2-3-2016

Blockade of Astrocytic Calcineurin/NFAT Signaling Helps to Normalize Hippocampal Synaptic Function and Plasticity in a Rat Model of Traumatic Brain Injury

Jennifer L. Furman  
*University of Kentucky, jlfurm2@uky.edu*

Pradoldej Sompol  
*University of Kentucky, pradoldej.sompol@uky.edu*

Susan D. Kraner  
*University of Kentucky, susan.kraner@uky.edu*

Melanie M. Pleiss  
*University of Kentucky, melanie.pleiss@gmail.com*

Esther J. Putman  
*University of Kentucky, esther.putman@uky.edu*

Follow this and additional works at: [https://uknowledge.uky.edu/pharmacol_facpub](https://uknowledge.uky.edu/pharmacol_facpub)

Part of the [Diseases Commons](https://uknowledge.uky.edu/diseases), [Medical Nutrition Commons](https://uknowledge.uky.edu/medical-nutrition), [Medical Pharmacology Commons](https://uknowledge.uky.edu/medical-pharmacology), [Neuroscience and Neurobiology Commons](https://uknowledge.uky.edu/neuroscience), and the [Pharmacology, Toxicology and Environmental Health Commons](https://uknowledge.uky.edu/pharmacology).
Blockade of Astrocytic Calcineurin/NFAT Signaling Helps to Normalize Hippocampal Synaptic Function and Plasticity in a Rat Model of Traumatic Brain Injury

Notes/Citation Information
Published in The Journal of Neuroscience, v. 36, issue 5, p. 1502-1515.

Copyright © 2016 the authors

This work is available to the public to copy, distribute, or display under the terms of the Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by/4.0/).

Digital Object Identifier (DOI)
Blockade of Astrocytic Calcineurin/NFAT Signaling Helps to Normalize Hippocampal Synaptic Function and Plasticity in a Rat Model of Traumatic Brain Injury

Jennifer L. Furman,1 Pradoldej Sompol,1* Susan D. Kraner,1 Melanie M. Pleiss,1,2 Esther J. Putman,2 Jacob Dunkerson,2 Hafiz Mohmmad Abdul,2 Kelly N. Roberts,2 Stephen W. Scheff,2,3 and Christopher M. Norris1,2

1Department of Pharmacology and Nutritional Sciences, 2Sanders-Brown Center on Aging, and 3Department of Anatomy and Neurobiology, University of Kentucky College of Medicine, Lexington, Kentucky 40536

Increasing evidence suggests that the calcineurin (CN)-dependent transcription factor NFAT (Nuclear Factor of Activated T cells) mediates deleterious effects of astrocytes in progressive neurodegenerative conditions. However, the impact of astrocytic CN/NFAT signaling on neural function/recovery after acute injury has not been investigated extensively. Using a controlled cortical impact (CCI) procedure in rats, we show that traumatic brain injury is associated with an increase in the activities of NFATs 1 and 4 in the hippocampus at 7 d after injury. NFAT4, but not NFAT1, exhibited extensive labeling in astrocytes and was found throughout the axon/dendrite layers of CA1 and the dentate gyrus. Blockade of the astrocytic CN/NFAT pathway in rats using adeno-associated virus (AAV) vectors expressing the astrocyte-specific promoter Gfa2 and the NFAT-inhibitory peptide VIVIT prevented the injury-related loss of basal CA1 synaptic strength and key synaptic proteins and reduced the susceptibility to induction of long-term depression. In conjunction with these seemingly beneficial effects, VIVIT treatment elicited a marked increase in the expression of the prosynaptogenic factor SPARCL1 (hevin), especially in hippocampal tissue ipsilateral to the CCI injury. However, in contrast to previous work on Alzheimer’s mouse models, AAV-Gfa2-VIVIT had no effects on the levels of GFAP and Iba1, suggesting that synaptic benefits of VIVIT were not attributable to a reduction in glial activation per se. Together, the results implicate the astrocytic CN/NFAT4 pathway as a key mechanism for disrupting synaptic remodeling and homeostasis in the hippocampus after acute injury.

Key words: astrocytes; brain injury; calcineurin; calcium; glia; synapse

Introduction

Astrocytes exhibit striking morphological changes with CNS injury and disease, which are collectively referred to as “astrocyte activation” (Pekny and Nilsson, 2005; Parpura et al., 2012; Anderson et al., 2014). Hypertrophy of astrocyte somata and processes, along with a corresponding increase in the expression of the glial fibrillary acidic protein (GFAP), are the most robust and recognizable biomarkers of activated astrocytes, yet the func-
Several key morphologic and biochemical features of astrocyte activation have been linked to the expression and/or hyperactivation of the Ca$^{2+}$/calmodulin-dependent protein phosphatase calcineurin (CN) (Norris et al., 2005; Fernandes et al., 2007; Canellada et al., 2008; Furman et al., 2012; Furman and Norris, 2014). Although weakly expressed in astrocytes in healthy adult nervous tissue, CN can appear at high levels in activated astrocytes associated with aging (Norris et al., 2005), acute injury (Hashimoto et al., 1998), and progressive amyloid pathology (Norris et al., 2005; Celsi et al., 2007; Abdul et al., 2009). In peripheral tissues, including lymphoid tissue, heart, vascular smooth muscle cells, and skeletal muscle, CN is known to strongly regulate the morphological and functional phenotypes of cells through the activation of NFAT (Nuclear Factor of Activated T cells) transcription factors (Crabtree and Olson, 2002; Horsley and Pavlath, 2002; Hogan et al., 2003; Wilkins and Molkentin, 2004; Hassoun et al., 2009). There are four CN-dependent NFAT isoforms (i.e., NFATs 1–4), all of which are expressed in mammalian brain (Nguyen and Di Giovanni, 2008; Vilma et al., 2008). Changes in NFAT expression/activity, especially for the NFAT1 and 4 isoforms, have been observed in astrocytes in progressive neurodegenerative conditions such as Alzheimer’s disease (AD) (Abdul et al., 2009) and Parkinson’s disease (Caraveo et al., 2014) and also after acute nervous system injury (Serrano-Pérez et al., 2011; Yan et al., 2014). Blockade of CN/NFAT interactions in astrocytes using adeno-associated virus (AAV) vectors and the CN docking site mimetic peptide VIVIT suppressed glial activation, reduced amyloid pathology, and improved synaptic function in a mouse model of AD (Furman et al., 2012), suggesting that astrocytic CN/NFAT signaling may be detrimental to neural function in chronic neurodegenerative conditions. Whether CN/NFAT plays a similarly deleterious role in activated astrocytes after acute neural injury is unknown.

Here, we investigated the DNA-binding activities of all four CN-dependent NFAT isoforms in rat hippocampus 7 d after a unilateral cortical contusion injury (CCI). Although we found evidence for increased expression of the NFAT1 and 4 isoforms, specific colocalization with GFAP-positive astrocytes was only observed for NFAT4. Intrahippocampal injection of AAV vectors containing the astrocyte-specific promoter Gfa2 and the NFAT inhibitory peptide VIVIT prevented the weakening of basal CA3–CA1 synaptic strength, reduced susceptibility to long-term synaptic depression (LTD), and protected against the loss of several key synaptic proteins. However, AAV-Gfa2-VIVIT did not appreciably alter astrocyte or microglial protein markers (i.e., GFAP and Iba-1, respectively). The results suggest that astrocytic CN/NFAT signaling can impair and/or prevent the recovery of synaptic function after traumatic brain injury without reducing astrocyte activation per se.

Materials and Methods

Animals and TBI. Adult (~2-month-old) male Sprague Dawley rats (250–275 g; Harlan Laboratories) were housed 2/cage on a 12 h light/dark cycle, provided access to food and water ad libitum, and used in accordance with the Institutional Animal Care and Use Committee of the University of Kentucky. Stereotoxic surgeries and delivery of traumatic brain injury was performed with slight modifications of our previous methods (Norris and Scheff, 2009; Ansari et al., 2013; Scheff et al., 2013). Briefly, rats were anesthetized with 2% isofluorane and immobilized in a Kopf stereotaxic frame with the incisor bar set at ~5. Body temperature for each rat was monitored and maintained at 36°C with a Space Gel heating pad (Braintree Scientific). After a midline incision and retraction of the skin, a 6-mm-diameter craniotomy was made lateral to midline and approximately midway between bregma and lambda with a Micle hand trephine and the skull disk was removed without disturbing the dura. An electronic controlled pneumatic impact device (TBI0310; Precision System) with a hard stop Bimba cylinder was used to deliver a moderate unilateral controlled cortical impact (CCI) injury, which compressed the cortex to a depth of 2.0 mm at a velocity of 3.5 m/s. After the injury, the craniotomy site was sealed with MASCOT adhesive (EMS) and an 8 mm disc formed from clear polyester (0.5 mm; Midwest Products). In some cases, rats were subjected to craniotomy, but did not receive a CCI, and served as sham controls. The surgical procedure was completed in ~15–20 min.

Electrophoretic mobility shift assay. As the EMSA probe, a duplex DNA oligonucleotide labeled on its 5’ ends with IRDye 700 (LI-COR) was obtained from Integrated DNA Technologies. The sequence of the strands were 5’-IRDye700-ACATTTGAAAAATTITATACACCA-3’ annealed to 5’-IRDye700-TGGTGTATAAATTTTCCCAATG-3’) and corresponded to the NFAT binding site from the IL4 promoter, with which previous high-quality EMSAs have been performed by others (Neal and Clipstone, 2001). Probe specificity was assessed using an unlabeled wild-type competitor of the same sequence or a mutant competitor containing an altered NFAT-binding site (TGGAAAA→CTTTAAA). When indicated, these competitors were included at a 100-fold molar excess in the EMSA-binding mix.

Whole cell extracts from frozen brain tissue at 7 d after CCI were made using a kit from Active Motif following the manufacturer’s instructions and protein assays performed using a Bradford assay. The binding reaction instructions for these experiments contained 10 m Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.05% NP40, 5% glycerol, 0.5 μg of poly dC, 5 μg of BSA, 10% 10x orange loading buffer (LI-COR), 1% protease inhibitor mixture III (EMD Millipore), and 1% phosphatase inhibitor mixture II (EMD Millipore), indicated extract, and 10 femtomoles of IL4 NFAT probe. To induce supershift/blockshift with NFAT antibodies, 3.3 μl of the indicated NFAT antibody was added to the mixture and 3.3 μl of PBS to the negative control. NFAT1 (Abcam, catalog #ab2272), NFAT2 (Santa Cruz Biotechnology, catalog sc-13033x), NFAT3 (Santa Cruz Biotechnology, catalog sc-13036x), and NFAT4 (Santa Cruz Biotechnology, catalog sc-8405x) were used individually. For some conditions, NFAT1 and NFAT4 antibodies were used in combination, as indicated. EMSA samples were incubated on ice for 2 h, covered with foil to protect fluorescent probe from photobleaching, and incubated at room temperature for 10 min before loading on gels. Electrophoresis was performed on chilled 5% nondenaturing polyacrylamide gels in ½X TBE running buffer in a cold room for 1 h and 50 min at 200 V, covering the apparatus with foil and keeping the lights out. The orange dye/free NFAT probe was close to the bottom of the gel at the end of the gel run. Images were obtained on an Odyssey scanner (LI-COR) using the 700 channel at the highest intensity and Image Studio 2.1 software (LI-COR) was used to quantify fluorescence of shifted bands. Protein concentrations used in these studies were optimized from titration assays (data not shown) and fell within the linear detection range of the Odyssey scanner. Control (contralateral side of the hippocampus) and injured (ipsilateral side of the hippocampus) tissue samples were run on the same gel for direct statistical comparisons.
Immunofluorescent labeling of NFAT isoforms in brain sections. On day 7 of recovery after treatment with cortical contusion injury, rats were deeply anesthetized and perfused with 30 ml of saline followed by 100 ml of a solution of 4% paraformaldehyde, pH 7.4. After perfusion, the brains were removed from the skull, further fixed for an additional 4 h, and then put in a 30% sucrose solution. Coronal brain sections (40 μm) were made and the sections stored in the sucrose solution in a −20°C freezer. As needed, sections were removed and stained as free-floating sections in 12-well tissue culture plates using a bent glass pipette to transfer brain section from well to well containing the appropriate solution. Brain sections were rinsed 2× with TBS buffer (100 mM Tris, pH 7.5, 0.85% saline), treated with H2O2 solution (3% H2O2, 10% methanol in TBS) for 60 min, and then rinsed again with TBS buffer. Sections were pretreated with TBS-T (TBS with 0.1% Triton X-100) and blocked with TBS-BSA (2% BSA in TBS-T). First antibodies, the same ones used for the EMSAs (either NFAT1, Abcam, catalog #ab2722, 1:100 dilution, or NFAT4, Santa Cruz Biotechnology, catalog sc-8405, 1:20 dilution) were diluted in TBS-BSA and brain sections were incubated overnight at 4°C with rotation on a shaker. The next morning, sections were washed 3× in TBS-T and then blocked in TBS-BSA. The secondary antibody (to the mouse primaries) and an Alexa Fluor 488-tetraimide from a tyramide signal amplification kit (Invitrogen, catalog #T20912) were used according to the manufacturer’s directions. After additional washes in TBS-T, the sections were blocked again in TBS-BSA and incubated with directly conjugated Alexa Fluor 394-monoclonal Ab to GFAP (Cell Signaling Technology, catalog #8152) at a 1:100 dilution overnight at 4°C. After washes in TBS-T, the sections were mounted on gelatin-coated microscope slides and coveredslips with DAPI-containing Prolong Gold Antifade Mounting Medium (Invitrogen, catalog #P36931). Fluorescent images were obtained on a confocal microscope (Nikon Eclipse Ti) and contralateral and ipsilateral sides were taken at the same settings so that quantitative comparisons could be made between the two sides. Images shown are single plane projections of focused stacks of images. NFAT4 nuclear/cytosolic localization images (taken with a 100× objective) were shown are single plane projections of focused stacks of images. NFAT4 nuclear/cytosolic localization images (taken with a 100× objective) were measured by personnel blind to the experimental condition. Cytosolic localization images were obtained from the UPENN virus core. Vectors expressed EGFP alone or EGFP fused to the NFAT inhibitory peptide VIVIT. Transgene expression was measured by personnel blind to the experimental condition. Cytosolic and nuclear NFAT4 fluorescence intensities were quantified by Imaris 8.1.3 software.

**AAV delivery.** AAV2/5 vectors constructed using rep and cap genes from AAV2 and AAV5 serotypes, respectively (Hildinger et al., 2001), were obtained from the UPENN virus core. Vectors expressed EGFP alone or EGFP fused to the NFAT inhibitory peptide VIVIT. Transgene expression was under the control of the human GFAP promoter (Gfα2) isolated and characterized by Dr. Michael Brenner’s laboratory at the University of Pennsylvania first with a 10× objective (Furman et al., 2012) and then with a 200× objective (Furman et al., 2012). Methods for the bilateral delivery of AAV vectors to rat hippocampus were adapted, with slight modifications, from our previous work on mice (Furman et al., 2012). Briefly, rats were placed in a stereotaxic frame under isoflurane anesthesia as described above. After a midline incision, holes were drilled bilaterally and needles attached to separate Hamilton syringes containing AAV vectors (1012 ifu/ml) were lowered into the hippocampus and injected at a rate of 0.2 μl/min (8 μl total) using a stereotaxic microinjector (Stoelting). Syringe needles were left in place for 2 min after completion to limit reflux. Coordinates for injection relative to bregma were +3.8 mm anteroposterior, +1.8 mm mediolateral, and −2.8 mm dorsoventral. Wounds were closed and rats were returned to their home cages for ~8 weeks before receiving a unilateral CCI injury, as described above.

**Astrocyte immunodepletion and AAV specificity.** At 4 weeks after AAV-Gfα2-EGFP injection, some rats were anesthetized under CO2 and decapitated, with hippocampi dissected away and stored in ice-cold Ca2+/Mg2+-free PBS solution. Hippocampal tissue then was minced finely in a Petri dish, treated with 0.125% trypsin-EDTA, and transferred to a conical tube (in a total volume of 2 ml) using a 100 μl pipet tip. After 15 min at room temperature, the cells were pelleted at 1000 g for 10 min, resuspended in 1 ml of 2× sample buffer, heated at 65°C for 20 min, and then transferred to separate lanes of a 4–20% Criterion gradient gel (Bio-Rad) for SDS-PAGE. The gel was then transferred to Immobilon-FL PVDF membranes (Millipore, catalog #IPFL 00010). Western blots were performed using primary antibodies to GFAP (rabbit; Cell Signaling Technology), EGFP (rabbit; Abcam), and β-actin (mouse; Sigma-Aldrich).

**Preparation of hippocampal slices.** Methods for preparing hippocampal slices from brain-injured rats are nearly identical to our previous work (Norris and Scheff, 2009). Briefly, at 7 d after CCI, rats were killed under CO2 anesthesia and decapitated. Brains were rapidly removed and placed in ice-cold, oxygenated (95% O2, 5% CO2) artificial CSF (ACSF) containing the following (in mM): 124 NaCl, 2 KCl, 1.25 KH2PO4, 2 MgSO4, 0.5 CaCl2, 26 NaHCO3, and 10 dextrose, pH 7.4. Hippocampi were bilaterally dissected and sectioned coronally into ~400–450 μm slices using a McIlwain Tissue Chopper (Stoelting). Slices were then transferred to a custom interface holding chamber (Mathis et al., 2011) and incubated with warmed (32°C) oxygenated ACSF containing 2 ms CaCl2 until electrophysiological recordings (usually 1.5–5 h).

**Electrophysiological analysis of CA1 synaptic function.** Slices were submerged in oxygenated ACSF (32°C) and perfused at a rate of 1–2 ml/min in an RC22 chamber (Warner Instrument). Schaffer collaterals were activated with a bipolar platinum/iridium electrode located in stratum radiatum near the CA3–CA1 border. Stimulus intensity was controlled by a constant current stimulus isolation unit (World Precision Instruments) and stimulus timing was controlled by Clampex 9.2 software (Molecular Devices). Field potentials were recorded in CA1 stratum radiatum using a glass micropipette (1–6 MΩ) and filled with ACSF containing an Ag/AgCl wire. Physiologic activity was amplified 1000×, Bessel-filtered at 1 kHz, and digitized at 10 kHz using a Multiclamp 700B amplifier and a Digidata 1320 digitizer (Molecular Devices).

To assess basal synaptic strength, 100 μs stimulus pulses were given at nine intensity levels (range 30–300 μA) at a rate of 0.1 Hz. Five field potentials at each level were averaged, and measurements of fiber volley (FV) amplitude (in millivolts) and EPSP slope (in millivolts/millisecond) were performed offline using Clampfit software (Molecular Devices). FV amplitudes were plotted against stimulation intensity to estimate the relative level of presynaptic excitability and EPSP slope measurements were plotted against their corresponding FV amplitudes to estimate the relative strength of CA3-CA1 synapses. Synaptic strength curves for each hemisphere were fit (SigmaPlot 12; Systat Software) with a sigmoidal equation of the form:
Where \( a \) equals the maximal amplitude of the curve, \( b \) equals the curve slope, \( x \) equals the FV amplitude, and \( x_0 \) equals the FV amplitude required for half-maximal EPSP amplitude. Dynaptic strength was also estimated by averaging the EPSP/FV ratio across the top three stimulus levels of the input output curve.

Methods for inducing and measuring LTD are similar to those used in our previous work (Norris et al., 1996; Sama et al., 2012; Norris et al., 2016). After input/output curves, single-stimulus pulses were delivered at a rate of 0.033 Hz to establish a baseline. Although the \( \frac{1}{2} \) maximal stimulus intensity from the input/output curve is often used for setting the baseline and LTD stimulation levels, we instead used the stimulus intensity necessary to elicit an \( \sim 1 \) mV EPSP. This procedure was implemented to minimize possible hemispheric differences in the level of postsynaptic depolarization achieved during LTD induction (see discussion in Norris and Scheff, 2009; Mathis et al., 2011). It is well established that the induction of both LTD and long-term potentiation (LTP) are strongly dependent on postsynaptic depolarization levels (Malinow and Miller, 1986; Wigstrom and Gustafsson, 1986; Mulkey and Malenka, 1992). We have found that, relative to the contralateral hemisphere, slices from the ipsilateral hippocampus typically show reduced EPSP amplitudes (less depolarization) in response to the same level of presynaptic stimulation at 7 d after CCI (Norris and Scheff, 2009). Using the \( \frac{1}{2} \) maximal stimulus intensity in the CCI model would therefore necessarily lead to the elicitation of smaller EPSP amplitudes during LTD stimulation for ipsilateral relative to contralateral slices, which may in turn negatively affect the extent to which postsynaptic plasticity mechanisms are engaged.

After a stable baseline period of no less than 20 min, slices received a 15 min train of 1 Hz stimulation at baseline stimulation intensity, followed by another 60 min baseline period (stimulation rate = 0.033 Hz). Within each hemisphere, EPSP measures from the last 10 min of the post-1 Hz stimulation baseline were averaged across slices and expressed as a percentage of the pre-1 Hz stimulation baseline level. Therefore, for all electrophysiologic biomarkers, \( n \) is equal to the number of rats used rather than the number of slices.

Western blot. On day 7 of recovery from CCI injury, hippocampi (contralateral control and ipsilateral injured) were harvested, put into Microfuge tubes, and snap frozen in liquid \( N_2 \). Samples were stored at \( \sim 80^\circ \)C until needed. Samples were homogenized in PBS with a panel of protease and phosphatase inhibitors as described previously (Ansari et al., 2013). Lowry protein assays were performed to normalize protein levels and 25–75 \( \mu \)g of protein was used per gel well (depending on the antibody used for Western analysis). Proteins were resolved on 4–20% Criterion gradient gels (Bio-Rad) and transferred to Immobilon-FL PVDF membranes (Millipore, catalog #IPFL 00010). After transfer, membranes were blocked in 50% Odyssey protein blocking reagent (LI-COR) in PBS with 0.2% Tween overnight at 4°C on a shaking platform. Westerns were performed with the following primary antibodies: GFAP (Cell Signaling Technology, catalog #36705), Iba1 (Wako, catalog #019-19741), PSD-95 (Cell Signaling Technology, catalog #24505), synapsin-1 (Cell Signaling Technology, catalog #67105), GluR1 (Millipore, catalog #AB5104), NR2A (Millipore 07-632), NR2B (Millipore, catalog #05-920), CNAta (Millipore, catalog #07-1492), GAPDH (Abbcom, catalog #ab9484), hevin (R&D Systems, catalog #MAB2836), and SPARC (R&D Systems, catalog #MAB942). Membranes were then washed in PBS/0.1% Tween, incubated in LI-COR 800-conjugated anti-mouse and 680-conjugated anti-rabbit secondary antibodies, washed, and imaged on an Odyssey Infrared Imaging System (LI-COR) on both the green (800) and red (680) channels. Quantification of bands was performed using Image Studio 2.1 software (LI-COR). Expression was normalized to loading control GAPDH for each sample.

Statistical analyses. Unless otherwise noted, outcome measures were assessed in both the contralateral (uninjured) and ipsilateral (injured) hemispheres and compared across treatment groups using repeated-measures ANOVA. When appropriate, Fisher’s protected least significant difference test was used for post hoc analyses. Significance for all statistical comparisons was set at \( p \leq 0.05 \).

Results

Upregulation of NFAT expression is isoform and cell-type specific

The role of CN/NFAT signaling in TBI was assessed using a unilateral CCI model, which results in a substantial loss of CA3 neurons (Baldwin et al., 1997) and CA3–CA1 synaptic contacts in stratum radiatum of the ipsilateral hemisphere, followed by the partial reinnervation of CA1 during the first few weeks after injury (Scheff et al., 2005). Significant changes in CN expression and/or activity have also been reported in this and other models of acute injury (Huang et al., 2005; Liu et al., 2005; Shioda et al., 2006; Bales et al., 2010; Rosenkranz et al., 2012), although the nature of the change shows considerable variability from study to study. All end point measures were taken at 7 d after CCI, at which time CA3 presynaptic input into CA1 of the ipsilateral hemisphere has plateaued (and is functionally comparable to the contralateral hemisphere), but CA1 postsynaptic strength is still impaired (Norris and Scheff, 2009; Norris et al., 2016).

Figure 1, A and B, shows the appearance of a 45–48 kDa CN fragment in the hippocampus, ipsilateral to CCI, at 7 d after injury \( p < 0.05, n = 5 \). This fragment is similar in size to the constitutively active CN (aCN) proteolytic fragment expressed in rat primary hippocampal cultures exposed to excitotoxic insults (Liu et al., 2005) or oligomeric Aβ peptides (Mohammad Abdul et al., 2011) (also see Fig. 1A, 45 kDa aCN lane). Previous work has shown that aCN can appear at high levels in nervous tissue of intact rodents as a result of acute injury (Huang et al., 2005; Liu et al., 2005; Shioda et al., 2006), elevated amyloid levels (Abdul et al., 2009), or progressive neurodegeneration (Wu et al., 2010; Mohammad Abdul et al., 2011), where it may contribute to synaptic deficits, cell death, and neuroinflammation, among other pathologic processes (Norris, 2014).

To investigate possible changes in the CN-dependent transcription factor NFAT, we used an electrophoretic mobility shift assay (EMSA) based on a well characterized NFAT-binding oligonucleotide probe derived from the IL4 enhancer region (Neal and Clipstone, 2001). Using IRDye-labeled probe, we observed a prominent protein-DNA complex (blue arrowheads, Fig. 1C) and a more variable and faint complex (black arrowheads, Fig. 1C) in hippocampal tissue fractions from both hemispheres of injured \( (n = 8) \) and sham \( (n = 5) \) rats at 7 d after injury. Both of these bands were displaced when EMSAs were performed in the presence of unlabeled wild-type probe, but not with a mutant probe, confirming that observed bands reflect NFAT-specific binding. The intensity of the higher-molecular-weight complex (blue arrowheads) was increased in the ipsilateral relative to the contralateral hemisphere of injured, but not sham-operated, rats at 7 d after CCI \( (p < 0.05) \). Supershift/blockshift experiments were then performed to identify which CN-dependent NFAT isoform(s) (NFATs 1–4) gave rise to the DNA-protein complexes. The NFAT1 antibody caused a clear supershift, indicated by the red asterisk in Figure 1C. The NFAT2 and 3 antibodies did not perceptibly alter the basic EMSA, indicating that they were not part of the complex. Finally, the NFAT4 antibody completely blocked the formation of the DNA–protein complex (see blue asterisk in Fig. 1C for where the complex normally migrates). For purposes of quantification, each “pair” of contralateral control and ipsilateral hippocampal homogenates was assayed on one gel.
Because the NFAT4 antibody entirely blocked the formation of the DNA-protein complex (indicated by the blue arrow), this band was quantified and used to estimate total NFAT4 binding. For NFAT1, the supershifted band, indicated by the red asterisks, was quantified for each sample set. The quantitative data are shown in Figure 1D. There was a clear and significant increase in the DNA-binding activities of NFATs 1 and 4 in the ipsilateral hippocampi of the CCI animals, with little change observed across hemispheres in the shams.

Previous work has shown that NFATs 1 and 4 can appear in astrocytes as the result of injury or disease (Abdul et al., 2009; Serrano-Pérez et al., 2011; Caraveo et al., 2014). To determine whether the expression differences for NFATs 1 and 4 revealed by EMSA analyses (Fig. 1C,D) are associated with activated astrocytes after CCI, we colabeled coronal sections from injured rats (7 d after CCI) with NFAT and GFAP antibodies and imaged sections using confocal microscopy. As shown in Figure 2, we observed a striking increase in GFAP labeling (shown in red) in the hippocampus of the ipsilateral hemisphere, consistent with robust injury-dependent astrocyte activation. Although both NFAT isoforms exhibited diffuse labeling in the hippocampus of the contralateral hemisphere, there were clear differences in the distribution of the two isoforms in regard to cell type expression and injury. NFAT1 showed very little colocalization with GFAP-positive astrocytes in any region of the hippocampus examined regardless of hemisphere (Fig. 2A). Interhemispheric differences in NFAT1 labeling were characterized primarily by a shift to a punctate labeling pattern in the ipsilateral hippocampus and may be associated with proliferating microglia or infiltrating peripheral cells. In contrast, NFAT4 showed extensive colocalization with GFAP-positive astrocytes, especially in CA1 (Fig. 2B). In the ipsilateral hippocampus, the labeling intensity for NFAT4 was generally increased in proportion to the increase in GFAP labeling intensity. There was little evidence for the expression of either NFAT isoform in neuronal cell bodies, particularly in pyramidal neurons.

Interestingly, NFAT4/GFAP colocalization showed marked heterogeneity across different regions of the hippocampus (Fig. 3). NFAT4 colocalization with GFAP was extensive in the stratum lacunosum/moleculare, stratum radiatum, and stratum oriens of CA1 (Fig. 3A–C), as well as the molecular layers and hilar regions of the dentate gyrus (Fig. 3M, N), but was much less apparent in the neuronal cell body layers (Fig. 3, cf. H vs I, N vs O). Moreover, in contrast to an earlier study that used a kainic acid insult (Serrano-Pérez et al., 2011), we did not observe extensive colocalization of NFAT4 in astrocytes of CA3 even though
numerous activated astrocytes were found in this region after injury (Fig. 3 B, J–L). This discrepancy may be attributable to differences in the type of cellular degeneration occurring after kainic acid treatment versus CCI. Regardless, these observations suggest possible phenotype differences between astrocytes in different hippocampal subregions.

Use of AAV-Gfa2-VIVIT to inhibit astrocytic CN/NFAT signaling selectively in intact rats

To investigate the impact of astrocytic CN/NFAT signaling on functional outcome after TBI, we used an AAV-mediated gene delivery approach. Adult rats received bilateral hippocampal injections of AAV2/5 vectors expressing the NFAT inhibitor VIVIT (coupled to EGFP) or EGFP alone (control, CT). Some rats received identical injections of vehicle as an additional control. At approximately 8 weeks after AAV treatment, rats were subjected to a moderate CCI injury (Fig. 4A). To limit transgene expression to astrocytes, AAV vectors expressed the human GFAP promoter (Gfa2) (de Leeuw et al., 2006; Lee et al., 2008). As shown in our earlier work, a single injection (8 µl) of high titer (10^{12} ifu/ml) AAV-Gfa2 vectors into the hippocampus results in wide spread infection across all hippocampal subregions (Fig. 4B). Because CN is ubiquitously expressed and found at particularly high levels in hippocampal neurons, it was important to verify that AAV-Gfa2-mediated transgene expression occurred exclusively in astrocytes. Confocal microscopy was used to inspect the possible colocalization of AAV-dependent EGFP expression with neurons (MAP2b; Fig. 4E,F) and astrocytes (GFAP; Fig. 4D,G). As shown in Figure 4G, EGFP expression in CA1 strongly colocalized with GFAP (Fig. 4G,H), but there was no overlap between EGFP and MAP2b (Fig. 4F,H). Nearly identical observations were made in other hippocampal subregions as well (data not shown).

To further confirm that AAV-Gfa2 vectors selectively targeted astrocytes, we performed immunodepletion experiments (Fig. 4I). Hippocampi from AAV-Gfa2-EGFP-infected rats were extracted and triturated into single-cell suspensions, which were then placed in a Microfuge tube and treated with GLAST antibodies (to tag astrocytes) coupled to metal Dynabeads. Tubes were then placed in a magnetic rack to pull out bead-coupled cells from the suspension. The fraction bound to the beads is referred to as AE, whereas the unbound fraction is referred to as AD. Some suspensions were passed through the same protocol, but were not treated with GLAST antibodies and served as MD and ME control fractions. Western blots were then performed to determine which fraction contained EGFP.

As shown in Figure 4J, the astrocyte-specific marker GFAP was found in the MD fraction (as expected) and also in the AE fraction, but not in the AD fraction. The prominent appearance of β-actin (which is expressed across all cell types) in the AD fraction demonstrates that the lack of GFAP expression was not attributable to a simple loss of total protein. These observations validate the successful immunodepletion of astrocytes in this protocol. If transgene expression after AAV-Gfa2 treatment is selective to astrocytes, then EGFP should show a similar expression pattern to GFAP, which is exactly what was observed: EGFP appeared in MD and AE fractions, but not in AD fractions. Essentially the same results were observed in four separate animals. Combined with the confocal images shown in Figure 4, C–H, these data demonstrate that AAV-Gfa2 vectors selectively target astrocytes.

The VIVIT peptide, expressed by AAV-Gfa2 vectors, is derived from a critical endogenous CN-docking site (P*IXIT) found in the regulatory subregion of NFATs 1–4 (Aramburu et al., 1999). Numerous studies have shown that VIVIT inhibits the nuclear localization or the transcriptional activity of NFATs in diverse tissues and cell types (Yu et al., 2007). Previously, we showed that VIVIT inhibited NFAT activity in primary astrocytes treated with proinflammatory or cytotoxic factors (Sama et al., 2008; Abdul et al., 2009). Confocal micrographs in Figure 4K show the cellular localization of NFAT4 (red) in EGFP- or VIVIT-expressing astrocytes of AAV-infected rats. Note that nuclei are labeled blue (DAPI) and that
the EGFP signal (green) has been removed for clarity. In EGFP-expressing astrocytes, NFAT4 is mostly present in the cytosol, but also appears throughout the nucleus (blue) in many cells (Fig. 4K, arrows). In contrast, NFAT4 expression in VIVIT-expressing astrocytes is largely excluded from the nucleus (Fig. 4K, arrowheads), consistent with NFAT inhibition. The average nuclear-to-cytosolic ratios for NFAT4 measured from 32–42 cells per treatment condition across three animals per group (10–16 cells per rat) are shown in Figure 4L. Therefore, AAV-Gfa2 vectors not only provide selective targeting of astrocytes (Fig. 4C–J), they provide an effective way to limit the nuclear localization and activation of NFATs in these cells.

**AAV-Gfa2-VIVIT protects synaptic function in ipsilateral hippocampus**

The rat unilateral CCI model is associated with extensive loss of synaptic contacts in the CA1 region of the ipsilateral hippocampus (Scheff et al., 2005). Although there is a remarkable degree of structural and functional recovery with time after injury, deficits in basal synaptic strength are still evident at 7 d after injury in this model (Norris and Scheff, 2009), providing a reasonable time point to investigate the impact of astrocytic CN/NFAT signaling on synaptic function. One advantage of the rat CCI model, compared with mouse models or fluid-percussion injury models, is that moderate injuries to the ipsilateral hemisphere in adult rats (as investigated here) cause very few measurable changes in the contralateral hemisphere. For instance, previous work on oxidative stress markers after unilateral CCI in rats found no changes in the contralateral hemisphere of injured rats compared with either hemisphere of sham-operated rats or even to naive rats (Gilmer et al., 2009). Multiple presynaptic and postsynaptic protein markers, found at reduced levels in the ipsilateral hippocampus of rats after CCI, are also unchanged in the contralateral hemisphere compared with sham-operated controls (Ansari et al., 2013; Scheff et al., 2013). Similarly, our previous work using electrophysiological measures of synaptic function taken at 2, 7, and 14 d after CCI showed comparable levels of basal synaptic strength and plasticity in the contralateral hemisphere of injured and sham-operated rats (Norris and Scheff, 2009). These results support the validity of using the contralateral hemisphere as a within-animal control for synaptic measures after unilateral CCI in rats.

To determine the extent to which astrocytic CN/NFAT signaling influences synaptic function after CCI, we measured basal hippocampal synaptic strength in slices from ipsilateral and contralateral hippocampi of AAV-treated rats (n = 6–8 rats/group) at 7 d after injury (Fig. 5A–D). Field potentials were recorded in stratum radiatum of area CA1 in response to electrical stimulation of CA3 Schaffer collaterals. Synaptic strength curves were generated by plotting EPSP slope values against FV amplitudes across increasing stimulus intensities. As shown in Table 1, all synaptic strength curve parameters investigated here were statistically comparable across the contralateral hemisphere of injured, sham-operated, and naive adult (~2-month-old) Sprague Dawley rats.

Hippocampal slices from the ipsilateral hemisphere of AAV-Gfa2-EGFP control rats showed a downward shift in the synaptic strength curve relative to the contralateral hemisphere within the same animals (Fig. 5A). Moreover, the EPSP-FV ratio for the ipsilateral hemisphere was reduced in AAV-Gfa2-EGFP control rats by ~130% relative to the contralateral side (p < 0.01), indicative of reduced synaptic strength (Fig. 5D). In contrast, synaptic strength curves were qualitatively and quantitatively similar across hemispheres in rats pretreated with AAV-Gfa2-VIVIT-EGFP (Fig. 5B) and strongly resembled synaptic strength curves recorded in untreated sham rats (Fig. 5C) and naive rats (Table 1). The EPSP-FV ratio in AAV-Gfa2-VIVIT-EGFP rats showed only a 23% reduction in the ipsilateral hemisphere, which was similar to the ipsilateral hemisphere of sham rats (~4% reduction) and significantly less (p < 0.05) than the reduction found in AAV-Gfa2-EGFP control rats (Fig. 5D).

We next investigated the extent to which astrocytic CN/NFAT signaling influences synaptic plasticity after TBI. Although LTP is commonly measured in injury and disease...
To confirm these observations, we used a similar 1 Hz stimulus paradigm to induce LTD in the ipsilateral and contralateral hemispheres of injured rats (7 d after CCI) and sham-operated rats in the absence of AAV treatment. As shown in Figure 5E, slices from sham rats and slices from the contralateral hemisphere of injured rats showed no significant depression (96% and 101% of baseline, respectively) after 1 Hz stimulation. In contrast, the same stimulus paradigm caused a modest (82% of baseline) but significant (p < 0.01) depression in ipsilateral slices from injured rats, confirming that CCI increases the susceptibility to LTD in the ipsilateral hemisphere. Similar to these results, ipsilateral slices from AAV-Gfa2-EGFP–treated rats showed significant LTD (~83% of baseline, p < 0.05) after 1 Hz stimulation (Fig. 5F). In contrast, LTD was completely prevented in ipsilateral slices from rats treated with AAV-Gfa2-VIVIT (103% of baseline). These results suggest that the astrocytic CN/NFAT pathway helps to drive synapse dysfunction in the ipsilateral hippocampus after CCI injury.

**AAV-Gfa2-VIVIT prevents the loss of synapse-related proteins in ipsilateral hippocampus**

In addition to impaired synaptic function and altered plasticity, CCI is also associated with the loss of several key synaptic proteins in the ipsilateral hippocampus within the first week after injury, including the postsynaptic anchoring protein PSD-95, the presynaptic vesicle docking protein synapsin 1, AMPA/kainate-type glutamate receptor isoforms (e.g., GluR1), and NMDA-type glutamate receptor...
Figure 5. Effects of AAV-Gfa2-VIVIT on synaptic strength and plasticity. A, B, CA1 synaptic strength curves constructed from the mean ± SEM EPSP slope versus the mean ± SEM FV amplitude for hippocampal slices from the contralateral and ipsilateral hemispheres of rats treated with control (CT) AAV-Gfa2-EGFP (A) or with AAV-Gfa2-VIVIT (B). Slices from contralateral and ipsilateral hemispheres of sham-operated rats (C) are also shown for comparison. Insets in A–C show representative EPSP waveforms in each hemisphere matched on the basis of FV amplitude. Note, that for AAV-Gfa2-EGFP CT rats (A), the synaptic strength curve exhibited a marked downward shift in the ipsilateral relative to the contralateral hemisphere. In contrast, synaptic strength curves across hemispheres are qualitatively and quantitatively similar in sham rats (C) and rats treated with AAV-Gfa2-VIVIT (B). D, Mean ± SD of the EPSP-to-FV ratio in the ipsilateral hemisphere of AAV-treated and sham rats expressed as percentage change from the contralateral hemisphere. A reduction in the EPSP-to-FV ratio is only observed for AAV-Gfa2-EGFP CT rats. \( p < 0.01 \) ipsilateral versus contralateral, Fisher’s PLSD test. E, Left, Time plots showing mean ± SEM EPSP slope values (% baseline) from slices collected from the contralateral and ipsilateral hemispheres of untreated rats (i.e., no AAV) and the ipsilateral hemisphere of sham-operated rats expressed as percentage change from the contralateral hemisphere. For controls, some rats (\( n = 6 \)) received bilateral injections of AAV-Gfa2 expressing EGFP alone, whereas other rats (\( n = 6 \)) received vehicle injections. Because the two control groups showed no significant differences on any of the outcome measures, statistical comparisons were made only for the AAV-Gfa2-EGFP and AAV-Gfa2-VIVIT treatment groups.

For AAV-Gfa2-EGFP-treated rats, the postsynaptic protein PSD-95 and the GluR1 AMPA receptor subunit were each significantly reduced, by 56% and 82%, respectively, in the ipsilateral, relative to the contralateral, hippocampus (Fig. 5A–D and C,F). However, the loss of PSD-95 and GluR1 protein was comparably small (19% and 27%, respectively) for rats treated with AAV-Gfa2-VIVIT. Although levels for synapsin 1 were not reduced in the ipsilateral hippocampus of AAV-Gfa2-EGFP at 7 d after CCI, the AAV-Gfa2-VIVIT group showed a significant 110% increase in the ipsilateral hemisphere (\( p < 0.05 \)) (Fig. 6B,E). Similar to PSD-95 and GluR1, we also observed a significant reduction in NR2A (\( 26 \pm 57\% \) mean ± SD, \( p < 0.05 \)) and a small, nonsignificant reduction in NR2B levels (\( 20 \pm 35\% \) mean ± SD, \( p > 0.05 \)) in AAV-Gfa2-EGFP rats (data not shown). However, these changes were not significantly altered by AAV-Gfa2-VIVIT (NR2A \( 26 \pm 27\% \) mean ± SD, \( p < 0.05 \); NR2B \( 23 \pm 24\% \) mean ± SD, \( p > 0.05 \)). Therefore, together with measures of basal synaptic strength (Fig. 5A–D), the results show that AAV-Gfa2-VIVIT protects against the loss of several key synaptic proteins after CCI.
Fernandez et al., 2007; Nagamoto-Combs and Combs, Sama). Several studies have reported an increase in SPARC and hevin levels in activated astrocytes after injury (Jones et al., 2014). Moreover, a microarray study from our group observed significant changes in hevin and SPARC gene transcripts in primary neuron/astrocyte cultures as a result of CN overexpression (Norris et al., 2005).

Recent advances in genetic approaches, including the use of cell-type-specific promoters to selectively target astrocyte signaling pathways, have greatly increased our understanding of the functional impact of activated astrocytes. Several targeted approaches to limiting astrocyte activation have been reported, including the suppression of astrocyte proliferation (Bush et al., 1999), the deletion of astrocyte intermediate filament proteins (Pekny et al., 1999; Lundkvist et al., 2004), and the knock-down/inhibition of key inflammatory signaling pathways (Okada et al., 2006; Herrmann et al., 2008; Wanner et al., 2013). These manipulations have revealed many apparently protective functions of activated astrocytes after acute CNS trauma; for example, the formation of protective glial scars, support

### Table 1. CA3–CA1 synaptic strength curve parameters (mean ± SD) for naive rats, sham-operated rats, and AAV-treated rats 7 d after CCI

<table>
<thead>
<tr>
<th>Condition</th>
<th>EPSP/FV ratio</th>
<th>Curve amplitude</th>
<th>Curve slope</th>
<th>Curve ½ max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>1.71 ± 0.72</td>
<td>1.31 ± 0.25</td>
<td>0.15 ± 0.05</td>
<td>0.35 ± 0.11</td>
</tr>
<tr>
<td>Sham contra</td>
<td>1.49 ± 0.27</td>
<td>1.20 ± 0.26</td>
<td>0.11 ± 0.03</td>
<td>0.28 ± 0.07</td>
</tr>
<tr>
<td>Sham ipsi</td>
<td>1.58 ± 0.19</td>
<td>1.34 ± 0.16</td>
<td>0.13 ± 0.03</td>
<td>0.33 ± 0.11</td>
</tr>
<tr>
<td>EGFP/CT contra</td>
<td>1.87 ± 0.30</td>
<td>1.40 ± 0.12</td>
<td>0.11 ± 0.03</td>
<td>0.29 ± 0.07</td>
</tr>
<tr>
<td>EGFP/CT ipsi</td>
<td>1.15 ± 0.65</td>
<td>0.78 ± 0.22</td>
<td>0.12 ± 0.06</td>
<td>0.30 ± 0.12</td>
</tr>
<tr>
<td>VIVIT contra</td>
<td>1.66 ± 0.23</td>
<td>1.42 ± 0.64</td>
<td>0.12 ± 0.05</td>
<td>0.32 ± 0.14</td>
</tr>
<tr>
<td>VIVIT ipsi</td>
<td>1.81 ± 0.43</td>
<td>1.41 ± 0.70</td>
<td>0.12 ± 0.06</td>
<td>0.37 ± 0.13</td>
</tr>
</tbody>
</table>

n = 6–10 rats/group.

*p < 0.05 versus EGFP/CT contra, Fisher’s PLSD.

**Figure 6.** AAV-Gfa2-VIVIT protects against the loss of synaptic proteins. A–C, Representative Western blots and mean ± SD levels (D–F) for hippocampal synaptic proteins in the contralateral and ipsilateral hemispheres of rats treated with AAV-Gfa2-EGFP (CT) or AAV-Gfa2-VIVIT. Protein levels in D–F are from the ipsilateral hemisphere and are expressed as the percentage change from the contralateral hemisphere. The percentage change in the ipsilateral versus contralateral hemisphere of sham controls is also provided. Levels for PSD95 and GluR1 showed significant reductions in the ipsilateral hemisphere of AAV-Gfa2-EGFP rats (*p < 0.05 ipsilateral versus contralateral, Fisher’s LSD test, but not in the ipsilateral hemisphere of VIVIT-treated rats. Although Syn1 was not significantly reduced with injury, protein levels in the ipsilateral hemisphere were significantly increased by pretreatment with AAV-Gfa2-VIVIT. *p < 0.05 ipsilateral versus contralateral, Fisher’s LSD test, n = 5–6 rats per group.

**Figure 7.** AAV-Gfa2-VIVIT does not reduce glial activation after CCI, but does modulate astrocyte-related proteins involved in synaptic remodeling.

Our work, and work from others, has shown that the CN/NFAT pathway is an important regulator of the activated phenotypes of both astrocytes (Norris et al., 2005; Fernandez et al., 2007; Sama et al., 2008; Serrano-Perez et al., 2011; Furman et al., 2012) and microglia (Kataoka et al., 2009; Nagamoto-Combs and Combs, 2010; Shiratori et al., 2010). We previously showed that AAV-Gfa2-VIVIT protects synaptic function in a chronic, progressive disease model (APP/PS1 mice) in parallel with reductions in GFAP and Iba1 (a microglial protein). As shown in Figure 7, A–D, we performed similar measures on GFAP and Iba1 protein levels in the rat CCI model. Although we did observe significant injury-related elevations for both proteins, consistent with robust glial activation, neither protein marker was sensitive to AAV-Gfa2-VIVIT treatment. These results suggest that the synaptic protective effects of AAV-Gfa2-VIVIT after acute injury do not require a reduction in glial activation.

**Discussion**

**Astrocyte activation and the CN/NFAT pathway after acute injury**

Recent advances in genetic approaches, including the use of cell-type-specific promoters to selectively target astrocyte signaling pathways, have greatly increased our understanding of the functional impact of activated astrocytes. Several targeted approaches to limiting astrocyte activation have been reported, including the suppression of astrocyte proliferation (Bush et al., 1999), the deletion of astrocyte intermediate filament proteins (Pekny et al., 1999; Lundkvist et al., 2004), and the knock-down/inhibition of key inflammatory signaling pathways (Okada et al., 2006; Herrmann et al., 2008; Wanner et al., 2013). These manipulations have revealed many apparently protective functions of activated astrocytes after acute CNS trauma; for example, the formation of protective glial scars, support
The present study sought to inhibit astrocyte activation in the rat CCI model by selectively inhibiting the CN/NFAT pathway in astrocytes. In many cell types, CN/NFATs are key transcriptional regulators capable of driving striking changes in cellular phenotype (Crabtree and Olson, 2002; Horsley and Pavlath, 2002; Hogan et al., 2003; Wilkins and Molkentin, 2004; Hassoun et al., 2009). Although less attention has focused on CN/NFAT signaling in the CNS, mounting evidence suggests that CN/NFATs are associated with phenotype changes (i.e., activation) in astrocytes (Fernandez et al., 2007; Sama et al., 2008; Serrano-Perez et al., 2011; Furman et al., 2012) and microglia (Kataoka et al., 2009; Nagamoto-Combs and Combs, 2010; Shiratori et al., 2010). In primary cultures, CN/NFAT induces numerous immune/inflammatory pathways, many of which are suppressed by the NFAT inhibitor VIVIT (Sama et al., 2008; Nagamoto-Combs and Combs, 2010). Previously, we showed that VIVIT expression in astrocytes over the course of 10–11 months using AAV-Gfa2 vectors was associated with a reduction in biochemical and morphological markers of glial activation in a mouse model (APP/PS1) of AD (Furman et al., 2012). Similar to cell culture studies, the effects of AAV-Gfa2-VIVIT in APP/PS1 mice were beneficial in nature and included improved synaptic function and cognition, suggesting that astrocyte activation can exacerbate neural dysfunction, at least in the context of progressive amyloid pathology.

Together, these studies provided seemingly strong rationale for using AAV-Gfa2-VIVIT to test the impact of activated astrocytes on neural function in an intact animal model of TBI. To our surprise, however, AAV-Gfa2-VIVIT had little to no effect on GFAP or Iba1 levels in brain-injured rats. If anything, AAV-Gfa2-VIVIT precipitated an increase in Iba1 levels in ipsilateral hippocampus, consistent with greater microglial activation. This apparent discrepancy with earlier findings in APP/PS1 mice could indicate fundamental differences in the activated glial phenotype(s) associated with acute injury versus progressive neuropathology (i.e., amyloid deposition). Indeed, there is strong evidence to suggest that multiple phenotypic effects of AAV-Gfa2-VIVIT, in the face of robust glial activation, neuroinflammation in general, is greater in chronic disease conditions relative to acute injury. Regardless, the synaptoprotective effects of AAV-Gfa2-VIVIT, in the face of robust glial activation, suggests that the astrocytic CN/NFAT pathway influences neural function through mechanisms that are independent of classic neuroinflammatory signaling mediators.

**Effects of AAV-Gfa2-VIVIT on synaptic function markers**

Although the impact of AAV-Gfa2-VIVIT on glial activation appeared markedly different in the rat CCI model compared with APP/PS1 mice, the beneficial effects of VIVIT treatment on synaptic function were highly similar. The large reduction in synaptic strength measured in ipsilateral slices from AAV-Gfa2-EGFP control rats was essentially absent in AAV-Gfa2-VIVIT-treated rats. AAV-Gfa2-VIVIT also prevented the loss (or hastened the recovery) of several synaptic proteins and reduced susceptibility to LTD in injured hippocampus. Although the specific mechanisms linking astrocytic CN/NFATs to impaired synaptic function remain unsettled, Western blots suggested a possibly important role for hevin, an astrocyte-secreted protein that regulates physical interactions between cells and the extracellular matrix in a variety of tissues (Sullivan et al., 2004; Jones and Bouvier, 2014). The dynamic interactions between hevin and SPARC have been proposed as a critical mechanism for fine tuning synaptic networks during development.

**Figure 7.** AAV-Gfa2-VIVIT does not alter GFAP or Iba1 levels, but causes an increase in hevin levels. Representative Western blots (A, C) and mean ± SD. GFAP and Iba1 protein levels (B, D) in the contralateral and ipsilateral hippocampus of AAV-treated rats at 7 d after CCI. Note that both glial markers showed a significant increase in the hippocampus of the ipsilateral hemisphere, but were not significantly altered by pretreatment with AAV-Gfa2-VIVIT. #p < 0.001 ipsilateral versus contralateral, Fisher’s PLSD. n = 5–6 rats. Group. E–H, Representative Western blots (E, G) and mean ± SD SPARC and hevin protein levels (F, H) in the contralateral and ipsilateral hippocampus of AAV-treated rats at 7 d after CCI. No virus or injury-dependent effects were observed for SPARC. In contrast, hevin was sensitive to both injury and AAV treatment. In both AAV groups, hevin was elevated in the ipsilateral relative to the contralateral hemisphere. Overall hevin levels were greater in the VIVIT-treated group regardless of hemisphere, but were highest in the injured hemisphere. *p < 0.05; #p < 0.001 ipsilateral versus contralateral, Fisher’s LSD, n = 5–6 rats.

of the blood–brain barrier, myelin preservation, and/or the protection of neuronal viability. In contrast, other reports have suggested that activated astrocytes have a detrimental impact on nervous tissue. For instance, suppression of astrocyte activation via the combined knock-down of GFAP and vimentin led to improved synaptic recovery in the outer molecular layer of the dentate gyrus after an entorhinal cortex lesion (Wilhelmsson et al., 2004). These observations highlight the complexity and possibly the heterogeneity of astrocyte activation during injury and disease.
In addition to hevin, there are many other possible astrocyte-based mechanisms that could link CN/NFAT signaling to synaptic function. For instance, complement components such as C3 are associated with astrocyte activation (Tomimoto et al., 1997; Cardinaux et al., 2000) and synapse loss (Stevens et al., 2007) and are sensitive to CN activity (Norris et al., 2005). Astrocytes also protect neuronal viability and fine tune synaptic transmission by removing excess glutamate from the extracellular milieu (Satller and Rothstein, 2006), a process that is protected by VIVIT in primary culture models of neuroinflammation and amyloid toxicity (Sama et al., 2008; Abdul et al., 2009). Alternatively, astrocytic CN/NFAT signaling may stimulate the release of excitotoxict factors that disrupt neuronal Ca$^{2+}$ dysregulation, which in turn lead to synapse dysfunction, particularly in regard to the expression of synaptic plasticity. LTD, for example, is normally absent in hippocampal slices from healthy adult rodents when the bath Ca$^{2+}$/Mg$^{2+}$ concentrations are at a one-to-one ratio (O’Dell and Kandel, 1994; Norris et al., 1996; Vouimba et al., 2000; Foster and Kumar, 2007; Shankar et al., 2008; Dumas, 2012), but is much easier to induce in slices under conditions that promote Ca$^{2+}$ dysregulation such as aging, injury, and elevated amyloid levels (Norris et al., 1996; Vouimba et al., 2000; Foster and Kumar, 2007; Shankar et al., 2008; Li et al., 2009; Ma et al., 2012; Sama et al., 2012; Norris et al., 2016). Clearly, further studies will be needed to fully characterize NFAT interactions with hevin and multiple other astrocyte-based signaling mechanisms and their roles in synaptic recovery and repair.

NFAT isoforms and astrocyte signaling after CCI

Similar to peripheral tissues, changes in glial signaling may hinge upon the expression of specific NFAT isoforms. NFATs 1, 2, and 4 have each been implicated in the production of proinflammatory cytokines and chemokines in glial cells (Canellada et al., 2008; Perez-Ortiz et al., 2008; Nagamoto-Combs and Combs, 2010; Neria et al., 2013). Previously, we observed elevated levels of NFAT1 activation in astrocytes in human hippocampus at early stages of cognitive decline associated with AD (Abdul et al., 2009). Other groups have observed an increase in the localization of NFAT4 to hippocampal astrocytes, particularly after acute injury (Serrano-Pérez et al., 2011; Neria et al., 2013; Yan et al., 2014). Consistent with these reports, the present study found that NFAT–DNA interactions were increased in the hippocampus after unilateral CCI and attributable largely to the activities of NFATs 1 and 4. However, confocal micrographs showed that the expression of NFAT4 is far more selective for astrocytes. Unlike our work on human tissue, we found little to no evidence linking NFAT1 to activated astrocytes in brain-injured rats. It’s possible that NFAT1 is primarily associated with microglia and/or infiltrating immune cells in rodents or that its activity is strongly stimulated in astrocytes at earlier time points after injury, but then recedes with time. There may also be significant differences in the expression and function of different NFAT isoforms across rodents and humans. Further work using biochemical and fluorescence microscopy techniques will be needed to evaluate these possibilities and to identify the cell types that express NFAT1 after injury.

References


Blakely PK, Hussain S, Carlin LE, Irani DN (2015) Astrocyte matricellular proteins that control excitatory synaptogenesis are regulated by inflam-
matory cytokines and correlate with paralysis severity during experimental autoimmune encephalomyelitis. Front Neurosci 9:344. CrossRef Medline


Norris CM (2014) Calpain interactions with the protein phosphatase cal-
cineurin in neurodegeneration. Advances in Biochemistry in Health and Disease 8:17–45.