosum and stratum moleculare of the dentate gyrus when corrected for cell loss. Furthermore, calbindin levels in individual neurons were also significantly reduced. In addition, the expression of calbindin mRNA was decreased in epileptic animals. Time course studies demonstrated that decreased calbindin expression was initially present 1 month following pilocarpine-induced SE and lasted for up to 2 years after the initial episode of SE. The results indicate that calbindin is essentially permanently decreased in the hippocampus in AE. This decrease in hippocampal calbindin may be a major contributing factor underlying some of the plasticity changes that occur in epileptogenesis and contribute to the alterations in Ca^{2+} homeostasis associated with AE.

Keywords

Acquired epilepsy; Calbindin; Pilocarpine; Calcium homeostasis; Status epilepticus; Hippocampus

Introduction

Epilepsy is a common neurological disorder affecting approximately 1–2% of the population worldwide (Hauser and Hesdorffer, 1990; McNamara, 1999) and is characterized by the occurrence of spontaneous recurrent seizures (SRSs). Acquired epilepsy (AE) is often caused

*Corresponding author at: Department of Neurology, Virginia Commonwealth University, PO Box 980599, Richmond, VA 23298, United States. Tel.: +1 804 828 8968; fax: +1 804 828 6432. rdeloren@vcu.edu (R.J. DeLorenzo).

by a known brain injury, such as status epilepticus (SE), stroke, or traumatic brain injury that induces long-lasting changes in plasticity in previously normal brain tissue. This process has been termed epileptogenesis and leads to the development of SRSs (Hauser and Hesdorffer, 1990). These long-term changes in plasticity that occur in surviving neurons induce essentially permanent alterations in these neurons that have been implicated in causing the long-term morbidity associated with AE (Delorenzo et al., 2005). The process of epileptogenesis may also be influenced by genetic predisposition (Scher, 2003; Chaix et al., 2007) and cause long-term genetic changes in the brain (Elliott et al., 2003; Lukasiuk et al., 2006). Understanding how these changes promote epileptogenesis and the development of AE is an important goal in developing rational therapeutic approaches to reducing the mortality and morbidity of AE.

Several studies have demonstrated that SE produces acute and chronic elevations in intracellular Ca^{2+} ($[Ca^{2+}]_i$) and alterations in Ca^{2+} homeostatic mechanisms in the hippocampus, suggesting that alterations in Ca^{2+} dynamics play a major role in the development of many of the neuronal plasticity changes associated with the induction of AE and the maintenance of the epileptic phenotype (DeLorenzo et al., 1998; Raza et al., 2001; Raza et al., 2004; Delorenzo et al., 2005). Thus, it is important to understand the effects of epileptogenesis on Ca^{2+} homeostatic mechanisms. Calcium plays a fundamental role in neurons as a second messenger governing cellular functions such as differentiation and growth, membrane excitability, exocytosis, and synaptic activity (Delorenzo et al., 2005). It has been shown that some of the long-term alterations in Ca^{2+} homeostatic mechanisms observed after epileptogenesis are mediated by long-term alterations in the function of the endoplasmic reticulum Ca^{2+} ATPase and IP_3 Ca^{2+} induced Ca^{2+} release system (Pal et al., 1999; Pal et al., 2000; Parsons et al., 2000; Pal et al., 2001; Parsons et al., 2001; Parsons et al., 2004). In addition, calbindin-D28k is one of the major calcium binding proteins in brain and 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; Tang et al., 2006). The expression of calbindin-D28k is altered by SE in granule cells of the dentate gyrus and CA1 neurons in the mouse pilocarpine model (Tang et al., 2006) and in the rat kindling model (Kohr et al., 1991). Alternatively, calbindin expression has been shown to increase immediately following acute seizures (Lowenstein et al., 1991; Lowenstein et al., 1994; Lee et al., 1997). These results indicate that SE causes significant changes in calbindin expression, but that these changes may vary depending on the time of observation, the region studied and the model employed for observation. Because of the important role of calbindin-D28k in buffering intracellular neuronal Ca^{2+} ions (Mattson et al., 1995), it is important to evaluate both the acute and long-term effects of SE on the expression of calbindin-D28k in the pilocarpine model of AE.

This study was initiated to evaluate changes in the expression of calbindin in principle cells of the hippocampus using the rat pilocarpine model of AE that employed 1 h of SE. Changes in the expression of calbindin were determined using immunohistochemical analysis and by Western blot evaluation. In epileptic rats, a predominant long-term decrease in calbindin expression was observed in the dentate gyrus, the CA3, and CA1 regions. Immediately after SE no major changes were observed in calbindin expression. The decrease in calbindin protein expression was also observed in animals as early as 1 month following SE and in animals that were epileptic for 2 years. This decrease in calbindin protein levels was associated with a significant decrease in the expression of calbindin mRNA. The observed decrease in hippocampal calbindin levels were essentially permanent and indicate that this long-term decrease in the expression of this major Ca^{2+} binding protein may contribute to the inability of the epileptic brain to regulate intracellular calcium.

Materials and methods

Pilocarpine-induced SE

Sprague–Dawley male rats (Harlan) weighing 200–250 g and having an age of 6 weeks were used in strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by Virginia Commonwealth University's Institutional Animal Care and Use Committee. 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 SE typically occurred within 20–40 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. 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). Sixty minutes after the onset of SE, rats were administered diazepam (5 mg/kg i.p., solubilized in 10% ethanol, 45% propylene glycol, and 45% H_2O) followed by additional diazepam injections at 3 and 5 h after the onset of SE to control further seizure activity. Control groups were composed of sham control animals that received methylscopolamine nitrate and diazepam injections only and naïve animals (three sham controls and two naïve controls). In preliminary experiments, there were no differences found between these two groups and therefore the data were pooled to form the control group for these studies. To screen for epilepsy, rats were video recorded for 96 h consecutively at 6 months post-SE for routine experiments and at both 12 and 24 months post-SE for long-term studies to confirm the presence of SRSs. To qualify as a behavioral seizure for this study, the event must score at least a 3 on the Racine seizure scale (Racine, 1972). Control rats did not exhibit any behavioral seizures.

Tissue preparations and immunohistochemistry

Pilocarpine-treated and control rats from each time point were anesthetized with ketamine/xylazine cocktail (75 mg/kg/7.5 mg/kg; i.p.) and transcardially perfused with 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4). Perfusion was continued until full body fixation was observed. Following completion of the perfusion, brains were removed and cryoprotected in a 30% sucrose solution and flash frozen in isopentane for storage at −80 °C. Cryostat sections (40 μm) were prepared for immunostaining using established techniques (Scharfman et al., 2002; Krsek et al., 2004). Briefly, floating sections were blocked in Superblock (Pierce) with 0.4% Triton-X100 for 1 h and then incubated with polyclonal calbindin antiserum (Sigma–Aldrich, St. Louis, MO) at a 1:5000 dilution for 48 h at 4°C. Antibodies were diluted in Superblock with 0.4% Triton X-100. Tissue slices were then washed in PBS with 0.4% Triton X-100, followed by biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) secondary antibody at 1:2000 dilution for 1 h at room temperature. Calbindin immunoreactivity was visualized by exposure to avidin–biotin complex and 3-3′-diaminobenzidine (Vector Laboratories, Burlingame, CA). The length of time the sections were exposed to 3-3′-diaminobenzidine was kept consistent for each section from control and epileptic animals. Sections were dehydrated and mounted on slides for visualization and analysis. Adjacent sections were Nissl stained for analysis of cell loss.

Images were visualized on an Olympus IX-70 inverted microscope (Olympus America) using a 20× water immersion lens and captured with the Hamamatsu ORCA-ER CCD camera (Hamamatsu Photonics K.K., Japan). High magnification images were visualized with a 40× oil immersion lens. Image acquisition and processing was controlled using UltraVIEW™ Imaging system software v5.2 (PerkinElmer).

Western blot analysis

Gel electrophoresis was carried out on rat hippocampal cytosolic fractions from control and epileptic animals as previously described (Wallace et al., 2003). Briefly, hippocampi were homogenized and crude membranes were removed by centrifugation (Morris et al., 2000). Protein samples were balanced to 8 μg prior to gel loading. Following electrophoresis, proteins were transferred to Immobilon nylon membrane (Millipore Corp., Bedford, MA) for immunodetection. Calbindin was detected with a mouse (polyclonal) antibody (Sigma–Aldrich, St. Louis, MO) at a dilution of 1:3000 and an anti-mouse IgG-horseradish peroxidase-conjugated secondary antibody (1:1000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). SuperSignal (Pierce Chemical, Rockford, IL) was used 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. Membranes were stripped and re-blotted with anti-β-actin antibody (Sigma–Aldrich, St. Louis, MO) at a dilution of 1:5000 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.

RNA isolation and quantitative real-time PCR

RNA was isolated from whole hippocampi from control and epileptic animals. For isolation, the RNA NOW-TC kit was used (Biogentex, Seabrook, TX). Briefly, whole hippocampi were homogenized with a Polytron PT 1200. Following homogenization, RNA was extracted using a phenol extraction buffer and chloroform. The solution was centrifuged at 10,000 × *g* for 10 min at 4 °C. The aqueous phase was carefully removed and added to equal volume of isopropanol for the precipitation of the RNA. Following another 10,000 × *g* spin at 4 °C for 10 min, the RNA pellet was washed in 75% ethanol, dried, and resuspended in DEPC water (Sigma–Aldrich, St. Louis, MO). RNA concentrations were determined using the SmartSpec 3000 (BioRad Laboratories, Hercules, CA).

Quantitative real-time PCR (RT-PCR) experiments were performed in the ABI Prism® 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) using the TaqMan® One Step PCR Master Mix Reagents Kit (P/N: 4309169). All the samples were tested in triplicate under the conditions recommended by the fabricant. The cycling conditions were: 48 °C/30 min; 95 °C/10 min; and 40 cycles of 95 °C/15 s and 60 °C/1 min. The cycle threshold was determined to provide the optimal standard curve values (0.98–1.0). The probes and primers were designed using the Primer Express® 3.0 version. Two sets of probes were constructed, a short probe from the 5′ end of the gene and a long probe from the middle of the gene. For experiments run with the short probe, the forward primer sequence was 5′-CGCTCAGCGCTCTCTCAAA-3′, the reverse primer sequence was 5′-GTGAGGCTGTGATCAGAGATGACT-3′, and the probe sequence was 5′-TAGCCGCTGCACCATGGCAGAA-3′. For experiments run with the long probe, the forward primer sequence was 5′-CCGAACAGATCTTGCCCTTATT-3′, the reverse primer sequence was 5′-GCGCACAGTTATGGTTTTAGATACA-3′, and the probe sequence was 5′-TGGTGGCCACAACCACTTGCTAGTGATAC-3′. The probes were labeled in the 5′ end with FAM (6-carboxyfluoresceine) and in the 3′ end with TAMRA (6-

carboxytetramethylrhodamine). The reactions and the synthesis of the probes and primers were performed in the VCU Nucleic Acid Research Facilities.

Data and statistical analyses

Pixel values for immunohistochemical analysis were obtained with Image J software. Individual hippocampal regions, including CA1 stratum pyramidale, CA3 stratum lucidum, stratum granulosum, stratum moleculare, and hilus, were analyzed. Background staining was obtained using a no primary antibody control and subtracted from stained regions. For analysis of calbindin expression in individual soma, cells were identified by the presence of nuclei. Comparisons of background-corrected pixel values between control and pilocarpine-treated rats were made with the Student's *t*-test (SigmaStat®, version 3.1, SPSS Inc., Chicago, IL). For uniform presentation between each study, pixel values were converted to percent decrease of calbindin expression and presented as percent of control. Standard techniques were employed to evaluate cell counts (Mello et al., 1993). Adjacent sections were Nissl stained, and high-resolution images were acquired for analysis. Cell counts were made using the Abercrombie correction in the stratum granulosum of the dentate gyrus, hilus, and stratum pyramidale of the CA1 and CA3 regions (Abercrombie and Johnson, 1946). Neurons clearly identifiable with nuclei were counted and cell densities were determined (cells/mm^3). Cell densities were compared between control and epileptic animals with ANOVA (SigmaStat®). Data were expressed as percent of control.

Densitometric analysis of the Western blot was obtained using Image J software. Bands were normalized with β-actin loading control and control and epileptic densities were compared using Student's *t*-test. RT-PCR data from control and epileptic tissue were normalized using the 18S internal control. Comparisons between control and epileptic tissue were made with the Student's *t*-test. $p \leq 0.05$ was considered statistically significant for all data analysis. Graphs were created in SigmaPlot® software (version 9.0, SPSS Inc., Chicago, IL).

Results

Decreased hippocampal calbindin protein levels in epileptic rats

Immunohistochemical staining was used to evaluate the anatomical distribution and expression of calbindin in the hippocampus of control ($n = 5$) and epileptic ($n = 5$) rats (Fig. 1). Calbindin staining was dramatically decreased throughout the hippocampus of epileptic brains by 61.3 ± 9.9% compared to control (Fig. 1, $p < 0.001$, Student's *t*-test). Using Western blot analysis, the observed decrease in hippocampal calbindin protein expression in epileptic rats was confirmed and quantified (Fig. 2). The cytosolic fractions from whole hippocampal homogenates from epileptic ($n = 5$) and control ($n = 5$) rats were compared, revealing a 63 ± 9.6% decrease in the protein levels of calbindin in the epileptic tissue (Fig. 2B, $p < 0.01$, Student's *t*-test).

Decreased hippocampal calbindin mRNA expression in epileptic rats

To determine if the observed decrease in calbindin protein levels was due to a down regulation of the expression of the calbindin gene, quantitative RT-PCR was performed. RNA was isolated from whole hippocampi from control ($n = 5$) and epileptic ($n = 5$) animals for RT-PCR analysis. mRNA levels from control and epileptic animals were normalized with an 18S endogenous control for comparison. Two sets of probes were used, one short probe from the 5′ end of the gene and a longer probe from the middle of the gene. Results from both sets of probes showed a 40 ± 7.9% decrease in calbindin mRNA expression in epileptic animals compared to control (Fig. 3, $p < 0.01$, Student's *t*-test). Therefore, the decrease in calbindin protein expression in epileptic animals was associated with a significant down regulation of the expression of the calbindin gene in the form of calbindin mRNA.

Regional hippocampal decrease in calbindin protein expression

When looking regionally within the hippocampus, calbindin is highly expressed in the stratum pyramidale of the CA1 region, stratum lucidum of the CA3 region, the hilar region, and in the stratum granulosum and stratum moleculare of the dentate gyrus. In the epileptic brain, calbindin staining was dramatically reduced in all of these regions. In the stratum pyramidale of the CA1, calbindin expression was reduced by 62.9 ± 17.3% (Fig. 4, $p < 0.05$, Student's *t*-test). Calbindin staining was decreased in the stratum lucidum of the CA3 by 66.8 ± 11.9% (Fig. 4, $p < 0.05$, Student's *t*-test). In the dentate gyrus, calbindin expression was decreased in the stratum granulosum, stratum moleculare, and hilar region by 63.7 ± 6.8%, 49.9 ± 7.3, and 61.6 ± 9.7%, respectively (Fig. 4, $p < 0.01$, Student's *t*-test). It is important to evaluate cell loss in each of these regions to determine that the observed decrease in calbindin was not due to neuronal loss.

Hippocampal cell loss has been characterized in the pilocarpine model of AE (Mello et al., 1993; Rice and DeLorenzo, 1998; Falenski et al., 2007). To ensure that the decrease in calbindin expression was not a result of hippocampal cell loss associated with this model of AE, we performed cell counts in adjacent Nissl stained sections used for this study using established techniques (Mello et al., 1993) and presented the data as a percent of control (Fig. 5A). The mean cell loss in the CA1 stratum pyramidale, the CA3 stratum pyramidale, the hilus, and the stratum granulosum of the dentate gyrus were 14.1 ± 5.3%, 4.8 ± 4.2, 18.9 ± 8.3%, and 4.5 ± 6.2%, respectively. Only the cell loss in the CA1 and hilus were significantly different from control (Fig. 5A, $p < 0.05$, ANOVA). The amount of cell loss observed in these regions could not fully account for the corresponding decrease in calbindin shown in Fig. 4.

To further evaluate the decrease in hippocampal calbindin expression in epileptic animals, levels of calbindin antibody stain were measured within individual neurons in the CA1 stratum pyramidale and the stratum granulosum in the dentate gyrus. By evaluating calbindin levels in individual neurons that survived epileptogenesis, we could more accurately determine changes in neuronal calbindin levels in the epileptic condition. The stratum pyramidale of the CA1 and stratum granulosum of the dentate gyrus were analyzed at higher magnification (40×) and calbindin levels were measured from a large sampling of 70–100 soma per animal. This analysis was conducted on a large number of cells from control and epileptic animals in order to provide a representative sampling of the neurons in these regions. Calbindin expression in surviving neurons in the CA1 stratum pyramidale from epileptic tissue was reduced by 61.9 ± 3.0% compared to control (Fig. 5B, $p < 0.01$, Student's *t*-test). In the stratum granulosum of the dentate gyrus, surviving neurons from epileptic tissue had 81.0 ± 2.4% less calbindin than control tissue (Fig. 5B, $p < 0.001$, Student's *t*-test). These results demonstrate that calbindin expression was decreased in surviving cells from epileptic tissue compared to controls. Thus, this data provides strong evidence that the calbindin decrease in the hippocampus of epileptic animals was not primarily due to cell loss, but rather due to a decrease in the expression of calbindin in individual neurons.

Hippocampal calbindin protein expression during SE and epileptogenesis

Since many plasticity changes occur at various times during the epileptogenic phase following SE, we examined the time course of the decrease in calbindin expression during epileptogenesis by evaluating calbindin levels at different times after SE. Rats were perfused at various time points following SE, including 1 h ($n = 4$), 24 h ($n = 4$), 7 days ($n = 5$) and 30 days ($n = 5$). Age-matched control rats were sacrificed and perfused at the same time points. Immunohistochemical analysis for calbindin was evaluated in the hippocampi of rats from each time point. Pixel values from stained tissue were acquired from whole hippocampus and converted to percent of control for comparative analysis. One hour following SE onset, calbindin staining did not change compared to control (93.9 ± 8.3%). This was of particular

importance because it indicates that the seizures during SE alone are not causing calbindin levels to decrease. At 1-day and 7 days post-SE calbindin staining patterns were indistinguishable from controls (99.3 ± 8.1% and 100.2 ± 2.1%, respectively) whereas at 30 days post-SE, there was a 40.6 ± 5.1% overall decrease in calbindin expression (Fig. 6, $p < 0.001$, Student's *t*-test). These results indicate that the decrease in calbindin expression occurred at least 1 week after SE and were maximal at 1 month. Thus, the observed decrease in calbindin expression was not an acute effect and corresponded more closely to the onset of SRSs in this model.

Epileptogenesis causes a permanent decrease in hippocampal calbindin expression

To determine if the observed decrease in calbindin protein expression was long lasting, immunohistochemical analysis was carried out on sections from rats that manifested the epileptic phenotype for 2 years. In age-matched controls, calbindin protein expression was still present throughout the hippocampus. In the 2-year epileptic animals, there was an overall 60.4 ± 4.9% reduction of hippocampal calbindin protein expression (Fig. 7). Calbindin expression was decreased 55.3 ± 5.6% in the stratum pyramidale of the CA1 ($p < 0.05$, Student's *t*-test); 61.3 ± 6.8% in the stratum lucidum of the CA3 region ($p < 0.01$, Student's *t*-test); 61.2 ± 4.1% in the stratum granulosum of the dentate gyrus ($p < 0.001$, Student's *t*-test); 56.4 ± 3.0% in the stratum moleculare of the dentate gyrus ($p < 0.001$, Student's *t*-test); and 67.4 ± 5.5% in the hilar region ($p < 0.01$, Student's *t*-test). These decreases are consistent with what was observed in younger rats, indicating that this decrease in calbindin associated with the epileptic phenotype is essentially permanent for the life of the animal.

It was also important to demonstrate that the characteristics of the pilocarpine model in our laboratory reflect the findings of other laboratories to demonstrate the universal applicability of the findings in this study. Epileptic rats used for this study were monitored by video recording for 96 h consecutively to determine a mean seizure frequency. Rats employed for the calbindin studies shown in Figs. 1–5 were monitored 6 months after the initial SE event and were found to have a mean seizure frequency of 2.1 ± 0.8 seizures (stages 3–5) per 6 h. Individual frequencies varied in this group of animals from two seizures per day to 12 seizures per day. One animal demonstrated a cluster of high seizure frequencies and skewed the data to reflect a higher mean seizure frequency for this group. For long-term studies, animals were monitored two times at 12 and 24 months following SE and were found to have a mean seizure frequency of 0.9 ± 0.4 seizures per 6 h. The seizure frequencies observed in this study were comparable to those seen in other studies employing the pilocarpine model (Goffin et al., 2007;Hernandez et al., 2002;Cha et al., 2004;Wallace et al., 2003).

To determine if a trend existed between seizure frequency and degree of calbindin reduction in individual epileptic rats, a linear regression analysis was performed comparing seizure frequency to calbindin levels in individual rats used for this study. With an r^2 value of 0.53 (data not shown), it can be concluded that there was not a correlation between seizure frequency and calbindin decrease and thus, the decrease in calbindin was independent of seizure frequency.

Discussion

The calcium binding protein, calbindin-D28k, 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). Calbindin buffering is one of several important mechanisms for neurons to maintain Ca^{2+} homeostasis. It has been well established that hippocampal Ca^{2+} homeostasis is disrupted following SE induced AE (DeLorenzo et al., 1998; Raza et al., 2001; Raza et al., 2004; Delorenzo et al., 2005). This study was initiated to determine if hippocampal calbindin levels were altered following SE in

association with epileptogenesis in this model of AE. The findings presented in this study demonstrate that a significant decrease in calbindin expression occurs with the epileptic phenotype for as long as 2 years following the initial injury. The data demonstrate that calbindin levels in the hippocampus, specifically in the stratum granulosum and stratum moleculare of the dentate gyrus, hilar region, stratum pyramidale of the CA1, and stratum lucidum of the CA3 region, are significantly decreased. The reduction of calbindin in these regions indicates that calbindin levels are affected primarily in CA1 pyramidal cells and dentate granule cells in epilepsy. The observed loss of calbindin in the stratum lucidum, stratum moleculare, and hilus are due to the granule cells, as the dendrites and axons of the granule cells are located in these regions. Sections from ventral hippocampi from control and epileptic animals were also evaluated for calbindin immunoreactivity, and the same decrease in calbindin expression was observed, indicating that this is not unique to the dorsal hippocampus. These decreases are evident by 1 month after the SE induced CNS insult/injury, a time at which the epileptic phenotype is established. Additionally, the decrease in hippocampal calbindin was found to be essentially permanent, and was still evident in rats 2 years after a single episode of 1 h of SE. Not only are the protein levels of calbindin decreased in this study, but the mRNA levels of calbindin were also found to be decreased in epileptic hippocampi. Further studies are needed to determine the mechanisms responsible for this time course of calbindin decrease. Decreased gene expression and gradual decrease in protein levels due to the half-life of the protein are the most likely explanations. Additionally, it is important to characterize the loss of calbindin in more specific cell types, including interneurons. This may help provide a better understanding for why the epilepsy affects calbindin regulation.

It has been documented that it is difficult to obtain accurate seizure frequency on epileptic rats with monitoring techniques that observe animals for only a few days and some studies monitor continuously while others only monitor for short durations each day (Goffin et al., 2007). In addition, the pilocarpine model of AE manifests a high variability of seizure frequencies between individual rats and studies have demonstrated epileptic rats experiencing clusters of very high seizure frequency contributing to this variability (Mello et al., 1993; Goffin et al., 2007). Mello et al. (1993) demonstrated that some rats had as many as six seizures in an 8 h time. The epileptic rats used for the 6-month study in this investigation had an average seizure frequency of 8.4 seizures per day and this was skewed to a higher frequency due to the short monitoring time and one animal that had a high seizure frequency. However, the 1- and 2-year animals were monitored for two separate time periods and had a mean seizure frequency of 3.6 seizures per day. Although these seizure frequencies are only estimates of the actual seizure frequency over long time periods, they are comparable with the observations from other major laboratories using the pilocarpine model of AE: Pitkanen and co-workers have reported a frequency of 2.6 seizures per day (Goffin et al., 2007); Holmes and co-workers have reported 5.2 seizures per 42 h (Cha et al., 2004), and Dudek and coworkers have reported 6.9 seizures per day (Hernandez et al., 2002). Seizure durations are relatively short (35–50 s) in the pilocarpine model and animals have been shown to tolerate several brief seizures per day without significant behavioral effects (Goffin et al., 2007). Since the seizure frequencies observed in this study were comparable to those described by other laboratories, the observed decrease in calbindin expression presented here in the epileptic animals was not due to a high seizure frequency. Furthermore, the linear regression analysis comparing seizure frequency and calbindin levels demonstrated no correlation between seizure frequency and calbindin expression. Thus, this study demonstrates that the epileptic phenotype in the pilocarpine model is associated with a decreased expression of one of this major calcium binding protein in neurons.

There have been many other studies evaluating the role of calbindin in epilepsy. Calbindin levels in the dentate granule cells from human epileptic hippocampi are decreased (Magloczky et al., 1997; Nagerl et al., 2000; Selke et al., 2006). In other animal models of epilepsy, including

kindling, kainic acid, and pilocarpine, calbindin expression is reduced in the stratum granulosum and CA1 stratum pyramidale in the few months following the initial injury (Baimbridge and Miller, 1984; Baimbridge et al., 1985; Shetty and Turner, 1995; Yang et al., 1997; Tang et al., 2006). Conversely, following acute seizures by electrical stimulation or kainic acid treatments, calbindin protein and mRNA levels are increased (Lowenstein et al., 1991; Lowenstein et al., 1994; Lee et al., 1997). Therefore, it is possible that a compensatory increase in calbindin occurs following acute seizures and this change may be related to neurogenesis or stimulated synthesis (Lowenstein et al., 1991; Lowenstein et al., 1994; Lee et al., 1997). The animals used in these studies were not determined to have the SRSs characteristic of epilepsy. Our study is the first to evaluate calbindin protein and gene expression in animals with the epileptic phenotype in different regions of the hippocampus over an extensive time frame from immediately following SE to as long as 2 years after SE. In this study, calbindin expression observed at early time points (1 h, 1 day, and 7 days post-SE) was not significantly different from controls whereas calbindin was found to decrease significantly throughout the hippocampus as early as 30 days after SE and remained decreased essentially for the life of the animals. This long-term decrease in calbindin expression may play a role in some of the long-term abnormalities observed in Ca^{2+} homeostasis observed in this model of AE (DeLorenzo et al., 1998; Raza et al., 2001; Raza et al., 2004; Delorenzo et al., 2005).

In the pilocarpine model of AE, there is regionally specific cell loss in the hippocampus (Mello et al., 1993; Rice and DeLorenzo, 1998; Falenski et al., 2007). Although the granule layer of the dentate gyrus is fairly resistant to cell loss, results from this study showed a dramatic decrease in calbindin expression. This finding is consistent with previous studies that have reported decreases in calbindin in the dentate granule cell layer of the hippocampus in epilepsy (Scharfman et al., 2002; Krsek et al., 2004; Tang et al., 2006). The CA1 pyramidal layer and hilus are more sensitive to pilocarpine-induced SE, with more severe necrosis and greater cell loss than other hippocampal regions (Klitgaard et al., 2002; Hamani and Mello, 2002). Therefore, it is possible that cell loss in these regions could be contributing to the profound decrease in calbindin expression observed in this study.

It is important to emphasize that epileptogenesis is a complex process, and there may be changes in 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 potentially important regulator of epileptogenesis (Delorenzo et al., 2005). The Ca^{2+} hypothesis of epileptogenesis postulates that the pathophysiological effects of excess Ca^{2+} on neuronal function may lie on a continuum. One end of the continuum is characterized by brief, controlled Ca^{2+} loads of normal function, and the other end is characterized by irreversible Ca^{2+} loads and neuronal death associated with excitotoxicity. The middle of the continuum is characterized by prolonged sub lethal, but reversible, elevations in $[Ca^{2+}]_i$ that trigger pathological plasticity changes associated with epileptogenesis. It is hypothesized that these plasticity changes lead 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. During both excitotoxicity and epileptogenesis, neurons endure large elevations of $[Ca^{2+}]_i$. In excitotoxicity, these elevations progress to an irreversible loss of Ca^{2+} homeostasis and neuronal death. In epileptogenesis, these elevations, though prolonged, are buffered over time and lead to permanent plasticity changes and neuronal hyperexcitability.

Another important finding in this study was the decrease in calbindin mRNA expression in the epileptic hippocampus. Few studies have been conducted to examine potential changes in calbindin gene expression in association with AE. It has been shown that calbindin gene

expression is reduced in a genetic model of epilepsy (Montpied et al., 1995). Another study evaluating calbindin gene expression following acute seizures found that calbindin protein expression was decreased in the hippocampus; however, the mRNA levels were unchanged (Sonnenberg et al., 1991). To our knowledge, this is the first study that has demonstrated that in pilocarpine-induced AE, the observed decrease in hippocampal calbindin protein is associated with a significant down regulation calbindin gene expression. This finding could lead to a potential new target for the development of antiepileptic therapies.

The major finding of this study was that hippocampal calbindin expression was significantly decreased by 1 month after pilocarpine-induced SE and remained decreased for a long time after the establishment of SRSs. The decreased calbindin expression displayed in the hippocampi of epileptic animals was regionally specific and occurred primarily in the dentate gyrus, CA1 pyramidal layer, and CA3 dendritic field. This decrease in calbindin expression was observed up to 2 years following 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.

Calbindin is expressed in many regions of the brain (Baimbridge et al., 1985; Mody et al., 1987), that are affected by epilepsy. Therefore, it is important to evaluate potential changes in calbindin levels in other regions using this model of AE to see if this same phenomenon is consistent throughout the brain. In addition to the principle cells evaluated in this study, calbindin is also prevalent in a sub-population of interneurons in the hippocampus (Sloviter et al., 1991; Wittner et al., 2002; Dinocourt et al., 2003). Several studies have addressed calbindin levels in populations of interneurons in epilepsy. Calbindin containing interneurons is found to be preserved in the CA1 and hilus in both human epilepsy (Sloviter et al., 1991; Wittner et al., 2002) and in the rat pilocarpine model of AE (Dinocourt et al., 2003). Additionally, Scharfman et al. (2002) have reported that in the pilocarpine model of AE, newly born hilar granule cells express calbindin. The findings from these studies and the current study suggest that the decrease in calbindin that occurs in epilepsy is primarily found in surviving principle cells in the hippocampus.

In the pilocarpine model of AE, morphological changes occur over the course of 2–4 weeks after the initial SE injury, suggesting that interventions during this time period or sooner could possibly be beneficial. The findings from this study show that calbindin, a major component of Ca^{2+} regulation, is altered in epilepsy. Although it is unlikely that restoring Ca^{2+} 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 discharges. 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 acquired epilepsy.

Acknowledgments

This study was supported by National Institute of Neurological Disorders and Stroke Grants RO1NS051505 and RO1NS052529, and award UO1NS058213 from the National Institutes of Health CounterACT Program through the National Institute of Neurological Disorders and Stroke to RJD. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the federal government. In addition, the Milton L. Markel Alzheimer's Disease Research Fund and the Sophie and Nathan Gumenick Neuroscience Research Fund also funded this work.

References

Abercrombie M, Johnson ML. Quantitative histology of Wallerian degeneration. I Nuclear population in rabbit sciatic nerve. J Anat 1946;80:37–50. [PubMed: 17104988]

Baimbridge KG, Miller JJ. Hippocampal calcium-binding protein during commissural kindling-induced epileptogenesis: progressive decline and effects of anticonvulsants. Brain Res 1984;324:85–90. [PubMed: 6518394]

Baimbridge KG, Mody I, Miller JJ. Reduction of rat hippocampal calcium-binding protein following commissural, amygdala, septal, perforant path, and olfactory bulb kindling. Epilepsia 1985;26:460–465. [PubMed: 4043015]

Cha BH, Akman C, Silveira DC, Liu X, Holmes GL. Spontaneous recurrent seizure following status epilepticus enhances dentate gyrus neurogenesis. Brain Dev 2004;26:394–397. [PubMed: 15275703]

Chaix Y, Ferraro TN, Lapouble E, Martin B. Chemoconvulsant-induced seizure susceptibility: toward a common genetic basis? Epilepsia 2007;48 (Suppl 5):48–52. [PubMed: 17910581]

DeLorenzo RJ, Pal S, Sombati S. Prolonged activation of the *N*-methyl-D-aspartate receptor-Ca^{2+} transduction pathway causes spontaneous recurrent epileptiform discharges in hippocampal neurons in culture. Proc Natl Acad Sci USA 1998;95:14482–14487. [PubMed: 9826726]

Delorenzo RJ, Sun DA, Deshpande LS. Cellular mechanisms underlying acquired epilepsy: the calcium hypothesis of the induction and maintainance of epilepsy. Pharmacol Ther 2005;105:229–266. [PubMed: 15737406]

Dinocourt C, Petanjek Z, Freund TF, Ben-Ari Y, Esclapez M. Loss of interneurons innervating pyramidal cell dendrites and axon initial segments in the CA1 region of the hippocampus following pilocarpine-induced seizures. J Comp Neurol 2003;459:407–425. [PubMed: 12687707]

Elliott RC, Miles MF, Lowenstein DH. Overlapping microarray profiles of dentate gyrus gene expression during development- and epilepsy-associated neurogenesis and axon outgrowth. J Neurosci 2003;23:2218–2227. [PubMed: 12657681]

Falenski KW, Blair RE, Sim-Selley LJ, Martin BR, DeLorenzo RJ. Status epilepticus causes a long-lasting redistribution of hippocampal cannabinoid type 1 receptor expression and function in the rat pilocarpine model of acquired epilepsy. Neuroscience 2007;146:1232–1244. [PubMed: 17433556]

Goffin K, Nissinen J, Van Laere K, Pitkanen A. Cyclicity of spontaneous recurrent seizures in pilocarpine model of temporal lobe epilepsy in rat. Exp Neurol 2007;205:501–505. [PubMed: 17442304]

Hamani C, Mello LE. Spontaneous recurrent seizures and neuropathology in the chronic phase of the pilocarpine and picrotoxin model epilepsy. Neurol Res 2002;24:199–209. [PubMed: 11877905]

Hauser, W.; Hesdorffer, D. Epilepsy: Frequency, Causes, and Consequences. Demos Publications; New York: 1990.

Hernandez EJ, Williams PA, Dudek FE. Effects of fluoxetine and TFMPP on spontaneous seizures in rats with pilocarpine-induced epilepsy. Epilepsia 2002;43:1337–1345. [PubMed: 12423383]

Klitgaard H, Matagne A, Vanneste-Goemaere J, Margineanu DG. Pilocarpine-induced epileptogenesis in the rat: impact of initial duration of status epilepticus on electrophysiological and neuropathological alterations. Epilepsy Res 2002;51:93–107. [PubMed: 12350385]

Kohr G, Lambert CE, Mody I. Calbindin-D28K (CaBP) levels and calcium currents in acutely dissociated epileptic neurons. Exp Brain Res 1991;85:543–551. [PubMed: 1655508]

Krsek P, Mikulecka A, Druga R, Kubova H, Hlinak Z, Suchomelova L, Mares P. Long-term behavioral and morphological consequences of nonconvulsive status epilepticus in rats. Epilepsy Behav 2004;5:180–191. [PubMed: 15123019]

Lee S, Williamson J, Lothman EW, Szele FG, Chesselet MF, Von Hagen S, Sapolsky RM, Mattson MP, Christakos S. Early induction of mRNA for calbindin-D28k and BDNF but not NT-3 in rat hippocampus after kainic acid treatment. Brain Res Mol Brain Res 1997;47:183–194. [PubMed: 9221916]

Lowenstein DH, Miles MF, Hatam F, McCabe T. Up regulation of calbindin-D28K mRNA in the rat hippocampus following focal stimulation of the perforant path. Neuron 1991;6:627–633. [PubMed: 2015095]

Lowenstein DH, Gwinn RP, Seren MS, Simon RP, McIntosh TK. Increased expression of mRNA encoding calbindin-D28K, the glucose-regulated proteins, or the 72 kDa heat-shock protein in three models of acute CNS injury. Brain Res Mol Brain Res 1994;22:299–308. [PubMed: 8015387]

Lukasiuk K, Dabrowski M, Adach A, Pitkanen A. Epileptogenesis-related genes revisited. Prog Brain Res 2006;158:223–241. [PubMed: 17027699]

Magloczky Z, Halasz P, Vajda J, Czirjak S, Freund TF. Loss of calbindin-D28K immunoreactivity from dentate granule cells in human temporal lobe epilepsy. Neuroscience 1997;76:377–385. [PubMed: 9015323]

Mattson MP, Cheng B, Baldwin SA, Smith-Swintosky VL, Keller J, Geddes JW, Scheff SW, Christakos S. Brain injury and tumor necrosis factors induce calbindin D-28k in astrocytes: evidence for a cytoprotective response. J Neurosci Res 1995;42:357–370. [PubMed: 8583504]

McNamara JO. Emerging insights into the genesis of epilepsy. Nature 1999;399:A15–A22. [PubMed: 10392576]

Mello LE, Cavalheiro EA, Tan AM, Kupfer WR, Pretorius JK, Babb TL, Finch DM. Circuit mechanisms of seizures in the pilocarpine model of chronic epilepsy: cell loss and mossy fiber sprouting. Epilepsia 1993;34:985–995. [PubMed: 7694849]

Mody I, Baimbridge KG, Miller JJ. Distribution of calbindin-D28K 1 (CaBP) in the cerebral cortex and hippocampus of the epileptic (El) mouse. Epilepsy Res 1987;1:46–52. [PubMed: 3504383]

Montpied P, Winsky L, Dailey JW, Jobe PC, Jacobowitz DM. Alteration in levels of expression of brain calbindin D-28k and calretinin mRNA in genetically epilepsy-prone rats. Epilepsia 1995;36:911–921. [PubMed: 7649131]

Morris TA, Jafari N, DeLorenzo RJ. Chronic DeltaFosB expression and increased AP-1 transcription factor binding are associated with the long term plasticity changes in epilepsy. Brain Res Mol Brain Res 2000;79:138–149. [PubMed: 10925151]

Nagerl UV, Mody I, Jeub M, Lie AA, Elger CE, Beck H. Surviving granule cells of the sclerotic human hippocampus have reduced Ca(2+) influx because of a loss of calbindin-D(28k) in temporal lobe epilepsy. J Neurosci 2000;20:1831–1836. [PubMed: 10684884]

Newman GC, Hospod FE, Patlak CS, Trowbridge SD, Wilke RJ, Fuhrmann M, Jones KW. Calcium compartments in brain. J Cereb Blood Flow Metab 2002;22:479–489. [PubMed: 11919519]

Pal S, Sombati S, Limbrick DD Jr, DeLorenzo RJ. In vitro status epilepticus causes sustained elevation of intracellular calcium levels in hippocampal neurons. Brain Res 1999;851:20–31. [PubMed: 10642824]

Pal S, Limbrick DD Jr, Rafiq A, DeLorenzo RJ. Induction of spontaneous recurrent epileptiform discharges causes long-term changes in intracellular calcium homeostatic mechanisms. Cell Calcium 2000;28:181–193. [PubMed: 11020380]

Pal S, Sun D, Limbrick D, Rafiq A, DeLorenzo RJ. Epileptogenesis induces long-term alterations in intracellular calcium release and sequestration mechanisms in the hippocampal neuronal culture model of epilepsy. Cell Calcium 2001;30:285–296. [PubMed: 11587552]

Parsons JT, Churn SB, Kochan LD, DeLorenzo RJ. Pilocarpine-induced status epilepticus causes *N*-methyl-D-aspartate receptor-dependent inhibition of microsomal Mg(2+)/Ca(2+) ATPase-mediated Ca(2+) uptake. J Neurochem 2000;75:1209–1218. [PubMed: 10936204]

Parsons JT, Churn SB, DeLorenzo RJ. Chronic inhibition of cortex microsomal Mg(2+)/Ca(2+) ATPase-mediated Ca(2+) uptake in the rat pilocarpine model following epileptogenesis. J Neurochem 2001;79:319–327. [PubMed: 11677260]

Parsons JT, Sun DA, DeLorenzo RJ, Churn SB. Neuronal-specific endoplasmic reticulum Mg(2+)/Ca(2+) ATPase Ca(2+) sequestration in mixed primary hippocampal culture homogenates. Anal Biochem 2004;330:130–139. [PubMed: 15183771]

Racine RJ. Modification of seizure activity by electrical stimulation. II Motor seizure. Electroencephalogr Clin Neurophysiol 1972;32:281–294. [PubMed: 4110397]

Raza M, Pal S, Rafiq A, DeLorenzo RJ. Long-term alteration of calcium homeostatic mechanisms in the pilocarpine model of temporal lobe epilepsy. Brain Res 2001;903:1–12. [PubMed: 11382382]

Raza M, Blair RE, Sombati S, Carter DS, Deshpande LS, DeLorenzo RJ. Evidence that injury-induced changes in hippocampal neuronal calcium dynamics during epileptogenesis cause acquired epilepsy. Proc Natl Acad Sci USA 2004;101:17522–17527. [PubMed: 15583136]

Rice AC, DeLorenzo RJ. NMDA receptor activation during status epilepticus is required for the development of epilepsy. Brain Res 1998;782:240–247. [PubMed: 9519269]

Scharfman HE, Sollas AL, Goodman JH. Spontaneous recurrent seizures after pilocarpine-induced status epilepticus activate calbindin-immunoreactive hilar cells of the rat dentate gyrus. Neuroscience 2002;111:71–81. [PubMed: 11955713]

Scher MS. Prenatal contributions to epilepsy: lessons from the bedside. Epileptic Disord 2003;5:77–91. [PubMed: 12875951]

Selke K, Muller A, Kukley M, Schramm J, Dietrich D. Firing pattern and calbindin-D28k content of human epileptic granule cells. Brain Res 2006;1120:191–201. [PubMed: 16997289]

Shetty AK, Turner DA. Intracerebroventricular kainic acid administration in adult rat alters hippocampal calbindin and non-phosphorylated neurofilament expression. J Comp Neurol 1995;363:581–599. [PubMed: 8847419]

Sloviter RS, Sollas AL, Barbaro NM, Laxer KD. Calcium-binding protein (calbindin-D28K) and parvalbumin immunocytochemistry in the normal and epileptic human hippocampus. J Comp Neurol 1991;308:381–396. [PubMed: 1865007]

Sonnenberg JL, Frantz GD, Lee S, Heick A, Chu C, Tobin AJ, Christakos S. Calcium binding protein (calbindin-D28k) and glutamate decarboxylase gene expression after kindling induced seizures. Brain Res Mol Brain Res 1991;9:179–190. [PubMed: 1709439]

Tang FR, Chia SC, Jiang FL, Ma DL, Chen PM, Tang YC. Calcium binding protein containing neurons in the gliotic mouse hippocampus with special reference to their afferents from the medial septum and the entorhinal cortex. Neuroscience 2006;140:1467–1479. [PubMed: 16650619]

Wallace MJ, Blair RE, Falenski KW, Martin BR, DeLorenzo RJ. The endogenous cannabinoid system regulates seizure frequency and duration in a model of temporal lobe epilepsy. J Pharmacol Exp Ther 2003;307:129–137. [PubMed: 12954810]

Wittner L, Eross L, Szabo Z, Toth S, Czirjak S, Halasz P, Freund TF, Magloczky ZS. Synaptic reorganization of calbindin-positive neurons in the human hippocampal CA1 region in temporal lobe epilepsy. Neuroscience 2002;115:961–978. [PubMed: 12435433]

Yang Q, Wang S, Hamberger A, Celio MR, Haglid KG. Delayed decrease of calbindin immunoreactivity in the granule cell-mossy fibers after kainic acid-induced seizures. Brain Res Bull 1997;43:551–559. [PubMed: 9254026]

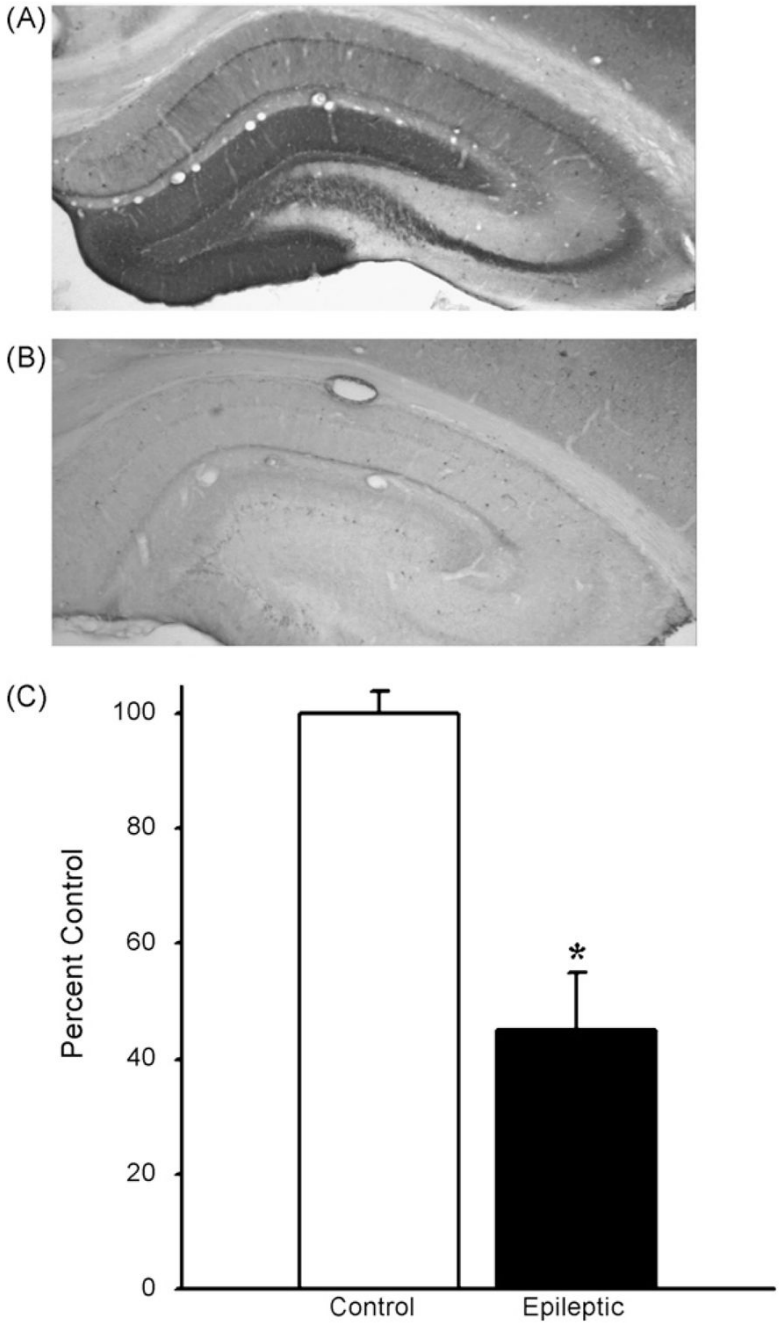

Figure 1.

Decrease in calbindin protein expression throughout the hippocampus in epileptic animals. Immunohistochemical detection of calbindin protein expression in hippocampi from control (A) and epileptic (B) animals. (C) Quantification of calbindin protein expression in control and epileptic hippocampi was converted to percent control for comparison. Calbindin expression in epileptic whole hippocampi was reduced by 55.1%. Data are presented as percent control ± S.E.M. *$p < 0.001$, Student's *t*-test.

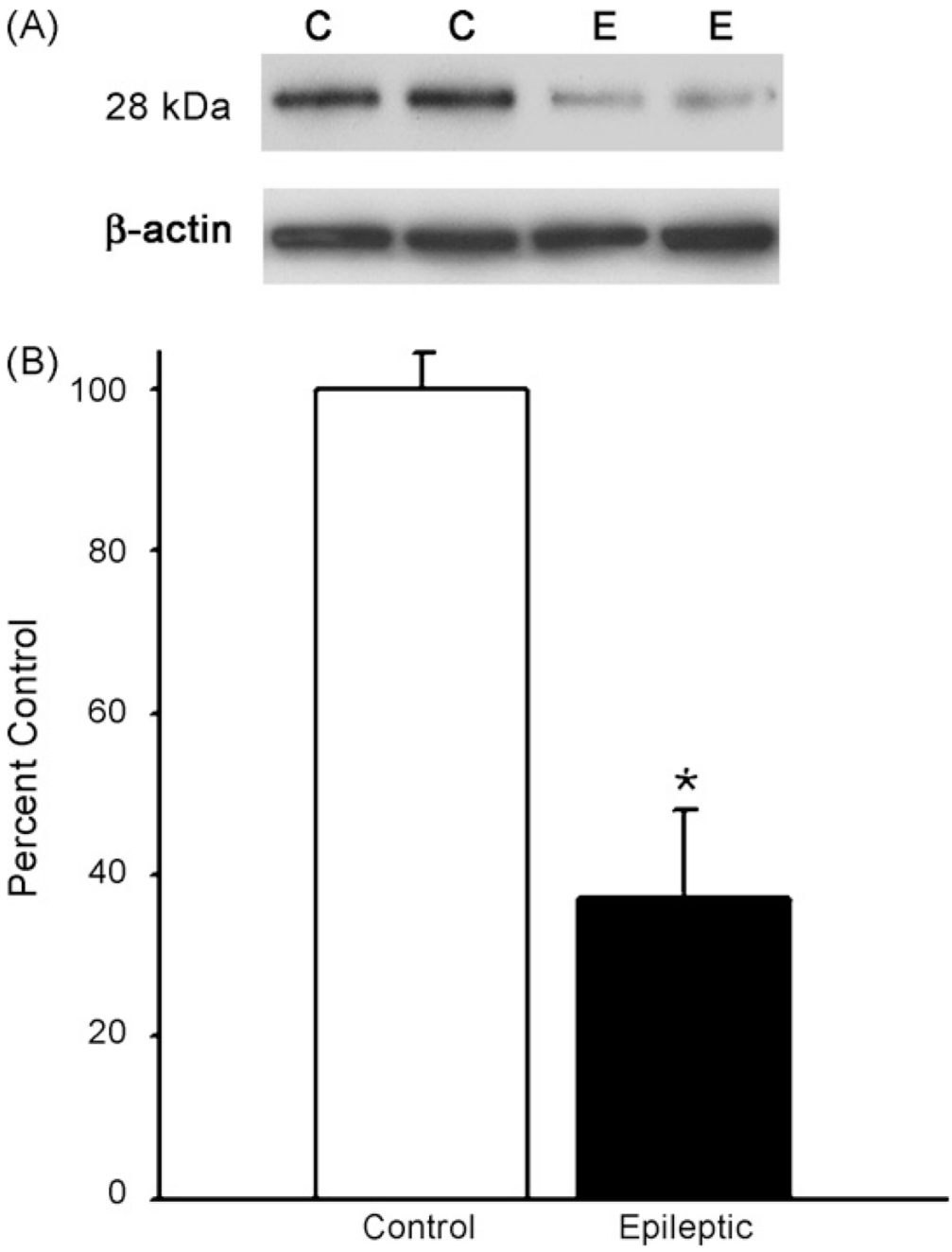

Figure 2.

Decrease in calbindin protein levels in the cytosol from whole hippocampi from epileptic animals. (A) Representative lanes from a Western blot showing calbindin protein levels from control (C) and epileptic (E) animals. β-actin was used as a loading control to confirm equal loading and normalize the optical densities from each protein band. (B) Densitometric analysis was performed to quantify and compare calbindin protein levels between control and epileptic animals. A 63 ± 9.6% decrease in calbindin protein levels was observed. Data were converted to percent control for comparison and are presented as percent control ± S.E.M. *$p < 0.01$, Student's *t*-test.

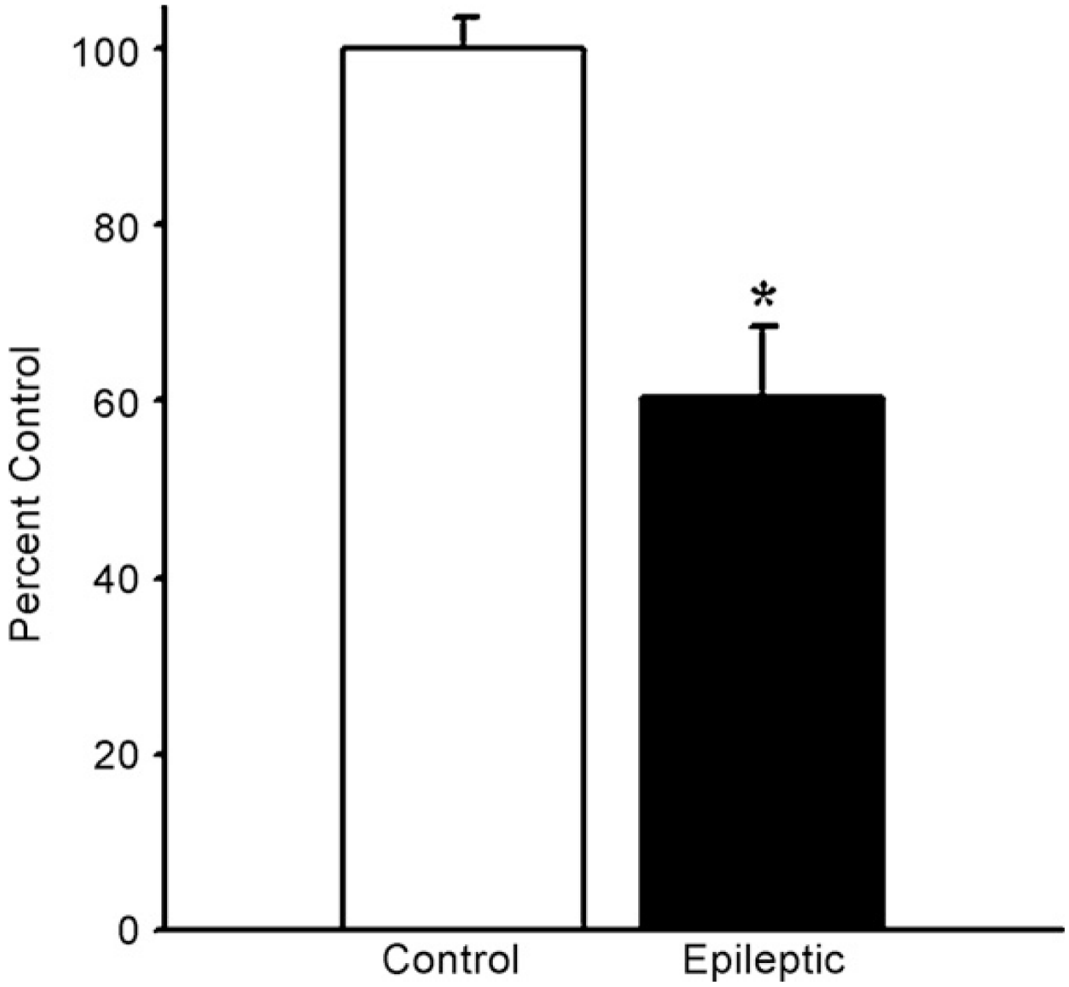

Figure 3.

Decrease in calbindin mRNA levels in whole hippocampal homogenates from epileptic animals. Quantitative RT-PCR was used to determine that the decrease in calbindin protein levels was due to a down regulation at the level of the gene expression. Results from RT-PCR revealed a 40% decrease in calbindin mRNA levels. Data were converted to percent control for comparison and are presented as percent control ± S.E.M. *$p < 0.01$, Student's *t*-test.

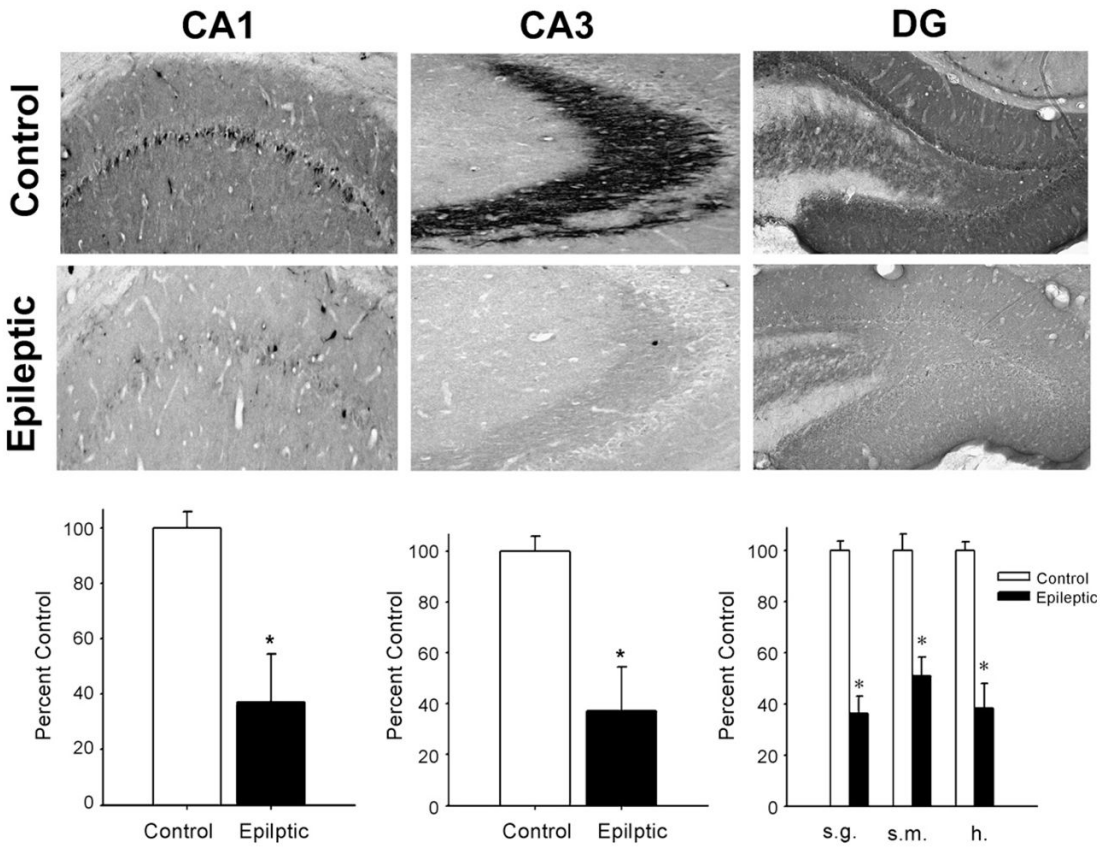

Figure 4.

Decrease in calbindin protein expression in the stratum pyramidale of the CA1, stratum lucidum of the CA3, and dentate gyrus. High magnification (20×) images of calbindin staining in control and epileptic CA1. Quantification of the mean pixel values for control and epileptic tissue were converted to percent control for comparison. Calbindin expression was reduced by 62.9 ± 17.3% in the CA1, 66.8 ± 11.9% in the CA3, and 63.7 ± 6.8%, 49.9 ± 7.3, and 61.6 ± 9.7% in the dentate stratum granulosum, stratum molecular, and hilus, respectively. Data are presented as percent control ± S.E.M. *$p < 0.05$, Student's *t*-test.

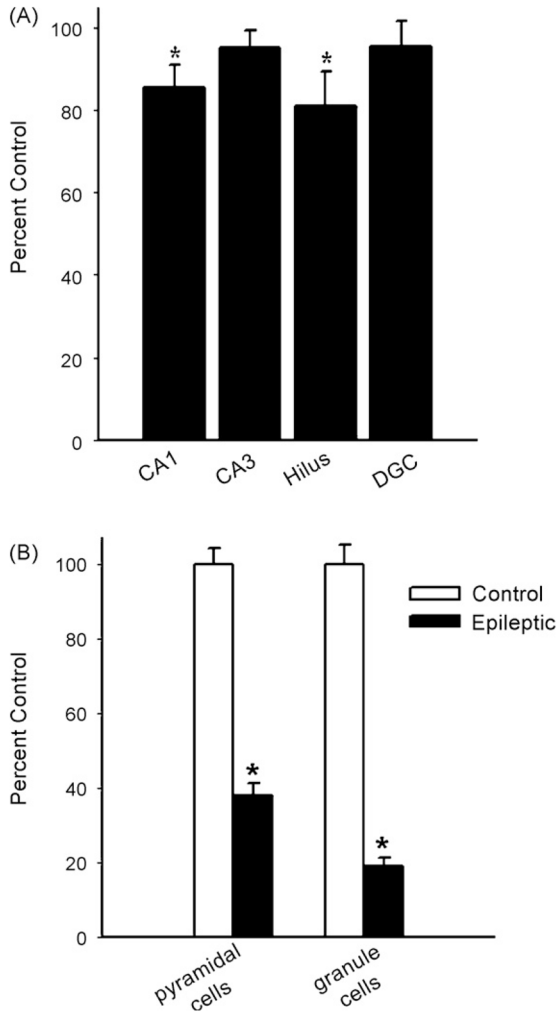

Figure 5.

(A) Mean cell loss in CA1 stratum pyramidale, CA3 stratum pyramidale, hilus, and stratum granulosum of the dentate gyrus (DGC). Standard techniques were employed to evaluate cell counts (Mello et al., 1993). Adjacent sections were Nissl stained and cell counts were made in the stratum granulosum of the dentate gyrus, hilus, and stratum pyramidale of the CA1 and CA3 regions. Neurons clearly identifiable with nuclei were counted and cell densities were determined (cells/mm^3) Comparison between control and epileptic animals was made with ANOVA (SigmaStat®). Data were converted to percent control for comparison and presented as percent control ± S.E.M. *$p < 0.05$, ANOVA. (B) Decrease in calbindin protein expression in individual soma from the straum pyramidale of the CA1 and stratum granulosum of the dentate gyrus. Denstiometric analysis was performed on individual cells (n = 70–100 per animal) and pixel values were converted to percent control. In the pyramidal cells, calbindin expression was reduced 61.9 ± 3.0% and 81.0 ± 2.4% in the granule cells. Data are presented as percent control ± S.E.M. *$p < 0.05$, Student's t-test.

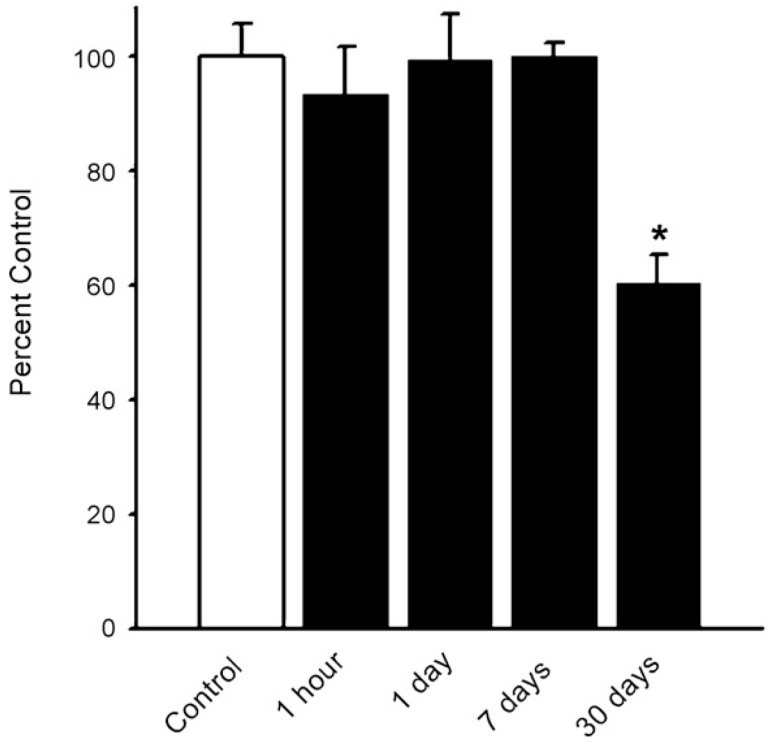

Figure 6.
Evaluation of changes in calbindin expression during epileptogenesis. Calbindin protein expression was evaluated in tissue obtained 1 h, 1 day, 7 days, and 30 days following SE onset. No changes in calbindin protein expression were detected until 30 days post-SE. Mean pixel values for each time point and corresponding control were obtained and converted to percent control and presented as percent control ± S.E.M. *$p < 0.001$, Student's *t*-test.

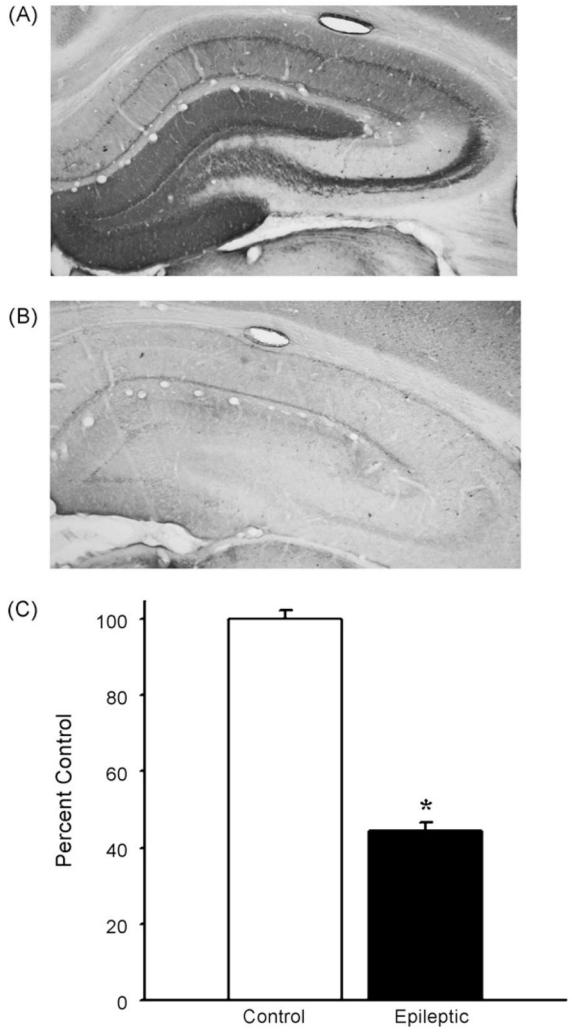

Figure 7.

Essentially permanent decrease in calbindin protein expression throughout the hippocampus in epileptic animals. Immunohistochemical detection of calbindin protein expression in hippocampi from age-matched control (A) and 2-year epileptic (B) animals. (C) Quantification of calbindin protein expression in control and epileptic hippocampi was converted to percent control for comparison. Calbindin expression in 2-year epileptic whole hippocampi was reduced by 55.7%. Data are presented as percent control ± S.E.M. *$p < 0.001$, Student's *t*-test.