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A Cellular Mechanism for Dendritic Spine Loss Following Traumautic Brain Injury in Rat

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A CELLULAR MECHANISM FOR DENDRITIC SPINE LOSS FOLLOWING

TRAUMATIC BRAIN INJURY IN RAT

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

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Traumatic brain injury is a leading cause of death and disability in the United States. The injury is often composed of two processes: the primary injury, which can involve irreversible loss of tissue, and the secondary injury, which involves a cascade of reactive processes such as excitotoxicity that occur in the hours and days after the initial insult. Excitotoxic stimulation of neuronal circuits can lead to cellular dysfunction and modulation of neuronal sensitivity. One mechanism of dysfunction involves the calcium-regulated phosphatase, calcineurin. Calcineurin has been shown to be involved in the

modulation of the neuronal post-synaptic structures known as dendritic spines. One means by which CaN regulates spine structure is through the dephosphorylation of the down-stream effector proteins such as, cofilin. This study tracks the changes in CaN activity levels as well as the phosphorylation state of cofilin in the cortex and hippocampus in each hemisphere of the laterally injured brain. We report that the lateral brain injury causes an increase in CaN activity in the hippocampus with a corresponding dephosphorylation of cofilin. Trauma-induced changes in CaN follow a slightly different time course in cortical tissue, as there is a biphasic modulation of cofilin that begins with an increased phosphorylation which is followed by an extended dephosphorylation. This dephosphorylation is partially prevented by a single post-injury injection of FK506, a calcineurin inhibitor. Since dephosphorylation of cofilin is a rate-limiting step in dendritic spine collapse, the results of this study demonstrate a potential cellular mechanism through which traumatic brain injury results in altered neuronal function.

INTRODUCTION

Traumatic Brain Injury

Traumatic brain injury (TBI) remains a major cause of death and disability in the United States and it is associated with a high rate of morbidity. It affects people of all ages, but occurrence peaks with children under the age of 5, between 15-24 years, and over 70 years (Kraus and McArthur, 1999). The 15-24 year age group is of particular concern as these individuals often lose their ability to fully contribute to society and can often become a financial burden. The injury can be caused by a variety of factors including blunt trauma, penetrating injury, and concussive force. Trauma can be complicated by skull fracture, intercranial hemorrhage, and contrecoup effects. However, the primary injury is not the only pathology associated with trauma; many patients suffer from a variety of secondary effects that manifest in the days, months, and years post-trauma. The secondary effects can be as mild as headache, dizziness, and nausea or much more severe, such as the development of epilepsy.

The causes of secondary effects have been difficult to elucidate and therefore, challenging to treat. While some pharmacological treatments have shown promise, treatment that is efficacious at all stages and in all injuries has proven to be elusive. In order to shed light

on this matter, the focus of research should be on identifying key pathways that yield secondary injury. By determining cellular and molecular mechanisms, we can begin identifying treatments that are effective in preventing the development of secondary pathologies.

Epilepsy

A seizure is caused by synchronous electrical activity in the brain, which may last anywhere from a few seconds to a few minutes. A person having at least two seizures that were not acutely caused by a medical condition is diagnosed with epilepsy. Epilepsy is one of the most common neurological disorders in the United States and it affects 0.5-2% of people worldwide (Pitkänen et al., 2006). While it is often controllable with chronic treatment, it has proven to be incurable. Still, even with effective therapy options, with over 40 varieties of epilepsy, some are non-responsive to treatment and completely uncontrolled.

Epilepsy is a disorder that can affect people of any age, but it is more likely to occur in young children or in people over the age of 65. On a yearly basis, epilepsy and seizures affect nearly 3 million Americans with approximately 200,000 new cases diagnosed annually. In addition to the social stigma attached to epilepsy, there is a high rate of morbidity, many people suffer from depression, anxiety, migraines, infertility, or reduced sexual libido. Furthermore, patients with epilepsy have a two to three times greater chance of mortality than the general population.

While the etiology of most epilepsy is unknown, one well-established cause is traumatic brain injury. However, epileptic seizures usually do not begin immediately after injury; rather they occur in the months and years after the initial trauma. This latency period, known as epileptogenesis, is common, but poorly understood. Therefore, research is needed to elucidate cellular markers of trauma-induced altered neuronal physiology.

Calcineurin and Cofilin

Two particular markers of interest are calcineurin (CaN) and its down-stream mediator, cofilin. CaN is a neuronally enriched calcium-regulated phosphatase that is implicated in learning and memory (Mulkey et al., 1994; Lu et al., 2000) and is localized to the post-synaptic structure (Kurz et al., 2005a). Cofilin is a downstream mediator of CaN activity and is also found within dendritic spines (Racz and Weinberg, 2006). In its dephosphorylated form, cofilin acts by binding the actin cytoskeleton and causing a structural breakdown by generating torsional forces (Bobkov et al., 2006). This results in a loss of the spiny formation and decreased interneuronal communication. CaN also regulates cellular processes such as apoptosis (Ankarcrona et al., 1996; Asai et al., 1999), gene transcription (Enslen and Soderling, 1994; Genazzani et al., 1999), and in neurons, membrane potential (Tong and Jahr, 1994). Due to its intricate involvement in so many processes, dysregulation of CaN activity is a potential mechanism for development and maintenance of pathology.

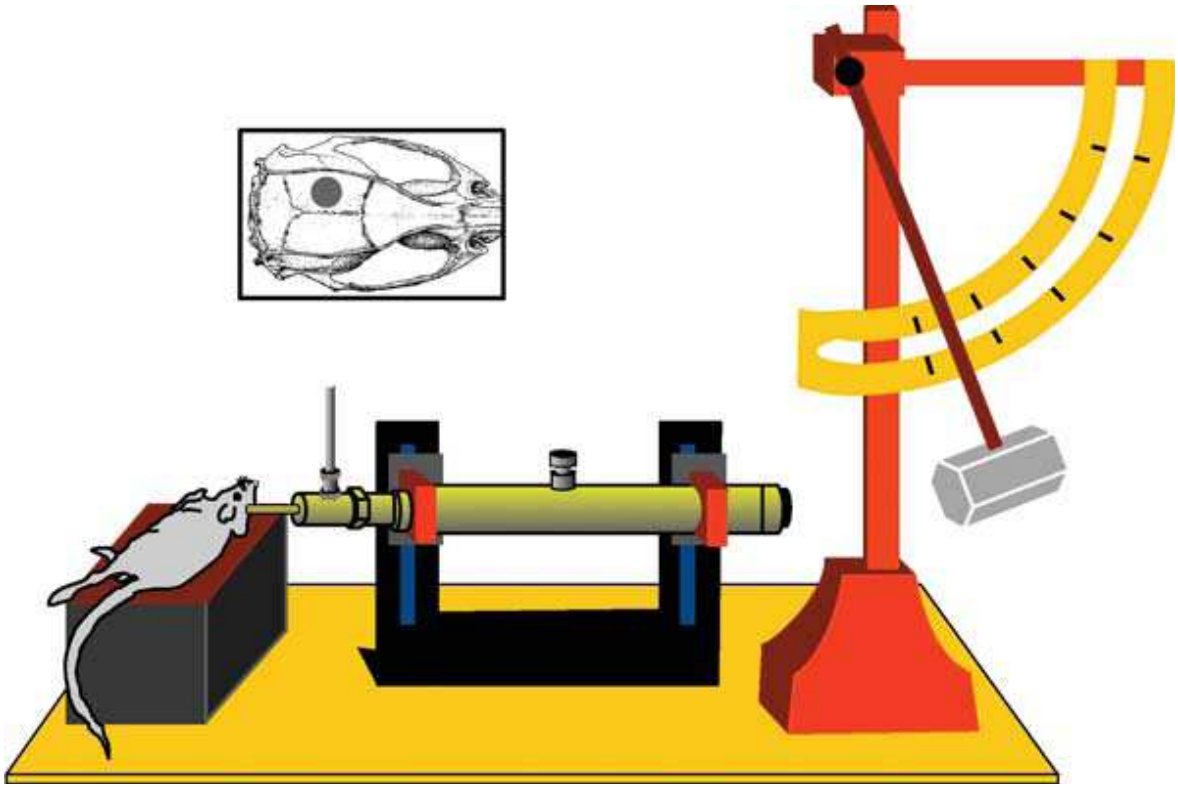
The Lateral Fluid Percussion Injury in Rat

There are a variety of experimental models that are used to model traumatic brain injury. As the brain is a complicated organ, it responds differently to assorted types of trauma. In the following study, rats were subjected to a lateral fluid percussion injury (LFPI) by first doing a craniectomy and attaching a hub to the skull in a lateral fashion. The rats were attached to the injury apparatus and a pressure wave was generated by a swinging metal pendulum striking the end of the injury device, injecting a small volume of isotonic saline into the surgically implanted hub (Figure 1). The saline caused a brief displacement of brain tissue and the time was noted as the primary injury. The hub was removed from the skull and the rats were allowed to recover for pre-determined lengths of time.

This study was conducted in order to characterize the cellular and molecular mechanisms that occur following LFPI. After injury, rats were decapitated at specific time points and post-mortem analysis was conducted. In previous studies, CaN and cofilin have been implicated in the loss of dendritic spines (Kurz et al., 2005a; Kurz et al., 2005b; Kurz et al., 2008). For this reason, biochemical assays were performed in order to examine the activity levels of CaN and the phosphorylation state of cofilin post-TBI. The data suggests that there is an increase in the activity level of CaN and a corresponding decrease in phosphorylated cofilin. Additionally, these changes are partially blocked by a single post-injury injection of the CaN inhibitor, FK506. The following chapters represent a paper that will be submitted for publication. The paper, A Cellular Mechanism for Dendritic Spine Loss Following Traumatic Brain Injury in Rat, will be submitted to the **Journal of**

Neurotrauma. Accordingly, the paper is formatted to conform with the guidelines set forth by the journal.

Figure 1. The lateral fluid percussion injury device. Representative placement and site of originally described lateral (parasaggital) fluid percussion brain injury (inset) and schematic of a fluid percussion brain injury device. A pendulum from a known height impacts the piston of a saline-filled reservoir, forcing a brief fluid bolus into the sealed cranial cavity (Thompson et al., 2005).



A CELLULAR MECHANISM FOR DENDRITIC SPINE LOSS FOLLOWING TRAUMATIC BRAIN INJURY IN RAT

Introduction

Traumatic brain injury (TBI) is a major cause of death and disability in the United States (Ghajar, 2000). The initial injury often involves an early, irreversible destruction of tissue; however, secondary, potentially preventable injury processes take place in the days and weeks following the initial insult. This secondary damage causes pathological changes in cells over a much greater area and time than the primary injury and is often responsible for many of the long lasting neurological deficits associated with TBI (Siesjo and Siesjo, 1996; Ghajar, 2000). The delayed nature of the secondary injury offers a potential window for treatment, making it an exciting area for both basic research and clinical trials. However, to develop treatment protocols, it is necessary to elucidate the cellular mechanisms through which TBI induces altered brain function.

Calcium-regulated systems have been implicated in the spread of neuronal damage following ischemia and TBI (Tymianski and Tator, 1996; Deshpande et al., 2008; Sun et al., 2008). One of the calcium-mediated systems of note involves the neuronally enriched calcium-regulated phosphatase, calcineurin (CaN) (Pallen and Wang, 1985). Among its

many actions, CaN plays a role in neuronal regulation of nitric oxide synthase (Dawson et al., 1993), glial apoptosis (Szydlowska et al., 2006), inflammatory processes (Norris et al., 2005), and expression of cytokines (Fernandez et al., 2007). In neurons, CaN has been shown to modulate neuronal excitability and alter interneuronal communication – e.g., (Lu et al., 2000; Groth et al., 2003; Park et al., 2006). For instance, within dendritic spines, changes in CaN activity have been shown to result in changes in dendritic spine morphology ((Kurz et al., 2008); Campbell, Kurz and Churn, 2009, *in press*) and decreased sensitivity to neurotransmitters by dephosphorylation of neurotransmitter receptors (Yuen et al., 2008). Since CaN is involved in so many important neuronal processes, changes in CaN activity are likely involved in the development of neuronal pathologies. Indeed, modulating CaN activity has been identified in pathologies such as status epilepticus (SE) (Kurz et al., 2008), stroke (Nagahiro et al., 1998), and TBI (Kurz et al., 2005a; Kurz et al., 2005b).

Changes in CaN activity and distribution have been implicated as a mechanism for dendritic spine loss in rodent models of SE through the modification of the actin binding protein, cofilin (Kurz et al., 2008). SE induces a post-translational modification of CaN that yields Ca^{2+}-independent activity in addition to intracellular translocation of CaN (Kurz et al., 2003). The activity change coupled with translocation may be a key component in the dendritic spine loss seen after SE (Kurz et al., 2008). Similarly, an increase in Ca^{2+}-independent CaN activity and an increase in the concentration of CaN in the post-synaptic

density of dendritic spines was observed with the central fluid percussion injury (Kurz et al., 2005a; Kurz et al., 2005b).

The present study investigated the temporal changes in CaN activity in rat brain homogenate from the hippocampus and cortex both ipsilateral and contralateral to a lateral fluid percussion injury. CaN activity was measured both enzymatically and by quantifying the phosphorylation state of down-stream substrates. The data indicate there was a transient increase in CaN activity as well as a long lasting dephosphorylation of the downstream effector, cofilin. The studies characterize the effect of LFPI on an important calcium-dependent signal transduction pathway. Together, with our companion paper (Campbell et al., 2009); the data demonstrate a cellular pathway through which LFPI results in altered spine density.

Materials and Methods

Surgical preparation and fluid percussion injury

All animal use procedures were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals described by the National Institutes of Health and were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee. Animal subjects received ad libitum access to food and water and were maintained on 12-hour light/dark cycles throughout the experiment. The fluid percussion injury was performed as previously described (Kurz et al., 2005a) with exceptions as described below.

Adult male Sprague-Dawley rats were anesthetized with sodium pentobarbital (54 mg/kg intraperitoneally) and placed into a stereotactic frame. The scalp was sagittally incised and a 4.8-mm hole was made with a trephine centered 4 mm caudal to bregma and - 3 mm lateral to the sagittal suture, approximately over the parietal cortex. A screw was placed over the opposite hemisphere to anchor the skull attachments. A modified female Luer-Loc syringe hub (2.6 mm inside diameter) was placed over the exposed dura and affixed with a cyanoacrylate adhesive. Dental acrylic was then applied around the hub and screws. The scalp was sutured, a topical antibiotic ointment was applied to the wound, and the animal was allowed to recover on a heating pad prior to being returned to its home cage. Within 24 hours following the surgery, the rats were anesthetized (4% isoflurane in a carrier gas mixture of 30% N_2O and 70% O_2) and subjected to a lateral FPI of moderate severity (2.2 ± 0.2 atm). Immediately after injury, the Luer-Loc fitting, screws, and dental

cement were then removed from the skull. The rat's scalp was then sutured closed and a topical antibiotic was applied to the sutured area. The times at which somatosensory and righting reflexes returned after FPI were recorded. Following recovery, rats were returned to their home cages and allowed to survive for 1 hr, 6 hrs, 12 hrs, 18 hrs, 24 hrs, 1 wk, 2 wks, or 4 wks.

Fluid percussion injury device

The FPI device used in these experiments is identical to that described by Dixon and coworkers (Dixon et al., 1987). Briefly, the device consisted of a Plexiglas cylinder reservoir 60 cm long and 4.5 cm in diameter. At one end of the cylinder was a rubber-covered Plexiglas piston mounted on O-rings. The opposite end of the cylinder was connected to a metal housing 2 cm long, containing a transducer. A 5-mm-long tube with a 2-mm inner diameter was fitted at the end of the metal housing. The tube terminated with a male Luer-Loc fitting. This fitting was connected to the female Luer-loc fitting that had been implanted over the exposed dura of the rat. The entire system was filled with sterile isotonic saline and a metal pendulum that struck the piston of the injury device produced the injury. The device injected a small volume of saline into the closed cranial cavity and produced a brief displacement of brain tissue. Varying the height from which the pendulum was released controlled the magnitude of the injury. The resulting pressure pulse was measured externally by a pressure transducer (model EPN-0300A*-100A, Entran Devices, Fairfield, NJ) and recorded on a storage oscilloscope (model 5111,

Tektronix, Beaverton, OR). Where stated, FK506 (5mg/kg; Fujisawa Chemical Company, Osaka, Japan) was administered intraperitoneally 1 hour after FPI.

Isolation and homogenization of brain regions

Rats were anesthetized using isoflurane and euthanized via decapitation 1 hr, 6 hrs, 12 hrs, 18 hrs, 24 hrs, 1 wk, 2 wks, or 4 wks after injury. Brains were rapidly dissected on a Petri dish on ice to reduce postmortem alteration of enzyme activity as described previously in detail (Kurz et al., 2005a). Ipsilateral and contralateral neocortical 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 in ice-cold homogenization buffer containing 7 mM EGTA, 5 mM EDTA, 1 mM dithiothreitol, 0.3 mM phenylmethylsulfonylfluoride, and 300 mM sucrose. Neocortical regions were homogenized in 4 mL of buffer, hippocampal regions in 2 mL, and cerebellar regions in 5 mL. The homogenate was aliquoted and stored at -80°C until use.

pNPP assay of CaN activity

CaN activity was quantified using the procedure of Pallen and Wang (1983) as described in detail by Kurz and coworkers (Kurz et al., 2001; Kurz et al., 2005b). Briefly, all reaction tubes were prepared on ice and contained the following: 25 mM MOPS (pH 7.0), 1 mM DTT, 2 mM p-nitrophenyl phosphate (pNPP) (Sigma-Aldrich). Some tubes used to measure basal CaN activity also contained 2 mM EGTA and 2 mM EDTA. Tubes used to

measure maximal CaN activity contained the same reagents as basal reactions, with the addition of 2 mM $MnCl_2$. Manganese activates CaN much more strongly than calcium in the pNPP assay and is therefore used to better visualize the effects of TBI on maximal CaN activity (Kurz et al., 2005b). Final reaction volumes were 1 mL. Prior to use, the protein concentrations of all homogenate samples were determined using the method of Bradford (Bradford, 1976). Reactions were initiated by the addition of 50 mg/mL brain homogenate. Reactions were incubated at 37°C for 30 minutes in a shaking water bath. Tubes were then removed from the water bath and placed in ice to stop the reaction. Absorbance of the reaction mixture was immediately measured at 405 nm in a spectrophotometer (UV-2101, Shimadzu Scientific Instruments, Inc., Columbia, MD). Absorbance units were converted to nmol of pNP produced by comparison to a pNP concentration standard absorption curve.

Western analysis

Brain samples were normalized using the Bradford method, resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Mini-Protean II system, BioRad, Hercules, CA), and transferred to a nitrocellulose membrane using the Trans-blot system (BioRad, Hercules, CA) with the plates in the high-intensity field configuration. Nitrocellulose was then twice immersed for 15 minutes 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 was then incubated with the appropriate antibody in blocking solution over night. Anti-

cofilin (Chemicon International, #3842, Temecula, CA, U.S.A.) and anti-phosphocofilin (Chemicon International, #3831) were diluted 1:500 for Western blot analysis. Membranes were then washed three times for 10 minutes each wash in PBS and Tween 20. Next, the nitrocellulose was reacted with horseradish peroxidase-conjugated secondary antibody in blocking solution for 45 minutes. Then the blots were washed three times in PBS and Tween 20 for 10 minutes each wash. Lastly, the blots were reacted with a luminol reagent for 5 minutes (Pierce Femto-sensitive, Pierce, Rockford, IL). Blots were immediately exposed to x-ray film (Kodak X-OMAT) and developed using a Kodak X-OMAT developer. Specific immunoreactive bands were quantified by computer-assisted densitometry (GS-800 Calibrated Densitometer, BioRad) and compared to a linear concentration curve as described previously (Churn et al., 1992).

Statistical analysis

All statistical analysis was performed using GraphPad Prism 4.0 (Graph-Pad Software, San Diego, CA). For single group comparisons, Student's t-test was used and for multiple group comparisons, ANOVA with Tukey post hoc analysis was used with a minimum significance level of $p < 0.05$. All data are presented as mean ± SEM unless otherwise noted.

Results

A total of 44 rats were injured used to complete the time points studied. Injury severity was determined both by pressure pulse and righting reflex. Amongst the 44 injured animals, the average pressure pulse was 2.226 ± 0.013 atm and the average righting time was 384 ± 12 seconds, which correspond to a moderate injury (Morehead et al., 1994; Schmidt and Grady, 1995; Carbonell et al., 1998; Hallam et al., 2004). All data is reported as a percentage of control and phosphocofilin data has been normalized to total cofilin levels to control for experimental error.

Effects of LFPI on CaN activity

In order to determine the effects of LFPI on calcineurin activity, we utilized the well characterized pNPP assay (Kurz et al., 2001; Kurz et al., 2003; Kurz et al., 2005b; Kurz et al., 2008) in whole cell homogenates from both control and injured tissue isolated at specific time points post-LFPI (see Materials and Methods). In hippocampal fractions, a delayed increase in basal CaN activity was observed following TBI (Figure 2). In the hippocampus located ipsilateral to the injury (Figure 2A), basal CaN activity levels were unchanged up to 1 hour post-injury (96 ± 8% of control, $p > 0.05$, ANOVA). However, by 6 hours post-injury, CaN activity was significantly increased (141 ± 13% of control, $p < 0.05$, ANOVA). Activity remained significantly elevated at 12 hours post-TBI (134 ± 8% of control, $p < 0.05$, ANOVA) and at 18 hours (154 ± 16% of control, $p < 0.01$, ANOVA). After 18 hours post-TBI, CaN activity decreased and returned to control levels by 24 hours (105 ± 7% of control, $p > 0.05$, ANOVA). At 1 week post-injury, CaN was slightly

elevated (133 ± 14% of control, $p < 0.05$, ANOVA), but did not differ significantly from control at 2 or 4 weeks (110 ± 7%; 85 ± 4% of control respectively, $p > 0.05$ for both, ANOVA).

LFPI resulted in a significant change in CaN activity in the contralateral hippocampus as well (Figure 2B). As observed in the ipsilateral hippocampus, basal CaN activity in the contralateral hippocampus was not significantly different from control levels at 1 hour post-LFPI (110 ± 7% of control, $p > 0.05$, ANOVA). However, TBI resulted in a near two-fold increase in activity by 6 hours (170 ± 9% of control, $p < 0.01$, ANOVA) and activity levels remained elevated at 12 hours (157 ± 13% of control, $p < 0.01$, ANOVA). At 18 hours post-TBI, activity levels were elevated, but not significantly (115 ± 12% of control, $p > 0.05$, ANOVA). The downward trend in activity continued through 24 hours when CaN was slightly decreased compared to control levels (82 ± 6% of control, $p < 0.05$, ANOVA). Activity levels slowly recovered to control levels, but did not significantly differ from control at 1 week, 2 weeks, or 4 weeks post-injury (77 ± 14%; 86 ± 18%; 107 ± 11% of control respectively, $p > 0.05$ for each, ANOVA). The data demonstrate a delayed, persistent increase in cation-independent CaN activity following TBI in both ipsilateral and contralateral hippocampi. This increase in basal CaN activity is consistent of a post-translational modification of the enzyme (Kurz et al., 2003).

As observed for basal CaN activity, LFPI resulted in increased maximal CaN activity in both the ipsilateral and contralateral hippocampus. Under maximal stimulation, CaN

activity in ipsilateral hippocampal brain regions (Figure 3A) was not significantly different from control at 1 or 6 hours post-injury (89 ± 6%; 107 ± 3% of control respectively, $p > 0.05$, ANOVA). By 12 hours, there was a significant increase in maximal CaN activity (128 ± 5% of control, $p < 0.01$, ANOVA), which persisted at 18 hours (125 ± 9% of control, $p < 0.05$, ANOVA). CaN activity returned to control levels by 24 hours (97 ± 5% of control, $p > 0.05$, ANOVA) and did not differ significantly from controls at 1 week, 2 weeks, or 4 weeks (113 ± 11%; 103 ± 6%; 85 ± 4% of control, respectively; $p > 0.05$, ANOVA).

In the contralateral hippocampus, changes in the maximal activity of CaN were similar to those observed of basal (Figure 3B). At 1 hour post-TBI, maximal CaN activity was not significantly different from control (104 ± 5% of control, $p > 0.05$, ANOVA), but there was a significant increase at 6 hours (131 ± 4% of control, $p < 0.05$, ANOVA) and 12 hours (129 ± 7% of control, $p < 0.05$, ANOVA). Maximal activity levels at 18 hours remained slightly elevated, but were not significantly so (108 ± 6% of control, $p > 0.05$, ANOVA). By 24 hours, there was a significant loss of maximal activity (88 ± 4% of control, $p < 0.05$, ANOVA) and, similar to basal activity, a restoring trend across the remaining time points studied: 1 week (91 ± 6% of control, $p > 0.05$, ANOVA), 2 weeks (92 ± 12% of control, $p > 0.05$, ANOVA), 4 weeks (108 ± 5% of control $p > 0.05$, ANOVA).

In cortical fractions, unlike hippocampal fractions, we saw no change in either basal or maximal CaN activity at any time point, relative to controls, in either the ipsilateral or contralateral cortex (Data not shown). This suggests the TBI-induced changes in CaN are not due to post-translation modification, but rather some other mechanism. Alternatively, the change in CaN may not be global and instead restricted to peri-contusional structures. Therefore, any changes in CaN activity are likely beyond the range of detection for the pNPP assay. Altered CaN activity is explored further below.

Cofilin dephosphorylation in LFPI

In order to determine the cellular consequences of LFPI-induced increases in CaN activity, our group examined the effects of LFPI on the phosphorylation state of cofilin, a downstream CaN-regulated protein, in both the ipsilateral and contralateral cortex and hippocampus. Using a phospho-specific cofilin antibody (Kurz et al., 2008), we quantified the levels of phosphorylated cofilin following LFPI both with and without the administration of CaN inhibitors. LFPI resulted in an altered phosphorylation state of cofilin in both ipsilateral and contralateral brain tissue.

In homogenates isolated from the ipsilateral hippocampus, there was an immediate dephosphorylation of cofilin following LFPI (Figure 4A). As soon as 1 hour post-TBI, compared to controls, an approximate 80% dephosphorylation was observed (21 ± 5% of control, $p < 0.05$, Student's t-test). At 12 and 24 hours post-injury, cofilin was still significantly dephosphorylated relative to controls (11 ± 4% of control, $p < 0.05$ and 30 ±

13% of control, $p < 0.05$, Student's t-test). There was an apparent renewal of cofilin phosphorylation at 1 week, but the change was not significantly different from control (144 ± 26% of control, $p > 0.05$, Student's t-test). Interestingly, cofilin phosphorylation was reduced again at 2 weeks (33 ± 6% of control, $p < 0.01$, Student's t-test) and 4 weeks post-TBI (22 ± 7% of control, $p > 0.01$, Student's t-test). The data suggest a dynamic regulation of cofilin activity following TBI.

Similarly, in homogenates of the contralateral hippocampus, LFPI caused a substantial dephosphorylation of cofilin (Figure 4B). At 1 hour post-TBI, cofilin was significantly dephosphorylated (28 ± 6% of control, $p < 0.01$, Student's t-test). At 12 hours post-injury, cofilin phosphorylation remained below that of controls (11 ± 4% of control, $p < 0.05$, Student's t-test) and was still down at 24 hours (26 ± 10% of control, $p < 0.01$, Student's t-test). Cofilin partially recovered by 1 week post-TBI (78 ± 7% of control, $p > 0.05$, Student's t-test) and remained comparable to controls at 2 weeks post-injury (112 ± 18% of control, $p > 0.05$, Student's t-test). Similar to ipsilateral phospho-cofilin expression, contralateral expression was significantly decreased 4 weeks post-TBI (37 ± 4% of control, $p < 0.05$, Student's t-test). Thus, LFPI resulted in similar, albeit not the same, modulation of cofilin in both ipsilateral and contralateral hippocampi.

Unlike the hippocampal fractions, cortical samples displayed a biphasic modulation of cofilin phosphorylation (Figure 5). In ipsilateral homogenates of the cortex (Figure 5A), there was a hyper-phosphorylation of cofilin by 1 hour post-TBI (221 ± 27% of control, p

< 0.01, Student's t-test). The data suggests that there is an acute hypofunction of cortical structures, which agrees with SPECT data (Abdel-Dayem et al., 1998). By 12 hours post-injury, however, cofilin was significantly dephosphorylated as compared to control (57 ± 7% of control, $p < 0.05$, Student's t-test). With the exception of the 1 week time point (76 ± 7% of control, $p > 0.05$, Student's t-test), cofilin was significantly dephosphorylated at every remaining time point thereafter: 24 hours (56 ± 17% of control, $p < 0.05$, Student's t-test), 2 weeks (42 ± 7% of control, $p < 0.01$, Student's t-test), and 4 weeks (46 ± 11% of control, $p < 0.01$, Student's t-test).

Phosphorylation of cofilin in the contralateral cortex was similar to that observed in the ipsilateral cortex (Figure 5B). Cofilin was hyper-phosphorylated at 1 hour (156 ± 18% of control, $p < 0.05$, Student's t-test), but returned to control levels between 6 and 12 hours post-TBI (107 ± 13%; 111 ± 5% of control respectively, $p > 0.05$ for both, Student's t-test). By 18 hours post-TBI, cofilin was significantly dephosphorylated (41 ± 13% of control, $p < 0.01$, Student's t-test), which persisted 24 hours post-injury (25 ± 9% of control, $p < 0.05$, Student's t-test), and through the 1, 2, and 4 week time points (50 ± 7% of control, $p < 0.05$; 60 ± 14% of control, $p < 0.05$; 52 ± 7% of control, $p < 0.01$, respectively, Student's t-test).

<u>FK506 decreases cofilin dephosphorylation in LFPI</u>

To determine the CaN-dependence of cofilin dephosphorylation, we administered the selective CaN-inhibitor, FK506 (Tacrolimus). Animals were injected with FK506 1 hour

post-injury and allowed to recover for 23 hours to determine if a single post-injury injection would preserve the phosphorylation state of cofilin. An age-matched control group also received an injection of FK506 but did not sustain injury. The animals were decapitated 23 hours after injection to match injury group conditions.

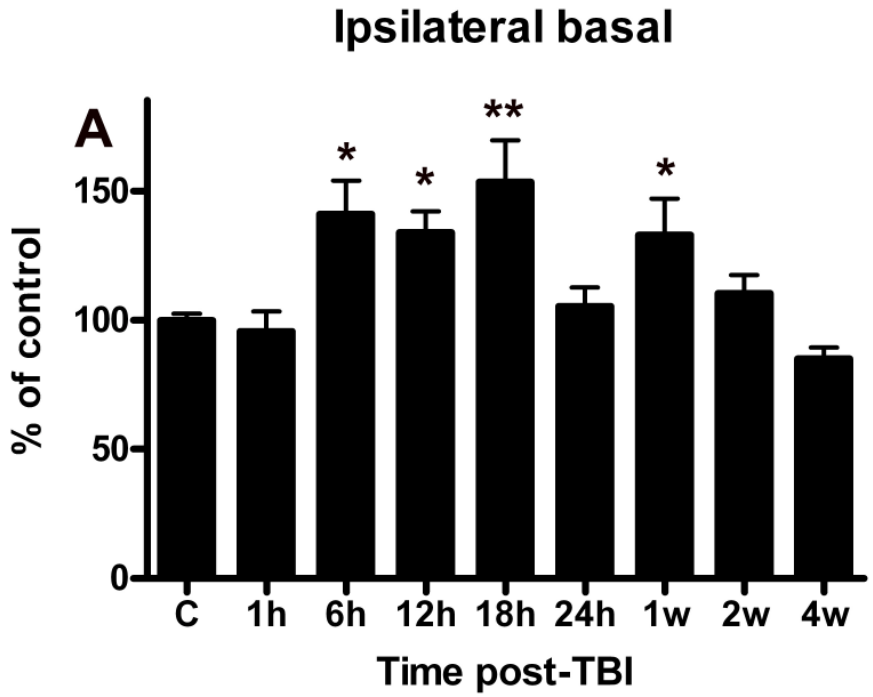
In all cortical and hippocampal regions, there was a slight, but not significant, increase in phosphocofilin levels in FK506-treated uninjured animals as compared to those that did not receive FK506. Therefore, FK506-treated trauma samples were compared to FK506-treated control samples. In the hippocampus, FK506 partially preserved cofilin phosphorylation levels, but did not prevent the dephosphorylation of cofilin after TBI. There was significant dephosphorylation of cofilin in ipsilateral fractions (Figure 6A, 33 ± 9% of control, $p < 0.001$, Student's t-test), but the contralateral hippocampus was mildly protected (Figure 6B, 51 ± 26% of control, $p > 0.05$, Student's t-test).

However, in cortical structures, FK506 treatment was more protective as it prevented the bilateral dephosphorylation of cofilin in the cortex following LFPI. In the ipsilateral cortex, phosphorylation of cofilin remained close to control levels (Figure 7A, 103 ± 11% of control, $p > 0.05$, Student's t-test). Similarly, in the contralateral cortex, cofilin was not significantly dephosphorylated relative to controls (Figure 7B, 107 ± 20% of control, $p > 0.05$, Student's t-test). These data demonstrate that CaN inhibition is sufficient to prevent the dephosphorylation of cofilin in the cortex, and partially in the hippocampus, following

lateral brain injury. The data suggests that the mechanism is CaN-dependent in the cortex, but in the hippocampus, there are other mechanisms concurrently working with CaN.

Overall, cofilin immunoreactivity was not significantly affected in any region of the brain nor at any time point. This indicates that the changes in phosphorylated cofilin levels were due to a change in phosphorylation rather than altered protein expression.

Figure 2. The LFPI-induced increase in basal CaN activity in the hippocampus is acute. Hippocampal homogenates from injured and control animals were isolated at specific time points post-TBI. Under basal reactions, CaN activity was significantly elevated in the hippocampus located ipsilateral to injury **(A)** at 6 hours, 12 hours, 18 hours, and 1 week post-trauma. Basal CaN activity was not significantly different from controls at 1 hour, 24 hours, 2 weeks, or 4 weeks. In the hippocampus located contralateral to injury, basal CaN activity was significantly elevated **(B)** at 6 and 12 hours post-injury and significantly decreased at 24 hours. At 1 hour, 18 hours, 1 week, 2 weeks, and 4 weeks, basal CaN activity did not significantly differ from controls (**$p < 0.01$, *$p < 0.05$, one-way ANOVA with Tukey post-hoc analysis).


Ipsilateral basal
A
% of control
150
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12h
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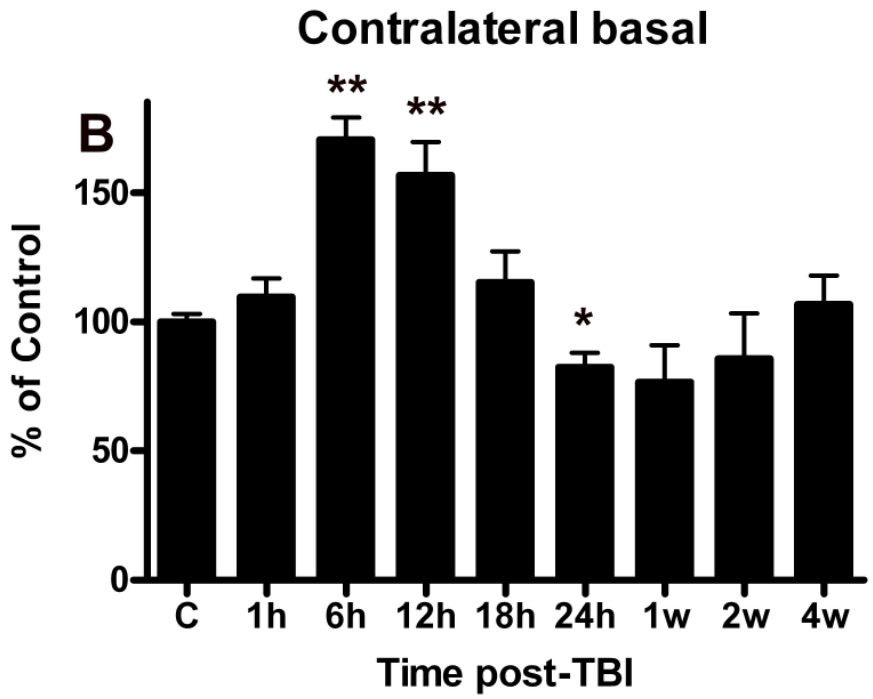
Time post-TBI


Contralateral basal
B
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1h
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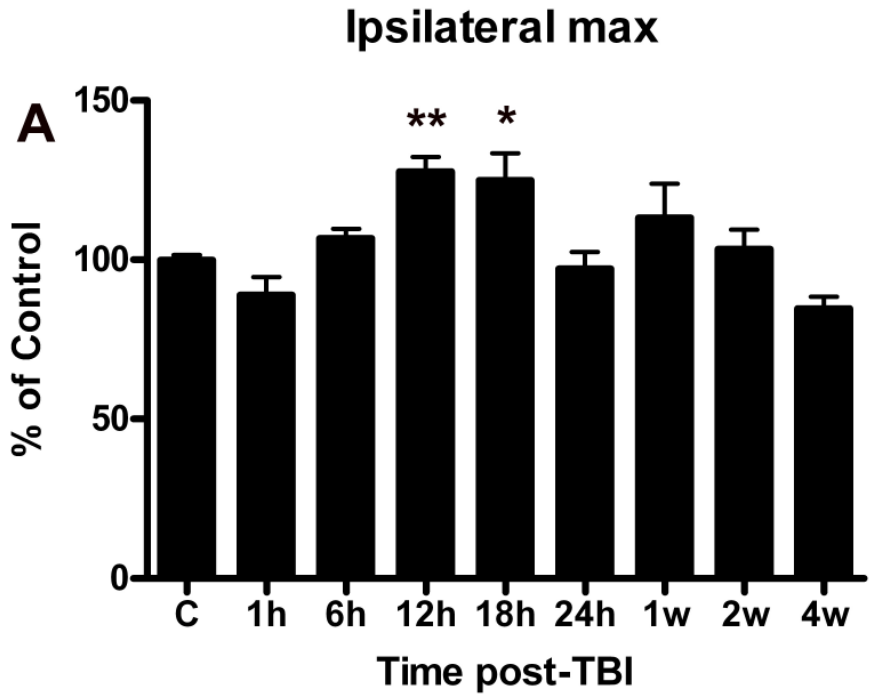
Time post-TBI

Figure 3. The LFPI-induced increase in maximal CaN activity in the hippocampus is acute. Hippocampal homogenates from injured and control animals were isolated at specific time points post-TBI. Under maximal reactions, CaN activity was significantly elevated in the hippocampus located ipsilateral to injury **(A)** at 12 hours and 18 hours. In the hippocampus located contralateral to injury, cation-stimulated CaN activity was significantly elevated **(B)** at 6 and 12 hours post-injury and significantly decreased at 24 hours post-trauma (**$p < 0.01$, *$p < 0.05$, one-way ANOVA with Tukey post-hoc analysis).


Ipsilateral max
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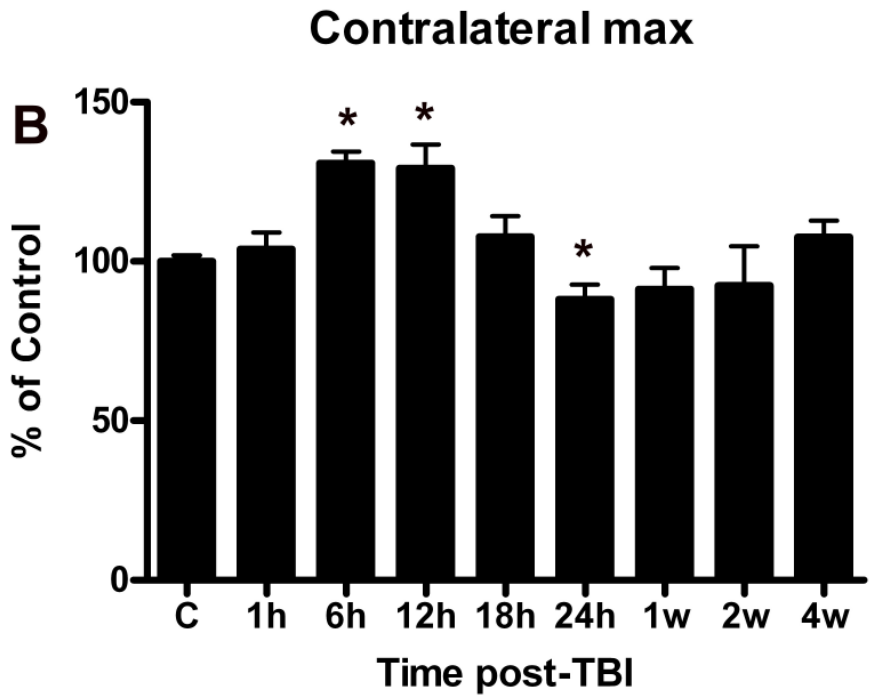
Time post-TBI


Contralateral max
B
150
100
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C
1h
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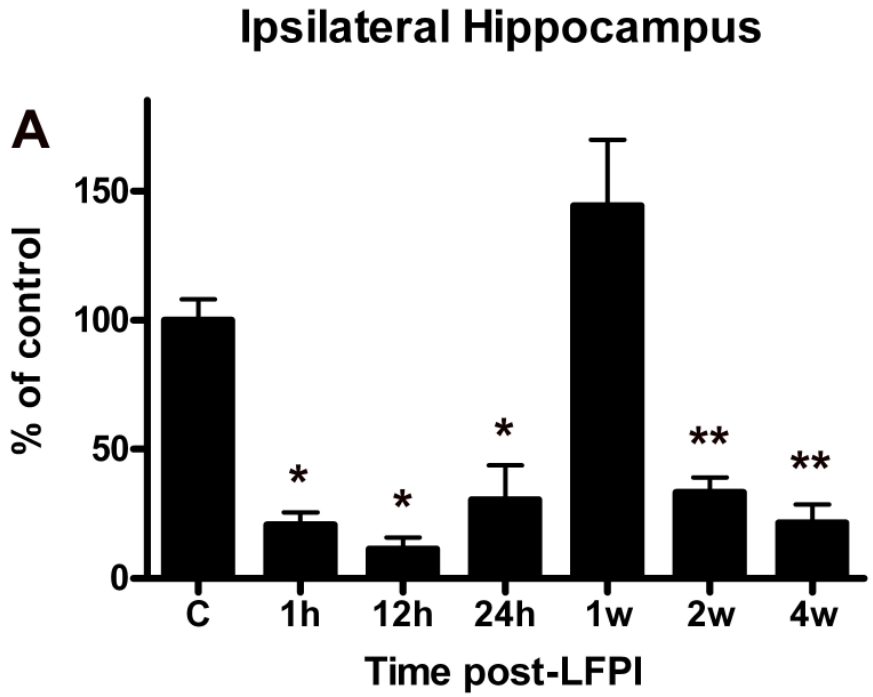
2w
4w
Time post-TBI

Figure 4. Temporal profile of cofilin dephosphorylation in the hippocampus following LFPI. Calibrated densitometry of phosphocofilin Western blots from the ipsilateral and contralateral hippocampus. In the ipsilateral hippocampus, cofilin phosphorylation was significantly decreased **(A)** at 1 hour, 12 hours, 24 hours, 2 weeks, and 4 weeks post-TBI. Contralateral to the injury site, cofilin was significantly dephosphorylated **(B)** 1 hour, 12 hours, 24 hours, and 4 weeks after trauma (**$p < 0.01$, *$p < 0.05$, Student's t-test for each time point).


Ipsilateral Hippocampus
A
% of control
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1h
12h
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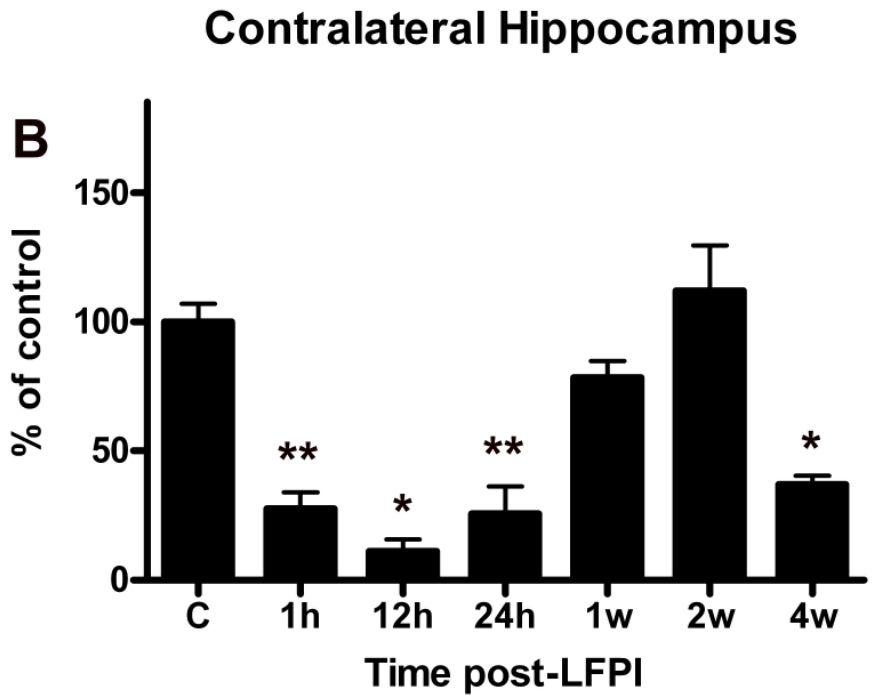
4w
Time post-LFPI


Contralateral Hippocampus
B
% of control
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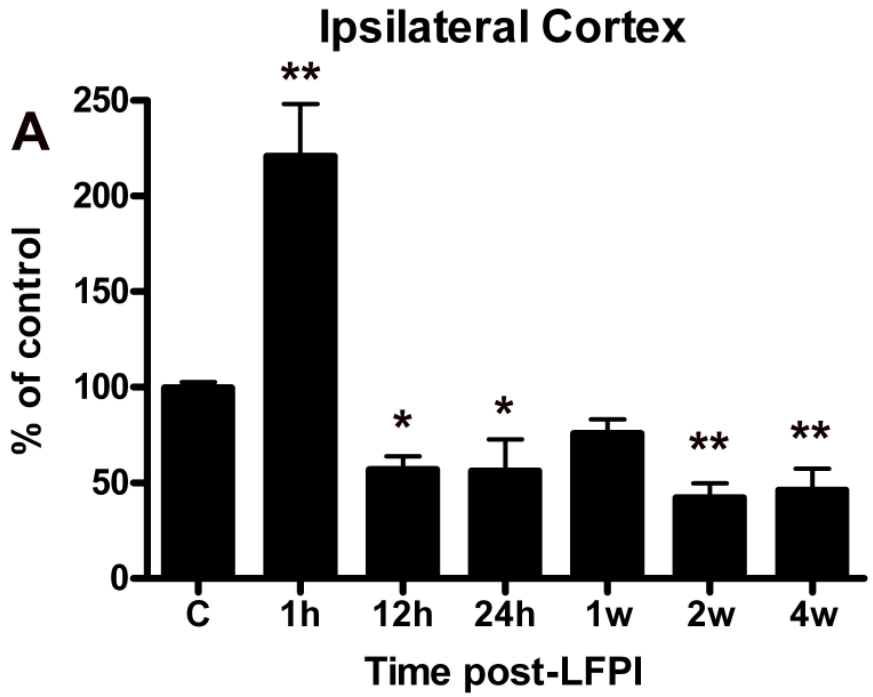
C
1h
12h
24h
1w
2w
4w
Time post-LFPI

Figure 5. Temporal profile of cofilin dephosphorylation in the cortex following LFPI. Calibrated densitometry of phosphocofilin Western blots from the ipsilateral and contralateral cortex. In the ipsilateral cortex, cofilin phosphorylation was significantly increased **(A)** at 1 hour and significantly decreased at 12 hours, 24 hours, 2 weeks, and 4 weeks post-TBI. Contralateral to the injury site, cofilin was hyper-phosphorylated **(B)** 1 hour post-injury and significantly decreased 24 hours, 1 week, 2 weeks, and 4 weeks after trauma (**$p < 0.01$, *$p < 0.05$, Student's t-test for each time point).


Ipsilateral Cortex
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% of control
250
200
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1h
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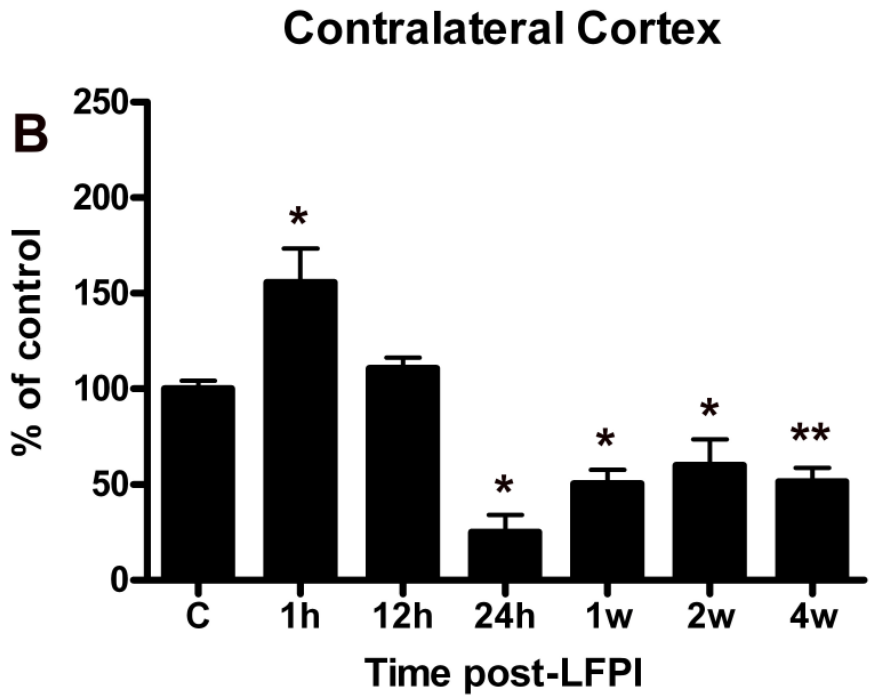
Time post-LFPI


Contralateral Cortex
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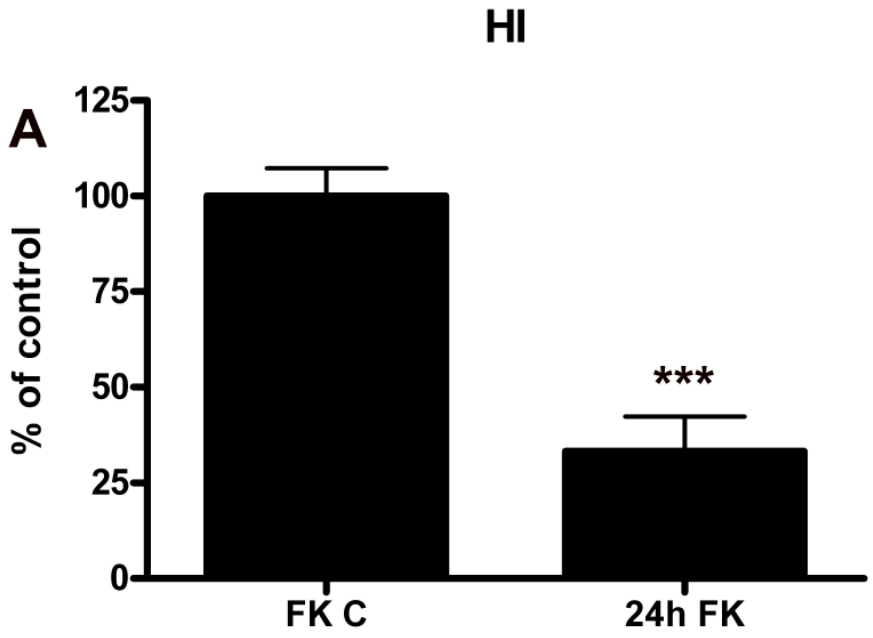
Time post-LFPI

Figure 6. FK506 partially blocks the CaN-dependent decrease in phosphocofilin in the hippocampus. One post-injury injection of FK506 did not prevent the dephosphorylation of cofilin in the ipsilateral hippocampus. **(A)** 24 hours post-injury cofilin was significantly dephosphorylated in the hippocampus located ipsilateral to injury as compared to control. Phosphocofilin levels **(B)** 24 hours post-TBI in the contralateral hippocampus did not significantly differ from control levels (***$p < 0.001$, Student's t-test).


HI
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% of control
125
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75
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FK C
24h FK

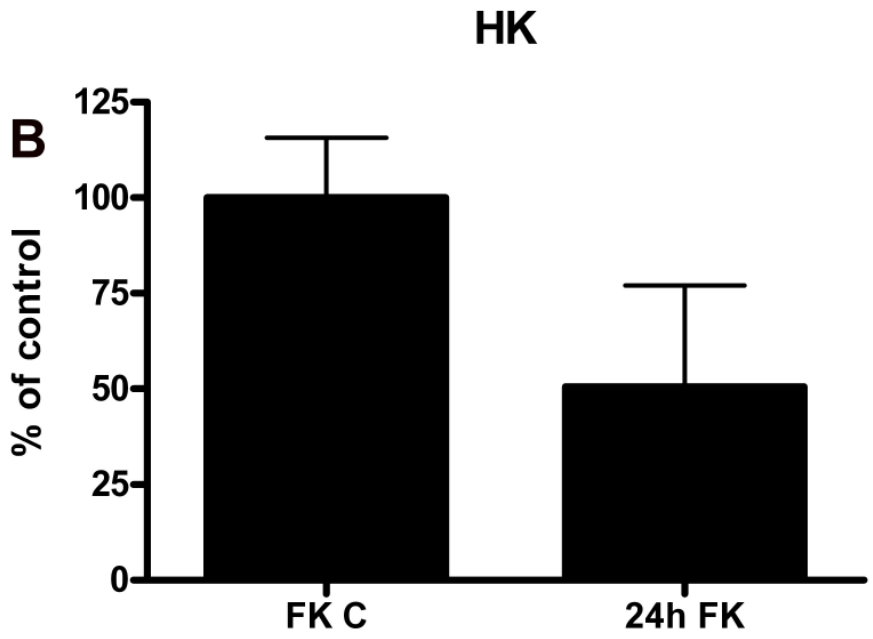
HK
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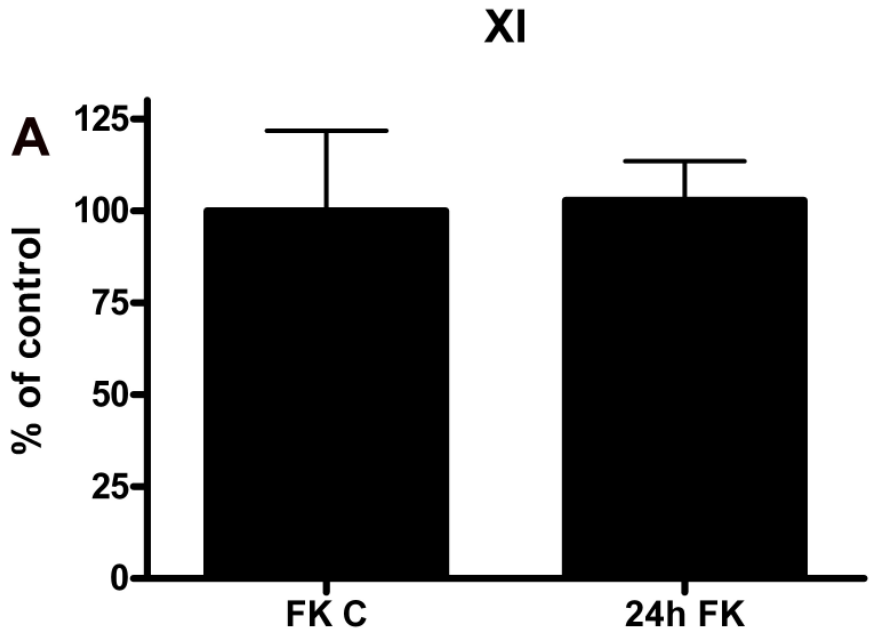
Figure 7. FK506 blocks the CaN-dependent decrease in phosphocofilin in the cortex. One post-injury injection of FK506 prevented the dephosphorylation of cofilin in the ipsilateral and contralateral cortex. **(A)** 24 hours post-injury phosphocofilin was not significantly dephosphorylated in the cortex located ipsilateral to injury as compared to control. Phosphocofilin levels **(B)** 24 hours post-TBI in the contralateral cortex also did not significantly differ from control levels (Student's t-test).


XI
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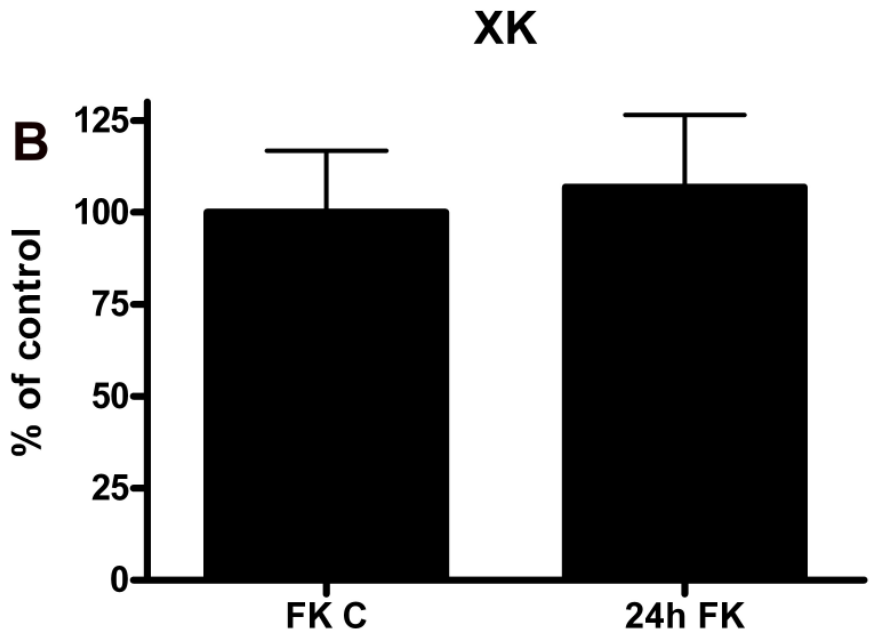
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Discussion

This manuscript describes a differential modulation of CaN activity following LFPI. In the hippocampus, there was a delayed increase in CaN activity when measured directly by an enzymatic assay or by tracking the phosphorylation of a downstream mediator. However, the increase in hippocampal CaN activity returned to control levels 24 hours post-TBI. In contrast, cortical tissue displayed biphasic changes in CaN activity following LFPI. An initial decrease in CaN activity was observed acutely, followed by a prolonged increase in CaN activity as measured by a downstream substrate of CaN, cofilin. The data demonstrate that LFPI results in significant modulation of CaN activity. Considering these results along with those of the companion histochemical study (Campbell et al., 2009), we believe that the increase in CaN activity is related to the subsequent loss of dendritic spines in the LFPI model, as has been previously shown in a status epilepticus model (Kurz et al., 2008).

One pathology associated with head trauma is altered cognitive function, which is especially manifest in hippocampal-dependent learning and memory models (Hamm et al., 1992; Hamm et al., 1993; Hamm et al., 1996; Wiley et al., 1996). CaN plays an important role in these processes by regulating long-term potentiation (LTP) and long-term depression (LTD) (Mansuy et al., 1998; Mansuy, 2003). CaN has been shown to be involved in synaptic plasticity under physiological circumstances such as in the dephosphorylation of AMPA receptors (Tong et al., 1995; Sanchez et al., 2005) or decreased spine stability (Halpain et al., 1998). Under pathological conditions, CaN has

been shown to translocate to the post-synaptic density following SE (Kurz et al., 2008) and central fluid percussion injury (Kurz et al., 2005a), where it may affect post-synaptic function and/or structure. As CaN is known to regulate learning and memory, and because TBI induces an increase in CaN activity (Kurz et al., 2005b), it is possible that the cognitive impairment associated with TBI is due in part to CaN dysregulation. Indeed, cognitive deficits are evident following a central injury (Hamm et al., 1992; Hamm et al., 1993; Hamm et al., 1996) and correspond to increased CaN activity and immunoreactivity in synaptoplasmic membrane fractions (Kurz et al., 2005a; Kurz et al., 2005b). These cognitive deficits may be explained by a loss of dendritic spines, such as that observed in models of SE (Kurz et al., 2008; Wong, 2008). A loss of dendritic spines is associated with cognitive impairment in other brain pathologies, including Alzheimer's disease (Knobloch and Mansuy, 2008). In our companion paper (Campbell et al., 2009); we observed a decrease in dendritic spine densities at 24 hours post-LFPI. Therefore, these companion studies together demonstrate a cellular and molecular mechanism through which TBI can alter learning and memory.

Another consequence of traumatic brain injury is the development of post-traumatic epilepsy (PTE). The risk of developing PTE correlates positively with the severity and type of injury (Annegers et al., 1998). This recurrent seizure disorder often appears many weeks or months following the initial injury (Frey, 2003; Agrawal et al., 2006). Furthermore, a rat model of PTE has been established in which a severe lateral fluid percussion injury induces epilepsy in 43%-50% of animals from 7 weeks to 1 year post-

injury (Kharatishvili et al., 2006; Pitkanen and McIntosh, 2006). This latency period suggests that the development of epilepsy involves more than an acute cell loss, but a delayed, chronic cellular response, such as the loss and subsequent regrowth of dendritic spines. With the loss of spines, there are three possible outcomes: the spines may not regrow (decreased learning and memory), the spines may re-establish neuronal networks (healing), or more interestingly, may connect aberrantly with pre-synaptic cells and thus create new networks. These new connections may upset the balance of excitability or inhibition in the neuronal networks, potentially facilitating epileptiform activity.

Compared to the central fluid percussion injury model (Kurz et al., 2005b), the modulation in CaN activity following LFPI proved to be more complex. With a central injury, basal and maximal activity levels were significantly increased 24 hours post-injury in both the hippocampus and cortex, as compared to controls. Furthermore, CaN activity was significantly increased at least 3 weeks post-injury in the hippocampus. In the cortex, both basal and maximal levels of CaN activity were increased through 2 weeks post-TBI (Kurz et al., 2005b). However, when the injury was located laterally, basal and maximal CaN activity changed between 6 and 18 hours in both hippocampi and returned to normal by 24 hours in ipsilateral regions and by 18 hours in regions located contralateral to the injury. When assessing cortical CaN activity levels, we recorded no changes in CaN activity following lateral injury, which may suggest that the effects on CaN activity are localized to the injury site as opposed to throughout the whole cortical hemisphere. Therefore, since

both studies were performed on the same FPI device, the data suggests that the injury site is critical in determining the severity and duration of TBI-related changes.

The bilateral effect of lateral brain injury on CaN activity was surprising, but not unprecedented. LFPI has been shown previously to have a bilateral effect on the phosphorylation state of calcium calmodulin-dependent protein kinase II (Folkerts et al., 2007). The bilaterality may be due to a contrecoup injury that results from the lateral injury, causing the contralateral regions to undergo changes similar to the ipsilateral regions. However, it is likely that the contralateral side is injured less severely as it is not exposed to the direct insult. If that is true, an unequal repair process could yield aberrant interneuronal connections, leading to further pathology.

A precipitating event for the changes reported by this study and its companion (Campbell et al., 2009) may be glutamate receptor-mediated cell injury. Extracellular glutamate is elevated immediately following the primary injury, causing excitotoxic damage to neurons by over-activating glutamate receptors (Faden et al., 1989; Katayama et al., 1990; Michaels and Rothman, 1990; Globus et al., 1995; Tymianski and Tator, 1996). Excessive stimulation of glutamate receptors leads to a large increase in intracellular calcium ($[Ca^{2+}]_i$) and the subsequent dysregulation of calcium (Ca^{2+}) signaling pathways (Choi, 1988b, a; Choi et al., 1988). The Ca^{2+} hypothesis of epileptogenesis suggests that this influx of Ca^{2+} triggers pathological plasticity, leading to a persistent elevation of $[Ca^{2+}]_i$ and the establishment of a new Ca^{2+} plateau, which in turn promotes epileptogenesis

(Delorenzo et al., 2005). Establishment of a new Ca^{2+} plateau has been observed in models of SE (Nagarkatti et al., 2009) and in central fluid percussion injuries (Sun et al., 2008). With the central fluid percussion injury, $[Ca^{2+}]_i$ re-establishes between 200-300 nM for at least one week following the injury (Sun et al., 2008). Interestingly, CaN has a K_m between 250-500 nM, which means that the new Ca^{2+} plateau after injury could constitutively activate CaN.

In the cortex following LFPI, we did not detect any changes in basal or maximal CaN activity levels utilizing a post-hoc assay. However, we did observe an acute increase in the phosphorylation state of cofilin in the cortex, which was followed by a sustained dephosphorylation. Generally, the sustained dephosphorylation of cofilin may be due to the establishment of a higher baseline $[Ca^{2+}]_i$ plateau by neural cells in the hours following injury(Sun et al., 2008; Nagarkatti et al., 2009). As indicated above, if the new Ca^{2+} plateau is within the K_m of CaN, there could be constitutive activation of CaN and a continuous dephosphorylation of cofilin.

The prolonged increase in cation-independent CaN activity may involve a post-translational modification of the enzyme (Kurz et al., 2003). Proteolysis of the CaN A subunit by calpain I can produce a calcium-independent form of CaN that is highly active (Tallant et al., 1988; Wang et al., 1999). However, in spite of these changes, CaN activity returns to baseline 1 to 2 weeks post-LFPI. It is possible that by this time, the modified CaN has been replaced by new, unmodified enzyme. This transient increase in CaN

activity, as observed in the hippocampus, may lead to possible aberrant regrowth of dendritic spines.

Interestingly, when we used a direct measurement of CaN activity, only the hippocampus showed a change in CaN activity. This change in CaN activity positively correlates with the dephosphorylation of cofilin in both hemispheres of the hippocampus. There are many potential outcomes of increased CaN activity including, but not limited to, apoptosis (Ankarcrona et al., 1996; Asai et al., 1999) and changes in dendritic spines (Kurz et al., 2008). CaN mediates cofilin indirectly through the activation of the cofilin phosphatase, slingshot (Wang et al., 2005). When cofilin is dephosphorylated, it regulates actin dynamics by binding actin filaments and causing torsional stress that severs the filamentous structure (Bobkov et al., 2006). Because actin filaments are the major cytoskeletal element of spines (Fifkova and Delay, 1982), the loss of actin filaments due to cofilin activation may lead to the collapse of dendritic spines.

A substantial problem with PTE is the lack of effective treatment. Many anti-epileptic drugs have proven ineffective or unreliable for terminating seizure activity and are not recommended as prophylactic treatment (Formisano et al., 2007; Teasell et al., 2007). Rather than initiating treatment at the onset of epilepsy, it may be more effective to combat the disease during its developmental stage, epileptogenesis. Conveniently, the latency period between injury and epilepsy suggests a wide window for therapeutic interaction. This potentially provides a large time frame in which to administer pharmacological

treatment. In this study, we have identified a cellular mechanism which occurs during the latency period following TBI, and which can be prevented by a post-injury injection of a commercially available drug, FK506. FK506 proved effective in stopping the dephosphorylation of cofilin in the cortex as well as preventing the loss of dendritic spines. If by preventing spine loss, FK506 preserved neuronal circuitry, this may reduce the risk of TBI-induced pathologies such as cognitive impairment and PTE.

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Literature Cited

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