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HIPPOCAMPAL SEIZURES CAUSE DEPOLYMERIZATION OF FILAMENTOUS ACTIN IN NEURONS INDEPENDENT OF ACUTE MORPHOLOGICAL CHANGES

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Abstract

Seizures may exert pathophysiological effects on dendritic spines, but the molecular mechanisms mediating these effects are poorly understood. Actin represents a major structural protein of dendritic spines, and actin filaments (F-actin) can be depolymerized by the regulatory molecule, cofilin, leading to structural or functional changes in spines in response to normal physiological activity. To investigate mechanisms by which pathophysiological stimuli may affect dendritic spine structure and function, we examined changes in F-actin and cofilin in hippocampus due to 4-aminopyridine (4-AP)-induced seizures/epileptiform activity in vivo and in vitro and investigated possible structural correlates of these changes in actin dynamics. Within an hour of induction, seizure activity caused both a significant decrease in F-actin labeling, indicating depolymerization of F-actin, and a corresponding decrease in phosphorylated cofilin, signifying an increase in cofilin activity. However, 4-AP seizures had no overt short-term structural effects on dendritic spine density. By comparison, high potassium caused a more dramatic decrease in cofilin and an immediate dendritic beading and loss of dendritic spines. These findings indicate that activation of cofilin and depolymerization of F-actin represent mechanisms by which seizures may exert pathophysiological modulation of dendritic spines. In addition to affecting non-structural functions of spines, the degree to which overt structural changes occur with actin depolymerization is dependent on the severity and type of the pathophysiological stimulus.

Keywords

seizure; epilepsy; actin; cofilin; dendrite; spine

1. Introduction

Patients with epilepsy often suffer from significant neurocognitive deficits, such as memory impairment, which may, in part, be caused by direct injurious effects of seizures on the brain (Dodrill, 2002; Elger et al., 2004). Many previous studies indicate that seizures can directly cause “excitotoxic” cell injury and neuronal death under some conditions (Holmes, 2002; Sutula et al., 2003); however, seizures may also induce more subtle “non-lethal”

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pathophysiological changes in neuronal structure and function, such as abnormalities in synaptic transmission. Dendritic spines receive a majority of the excitatory synaptic inputs to cortical neurons and are strongly implicated in mechanisms of synaptic plasticity and learning (Carlisle and Kennedy, 2005; Segal, 2005). Studies of human epilepsy and animal models demonstrate that seizures may directly affect the morphological and functional properties of dendritic spines (reviewed in Swann et al., 2000; Wong, 2005), suggesting that seizure-related changes in spines may represent a mechanistic basis for cognitive deficits in epilepsy.

The molecular mechanisms by which physiological and pathological stimuli modulate dendritic spine structure and function are incompletely understood, but may involve regulation of the actin cytoskeleton (Carlisle and Kennedy, 2005). Filamentous actin (F-actin), which is highly concentrated in dendritic spines (Matus et al., 1982; Allison et al., 1998; Capani et al., 2001), forms complex networks that provide structural support for dendrites and dendritic spines and also mediate non-structural spine functions, such as biochemical compartmentalization, gating of protein translocation into spines, and receptor trafficking and anchoring (Allison et al. 1998; Kennedy et al., 2005; Oertner and Matus, 2005; Ouyang et al., 2005). Previous studies have shown that physiological activation of neurons or induction of long-term potentiation (LTP) can cause changes in actin dynamics that may mediate synaptic plasticity (Kim and Lisman, 1999; Krucker et al., 2000; Fukazawa et al., 2003; Okamoto et al., 2004; Lin et al., 2005; Ouyang et al., 2005; Kramar et al., 2006). This activity-dependent regulation of actin dynamics involves modulation of actin-binding proteins, such as cofilin (Sarmiere and Bamberg, 2004), which causes changes in the polymerization state of actin and may result in either structural changes or non-structural, functional changes in dendritic spines.

In contrast to the regulation of actin dynamics by physiological stimuli, relatively little is known about the effects of more pathological activity, such as seizures, on actin networks. In the present study, we have investigated the short-term effects of pathophysiological seizure stimuli, as well as elevated potassium, on actin and cofilin dynamics in the mouse hippocampus *in vivo* and *in vitro* and examined by modern cellular imaging methods whether there is a gross structural correlate to these effects in dendritic spines. We report that seizures can modulate actin and cofilin dynamics acutely, although the ultimate structural or functional consequences of these changes may be dependent on the type and severity of the pathophysiological stimulus.

2. Results

2.1 Hippocampal seizures *in vivo* and *in vitro* cause depolymerization of F-actin

For *in vivo* seizure experiments, stereotypical clinical-electrographic seizures were induced by injection of 4-aminopyridine (4-AP) into the CA1 region of hippocampus of mice (Fig. 1A). Behaviorally, within minutes of recovery from anesthesia after 4-AP injection, mice exhibited rapidly repetitive or continuous clinical symptoms, including head nodding, forearm clonus, rearing and falling, and occasional wild running and generalized convulsive activity (Racine stage 5), lasting one hour until the mice were sacrificed. On EEG, repetitive electrographic seizures or continuous spike activity, which always evolved into bilateral cortical discharges, was observed during the entire hour (Fig. 1B). In hippocampal slices *in vitro*, although discrete electrographic seizures were rare, repetitive epileptiform spike discharges could be reliably elicited by superfusion of 100 μ M 4-AP (Fig. 1C).

When examining the effect of 4-AP seizures on F-actin levels as assayed by rhodamine-phalloidin labeling, we found that one hour of *in vivo* hippocampal seizure activity led to a significant decrease in F-actin (Fig. 2A). Compared to ACSF-injected controls, F-actin labeling was decreased by 29.8% in the CA1 region of mice following 4-AP hippocampal seizures *in vivo* (Fig. 2B). Similarly, 4-AP applied to hippocampal slices *in vitro* caused a more modest

(13.9%), but significant, decrease in F-actin labeling (Fig. 2C). Similar to results reported previously (Ouyang et al. 2005), the punctate pattern of rhodamine-phalloidin labeling (Fig. 2A) and double-labeling experiments with MAP2 and PSD95 (data not shown) confirmed the localization of F-actin in dendrites and dendritic spines in the stratum radiatum. Overall, these results indicate that 4-AP seizures can induce depolymerization of actin filaments in dendrites.

2.2 Hippocampal seizures increase cofilin activation

F-actin can be depolymerized by the regulatory actin-binding protein, cofilin. As cofilin is inactivated by phosphorylation, dephosphorylation of cofilin at the Ser3 residue leads to cofilin activation, which can trigger depolymerization of F-actin and may serve as a more sensitive marker of F-actin depolymerization. Thus, we tested the hypothesis that seizure-induced depolymerization of F-actin is related to a decrease in the phosphorylated form of cofilin (p-cofilin). By Western blot analysis, in vivo hippocampal seizures for one hour had no effect on total actin or cofilin levels, but caused a significant decrease (63.4%) in p-cofilin in the hippocampus ipsilateral to the 4-AP injection (Fig. 3A). p-cofilin expression was also decreased in the contralateral hippocampus (58.0%), indicating that these effects were related to seizure activity that had spread, not due to direct pharmacological effects of 4-AP. Similarly, 4-AP-induced epileptiform activity in hippocampal slices in vitro caused a decrease in p-cofilin (46.4%), but not total actin or cofilin, levels (Fig. 3B).

2.3 Hippocampal seizures do not affect dendritic spine number within one hour

Changes in F-actin and p-cofilin may cause both structural and non-structural/functional changes in dendritic spines. To determine whether modulation of F-actin and p-cofilin dynamics by 4-AP seizures was associated with immediate structural alterations in dendritic spines, we examined the effect of 4-AP seizures on dendritic spine number/density in hippocampus by different in vivo and in vitro methods using transgenic mice expressing green fluorescent protein (GFP) in hippocampal neurons (Feng et al., 2001). In conventional fixed-tissue in vivo studies, dendritic spine density of CA1 pyramidal neurons in hippocampus was not significantly different in mice after one hour of seizure activity (1.40 ± 0.05 spines/ μm , $n = 28$ dendrites from 7 mice) compared to ACSF-injected controls (1.44 ± 0.03 spines/ μm , $n = 24$ dendrites from 6 mice; Fig. 4A). In time-lapse multiphoton imaging of individual living dendrites and spines in hippocampal slices in vitro, 4-AP epileptiform activity had no significant effect on spine number over a one hour period (Fig. 4B). There was <3% loss or gain of total spines in both control conditions and during 4-AP exposure (197 of 202 spines and 223 of 227 spines were stable over one hour in control and 4-AP conditions, respectively; 6 slices from 4 mice in each group).

2.4 Elevated extracellular potassium causes dramatically increased cofilin activation and dendritic spine loss

While pathophysiological seizure activity over a relatively short time scale may cause changes in actin and cofilin dynamics without obvious structural changes in dendritic spines, it is likely that more severe pathological stimuli will lead to more pronounced effects on actin polymerization that lead to gross changes in dendritic structure. As a positive control, we tested the effects of elevated extracellular potassium at 50 mM, which is commonly used experimentally to induce “neuronal activation”, on p-cofilin levels and dendritic structure. Within a few minutes of exposure of live hippocampal slices to 50 mM potassium chloride, dendrites exhibited a dramatic swelling of processes and loss of spines (Fig. 5A). This was associated with a very strong decrease in p-cofilin levels (92.6%), but unchanged total cofilin and actin levels (Fig. 5B,C). In comparison with the 4-AP seizures, these results suggest that

severe pathological activating conditions may cause more dramatic effects on cofilin/actin dynamics associated with overt structural changes in dendrites.

3. Discussion

Studies of both human epilepsy and animal models suggest that seizures may exert pathophysiological effects on dendritic spines (Swann et al., 2000; Wong, 2005), which could contribute to cognitive deficits of epilepsy patients. Although previous studies have demonstrated that seizures may activate a variety of types and mechanisms of brain injury involving dendrites, in the present study we focused on the hypothesis that seizures might disrupt dendritic structure or function, at least in part, via effects on the actin cytoskeleton. We found that seizure activity causes activation (dephosphorylation) of cofilin and an associated depolymerization of F-actin, even in the absence of major structural changes to the dendrites. While a recent study found that disruption of actin networks may alter seizure threshold (Sierra-Paredes et al., 2006) and other studies indicate that seizures may affect actin-binding proteins (Roth et al., 2001; Ferhat et al., 2003), the present study is among the first to directly show that seizures can modulate actin polymerization, as well as cofilin activation. While it is possible that the actin depolymerization might be a direct pharmacological effect of 4-AP rather than the seizure activity, preliminary data from our and other labs suggest similar effects in other seizure models (Wong, unpublished data), and p-cofilin was decreased bilaterally in this study, despite a unilateral 4-AP injection, consistent with seizure-mediated actions.

Although there is little in the prior literature about direct seizure effects on actin networks, it is perhaps not surprising that seizures could regulate actin and cofilin dynamics, given previous studies documenting the effect of more physiological forms of neuronal activation on actin. Many studies have found that tetanic electrical stimulation to induce LTP leads to an increase in F-actin, suggesting that the formation of new filamentous actin networks in dendritic spines could mediate the increased synaptic efficacy and consolidation of LTP (Fukazawa et al., 2003; Okamoto et al., 2004; Lin et al., 2005; Ouyang et al., 2005; Kramar et al., 2006). In contrast, other data suggest that depolymerization of actin may also be triggered by neuronal activation and LTP (Kim and Lisman, 1999; Ouyang et al., 2005; Shen and Meyer, 1999). We recently reported evidence that LTP causes a biphasic response, involving an initial transient phase of actin depolymerization, which may allow for plasticity and motility of dendritic structure or function, followed by subsequent polymerization of F-actin, which could lead to long-term stabilization or consolidation of dendritic changes (Ouyang et al., 2005). In contrast, pathological activation, such as with seizures, has the potential to disrupt this finely-regulated, dynamic system of dendritic actin networks.

The ultimate structural and functional consequences of physiological or pathological regulation of actin dynamics in dendrites are variable and incompletely understood. Modulation of actin networks may result in either morphological changes in dendrites or non-structural functional changes related to signal transduction. For instance, LTP has been reported to induce formation of new dendritic spines or morphological changes in existing spines (Engert and Bonhoeffer, 1999; Lang et al., 2004; Maletic-Savatic et al., 1999; Matsukazi et al., 2004). However, in some cases, these structural changes were either transient or occurred in a very small percentage of spines, suggesting that the regulation of actin dynamics that occurs with LTP could have other functional consequences. Accordingly, activity-dependent regulation of actin, such as during LTP, may also be involved in other, non-structural functions, such as biochemical compartmentalization and signaling, translocation of proteins into spines, and trafficking and insertion of receptors within spines (Allison et al., 1998; Shi et al., 1999; Kennedy et al., 2005; Oertner and Matus, 2005; Ouyang et al., 2005), in the absence of obvious structural changes to spines.

Similarly, modulation of actin dynamics by seizures could have both structural and non-structural effects on dendritic spines. In the present study we found that hippocampal seizures could regulate actin dynamics, in particular by decreasing F-actin and p-cofilin levels, over a relatively short-time period and in the absence of major structural changes in dendrites (i.e. change in spine number or density). Previous clinical and animal studies using conventional histological techniques indicate that epilepsy is often associated with moderate (often reversible) dendritic spine loss (Muller et al., 1993; Isokawa, 1998; Jiang et al., 1998; Swann et al., 2000) or more severe “excitotoxic” injury to dendrites/neurons (Collins and Olney; 1982; Sloviter, 1983; Olney et al., 1983), whereas other studies have reported an increase in spines (Bundman et al., 1994; Spiegelman et al., 1998) or minimal acute effects on spines (Mizrahi et al., 2004; Rensing et al., 2005). This reported variability in the effects of seizures on dendrites likely relates to a number of factors, such as the specific seizure model, time course, experimental approach, and concurrent modulatory conditions. In the present study, the lack of obvious structural changes in dendrites with seizures over a short time is consistent with previous studies using a similar 4-AP model (Yang and Rothman, 2001) and with other recent studies utilizing modern time-lapse imaging methods to directly assess spine changes during seizures in living tissue in vivo (Mizrahi et al., 2004; Rensing et al., 2005). Besides variability between different seizure models, this lack of acute seizure-induced effects on dendritic morphology in the present study may be attributed to a number of reasons and subject to some caveats. First, although seizure-induced neuronal death has been reported in a number of seizure models, mice with a C57/BL6 genetic background, as used in the present studies, are relatively resistant to seizure-induced excitotoxic injury (Schaunecker and Steward, 1997). Second, as the present study focused on immediate changes (within an hour) in actin dynamics analogous to the effects of LTP, it is still possible that these acute seizure-induced effects on actin could be associated with dendritic spine loss that may develop over a longer time period. Alternatively, rather than gross changes in spine number, this modulation of actin dynamics could cause more subtle changes in existing spine morphology, which could not be adequately assessed with the experimental design and imaging methods of this study. Finally, it is possible that the seizures in our models were not severe or wide-spread enough to activate overt structural damage. For example, although the in vivo 4-AP seizures were very robust clinically and electrographically, the epileptiform activity in slices usually just consisted of frequent spikes, but not discrete seizures. On the other hand, the fact that both the in vivo and in vitro models showed significant effects on actin/cofilin indicates that the seizure stimuli were sufficient to activate dendritic changes and reinforces the validity of these changes. Thus, despite these caveats, it appears that the potentially detrimental effects of seizures on F-actin and p-cofilin in the present study may not involve overt structural changes, but may relate more to the non-structural roles of actin in spines, such as biochemical compartmentalization and signaling, protein translocation, and receptor trafficking (Naylor et al., 2005).

In contrast to 4-AP seizures, elevated potassium induced a larger effect on cofilin and corresponding gross changes in dendritic morphology. As increased potassium concentration at this level (50 mM) leads to depolarization block and spreading depression, these biochemical and morphological changes are probably not related to activity/seizure-dependent mechanisms, but might relate more to osmotic effects. Thus, it is difficult to directly compare the 4-AP and high potassium results quantitatively, but the high potassium studies serve as a positive control, demonstrating that severe perturbations in actin dynamics can relate directly to overt structural abnormalities in dendrites and suggesting that actin may be a “final common pathway” for modulation of dendritic structure or function by multiple physiological or pathological mechanisms. In addition, these findings may have direct relevance for the interpretation of other studies that commonly utilize similarly elevated potassium concentrations to induce “neuronal activation” experimentally, as well as for pathophysiological states where potassium may locally or transiently achieve such concentrations.

Overall, we propose that pathological activity-dependent changes in the actin cytoskeleton represent mechanisms that may be relevant to both structural and non-structural abnormalities in dendritic function. We hypothesize that the degree to which overt structural changes occur is dependent on the severity and type of the pathophysiological stimulus. Future studies are required to better define the functional and clinical relevance of the seizure-induced changes in actin polymerization. More detailed information about the specific regulatory mechanisms involved may ultimately lead to novel therapeutic strategies either for treating seizures or preventing neurocognitive deficits in epilepsy. While most drugs for epilepsy have targeted neurotransmitter receptors and ion channels, an innovative therapeutic approach would be to modulate actin-based spine motility in an activity-dependent fashion. It is conceivable that selective stabilization of the dendritic actin cytoskeleton during seizures by drugs that regulate actin polymerization (Ackermann and Matus, 2003) could help prevent seizure-induced spine changes and potentially reduce resultant neurocognitive deficits. Thus, better insights into the mechanisms of modulation of actin-based spine dynamics by seizures could have significant impact in reducing the long-term negative consequences of epilepsy.

4. Experimental procedures

4.1 Animals and reagents

Two to three month old male C57/BL6 wild-type mice (Charles River) and transgenic mice (with a C57/BL6 background) expressing green-fluorescent protein (GFP) in subpopulations of hippocampal neurons (line GFP-M, Feng et al. 2001) were used in this study. Wild-type C57/BL6 mice were used in experiments for rhodamine-phalloidin labeling and Western blot analysis of actin and cofilin. GFP-M transgenic mice were utilized in imaging experiments involving analysis of dendritic spine density. Care and use of animals conformed to a protocol approved by the Washington University School of Medicine Animal Studies Committee.

Anti-cofilin antibody and anti-phosphocofilin (Ser3) antibody were obtained from Cytoskeleton (Denver, CO) and Cell Signaling (Beverly, MA), respectively. Rhodamine-phalloidin and 3 kD dextran conjugated with tetramethylrhodamine were obtained from Molecular Probes (Eugene, Oregon). Anti-MAP2 and anti-PSD95 antibodies, 4-AP, and all other reagents were purchased from Sigma (St. Louis, MO).

4.2 In vivo 4-aminopyridine induced seizures and video-EEG recording

Mice were anesthetized with halothane (2%) and placed on a stereotaxic frame (David Kopf Instruments, Tujunga, CA). A burr hole was drilled over the right hemisphere at a site 2.5 mm posterior to bregma and 2 mm lateral from midline using a dental drill. 4-AP (0.25 to 0.5 μ l of 25 mM 4-AP in ACSF, Sigma; both volumes elicited similar stage seizures) was injected stereotactically into hippocampus 2 mm ventral to the cortical surface through a glass micropipette connected to an oocyte injection system (Drummond Scientific). Injections occurred over 5 minutes to minimize physical damage. Control mice were injected with ACSF in a similar manner. As the micropipette usually produced minimal physical damage, to confirm the injection sites, 4-AP or ACSF was mixed with 3 kD dextran conjugated with tetramethylrhodamine in some experiments. After surgery, the mice were allowed to recover from anesthesia and transferred to cages to monitor for seizures by video-EEG recording.

Behavioral rating of clinical seizure activity was performed using a modified Racine scale (Racine, 1972). For EEG recording, two screw electrodes were placed over each hemisphere to differentially record EEG compared to a midline reference screw electrode, using standard AC EEG amplifiers (Astro-Med, West Warwick, RI). The EEG was filtered (high frequency 100 Hz), digitized (200 Hz) and stored using PC-based commercial hardware and software

(Digidata and Axoscope, Molecular Devices, Sunnyvale, CA). Mouse behavior was recorded simultaneously with a PC-based video camera system (Sanyo camera coupled to an MPEG1 encoder from Darim Vision, Ltd). Mice were monitored for behavioral and electrographic seizures for one hour before they were sacrificed.

One hour after seizures were induced with 4-AP injection, the mice were anesthetized with halothane and sacrificed. For Western blot analysis, the brains were removed and the hippocampi were dissected out and homogenized individually. For F-actin labeling or dendritic imaging, other mice were perfused with ACSF and fixed with 4% paraformaldehyde and 0.5% glutaraldehyde in phosphate buffered saline (PBS, pH 7.0). Brains were then removed and post-fixed overnight in 4% paraformaldehyde. Coronal brain sections (50 μ m) between 200 μ m anterior and posterior to the injection site were cut with a vibratome and mounted on slides for F-actin labeling or dendritic imaging.

4.3 In vitro epileptiform activity in hippocampal slices

For in vitro slice experiments, hippocampal slices (500 μ m) were prepared with a tissue chopper or vibratome, as described previously (Ouyang et al., 1997;2005). Slices were placed on filter paper with ACSF in an oxygenated chamber for at least one hour. Slices were then exposed to control ACSF or drugs (100 μ M 4-AP or 50 mM KCl) in ACSF for one hour. In most experiments, extracellular field potentials were recorded with a glass electrode in the CA1 region and Axoclamp-2A amplifier in bridge mode and digitized and saved at a sampling frequency of 1 kHz by an Axon Digidata and Axoscope software. After one hour of drug or control exposure, slices were either homogenized for Western blotting or fixed in 4% paraformaldehyde and 0.5% glutaraldehyde in PBS for one hour and then transferred and stored overnight in PBS. The fixed 500 μ m slices were then cut into 50 μ m sections and mounted on slides for F-actin labeling.

In other experiments for live multiphoton imaging of dendritic spines, coronal hippocampal slices (500 μ m) from GFP-M transgenic mice were cut with a vibratome and placed in a submerged chamber on the platform of the multiphoton microscope. Slices were superfused with oxygenized ACSF, which could then be replaced with 100 μ M 4-AP in ACSF to induce epileptiform activity, while recording extracellular field potentials in the CA1 region as described above.

4.4 Imaging of dendritic spines in vivo and in vitro

For the in vivo seizure experiments, fixed 50 μ m sections from control (ACSF-injected) and seizure (4-AP-injected) GFP-M mice were used for analysis of dendritic spine density by confocal microscopy. GFP-positive secondary dendrites from pyramidal neurons of the CA1 region of hippocampus were selected in a blinded fashion at low magnification (10x). At high magnification (40x, 0.9 NA) with 3x digital zoom, Z-stacks (1 μ m intervals) of individual dendrites with spines were imaged and saved with a Zeiss LSM PASCAL confocal microscope. Spine density (# of spines/dendrite length) was calculated from each dendrite in a blinded fashion and average spine density was compared between the control and seizure groups.

For the in vitro slice experiments, the effect of 4-AP-induced epileptiform activity on dendritic spine number was assessed directly in living slices from GFP-M mice with time-lapse multiphoton imaging. First under control conditions, Z-stacks (1 μ m intervals) of GFP-positive secondary dendrites and spines were imaged at high magnification (40x, 0.9 NA, 3-5x digital zoom) with a Zeiss NLO Meta multiphoton microscope. After application of 4-AP to induce epileptiform activity, the same dendrites and spines were followed over 60 minutes to monitor for changes in total spine number and a loss or gain of individual spines, similar to techniques

we have used with time-lapse imaging of spines during electrographic seizures in vivo (Rensing et al., 2005).

4.5 F-actin labeling

The rhodamine-phalloidin labeling method was used as described previously (Ouyang et al., 2005). Phalloidin labeling has a high affinity for F-actin and is selectively concentrated in dendritic spines of neurons (Capani et al. 2001). Briefly, fixed sections were treated with 0.7% Triton X-100 in 10 mM PBS (pH 7.2) for one hour. Sections were then incubated with rhodamine-phalloidin (Molecular Probes) for one hour. Sections were washed three times and mounted with anti-fade medium for confocal imaging. Images of the stratum radiatum of CA1 regions of hippocampus were acquired with a Zeiss LSM PASCAL confocal microscope, using consistent microscope parameters by a person blinded to the identity of the sections. A high power objective (63x, 1.2 NA) was used to confirm the punctuate labeling typical of spines (Ouyang et al., 2005) and a low power objective (25x, 0.8 NA) was used to obtain images for regional F-actin intensity measurements. Regions of interest from images were selected within the stratum radiatum of CA1 to measure the average brightness of F-actin labeling using MetaMorph analysis software. Double-labeling experiments involving immunolabeling of MAP2 and PSD95 with F-actin staining were performed to confirm the primary dendritic localization of F-actin in these studies.

4.6 Western blotting

Dissected hippocampi from the in vivo experiments or hippocampal slices from the in vitro experiments were homogenized in SDS-PAGE sample buffer containing 3% SDS, 2% β -mercaptoethanol, and 5% glycerol in 60 M Tris buffer, pH 6.7 as described previously (Ouyang et al., 2005). Samples were boiled for 5 min and stored at -20°C . Protein concentration was determined with the Lowry method. Equal amounts of protein were loaded in triplicate on gels for SDS-PAGE. Electrophoresed proteins were transferred to polyvinylidene difluoride membranes. After incubation with a primary antibody (1:1000) that recognized phosphorylated cofilin (p-cofilin) at Ser3, total cofilin, and actin, the membranes were labeled with secondary antibody conjugated to alkaline phosphatase for DAB reaction and scanned for quantitative analysis with MetaMorph.

4.7 Statistics

In separate experiments, two-sided Student's t-test was used for parametric comparisons of mean spine density, F-actin intensity, and quantified protein expression between control and 4-AP-treated groups. All data are expressed as mean $\pm$ standard error of the mean.

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Fig 1.

4-AP induces hippocampal epileptiform activity in vivo and in vitro. (A) Seizures were induced by stereotaxic injection of 4-AP into hippocampus in vivo. This fixed section of hippocampus from a GFP-M mouse shows a representative 4-AP injection site, as marked in red by dextran conjugated with tetramethylrhodamine, which in this experiment was included in the injection solution along with 4-AP. The typical distribution of GFP-positive neurons in hippocampus of GFP-M mice is shown in green. Scale bar = 200 μ m. (B) EEG recordings demonstrated in vivo electrographic seizures resulting from hippocampal 4-AP injection. Despite unilateral (right-sided) 4-AP injection, seizures quickly progressed into repetitive bilateral cortical discharges within minutes. (C) 4-AP (100 μ M) also induced repetitive epileptiform spike discharges in the CA1 region of hippocampal slices.

Fig 2.

Hippocampal seizures/epileptiform activity causes depolymerization of F-actin. (A) Representative images of F-actin labeling, assayed by the rhodamine-phalloidin method, are shown in the stratum radiatum of CA1 region of a control (ACSF-injected) mouse and a mouse following one hour of in vivo 4-AP-induced seizure activity. The same imaging parameters were used for all experiments in a blinded fashion. The punctate labeling in the control image is typical of dendritic spine localization of F-actin. The blood vessel in the lower left corner of the 4-AP image serves as a positive control for F-actin staining. Scale bar = 5 μ m. (B) Summarized data for all in vivo experiments show relative F-actin labeling following 4-AP seizures normalized to the ACSF-injected control group. The relative intensity of F-actin labeling was significantly decreased in the stratum radiatum of the hippocampal CA1 region of mice following 4-AP seizures. * $p < 0.05$ by t-test ($n = 9$ sections from 3 mice each in 4-AP and control groups). (C) 4-AP-induced epileptiform activity also causes depolymerization of F-actin in hippocampal slices. The relative intensity of F-actin labeling was significantly decreased in the stratum radiatum of CA1 region of hippocampal slices exposed to 4-AP compared to control slices. * $p < 0.02$ by t-test ($n = 9$ sections from 3 mice each in 4-AP and control groups).

Fig 3.

Hippocampal seizures/epileptiform activity increase cofilin activation (A) Phosphorylated cofilin (p-cofilin), total cofilin, and actin were assayed by Western blotting of hippocampi from control (ACSF-injected) mice and mice following one hour of in vivo 4-AP- induced seizure activity. A representative Western blot from two sets of mice is shown (top). Summarized data for all experiments show the relative protein expression of actin, total cofilin, and p-cofilin in animals with 4-AP seizures normalized to the ACSF-injected control group for each protein (bottom). The expression of p-cofilin was significantly decreased in the hippocampus of mice following 4-AP seizures. In contrast, no changes were detected in total actin and cofilin, implying a corresponding increase in unphosphorylated (activated) cofilin. * $p < 0.05$ by t-test ($n = 4$ mice in each group). (B) Phosphorylated cofilin (p-cofilin), total cofilin, and actin were assayed by Western blotting of hippocampal slices incubated in 4-AP or ACSF. A representative Western blot from one experiment with different sets of slices is shown (top). Summarized data for all experiments show the relative protein expression of actin, total cofilin, and p-cofilin in hippocampal slices treated with 4-AP normalized for each protein to the control slices exposed only to ACSF (bottom). The expression of p-cofilin was significantly decreased in the hippocampal slices treated with 4-AP. In contrast, no changes were detected in total actin and cofilin, implying a corresponding increase in unphosphorylated (activated) cofilin.

* $p < 0.05$ by t-test ($n = 8$ slices from 3 mice in each group). A – ACSF, 4 – 4-AP.

Fig 4.

Hippocampal seizures/epileptiform activity have no effect on dendritic spine density over a one hour period. (A) Representative images of GFP-positive dendrites and dendritic spines of CA1 neurons in fixed sections of hippocampus of mice following 4-AP-induced seizures or ACSF injection in vivo are shown. There was no significant effect of 4-AP seizures on dendritic spine density over a one hour period (See text for quantification of all experiments). Scale bar = 10 μ m. (B) Time-lapse multiple-photon imaging was performed on GFP-positive dendrites of CA1 neurons in living hippocampal slices from GFP-M mice before and during 4-AP exposure. Representative images from a dendrite revealed no significant effect of 4-AP epileptiform activity on dendritic spines over a one hour period (See text for quantification of all experiments). Scale bar = 5 μ m.

Fig 5.

Elevated extracellular potassium causes loss of dendritic spines and increased cofilin activation. (A) Time-lapse multiple-photon imaging was performed on GFP-positive dendrites of CA1 neurons in live hippocampal slices from GFP-M mice before and during superfusion of 50 mM KCl. Within minutes of application of 50 mM KCl, dendrites exhibited a swelling of processes and loss of spines. Scale bar = 10 μ m. (B) Phosphorylated cofilin (p-cofilin), total cofilin, and actin were assayed by Western blotting of hippocampal slices incubated in 50 mM KCl or ACSF. A representative Western blot from one experiment is shown. (C) Summarized data for all experiments show the relative protein expression of actin, total cofilin, and p-cofilin in hippocampal slices treated with 50 mM KCl normalized for each protein to the control slices exposed only to ACSF. The expression of p-cofilin was significantly decreased in the hippocampal slices treated with 50 mM KCl. In contrast, no changes were detected in total actin and cofilin, implying a corresponding increase in unphosphorylated (activated) cofilin. * $p < 0.05$ by t-test ($n = 16$ slices from 4 mice in each group). A – ACSF, K – 50 mM KCl.