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A Cellular Mechanism for Dendritic Spine Loss in the Pilocarpine Model of Status Epilepticus

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Abstract

Purpose—Previous studies have documented a synaptic translocation of calcineurin (CaN) and increased CaN activity following status epilepticus (SE), however the cellular effect of these changes in CaN in the pathology of SE remains to be elucidated. This study examined a CaN-dependent modification of the dendritic cytoskeleton. CaN has been shown to induce dephosphorylation of cofilin, an actin depolymerization factor. The ensuing actin depolymerization can lead to a number of physiological changes that are of interest in SE.

Methods—SE was induced by pilocarpine injection, and seizure activity was monitored by video-EEG. Subcellular fractions were isolated by differential centrifugation. CaN activity was assayed using a para-nitrophenol phosphate assay protocol. Cofilin phosphorylation was assessed using phosphocofilin-specific antibodies. Cofilin-actin binding was determined by co-immunoprecipitation, and actin polymerization was measured using a triton-solubilization protocol. Spines were visualized using a single-section rapid Golgi impregnation procedure.

Results—The immunoreactivity of phosphocofilin decreased significantly in hippocampal and cortical synaptosomal samples after SE. SE-induced cofilin dephosphorylation could be partially blocked by the pre-injection of CaN inhibitors. Cofilin activation could be further demonstrated by increased actin-cofilin binding and a significant depolymerization of neuronal actin, both of which were also blocked by CaN inhibitors. Finally, we demonstrated a CaN-dependent loss of dendritic spines histologically.

Discussion—The data demonstrate a CaN-dependent, cellular mechanism through which prolonged seizure activity results in loss of dendritic spines via cofilin activation. Further research into this area may provide useful insights into the pathology of SE and epileptogenic mechanisms.

Keywords

Calcineurin; epilepsy; cofilin; epileptogenesis; cognitive function

Introduction

Status epilepticus (SE) is a severe neurological emergency characterized by persistent, continuous seizure activity (Lowenstein and Alldredge, 1998). Patients experiencing SE are

at risk for a number of chronic neurological pathologies as a consequence of the seizure episode (Bleck, 1991; Rice et al., 1998; Fountain, 2000). A number of SE-induced biochemical and morphological changes in the brain have been investigated as possible causes of this long-term pathology. One recent finding that may have relevance to this subject is the SE-induced loss of dendritic spines (Wong, 2005).

Dendritic spines are small, dynamic protuberances from the dendritic shaft that are critical for synaptic transmission throughout the CNS, representing the primary location of excitatory glutamatergic neurotransmission (Gray, 1959). The presence of dendritic spines is thought to aid neurotransmission in several ways, both by increasing the surface area for synaptic contact and by providing a specialized compartment for the excitatory postsynaptic machinery (Hayashi and Majewska, 2005). These factors, as well as the apparent ability of neurons to alter spine density, size and shape in response to synaptic input, makes them an ideal location for regulation of synaptic strength. In fact, spine plasticity has been noted as a possible mechanism of long-term potentiation/depression (Zhou et al., 2004) and has been implicated in several models of learning and memory (O'Malley et al., 2000; Leuner et al., 2003; Leuner and Shors, 2004).

Interestingly, profound decreases in dendritic spine density, as well as alterations in spine shape and size, have been detected in both SE and several models of chronic epilepsy (Isokawa, 1998, 2000; Ferhat et al., 2003; Wong, 2005). Although the precise physiological consequence of this pathological spine plasticity has not yet been elucidated, several possible theories have been proposed. SE and epilepsy have both been associated with long-term cognitive deficiencies (Rice et al., 1998), and a widespread and chronic loss of dendritic spine density could certainly play a major role in this pathology. On the other hand, SE-induced spine plasticity may also represent a potential mechanism of epileptogenesis, in which the loss of dendritic spines is part of a pathological reorganization of synaptic networks leading to an overall increase in neuronal excitability (Sierra-Paredes et al., 2006). In spite of the uncertainty about the pathological consequences of SE-induced spine plasticity, such a widespread alteration in the structure of excitatory synapses remains an important and highly interesting consequence of SE and certainly merits continued investigation.

Unfortunately, the biological mechanisms responsible for this SE-induced spine plasticity remain poorly understood. One potential mechanism involves the calcium-regulated phosphatase, calcineurin (CaN). The cytoskeleton of spines is composed primarily of actin filaments, and CaN has been shown to regulate actin stability in a number of neuronal settings, including axonal growth cones and dendrites. Additionally, activation of CaN by NMDA-receptor associated calcium influx has been shown to destabilize F-actin in dendritic spines, and CaN and F-actin are co-localized in dendritic spines under these conditions (Halpain et al., 1998). Finally, recent research has demonstrated an SE-induced increase in CaN activity and enzyme concentration in post-synaptic regions of neurons (Kurz et al., 2001; Kurz et al., 2003). Thus an enzyme capable of regulating the structure of dendritic spines is both activated and localized into the vicinity of the spines in SE, placing CaN in an ideal position to play a role in SE-induced spine plasticity.

The present study investigated the CaN-dependent regulation of actin stability in the context of SE-induced dendritic spine loss. We demonstrate an SE-dependent dephosphorylation of cofilin, and a subsequent increase in cofilin-actin binding and actin depolymerization. All of these SE-induced cytoskeletal events were shown to be blocked by pre-administration of the CaN inhibitors, FK506 (tacrolimus) and cyclosporin A, strongly suggesting a CaN-dependent mechanism. Finally, SE-induced dendritic spine loss was demonstrated histologically in several brain regions, this spine loss was also blocked by the administration

of CaN inhibitors. These findings implicate CaN in a widespread, SE-induced alteration in the structure of synaptic contacts, potentially furthering our understanding of the cellular mechanisms underlying chronic SE-induced neurological pathology.

Methods and Materials

Pilocarpine model of status epilepticus

All animal-use procedures were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee. Adult Sprague-Dawley rats were handled after arrival from Harlan Laboratories, for acclimation to handling before drug treatment. One week before the induction of SE, four surface electrodes were implanted into the skulls of rats under ketamine anesthesia, as described previously (Singleton et al., 2005). Two frontal electrodes were implanted over frontal cortex [3.5 mm anterior to bregma, ± 2.5 mm L/R (F1/F2)]. Two posterior electrodes were implanted over parietal cortex and hippocampus [2.0 mm posterior to bregma, ± 2.5 mm L/R (P1/P2)]. A fifth electrode was fixed onto the surface of the skull as a ground. The electrodes were secured in place with dental acrylate, and the animals were allowed 5 days to recover from surgery before experiments were performed.

Twenty minutes before the injection of pilocarpine, methylscopolamine, a muscarinic antagonist, was administered i.p. (1 mg/kg) to reduce adverse peripheral effects of the pilocarpine. Where appropriate, 10 mg/kg cyclosporin A (Sigma) or 5 mg/kg FK506 (Fujisawa) was administered i.v. by tail injection, 3 hours prior to the induction of SE. Control and sham-surgery animals were attached to video-EEG machines (BMSI 5000; Nicolet, Madison, WI), and baseline EEG recordings were obtained for 10 min after scopolamine injection. SE was induced in experimental animals by i.p. injection of 375 mg/kg pilocarpine HCl, a muscarinic agonist. Behavioral and encephalographic activities were recorded throughout the procedure (Singleton et al., 2005). Once initial seizure activity was observed, the time was noted, and rats were allowed to seize for specific amounts of time before the animals were processed. These time points consisted of 10, 15, 20, 30, 40, 50, 60, and 70 min after the first discrete seizure. Since our laboratory has previously characterized SE onset as being 10 min after the first discrete seizure (Singleton et al., 2005), these times approximate 0, 5, 10, 20, 30, 40, 50, and 60 min of SE, respectively. Behavioral seizures were assessed according to the scale of Racine (Racine, 1972).

Brain region isolation and subcellular fractionation

Rats were rapidly decapitated after specific durations of SE. Brains were rapidly dissected on a petri dish on ice to preserve postmortem enzyme activity. Cortical and hippocampal brain regions were quickly isolated and immediately homogenized with 10 strokes (up and down) at 12,000 rpm, using a motorized homogenizer (TRI-R Instruments, Inc., Rockville Center, NY). Brain regions were homogenized into an ice-cold isotonic homogenization buffer containing 10 mM Tris-HCl (pH 7.0), 7 mM EGTA, 5 mM EDTA, 1 mM dithiothreitol (DTT), 0.3 mM PMSF, and 187 mM sucrose. Cortical regions were homogenized into 7 ml of buffer and hippocampal regions into 3 ml. All brain homogenates were then subjected to the differential centrifugation procedure described below.

Isolation of crude synaptoplasmic membrane fractions was achieved using a differential centrifugation procedure (Edelman et al., 1985) modified as described previously (Kurz et al., 2003; Kurz et al., 2005b). Briefly, brain region homogenates were centrifuged at 5000g for 10 min to produce a crude nuclear pellet (P1) and a supernatant (S1). The P1 pellet was resuspended in homogenization buffer, separated into aliquots, and stored at -80°C . The S1

was centrifuged for 30 min at 18,000g to produce the crude synaptoplasmic membrane/mitochondrial pellet (P2) and a supernatant (S2). The P2 pellet was resuspended in 1/10 the original volume of the homogenate (700 μ l for cortex, 300 μ l for hippocampus), separated into aliquots, and stored at -80°C until used.

Western analysis

Western analysis was performed in a manner similar to that described previously (Kurz et al., 2003). Briefly, fractions were balanced for protein using the Bradford method (Bradford, 1976), resolved on SDS-PAGE, and transferred to a nitrocellulose membrane using the Trans-blot system with the plates in the high-intensity field configuration (BioRad, Richmond, CA). Nitrocellulose was then immersed for 1 h in blocking solution containing phosphate buffered saline (PBS, pH 7.4), 0.05% (v/v) polyoxyethylene sorbitan monolaurate (Tween 20), and 2.5% Bio-Rad blotting grade dry milk. The nitrocellulose membrane was then incubated with the appropriate primary antibody in blocking solution for 1 h. Anti-Ca N A antibody (clone CN-A1, mouse monoclonal IgG, Sigma Chemical Co.) was diluted 1:10,000, anti-actin antibody was diluted 1:5000, DARRP-32 antibody was diluted 1:1000, and anti-cofilin (Chemicon) and anti-phosphocofilin (Chemicon) were diluted 1:500 for western analysis. Membranes were then washed three times in a wash solution containing PBS, Tween 20, and dry milk. Next, nitrocellulose was reacted with the appropriate HRP-conjugated secondary antibody in blocking solution for 30 min, then washed 3 $\times$ in PBS/Tween for 10 minutes each wash. Finally, blots were reacted with a luminol reagent for 5 minutes (Pierce Pico-sensitive reagent for Ca N A and actin studies, Pierce Femto-sensitive reagent for cofilin and phospho-cofilin staining). Blots were immediately exposed to x-ray film (Kodak X-OMAT). Films were developed using a Kodak X-OMAT developer. Specific immunoreactive bands were quantified by computer-assisted densitometry (GS-800 calibrated densitometer and Quantity One software, BioRad) and compared to a linear concentration curve as described previously (Churn et al., 1992).

Immunoprecipitation

Homogenates were spun at 5000 $\times$ g for 5 min to clear large insoluble material. All samples were balanced to ensure equivalent protein content, 500 μ g of protein was incubated on a rotating rack with anti-cofilin antibody diluted 1:50 (Chemicon) overnight at 4° C. 30 μ L of protein-A agarose beads were then added to the sample and incubated (rotating) for 3h at 4° C. The beads were then precipitated by spinning the samples at 2000 $\times$ g for 2 minutes. The resulting pellet was washed 3 $\times$ in phosphate buffered saline with 1% triton $\times$ -100. The pellet was then suspended in SDS sample buffer, placed in a boiling water bath for 5 minutes, and subjected to SDS-PAGE.

Actin polymerization assay

Actin polymerization was assayed using the differential solubility of F-actin and G-actin in 1% triton. Samples were incubated in 1% triton for 1h at 4° C, then subjected to a 1h centrifugation at 100,000 $\times$ g. The resulting supernatant and pellet were balanced for protein concentration, then run on a 10% SDS-PAGE gel and subjected to western analysis as described previously. The ratio of the amounts of actin present in these two fractions was used to provide an estimate of the actin polymerization state in different brain region isolates.

Rapid Golgi impregnation

Visualization of dendritic spines was accomplished using a commercially available Golgi-Cox preparation (FD Rapid Golgistain Kit, FD Neurotechnologies, Inc. Baltimore, MD). Brains were blocked into 1 cm sections containing the ventral hippocampus, and were then

processed according to the kit instructions. After staining, 200 micron sections were then obtained using a vibratome (Leica Wetzlar, Germany) and mounted onto gelatin-coated glass slides. Sections were then dehydrated through a series of increasing ethanol concentrations, cleared with xylene, and allowed to air-dry before being covered with glass coverslips using Permount. Slides were examined under 40× and 100× oil immersion objectives (400× and 1000× final magnification) as described below. Photographic images were obtained with a TCS-SP2 AOBS inverted confocal microscope (Leica, Wetzlar, Germany). For spine density quantification, Golgi-impregnated spines were visualized using a light microscope (Nikon Optiphot-2) and the data plotted using a digitizing stage controlled by Neurolucida software (MicroBrightField, Inc). Spine density was estimated by quantifying 20-35 μm sections of at least 3 well-defined dendrites per slide. For spine quantification, a region at least 30 μm from the soma was utilized. This ensured that similar regions in all cells were utilized and avoid any confounds of decreased spine density in proximal dendrites. For all data presented, at least 3 slide sections were averaged to estimate spine density per animal. The least number of animals necessary to obtain statistical significance were used.

Statistical analysis

EEG review and analysis was performed using Insight II software (Persyst, Prescott, AZ). All statistical analysis was performed using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). For multiple group comparisons, ANOVA with Tukey post-hoc analysis was used with a minimum significance level of $p < 0.05$. All data is presented as mean $\pm$ SEM unless otherwise noted.

Results

SE induces a rapid increase in CaN activity and concentration in post-synaptic regions

Previous studies in our laboratory described a dramatic increase in CaN immunoreactivity in post-synaptic neuronal regions after 1 hour of SE (Kurz et al., 2003). This places highly elevated amounts of CaN in an ideal location to regulate synaptic transmission through a number of post-synaptic mechanisms, including alteration of the dendritic cytoskeleton. The present study expands upon these results through the use of video-EEG monitoring, which allowed us to obtain tissue at highly precise time-points during SE, thus providing a temporal profile of SE-induced alterations in CaN activity and distribution. Detailed electrographic and behavioral analysis, as described previously (Singleton, 2004), ensured that all animals included had a similar seizure profile. Animals that experienced discrete seizures but never developed SE were excluded, as were animals that developed continuous seizure activity much more quickly or more slowly than the average (difference of more than one standard deviation from the average time from first seizure to SE of 10:41). These analyses ensured that animals obtained at the same time point would have experienced approximately equivalent seizure activity.

Crude synaptoplasmic membrane (SPM) fractions were isolated at specific times after the first discrete seizure as described previously (Singleton et al., 2005), and western analysis for CaN was carried out to determine the CaN content of SPM isolated from both control and SE animals at several time-points. Fractions were analyzed from both cortical and hippocampal tissues, as these were the brain regions previously shown to demonstrate an increase in SPM CaN immunoreactivity. In both cortical and hippocampal samples, SPM CaN immunoreactivity was not significantly different from control at 10 minutes post-first discrete seizure (a time-point which approximates the onset of status epilepticus). However, at 15 minutes post-first discrete seizure and all subsequent time points, CaN

immunoreactivity was significantly increased over control values in both cortical and hippocampal SPM (Fig 1).

Using a highly characterized, para-nitrophenol phosphate (pNPP)-based phosphatase assay as described previously (Kurz et al., 2001), we also examined CaN phosphatase activity at the above time-points in cortical and hippocampal crude SPM fractions (Fig 2). Similar to the increase in CaN concentration, CaN activity approximately doubled shortly after the onset of continuous seizure activity in both cortical and hippocampal crude SPM. This increase in activity is of roughly the same magnitude as the increase in CaN concentration described above, and is likely due to the increased quantity of the enzyme present in this fraction after the onset of SE. Interestingly, an additional increase in CaN activity – beyond the 2-fold increase observed at 10 minutes – was observed in cortical and hippocampal SPM fractions 30 minutes after the first discrete seizure. This increase in activity did not correspond to a further increase in CaN immunoreactivity in the crude SPM fraction. A similar increase in CaN activity is also observed in whole cell homogenates at this later time point (Kurz et al., 2005a); we have previously hypothesized that this late increase in CaN activity in the whole cell homogenate represents a post-translational modification of the enzyme brought on by SE (Kurz et al., 2001). This post-translational modification may also explain the increasing trend in SPM CaN activity with longer durations of SE.

To further characterize the SE-induced increase in synaptic CaN concentration and activity, we examined the phosphorylation state of a known neuronal CaN substrate, DARRP-32 (Fig 3). In both cortical and hippocampal fractions, SE induced a profound dephosphorylation of DARRP-32. This dephosphorylation could be blocked by administration of FK506 or cyclosporin A prior to the induction of SE, indicating that SE-induced dephosphorylation of these substrates was CaN-dependent. In cortical SPM, phospho-DARRP-32 immunoreactivity was $12.3 \pm 10.2\%$ of control after SE. Pretreatment with FK506 preserved a significant phosphorylation of DARRP-32 ($62.1 \pm 7.3\%$ of control). Pretreatment with cyclosporin A also resulted in a significant preservation of phosphorylation compared to SE animals ($45.8 \pm 9.1\%$ of control). Similar results were achieved in hippocampal tissue (data not shown). The CaN-dependent, SE-induced dephosphorylation of this substrate further demonstrates the dramatic increase in synaptic CaN activity that is present in SE, and confirms the efficacy of the CaN inhibitors used in this study.

The above results, taken together with our previous studies, demonstrate a significant increase in CaN activity and concentration in the post-synaptic regions of neurons shortly after the onset of continuous seizure activity.

CaN-dependent cofilin dephosphorylation in SE

To determine the cellular consequences of the SE-induced increase in post-synaptic CaN activity, we examined the effect of SE on the stability of the dendritic cytoskeleton, and consequently on dendritic spine plasticity. CaN may regulate the stability of dendritic actin via cofilin, a CaN-regulated actin depolymerization factor. Through the use of a phospho-specific cofilin antibody we were able to assess the phosphorylation state of cofilin after SE, both with and without the administration of CaN inhibitors. In both homogenates and crude SPM fractions isolated from SE animals, we found a significant dephosphorylation of cofilin in cortical and hippocampal samples. In cortical SPM, phospho-cofilin immunoreactivity was $45.8 \pm 12.3\%$ of control after 1h of SE (Fig 4A), while in hippocampal SPM phospho-cofilin levels decreased to $25.9 \pm 13.2\%$ of control (Fig 4B). A similar SE-induced decrease in cofilin phosphorylation level was observed in homogenates from these brain regions, indicating that the effect was not an artifact of the SPM isolation procedure (data not shown). Overall cofilin immunoreactivity was not significantly affected by SE in any fraction tested (Fig 4C, D), indicating that the observed decrease in phospho-cofilin was

likely due to dephosphorylation of the protein rather than a decrease in the amount of cofilin present in the neuron.

To determine if the SE-induced dephosphorylation of cofilin was CaN-dependent, we administered CaN inhibitors, FK506 or cyclosporin A, to animals prior to induction of SE. The SE-induced dephosphorylation of cofilin was significantly reduced by either FK506 or cyclosporin pre-injection. In FK506-treated SE animals, phospho-cofilin immunoreactivity was $81.8 \pm 13.0\%$ of control in cortical SPM (Fig 4A), and $62.9 \pm 14.6\%$ of control in hippocampal SPM (Fig 4B). While SE still induced some cofilin dephosphorylation in these FK506-treated animals, phospho-cofilin immunoreactivity was significantly greater in SPM samples from FK506-treated animals than in untreated SE animals, indicating that a significant, CaN-dependent cofilin dephosphorylation occurs with prolonged seizure activity. Similar results were achieved by treating animals with cyclosporin A prior to SE. Phosphocofilin immunoreactivity was $67.1 \pm 9.7\%$ of control in cortical SPM and $61.4 \pm 11.7\%$ of control in hippocampal SPM isolated from cyclosporin A animals (Fig 4A, B). These values were significantly greater than the cofilin phosphorylation levels in untreated SE animals. The fact that two different CaN inhibitors (with different mechanisms of action) were effective in preventing SE-induced cofilin dephosphorylation provides a strong argument that CaN inhibition was indeed the important factor in preventing cofilin dephosphorylation, rather than some side-effect of the inhibitors. This data suggests that increased dendritic CaN activity leads to cofilin dephosphorylation after SE. While this study is the first to present such a finding, the data corresponds well with previous research detailing CaN's effects on cofilin and actin stability (Halpain et al., 1998; Wang et al., 2005).

Time-course of cofilin dephosphorylation

Western blot analysis was used to determine the phosphocofilin immunoreactivity at specific time-points post-first discrete seizure. Both cortical and hippocampal crude SPM isolates were examined, using control and pilocarpine-treated animals. In both cortical and hippocampal samples, cofilin phosphorylation levels decreased significantly from control levels after just 10 minutes of seizures, a time point which generally corresponded with the onset of continuous seizure activity. At 10 minutes post-first discrete seizure, phosphocofilin immunoreactivity was $60.2 \pm 6.7\%$ of control in cortical samples and $39.9 \pm 8.3\%$ of control in hippocampal samples (Fig 5). At the 20 and 40 minute time points, phosphocofilin immunoreactivity was indistinguishable from the 70 minute time point presented earlier. It is interesting to note the speed with which cofilin phosphorylation responds to seizure activity, suggesting that even a few minutes of continuous seizure activity may be sufficient to induce widespread activation of this protein. We also noted that the temporal profile of cofilin dephosphorylation correlates well with the timing of the increase in dendritic CaN activity, providing further support for the idea that CaN is partially responsible for SE-induced cofilin activation.

SE increases cofilin-actin binding

After one hour of SE, there was a profound increase in cofilin-actin co-immunoprecipitation. In cortical samples, actin immunoreactivity was $171.7 \pm 11.3\%$ of control values in the precipitate (Fig 6A, B), suggesting a greater than 1.5-fold increase in cofilin-actin binding. Similar results were achieved in hippocampal samples, with actin immunoreactivity increased to $183.4 \pm 14.9\%$ in cofilin immunoprecipitates from SE animals (Fig 6C, D). When the precipitation procedure was carried out in the absence of anti-cofilin antibody, a minimal amount of actin was present in the precipitate, indicating that the primary source of actin immunoreactivity was actin that was bound to cofilin and co-precipitated with it.

Both FK506 and cyclosporin A significantly reduced cofilin-actin co-immunoprecipitation. Pretreatment of animals with FK506 resulted in a SE-induced cofilin-actin co-immunoprecipitation that was $122.3 \pm 6\%$ of control in cortical samples (Fig 6A, B) and $131.4 \pm 7.9\%$ of control in hippocampal samples (Fig 6C, D). Both of these values are significantly lower than those of untreated SE animals, suggesting that CaN inhibition prevents cofilin-actin binding. Similar results were achieved with cyclosporin A. This data complements our finding that CaN inhibition prevents SE-induced cofilin dephosphorylation, since dephosphorylation of cofilin is required for the protein to bind to actin.

SE induced actin depolymerization is CaN-dependent

In crude SPM isolated from SE cortex, Triton-soluble actin immunoreactivity was $123.2 \pm 8.1\%$ of control (Fig 7A), while Triton-insoluble actin immunoreactivity was $79.3 \pm 6.7\%$ of control (Fig 7B), indicating an SE-induced shift from F-actin to G-actin in the crude SPM. A similar change was noted in hippocampal samples, with Triton-soluble actin immunoreactivity $121.9 \pm 7.4\%$ of control (Fig 7C), while insoluble actin immunoreactivity was $71.3 \pm 5.4\%$ of control (Fig 7D), again demonstrating a decrease in F-actin and an increase in G-actin. Overall actin immunoreactivity in this fraction was unchanged by SE. In both cortical and hippocampal homogenates, FK506 significantly reduced this actin depolymerization, partially preventing the SE-induced increases in Triton-soluble actin immunoreactivity (G-actin) and the decreases in Triton-insoluble actin immunoreactivity (F-actin). Thus, by blocking the first step in this pathway – the CaN-induced dephosphorylation of cofilin – we prevented the final biochemical steps of actin depolymerization.

CaN inhibitors prevent dendritic spine loss in SE

The Golgi-Cox staining procedure was utilized to determine the cellular consequences of SE-induced CaN activation. Confocal images of dentate gyrus granule cells from control and SE + FK506-treated animals are presented in Figure 8 (D-F), and corresponding 2D light microscopic images are presented in Figure 8 (A-C). Granule cells with well stained dendritic arbors were identified during this step for spine quantification, areas of dendrite selected were all in the stratum moleculare of the dentate gyrus, and only included neurons that were well stained and free from any artifact from the staining procedure (see Methods and Materials). Dendritic processes from control animals displayed multiple dendritic spine processes in all planes. Dendritic processes visualized from animals subjected to SE displayed a substantial decrease in dendritic spine density under both light and confocal microscopy. However, pretreatment with FK-506 appeared to prevent the SE-induced loss of dendritic spines.

To further characterize the SE-induced dendritic spine loss, spine density was quantified under light microscopic conditions (Nikon Optiphot-2) using a digitizing stage controlled by NeuroLucida software (MicroBrightField, Inc). At least 3 individual neurons were counted on each slide, and a minimum of 3 slides were used from each animal (Fig 9). The average spine density in control animals was 0.51 ± 0.02 spines/micron. Following SE, spine density decreased to 0.21 ± 0.02 spines/um, or 41.2% of control ($p < 0.001$ compared to control, $n = 3$ animals, ANOVA with Tukey post-test). This result is consistent with previous studies (Isokawa, 1998) and confirms that prolonged seizure activity results in a significant reduction of dendritic spines. The SE-induced loss of dendritic spine density could be prevented by pre-administration of the CaN inhibitor, FK-506. FK506-treated SE animals had an observed spine density of 0.46 ± 0.07 spines/um, or 82.3% of control ($p > 0.05$ compared to control, $p < 0.01$ compared to SE, $n = 3$ animals), suggesting a CaN-dependent mechanism of spine loss in SE, such as the CaN-dependent activation of cofilin described above.

To determine if SE resulted in a CaN-dependent spine loss in pyramidal neurons, hippocampal CA1 and cortical layer 2-3 neurons were examined for spine density as performed for dentate gyrus. In hippocampal CA1, pyramidal neurons had an average of 0.49 ± 0.03 spines/um which was similar to dentate gyrus. SE resulted in a 71% loss of dendritic spines (0.142 ± 0.02 spines/um, $p < 0.001$). The SE-induced spine loss was prevented by the pre-administration of FK-506 (0.50 ± 0.03 spines/um, $p = 0.93$ compared to control group). Similar results were observed in cortical neurons. Pyramidal neurons in sections obtained from control animals displayed an average spine density of 0.50 ± 0.03 spines/um. SE resulted in a 62% loss of spine density ($0.19 \pm .02$ spines, um, $p < 0.001$). As observed in both pyramidal and gyrus hippocampal neurons, pretreatment with FK-506 results in almost complete preservation of cortical spine density ($0.44 \pm .02$ spines/um) and animals that experienced SE in the presence of FK-506 were not significantly different from control ($p = .0996$). The data demonstrate that inhibition of CaN activity results in a significant preservation of neuronal spine density in all cortical areas studied.

Discussion

The results presented in this study demonstrate a CaN-dependent mechanism of dendritic spine loss in the pilocarpine model of SE. A dramatic increase in CaN activity and concentration occurs in the crude SPM fraction of hippocampal and cortical tissues at or near the onset of continuous seizure activity. Coupled with our previous histochemical results (Kurz et al., 2003), this demonstrates an increase in the effective amount of CaN phosphatase activity in dendritic regions of neurons. This study elucidated one pathological consequence of this increased post-synaptic CaN activity by examining CaN's effects on dendritic actin stability. First, we have shown a CaN-dependent dephosphorylation of the actin-depolymerizing factor, cofilin. Like the increase in CaN concentration and activity, this dephosphorylation develops shortly after the onset of continuous seizure activity. The expected biochemical consequences of an SE-induced activation of cofilin were also found to occur in a CaN-regulated manner, with a SE-dependent increase in cofilin-actin binding and a subsequent SE-induced depolymerization of dendritic actin. Finally, the CaN-dependent depolymerization of dendritic actin led to SE-induced dendritic spine loss, which was demonstrated histologically in several brain regions and was blocked with the CaN inhibitor FK506. These findings demonstrate a cellular pathway, through which SE induces dendritic spine loss.

SE and Spine Loss

The observed SE-induced reduction in dendritic spine density may be part of the long-term neurological pathology that is associated with SE. As the primary site of excitatory neuronal synapses, and given their importance in models of learning and memory, spines appear to be critical for normal cognition. Studies in animal models have documented cognitive difficulties associated with SE, both in adult and developing animals (Rice et al., 1998). While SE-induced neuronal death undoubtedly accounts for some of this loss of cognitive function, other, more subtle, mechanisms – such as spine loss – are likely responsible as well. Depending on the duration of the SE-induced decrease in dendritic spine density, the mechanism described in this study could be a mechanism underlying SE-induced cognitive dysfunction. In one previous study, dendritic spine density rebounded in the weeks following SE, although with significant alterations in spine morphology and location. Spine density then decreased again once chronic seizure activity ensued (spontaneous recurrent seizures are a common consequence of the pilocarpine model of SE) (Isokawa, 1998). These findings tend to argue against the acute spine loss described in this study as the cause of long-term cognitive dysfunction, as it does not persist long enough to account for chronic deficits of cognition. However, the long-term decrease in spine density seen in many models

of recurrent seizures may involve a chronic, lower-intensity activation of the CaN-regulated mechanism described above. This persistent decrease in spine density could be involved in chronic deficits of cognitive function. More study is needed to determine the role of CaN in chronic epileptic states. On the other hand, the acute, CaN-regulated spine loss described above may be involved in another SE-associated pathology, epileptogenesis.

In the pilocarpine model of SE, spontaneous recurrent seizures typically begin approximately 2 weeks after the prolonged seizure episode (Turski et al., 1989). No epileptic activity is seen during this quiescent period, but there is a great deal happening on a cellular level. It is thought that the biochemical and structural changes that lead to recurrent seizure activity are being completed during this time. While the exact mechanisms responsible for epileptogenesis remain the subject of intense study and debate, dendritic spine plasticity certainly is a promising candidate for this role. The loss and subsequent regrowth of dendritic spines could represent a pathological reorganization of synaptic networks leading to the formation of epileptic foci in the brain. It is especially interesting that spine loss has been documented in the dentate gyrus of the hippocampus. Network reorganization and neuronal hyperexcitability in this region are frequently proposed as possible mechanisms of epileptogenesis (Sloviter, 1999; Bragin et al., 2000). Furthermore, after the SE-associated loss of spines, it has been shown that their regrowth in this region is colocalized with sites of mossy fiber sprouting (Isokawa, 2000). Thus an outgrowth of axonal structures that has been previously implicated in epileptogenesis seems to coincide with a reorganization of post-synaptic structures, all of which is occurring in a region that seems to be critical in the development of temporal lobe epilepsy. Considered in the context of epileptogenesis, the CaN-mediated mechanism of spine loss presented in this paper may help explain the results of several other studies. FK506 ameliorated spine loss in the present study, while other researchers have documented that CaN inhibitors can prevent epileptogenesis in both a kainic acid model of SE (Moriwaki et al., 1998) and a kindling model of epilepsy (Moia et al., 1994; Moriwaki et al., 1996). Further research may determine if these two effects are related.

Cellular Mechanism of Spine Plasticity

Spine plasticity has long been observed in both chronic epilepsy and acute SE models (Wong, 2005), although the mechanisms that underlie this spine loss have not yet been determined. A great deal of research suggests that CaN modulates neuronal spine density and morphology under normal conditions, with the enzyme having been previously shown to cause calcium-stimulated spine plasticity in several non-pathological model systems. Halpain et al. demonstrated a loss of spines in cultured hippocampal neurons in response to NMDA application. CaN was shown to co-localize with F-actin at synapses, and CaN inhibitors blocked NMDA-mediated spine loss in these neurons, strongly suggesting a CaN-mediated spine loss (Halpain et al., 1998). Similarly, Zhou et al. described an LTD-associated spine shrinkage that required both NMDA and CaN to occur (Zhou et al., 2004). Under physiological conditions, it is highly probable that CaN-mediated spine plasticity is part of a learning and memory mechanism, considering the importance of both CaN and spines to learning models. However, with the profound increase in CaN levels at the synapse that we have observed in SE, this regulation of spine plasticity may become pathologically active, resulting in a detrimental loss, and then reorganization, of synaptic contacts.

The present study documented a profound increase in CaN concentration and activity in the crude SPM fraction of neuronal tissue of animals that had undergone as little as 5-10 minutes of continuous seizure activity. As we have described previously, continuous seizure activity typically began approximately 10 minutes after the first discrete seizure in this model (Singleton et al., 2005). No detectable increase was seen, however, in animals that experienced discrete, but not continuous, seizure activity. There are two possible

explanations for this. The onset of SE may be essential for significant changes in CaN to occur, or these discrete seizures may simply not have been of sufficient duration to induce a measurable translocation of the enzyme (although a longer, but still isolated, seizure may prove sufficient). Regardless, CaN translocation seems to be a neuronal reaction to prolonged seizure activity. Such a translocation of CaN to the synapse may initially be a physiological defense mechanism for the neuron in the face of excessive excitation, as CaN is known to negatively modulate neurotransmission through the AMPA and NMDA subtypes of glutamate receptor. Perhaps such a mechanism, occurring near the onset of continuous seizures, represents a pathway by which seizure activity can be terminated in brains that are not prone to SE. However, as SE progresses and worsens, this sustained increase in synaptic CaN activity also could play a pathological role in neurons. A number of recent studies support such a hypothesis. For example, it has long been known that administration of CaN inhibitors prior to SE prevents chronic SE-related behavioral pathologies, such as a SE-induced loss of cognitive abilities and the development of spontaneous continuous seizures (Moriwaki et al., 1998). Several mechanisms have been proposed as pathological roles for CaN in seizure disorders, including negative modulation of the GABA receptor (Sanchez et al., 2005; McNamara et al., 2006) and – the focus of the current study – modulation of dendritic spines.

CaN may perform this pathological modulation of spine morphology via its regulation of the actin-depolymerizing factor, cofilin. Cofilin is a small peptide that, when dephosphorylated, binds to F-actin and causes its depolymerization (Agnew et al., 1995; Bamburg, 1999). Cofilin has been shown to regulate the structure of dendritic spines by inducing the depolymerization of their actin cytoskeleton (Meng et al., 2004; Sarmiere and Bamburg, 2004; Zhou et al., 2004). Recent studies have shown that CaN induces cofilin dephosphorylation (and thus subsequent actin depolymerization) either directly (Meberg et al., 1998), or, more likely, indirectly via an intermediary phosphatase known as slingshot (Wang et al., 2005), thus making a mechanism involving CaN activation of cofilin and subsequent spine loss quite plausible. In fact, one recent study has already shown spine regulation under some physiological conditions through a mechanism dependent on both CaN and cofilin (Zhou et al., 2004).

The data described in this study clearly indicate a CaN-dependent regulation of cofilin in SE. First, the SE-induced dephosphorylation of cofilin required the onset of continuous seizure activity, a requirement shared with the increase in SPM CaN and SE-induced dendritic spine loss. In fact, the timing of this loss of SPM cofilin phosphorylation coincides perfectly with the increase in SPM CaN concentration and activity. Furthermore, both of the CaN inhibitors cyclosporin A and FK506 blocked SE-induced cofilin dephosphorylation. The fact that two different CaN inhibitors blocked SE-induced cofilin dephosphorylation strongly argues in favor of a CaN-dependent mechanism, although some modest SE-induced dephosphorylation did occur in the presence of effective doses of each inhibitor. This is not in itself surprising, as a number of other mechanisms exist for the regulation of cofilin phosphorylation. Activation of these pathways in SE is certainly possible, and merits future study. However, CaN-mediated dephosphorylation represented a major portion of the observed decrease in phosphocofilin immunoreactivity. This CaN-dependent dephosphorylation of cofilin did indeed activate the molecule, as SE was also shown to lead to a CaN-dependent increase in cofilin-actin binding and a CaN-dependent depolymerization of actin. While the observed change in the F/G actin ratio was relatively modest, when one considers the prevalence of actin in all cell types and cellular structures in the brain, even a 20% difference in the relative amounts of F- and G- actually represents a quite profound structural change. This structural change manifested itself in a CaN-dependent loss of dendritic spines. As other researchers have previously, we observed a SE-dependent loss of dendritic spines after 1 hour of SE. Considering the timing of the CaN-induced cofilin

dephosphorylation, this loss of spines may, in fact, have occurred even earlier in SE, and future studies should certainly explore synaptic changes at these earlier time-points.

The novel findings presented in this study describe a cellular mechanism for actin depolymerization and dendritic spine loss in the pilocarpine model of SE. This spine loss is widespread throughout the forebrain after SE, and is CaN-dependent, involving the actin depolymerizing factor, cofilin. Further research in this area may help elucidate the role of dendritic spine plasticity in SE-associated neuronal pathologies, potentially provide insight into some of the mechanisms underlying epileptogenesis and providing the basis for future treatment options.

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Figure 1. The seizure-induced translocation of CaN to synaptic membrane occurs early in the duration of SE

CaN immunoreactivity was measured in crude SPM fractions isolated from cortex (A) and hippocampus (B). In both fractions, CaN immunoreactivity was found to be increased significantly above control values within 15 minutes post-first discrete seizure, or approximately 5 minutes after the onset of continuous seizure activity. CaN immunoreactivity doubled at this time-point in both cortical and hippocampal SPM, and remained similarly elevated at all subsequent time-points. (** $p < 0.01$, $n = 3$ per time-point, Student's t-test).

Figure 2. Temporal profile of increased CaN synaptic membrane CaN activity in cortical and hippocampal samples

In both cortex (A, B) and hippocampus (C, D) CaN activity in the SPM increased with a temporal profile similar to the SE-induced increase in CaN immunoreactivity noted above. Both basal (A, C) and cation-stimulated (B, D) activity increased significantly by 20 minutes post-first discrete seizure, with the magnitude of the increase being roughly similar to the magnitude of the increase in CaN immunoreactivity via western blot. As seizure activity progressed, a further increase in CaN activity was observed in all fractions and in all reaction conditions (although this increase is most noticeable in under the basal reaction conditions). The timing of this later increase corresponds to an increase in CaN activity that is observed in the overall homogenate and is discussed previously (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 3. SE leads to increased CaN-dependent dephosphorylation of DARRP-32

Phospho-DARRP-32 immunoreactivity was determined via western analysis in cortical homogenates. SE was found to induce a significant dephosphorylation of the protein (* $p < 0.05$, $n=3$), a finding that is consistent with increased CaN activity. Pretreatment of animals with either CaN inhibitor reduced DARRP dephosphorylation significantly (* $p < 0.05$ compared to SE, $n=3$), indicating that the inhibitors were reaching the brain in sufficient concentrations to inhibit CaN activity.

Figure 4. SE induces a CaN-dependent dephosphorylation of cofilin

SE induced a significant dephosphorylation of cofilin in crude SPM isolated from cortical (A) and hippocampal (B) regions, as measured by immunoreactivity on western analysis using a phosphocofilin antibody ($p < 0.01$, $n=10$ in both regions). Administration of either of the immunosuppressant CaN inhibitors (FK506 or cyclosporin A) prior to SE partially blocked this dephosphorylation ($p < 0.05$ when compared to SE, $n = 6$ in both cortex and hippocampus). Changes in phosphocofilin immunoreactivity were not due to changes in overall cofilin concentration, as western analysis with a cofilin antibody revealed no change in overall levels of the protein in either cortical (C) or hippocampal (D) SPM.

Figure 5. Temporal profile of cofilin dephosphorylation in the pilocarpine model of SE
 (A) Western analysis for phosphocofilin in cortical (left) and hippocampal (right) crude SPM at 10, 20 and 40 minutes post-first discrete seizure. Cofilin phosphorylation was detected as early as 10 minutes post-first discrete seizure in both brain regions. This time point corresponds approximately to the onset of continuous seizure activity in this model.
 (B) Calibrated densitometry of phosphocofilin western blots in cortical (left) and hippocampal (right) crude SPM fractions. Cofilin phosphorylation was significantly decreased at 10 minutes and all subsequent time points (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n=3$ at each time-point).

Figure 6. SE induces a CaN-dependent binding of cofilin to actin as detected by coimmunoprecipitation

Cofilin was immunoprecipitated from cortical (A,B) and hippocampal (C,D) homogenates, then the actin content of the precipitate was determined by western analysis. Very little actin was precipitated in the absence of cofilin primary antibody (A, far left), confirming the specificity of the assay procedure. In both cortical (A, B) and hippocampal (C,D) samples, SE led to a significant ($p < 0.05$) increase in cofilin-actin coprecipitation. This association of the two proteins was significantly blocked by calcineurin inhibitors ($p < 0.05$ when compared to SE value).

Figure 7. SE induces CaN dependent actin depolymerization

F-actin and G-actin were separated by solubility in 1% triton, then assayed by western analysis in cortical (A, B) and hippocampal (C,D) crude SPM. In cortical SPM, SE caused a significant ($p < 0.05$) increase in the amount of actin present in the triton-soluble fraction (A). G-actin is soluble in triton while F-actin is not, and thus this increase in soluble actin likely represents an SE-induced depolymerization of actin. A similar decrease in triton-insoluble actin (F-actin) was observed in cortical homogenates following SE (B). The CaN inhibitor FK506 blocked the shift of actin from the soluble to insoluble fraction, suggesting that the observed actin depolymerization was CaN-dependent. Similar effects were observed in hippocampal triton-soluble (C) and triton-insoluble (D) fractions isolated from crude SPM ($n = 3$ for all groups).

Figure 8. FK506 blocks SE-induced dendritic spine loss in granule cells of the dentate gyrus
 Rat brains from control animals and animals subjected to SE $\pm$ FK-506 injection were blocked and coronal sections (200 μ m thick) sliced using a vibratome and neurons stained using the golgi method (see Materials and Methods). Dendritic spines were visualized in hippocampal gyrus neurons under light (A-C) and confocal microscopy (D-F). Numerous mature spines were observed in sections isolated from control animals (panel A/D). However, prolonged seizure activity in SE results in a substantial reduction in spine density (Panel B/E). Spine density was preserved in animals pre-treated with FK-506 (panels C/F). (Representative sections shown, n = 3 animals per group).

Figure 9. SE induced a significant, FK-506 sensitive loss of dendritic spine density

Spine density was quantified in hippocampal gyrus neurons using Neurolucida software (See Materials and Methods). Control animals expressed an average of 0.51 ± 0.02 spines/um. Spine density in animals subjected to SE was reduced almost 60% (0.21 ± 0.02 spines/um; $p < 0.001$ 1-way ANOVA, $n = 3$ animals). Pretreatment with FK-506 significantly preserved spine density (0.46 ± 0.07 spines/um. Mature spine density in SE-FK506 animals were not significantly different from control. The data demonstrate that inhibition of CaN activity will prevent SE-induced dendritic spine loss.