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Differential Effect of Traumatic Brain Injury on the Nuclear Factor of Activated T Cells C3 and C4 Isoforms in the Rat Hippocampus

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Abstract

The interaction between the phosphatase calcineurin and transcription factor nuclear factor of activated T cells (NFAT) plays an important role numerous signaling and the regulatory events. Although NFAT is mostly known for its transcription function in the immune system, NFAT also has essential functions even in the central nervous system (CNS). The effects of traumatic brain injury (TBI) on NFAT are currently unknown. To determine if there is an alteration in NFAT after TBI, we examined NFATc3 and c4 levels at 6 hours, 1 day, 1 week, 2 weeks and 4 weeks post injury. Rats were anesthetized and surgically prepared for controlled cortical impact (CCI) injury or sham surgery. Semi-quantitative measurements of NFATc3 and c4 in the hippocampal homogenates from injured and sham rats sacrificed at the appropriate time after injury were assessed using Western blot analysis. After TBI insult, in the hippocampus ipsilateral to the injury, NFATc3 expression levels were decreased both in the cytoplasmic and nuclear fractions. However, NFATc4 expression levels were increased in the cytoplasmic fraction but decreased in the nuclear fraction. Double labeling (with NeuN and GFAP) immunohistochemistry revealed that NFATc3 was expressed in subset of astrocytes and NFATc4 was expressed primarily in neurons. These differential responses in NFATc3 and c4 expression after TBI insult may indicate long-term changes in hippocampal excitability and may contribute to behavioral deficits. Further study is warranted to illustrate the role of NFATc3 and c4 in the setting of TBI.

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Keywords

Nuclear factor of activated T cells (NFAT); Immunohistochemistry; Rat; Traumatic brain injury (TBI); Calcineurin

1. Introduction

Nuclear factor of activated T cells (NFAT), a family of transcription factors activated by intracellular increase in calcium (Ca^{2+}) levels, integrates multiple intracellular signaling pathways, and has an important role in the differentiations of various cell types. Traumatic brain injury (TBI) has been documented to produce dysregulation of calcium and downstream signaling cascades (Wallace and Porter, 2011; Zipfel et al., 2000). Also, several studies have reported changes in calcineurin following TBI (Kurz et al., 2005a; Kurz et al., 2005b), but its downstream target transcription factor NFAT, which has an important role in apoptosis (Asai et al., 1999), as well as neuronal survival (Benedito et al., 2005) has not been studied in the setting of TBI.

Currently, five isoforms of NFAT have been reported in the literature: NFATc1-c4, as well as the primordial form of NFAT named NFAT5 (Macian, 2005) Whereas NFATc1-c4 contain Ca^{2+} sensor domain that are regulated by calcium levels (Graef et al., 2001b), NFAT5 is activated by cytokines such as tumor necrosis factor (TNF) or lymphotoxin-β in the setting of osmotic stress (Lopez-Rodriguez et al., 2001), Despite the differences, all the isoforms of NFAT have highly conserved DNA-binding domains (Macian, 2005).

Although the NFAT function in regulation of T cells and immune system has been well characterized (Rao et al., 1997; Serfling et al., 2000), several studies have also shown their important effect on neurons (Graef et al., 2003; Nguyen and Di Giovanni, 2008). NFAT signaling has a crucial role in axonal projection and growth, as mice lacking calcineurin or NFAT c2/c3/c4 had major defects in axonal outgrowth (Graef et al., 2003). Also, NFAT signaling was shown to be an important component in BNDF-induced transcription leading to synaptic plasticity (Groth and Mermelstein, 2003).

The activation of NFAT is regulated by intracellular Ca^{2+} level. Intracellular Ca^{2+} increase can occur via influx though L-type calcium channels (Graef et al., 1999) or influx through N-methyl-D-aspartate receptors. Otherwise, activation of Trk receptors by neurotrophins or netrin receptor can lead to phospholipase C activation (Graef et al., 2003; Groth et al., 2007), which leads to hydrolysis of phosphatidylinositol 4,5-bisphophate to form inositol 1,4,5-triphosphate (IP_3). IP_3 then leads to release of Ca^{2+} from intracellular stores such as endoplasmic reticulum. This intracellular Ca^{2+} binds to calmodulin, which then subsequently activates protein serine/threonine phosphatase calcineurin. Activated calcineurin dephosphorylate NFAT and activates it. Yet, the activation of NFAT can be opposed by various kinases (Graef et al., 2001a) such as glycogen synthase kinase-3 (Neal and Clipstone, 2001).

Although NFAT in a resting cell is phosphorylated and found in the cytoplasm, stimulation by calcineurin leads to dephosphorylation and translocation to the nucleus. It is then

transcriptionally active in the nucleus and regulates gene transcription (Hogan et al., 2003). In the nucleus, NFAT can interact with other transcription factors such as AP-1 (Chen et al., 1998), ICER (Bodor et al., 2000), and EGR (Decker et al., 2003) among many others (Lopez-Rodriguez et al., 2001). The activity of NFAT can then lead to activation of numerous genes (Kiani et al., 2000; Rao et al., 1997). Thus, by integration of the multiple upstream signaling pathways leading to intracellular Ca^{2+} increase, NFAT leads to changes in gene expression. This change, in turn, can either lead to activation or deactivation of cellular reactions (Im and Rao, 2004).

As previously described, there is evidence that NFAT regulates cell differentiation programs in cell types other than immune cells, and NFATc4 may be an important mediator of BDNF-induced plasticity. Although prior studies have examined calcineurin and NFAT translocation in hippocampus of mice with ischemic injury (Shioda et al, 2006, 2007), the role of NFAT in CNS injuries such as stroke or TBI is incomplete. In the current TBI study we looked specifically at.NFATc3 and NFACTc4 since previous studies showed their response to injury in cells of the CNS (Fernandez et al., 2007; Serrano-Perez et al., 2011; Vashishta et al., 2009) and differential properties of NFATc3 and c4 to stimuli (Ulrich et al., 2012; Vashishta et al., 2009). We examined NFATc3 and NFAT c4 levels at 6 hours, 1 day, 1 week, 2 weeks and 4 weeks post-surgery by utilizing commercially available antibodies. Semi-quantitative measurements of NFATc3 and NFATc4 in the hippocampal cytoplasm and nuclear portion from injured and sham rats sacrificed at the appropriate time after injury was assessed using Western blot analysis. Double labeling (with NeuN and GFAP) immunohistochemistry was used to identify the cell type to express NFATc3 or NFATc4, respectively.

2. Results

2.1 NFATc3

Western blot analysis was performed to evaluate the expression of NFATc3 protein in the cytoplasm and nuclear portion of the hippocampus at 6 hours, 1 day, 1 week, 2 weeks and 4 weeks after TBI in rats. Western blot analysis revealed that NFATc3 signal was seen as dual bands of approximately 120 kDa and 80 kDa (Figure 1A & 2A). It is currently unknown if these two bands for NFATc3 might represent two isoforms (i.e. a ‘pro’ form that is cleaved to become active). However, both bands have a similar response to TBI. NFATc3 expression levels in the hippocampus ipsilateral to the injury were decreased in the cytoplasmic fraction as early as 6 hours and lasting up to 1 week after TBI (Figure 3, $P \leq 0.05$). At 2 and 4 weeks after TBI, there were no significant differences. In contrast, NFATc3 levels in the nuclear fraction were significantly reduced in the ipsilateral hippocampus at all measured time points after TBI (Figure 2). No statistically significant difference of NFATc3 expression was observed in the contralateral hippocampal extracts in the injured animals compared with the sham controls. Double labeling NFATc3 and GFAP immunohistochemistry revealed that at 1 week post injury NFATc3 expression primarily colocalized with GFAP-positive cells (Figure 5B). However not all GFAP-positive cells appeared NFATc3-positive (Figure 5B). We did not observe NFATc3 positive cells colocalized with NeuN-positive cells (Figure 5A). Confocal immunohistochemical

quantification revealed that NFATc3 had significantly ($p<0.001$) greater mean correlation coefficients to GFAP than NeuN under both injured (0.38 ± 0.05 vs. 0.19 ± 0.04) and sham (0.45 ± 0.01 vs. 0.12 ± 0.02) conditions in CA3 hippocampal regions. Thus, the majority NFATc3 are expressed in the astrocytes but not neurons.

2.2 NFATc4

Western blot analysis was performed to evaluate the expression NFATc4 protein in the cytoplasm and nuclear portion of the hippocampus at 6 hours, 1 day, 1 week, 2 weeks and 4 weeks after TBI in rats. NFATc4 signal was seen as a single band of approximately 140kDa (Figure 3A & 4A). In the cytoplasmic faction, there were no differences in NFATc4 expression between any of the groups at 6 hours and 1 day after TBI. However, at 1 week, 2 weeks, and 4 weeks after TBI, the ipsilateral hippocampal,NFATc4 expression levels were increased in the cytoplasmic fraction. In the nuclear faction, there were no differences NFATc4 expression between any of the groups at 6 hours and 1 day after TBI. At 1 week post injury NFATc4 expression was significantly reduced in both the ipsilateral and contralateral nuclear fractions. Trends toward a reduction of ipsilateral hippocampal reduction at weeks 2 and 4 were not significant. Double labeling with NFATc4 and NeuN immunohistochemistry revealed that at 1 week post injury NFATc4 expression primarily colocalized with NeuN-positive cells (Figure 6A). However, not all NeuN-positive cells were NFATc4-positive (Figure 6A). We did not visually observe NFATc4 positive cells colocalized with GFAP-positive cells (Figure 6B). Confocal immunohistochemical quantification revealed that NFATc4 had significantly ($p<0.001$) greater mean colocalization to NeuN compared to GFAP under both injured (0.45 ± 0.03 vs. 0.14 ± 0.04) and sham (0.50 ± 0.01 vs. 0.12 ± 0.02) conditions in CA3 hippocampal regions. Thus, the majority of NFATc4 are expressed in neurons.

3. Discussion

Each NFAT isoform is believed to have distinct but overlapping functions, based on unique phenotypic defects in knockout mice of each NFAT isoform (Horsley and Pavlath, 2002; Macian, 2005) and the fact that individual knockout mice have mild effects. In this study, we focused on TBI induced activation properties of NFAT, by using Western blotting to determine protein levels in cytoplasmic and nuclear fractions, as well as use of immunohistochemistry to detect its presence in neurons and astroglia.

We found that whereas NFATc3 was decreased acutely from 6 hours to 1 week in the cytoplasmic fraction but decreased from 6 hours to 4 weeks after TBI in nuclear fraction. NFATc4 was found to be increased in the cytoplasmic fraction from 1 week to 4 weeks but decreased in nuclear fraction at 1 week after TBI, In macrophages, NFATc3 and NFATc4 have constitutive activity without any stimulation, shown by their localization to the nucleus (Minematsu et al., 2011). Stimulation using lipopolysaccharide or synthetic bacterial lipoprotein did not alter their nuclear location in macrophages. However, NFATc3 and NFATc4 in the CNS have a different response to injury. Lipopolysaccharide treated astrocyte cultures show increased NFATc3 levels and activity (Fernandez et al., 2007), and NFATc3 is specifically induced in astrocytes after kainic acid or stab wound injury

(Serrano-Perez et al., 2011). Similarly, NFATc4 in cerebellar granule neurons is induced to higher levels in proapoptotic conditions with serum and K+ deprivation (Benedito et al., 2005). Thus, in the CNS, NFATc3 and c4 isoforms are induced by injury stimuli, and may exhibit cell specific expression in astrocytes or neurons.

In this study, we used Western blot analysis to assess NFATc3 and c4 levels in the cytoplasmic vs. nuclear fractions. Compared to the previous studies that reported injury induced changes in NFAT by assessing the percentage of cells containing nuclear NFATc4 or the number of hippocampal NFATc3 positive cells (Benedito et al., 2005; Serrano-Perez et al., 2011), our assessment using cytosolic and nuclear fractions and quantifying NFAT levels do not give information about the specific spatial distribution of the protein in the hippocampus. While nuclear transportation of NFAT was not directly assessed, the present Western blot analysis provides new information about NFAT changes in different subcellular compartments after TBI. NFATc4's mediation of the survival of neurons has been previously reported (Benedito et al., 2005; Luoma and Zirpel, 2008; Quadrato et al., 2012; Vashishta et al., 2009). Inhibition of NFATc4 function prevented neuroprotection from NMDA induced apoptosis, and knockdown of NFATc4 lead to neuronal apoptosis (Benedito et al., 2005; Vashishta et al., 2009). The decrease in the nuclear fraction and increase in the cytoplasmic fraction of NFATc4 from 1 week to 4 weeks is likely due to its decreased nuclear transportation. This possibility is corroborated by the fact that both increase in cytoplasmic fraction and decrease in nuclear fraction occur at the same time, at 1 week time point. Since this change was not detectable at earlier time points of 6 hours or 1 day after injury, it is likely not due to the direct effect of the injury but may reflect subacute compensatory changes. The potential decrease in nuclear transport of NFATc4 could have important implications on the activity of its upstream mediator calcineurin. One possible explanation may be the decreased calcineurin activity in the hippocampus at subacute to chronic time points, leading to decreased activity and reduction of nuclear transport of NFATc4.

If there is a reduction in NFATc4 nuclear transport after TBI, this would be contrary to the previously reported studies of calcineurin. A direct measurement of calcineurin activity by Kurz et al. (Kurz et al., 2005a; Kurz et al., 2005b) demonstrated increased calcineurin activity at 24 hours, and gradually reducing activity up to 4 weeks after FPI. In agreement with this, calcium accumulation has been noted immediately after FPI up to 4 days in the cerebral cortex (Osteen et al., 2001), and a delayed increase from 2-4 days to 14 days in the thalamus. Also, the use pharmacological agents that inhibit calcineurin activity were neuroprotective in a rat model of TBI. Single dose of FK506 around the time of injury in a fluid percussion injury (FPI) model reduces loss of dendritic spines (Campbell et al., 2012), preserves compound action potentials (Reeves et al., 2007), and reduces axonal injury (Singleton et al., 2001). However, these reports used the FPI model of TBI, which has more diffuse nature of mechanical injury compared to the CCI. Although both models can produce cortical contusion and diffuse axonal injury, the CCI model generally produces much more focal pattern of injury (Albert-Weissenberger and Siren, 2010; Morales et al., 2005). Thus, a potential deficit in NFATc4 nuclear translocation may be associated with model differences in severity of injury to the hippocampus. CCI may result in initial increase in calcineurin activity followed by decreased activity at a delayed time point, leading to

decreased NFATc4 nuclear translocation. However, FPI results in an initial increase in calcineurin activity followed by decrease to previous levels. Since injury-induced shuttling of NFAT from cytoplasm to nucleus can be fairly rapid, the sampling times in this study might be too late to detect an injury-induced reduction of cytoplasmic NFAT concomitant with a nuclear increase in the CCI model.

Although the calcium levels as well as calcineurin activity has been studied in FPI, the time course of calcineurin activity using a CCI model has not been reported. We have previously reported upregulation in CA1/CA2 regions of the hippocampus and downregulation in the dentate gyrus of calcineurin subunit B (Bales et al., 2010b), as well as downregulation of subunit A in CA1/CA2 regions after CCI (Bales et al., 2010a). However, it is recognized that calcineurin protein levels may not directly reflect its activity, and calcineurin is only active when bound to Ca^{2+}/calmodulin. In order to clarify the chronic effects of TBI in calcineurin signaling and its downstream targets, future studies are planned to examine the time course of calcineurin activity in the CCI model.

The function of NFATc3 in the CNS has not been described in detail, but a study using methamphetamine stimulus showed that it may be involved in Fas ligand expression to induce apoptosis (Jayanthi et al., 2005). NFATc3 is specific to astrocyte population and is not found in microglia or neurons, As shown by previous studies of brain injury induced by kainic acid excitotoxicity or stab wound (Neria et al., 2013; Serrano-Perez et al., 2011), NFATc3 appears to be specific to the astrocyte population and was not found in microglia or neurons. Specifically, NFATc3 expressed in a subset of astrocytes near the stab wound edge where high levels of astrogliosis and intense neuronal degeneration takes place. The decrease in both the cytosolic and nuclear fractions from 6 hours to 4 weeks after injury in the current study is in contrast to the inflammation and glial reaction one would expect after TBI. This decrease is not likely due to translocation, since we would expect an increase in one fraction and decrease in another, as we have noted in the NFATc4 data. Rather, increased degradation or decreased expression of this transcription factor is possible, bringing the general level of NFATc3 down. Downregulation of pro-apoptotic NFATc3 may be important step in attenuating the damaging effects of TBI. At 4 weeks post-injury, there was no significant difference between sham and injured groups, in the cytosolic fraction, possibly due to returning function of NFATc3 to the preinjury condition. The localization of NFATc3 and NFATc4 was studied using confocal microscopy at 1 week after TBI. Double labeling of NFATc4 with either NeuN or GFAP showed that NFATc4 localizes primarily in the neurons and not astroglia (Figure 6). The expression of NFATc4 was limited to a subset of neurons, as not all neurons colocalized with NFATc4. Also, as NFATc3 upregulation was proposed to be a marker of upregulation of astrogliosis in the previous studies (Neria et al., 2013; Serrano-Perez et al., 2011), we labeled hippocampal sections using NFATc3 and astroglial marker GFAP in order to examine the effect of TBI. Thus, our finding of limited expression of NFATc3 to a subset of astrocytes is in agreement with Neria et al., (2013) and Serrano-Perez et al., (2011), although others have observed NFAT3 in hippocampal neurons (Abdul et al, 2009; Ulrich et al, 2012).

The present study demonstrates that after TBI insult, in the hippocampus ipsilateral to the injury, NFATc3 expression levels were decreased both in the cytoplasm and nuclear

portions and NFATc4 expression levels were increased in the cytoplasm portion but decreased in the nuclear portion. Double labeling immunohistochemistry reveals that NFATc3 is mostly expressed in astrocytes and NFATc4 predominantly expresses in neurons but not in astrocytes. To our knowledge, this is the first time that the NFATc3 and NFATc4 protein levels have been evaluated in the hippocampus after TBI. Subsequent studies are needed to provide more direct evidence of a TBI-induced alteration in NFAT nuclear translocation and to assess the result of these changes in determining the transcriptional outcome and possible roles of this signaling pathway in clinical pathology after TBI.

4. Experimental Procedure

4.1 Controlled cortical injury

Male Sprague-Dawley rats weighing 250-275 grams were purchased from Hilltop Laboratories (Scattsdale, PA) and housed in standard steel/wire mesh cages at room temperature (22 ± 2°C) under 12-h light/dark cycles. Food and water was given *ad libitum*, and 2 rats were housed per cage. All of the experiments in this study were first approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. The animals were injured by the controlled cortical impact injury (CCI) device as previously described (Dixon et al., 1991). The CCI device (PPI, Inc. Pittsburgh, PA) was equipped with a small (1.975 cm) bore, double-acting, stroke-constrained, pneumatic cylinder with a 5.0 cm stroke.

The rats were anesthetized using 4% isoflurane with a 2:1 N_2O/O_2 mixture before intbation. After endotracheal intubation, rats were mechanically ventilated with a 1-1.5% isoflurane mixture. Animals were mounted on a stereotaxic frame to perform a 7 mm craniotomy centered 5 mm right from the central suture midway between bregma and lambda. The rats were injured with an impact velocity of 4 meters/sec and a tissue deformation of 2.7 mm. For sham group, the rats received craniotomy without CCI. Core body temperature was monitored using a rectal thermistor probe and maintained at 37 ± 0.5° C using a heating pad.

4.2 Western blot

Sixty animals were sacrificed for Western blot at 6 hours, 1 day, 7 days, 14 days and 28 days after surgery (n = 6 in each group for each time point). Rats were sacrificed after being anesthetized with pentobarbital (Nembutal, 100 mg/kg; Abbott Laboratories, North Chicago, IL), and the brains were quickly removed and chilled on ice. Hippocampal tissues were dissected out and frozen in liquid nitrogen immediately and stored at −80°C. On the day of Western blotting, tissues were washed with ice cold 0.1 M PBS then homogenized and processed for cytoplasmic and nuclear extracts using a commercial available kit (NE-PER Nuclear and Cytoplasmic Extraction Reagents, PIERCE, Rockford, IL). Protease Inhibitor Cocktail Kit (Halt, PIERCE, Rockford, IL) were added into the reagents during the preparation of the tissue samples. The protein concentrations of the hippocampal cytoplasmic and nuclear extracts were determined by a 96-well micro-plate reader (Spectra Max 340; Molecular Devices, Sunnyvale, CA) using a BCA protein Assay Kit (PIERCE, Rockford, IL). Samples containing 50-100 Pg of protein of the hippocampal cytoplasmic and nuclear extracts were separated by electrophoresis on a 10 % SDS-polyacrylamide gel

and transferred to nitrocellulose membranes. The membranes were blocked in 5% non-fat dry milk in 0.1 M PBS with 0.05% Tween-20 (PBST) at room temperature for one hour, and immunolabeled with rabbit anti- NFATc4 polyclonal or mouse anti- NFATc3 monoclonal antibodies (SC-13036 or SC-8405 respectively, 1:500, Santa Cruz Biotechnology, Inc. Santa Cruz, CA) at 4°C overnight followed by secondary antibody at room temperature for one hour. Proteins were visualized with a chemiluminescence detection system (SuperSignal, PIERCE, Rockford, IL).

To confirm equal loading and/or normalize to protein content, the membrane blots re-striped and re-blotted with mouse anti-β-actin monoclonal antibody or rabbit anti-β-actin polyclonal antibody (1:10,000, Sigma, St. Louis, MO). The injured tissue and the sham control were loaded together in the same gel for comparison. Blots were exposed to autoradiographic X-ray film for 10 sec to 1 min and bands were quantitated using SCION ImagePC (Frederick, MD) software. Values are given as a ratio (percentage change) of optical density of injured samples versus sham control within individual blots for each hemisphere. Data are expressed as the group means ± standard error of the mean (SEM). For each time point, a 2 × 2 ANOVA (injury vs sham × ipsilateral vs contralateral hemisphere) was performed to determine expression differences (Systat Software, Inc. Chicago, IL). If statistical significance was found, a Tukey post-hoc test was used to compare each group. A significance level of $P \leq 0.05$ was used for all tests.

4.3 Immunohistochemistry

Confocal immunohistochemistry was performed to determine the cell type specificity of the NFAT isoform antibodies, and not to semi-quantitate TBI-induced changes. The one week time point was chosen based on the time course Western blot results. Anesthetized rats were sacrificed for immunohistochemistry at 1 week after CCI injury and sham surgery (n = 6 in each group for each time point). Tissue preparation and immunohistochemistry procedures were as previously reported (Yan et al., 2001; Yan et al., 2002; Yan et al., 2007). Animals were first anesthetized with pentobarbital (Nembutal, 100 mg/kg; Abbott Laboratories, North Chicago, IL) then transcardially perfused with 100 ml 0.1 M phosphate buffered saline (PBS) with 50 U/ml heparin pH 7.4. Perfusion was continued using 500 ml 4% paraformaldehyde with 15% saturated picric acid in 0.1 M PBS. The brains were then removed and immersed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 at 4°C overnight. The next day, brains were transferred to 15% sucrose in 0.1 M PBS and maintained for 24 hours then to 30% sucrose in 0.1 M PBS until for the next few days until the brains sunk to the bottom. The cryoprotected rat brains were frozen sectioned with a cryostat (Jung CM 1800; Brodersen Instrument, Valencia, PA). Coronal sections of brain tissue through the dorsal hippocampus were cut in 35 Pm thickness and collected in 24-well culture plates contained with 0.1 M PBS.

Immunohistochemistry was performed as we have previously reported (Bales et al., 2011) and conducted in 24-well culture plates by free floating technique. Sections were pre-blocked with 10% normal goat serum (NGS) and 0.1% Triton X-100 in 0.1 M PBS. Sections were then incubated with primary antibody, NFATc3 or NFATc4 with mouse anti-NeuN (1:600, Chemicon International) monoclonal or rabbit anti-GFAP (1:6,000, Chemicon

International) polyclonal antibodies at 4qC for 16-24 hours. Alexa Fluor dyes (488, 594**)** were used for NFATc4, NFATc3, NeuN, or glial fibrillary acidic protein (GFAP) double labeling study. At least three sections of brain tissue through the dorsal hippocampus were processed for the NFATc3 or NFATc4 immunoreactivity either for NeuN or GFAP double labeling experiment for each rat. After rinsing three times with 0.1% Triton X-100 in 0.1M PBS, sections were incubated with secondary antibodies with 5% normal serum and 0.1% Triton X-100 in 0.1 M PBS at 4°C for 2 hours on a shaker. Tissues were rinsed between all steps with 0.1% Triton X-100 in 0.1 M PBS three times for at least 10 min each time. Control experiments were run in parallel to confirm specificity. Primary antibody was either omitted or replaced by normal rabbit serum at 4°C for 16 hours incubation. Images were captured using a confocal microscope (LSM 510; Zeiss, Jena, Germany). The sections were analyzed using a Nikon Eclipse 90i with the Nikon C2 confocal microscope system. Images of hippocampal CA3 regions were taken using a Plan APO 20x objective. Colocalization analysis was done using Nikon's NIS-Elements AR4.10.01 software. Pearson's correlation values were obtained for the whole 20X image.

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Highlights

- Traumatic brain injury produced differential effects on hippocampal NFAT C3 and C4 isoforms levels.
- NFATc3 levels were decreased in both cytoplasmic and nuclear hippocampal fractions after TBI.
- NFACc4 levels were increased in the cytoplasm but decreased in the nuclear fraction after TBI.

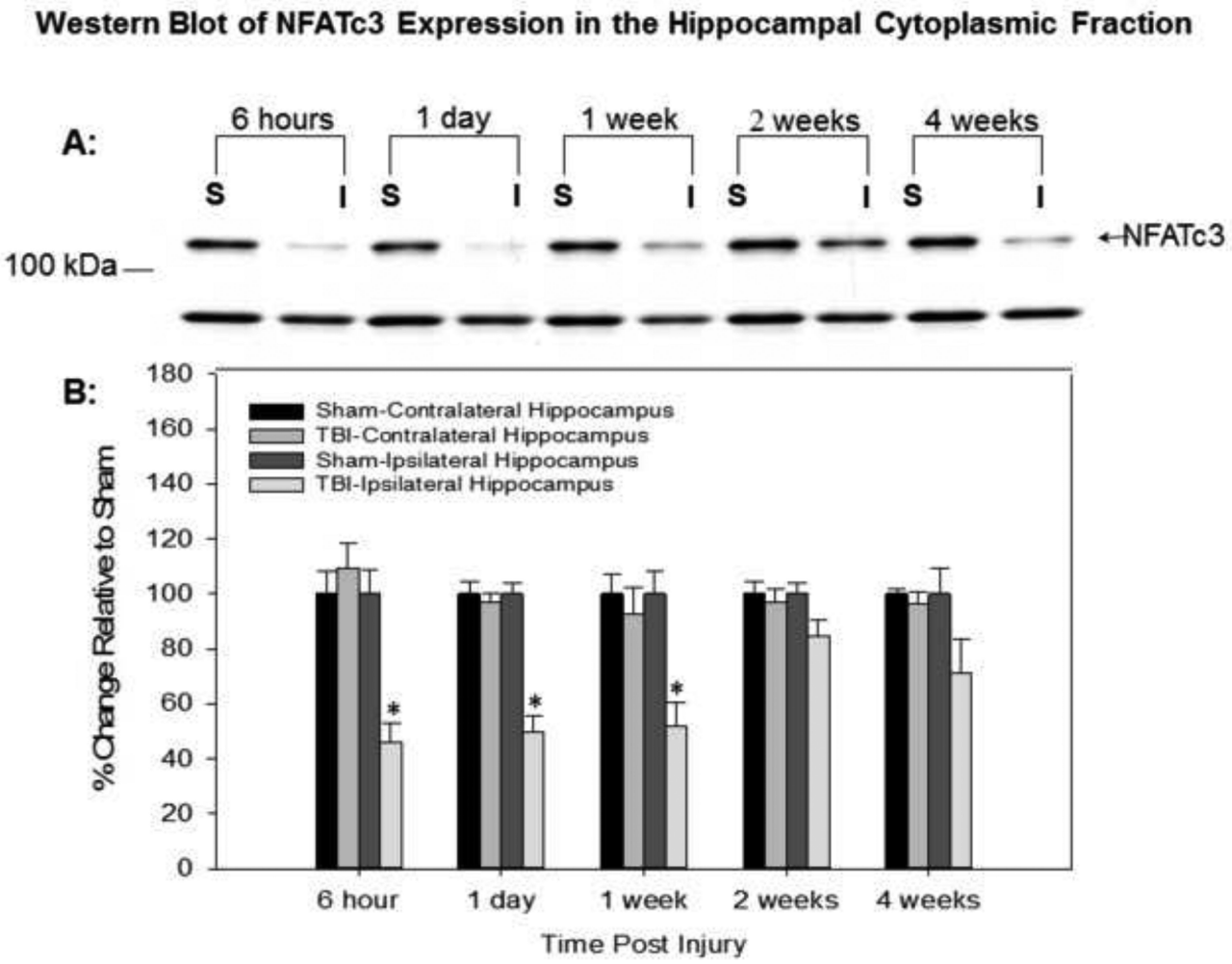

Fig. 1.

Western blot semiquantitative measurements of the hippocampal cytoplasmic fraction. The Western blot gel bands (A) of NFATc3 in the ipsilateral hippocampus showed a double band at ~80 and ~120kDa range. When the optical densities were quantified (B), there were significant decreases in TBI rats compared to shams at earlier time points from 6 hours to 1 week. * $P < 0.05$.

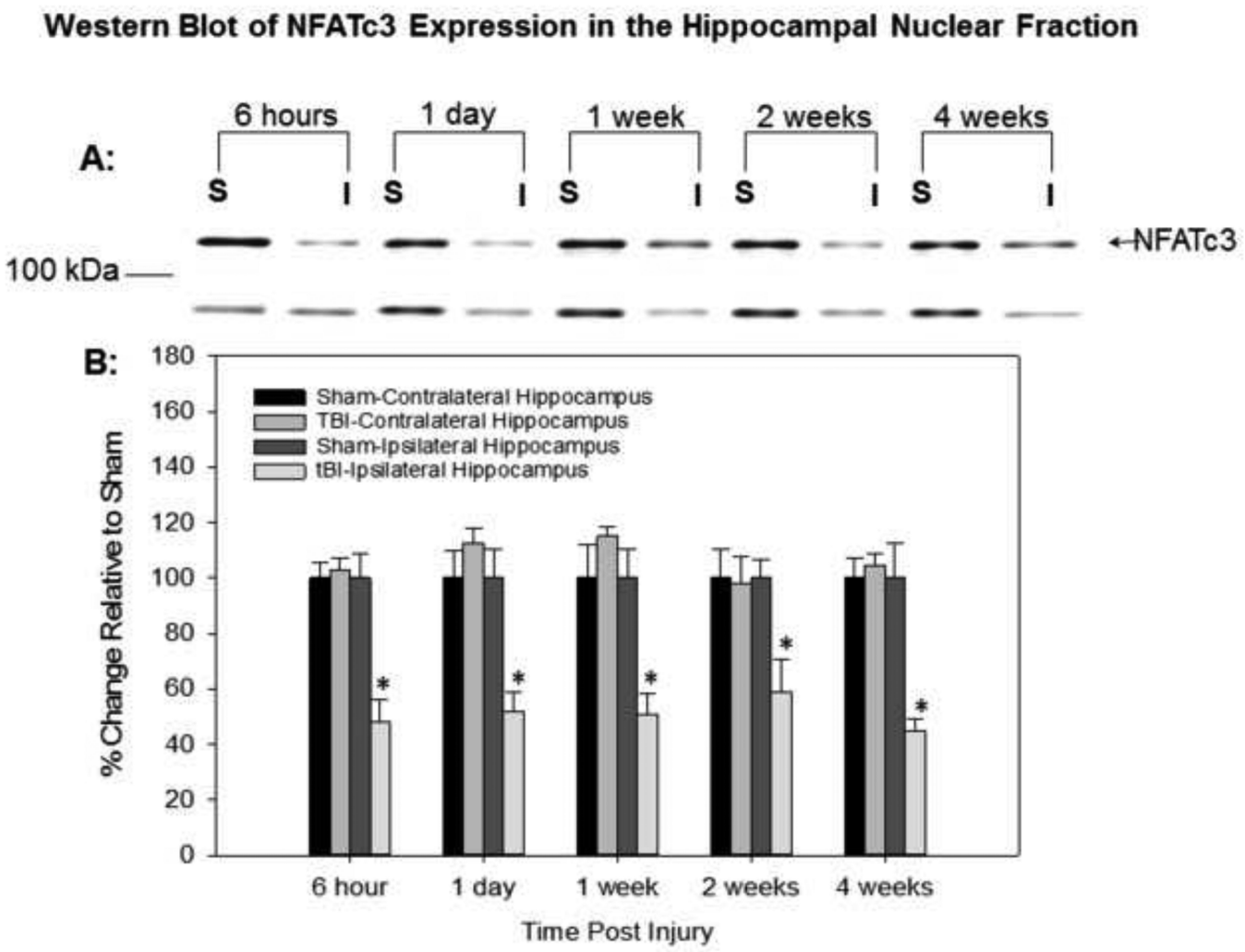

Fig. 2.

Western blot semiquantitative measurements of the hippocampal nuclear fraction. The Western blot gel bands (A) of NFATc3 in the ipsilateral hippocampus shows decreased levels in injured rats. Quantification of optical densities (B) showed significant decreases in TBI rats compared to shams at all the time points. * $P < 0.05$.

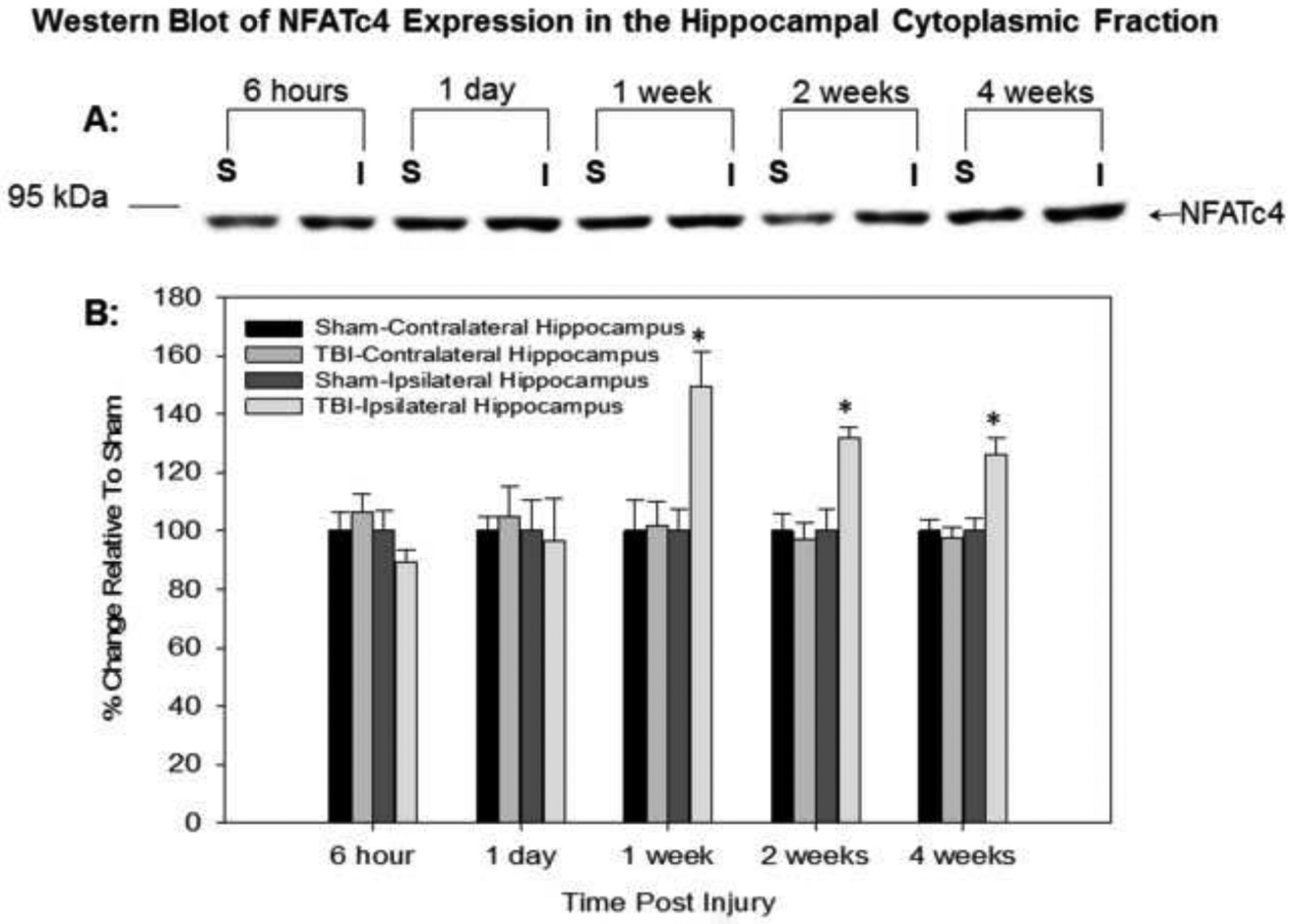

Fig. 3.

Western blot semiquantitative measurements of NFATc4 in hippocampal cytoplasmic fraction. Western blot gel bands (A) of NFATc4 in the ipsilateral hippocampus showed a single band around 120-140 kDa range. Quantification of the blots (B) showed increased levels in injured rats at 1 week, 2 weeks, and 4 weeks after injury. * $P < 0.05$.

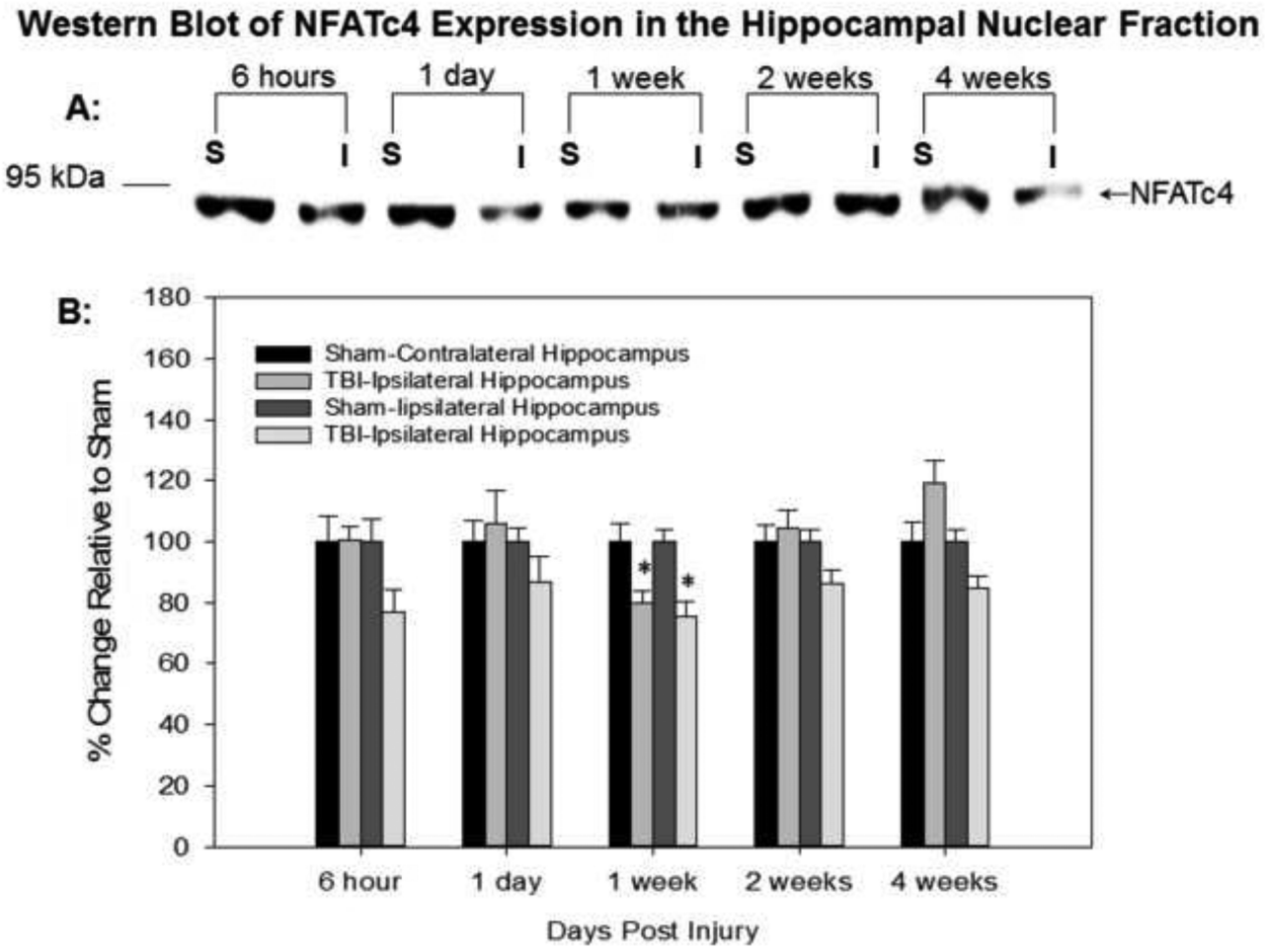

Fig. 4.

Western blot semiquantitative measurements of NFATc4 in ipsilateral hippocampal nuclear fraction. Western blot gel bands (A) confirms the same location of NFATc4 band as in Figure 3. Quantification of the blots (B) showed decrease levels in injured rats at one week after TBI. * $P < 0.05$.

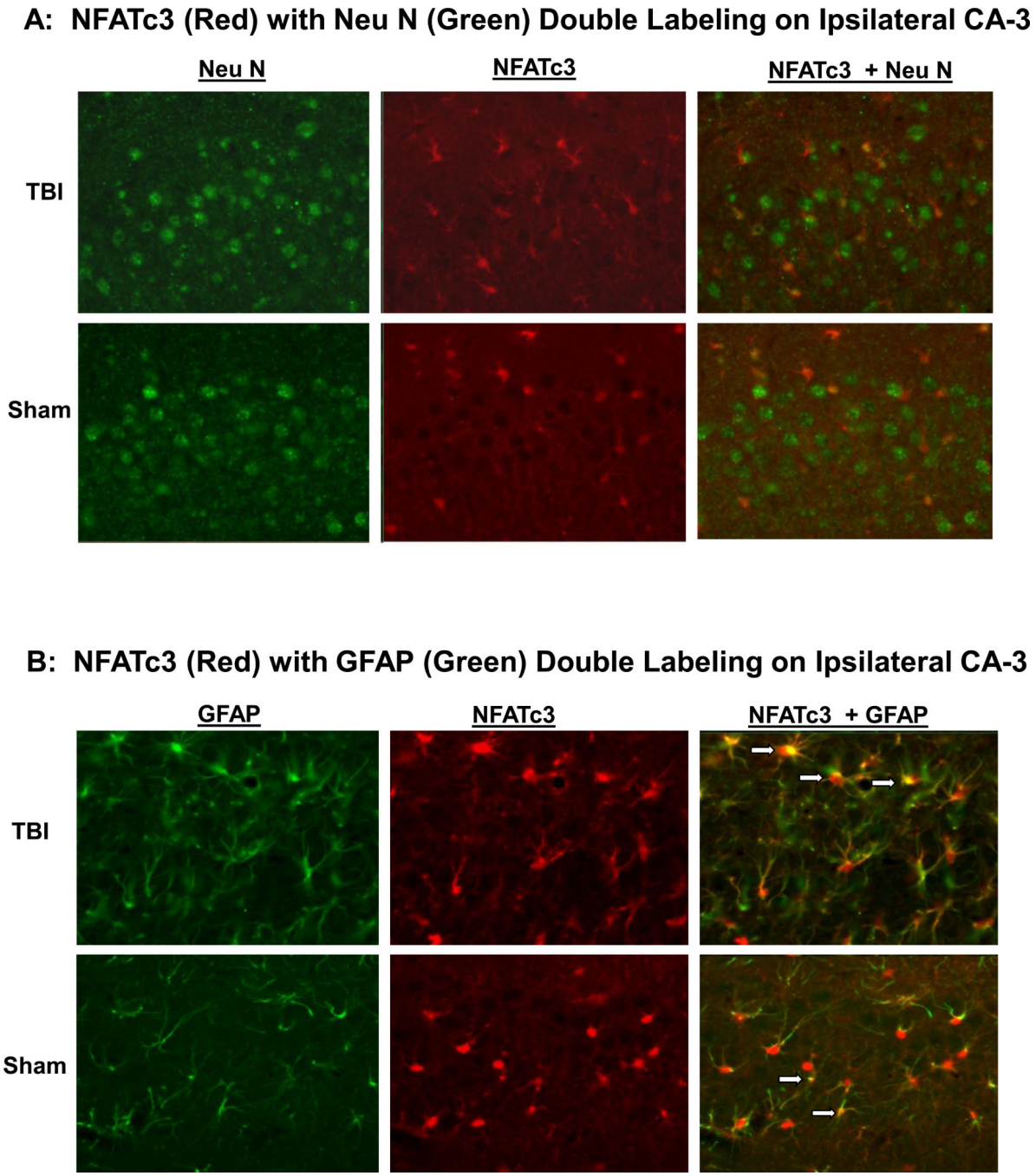

Fig. 5.

Representative microscopic photos of double-label immunofluorescent staining from rat ipsilateral hippocampal CA3 region for NFATc3 (red, Alexa Fluor 594) with astrocytes marker, GFAP (B, green, Alexa Fluor 488) or neuronal marker NeuN (A, green, Alexa Fluor 488) at 1 week after TBI or sham operation. Microscopic photos illustrate that NFATc3 is positive in astrocytes (GFAP positive). The arrows indicate select examples NFATc3 positive double-labeled with GFAP-positive cells.

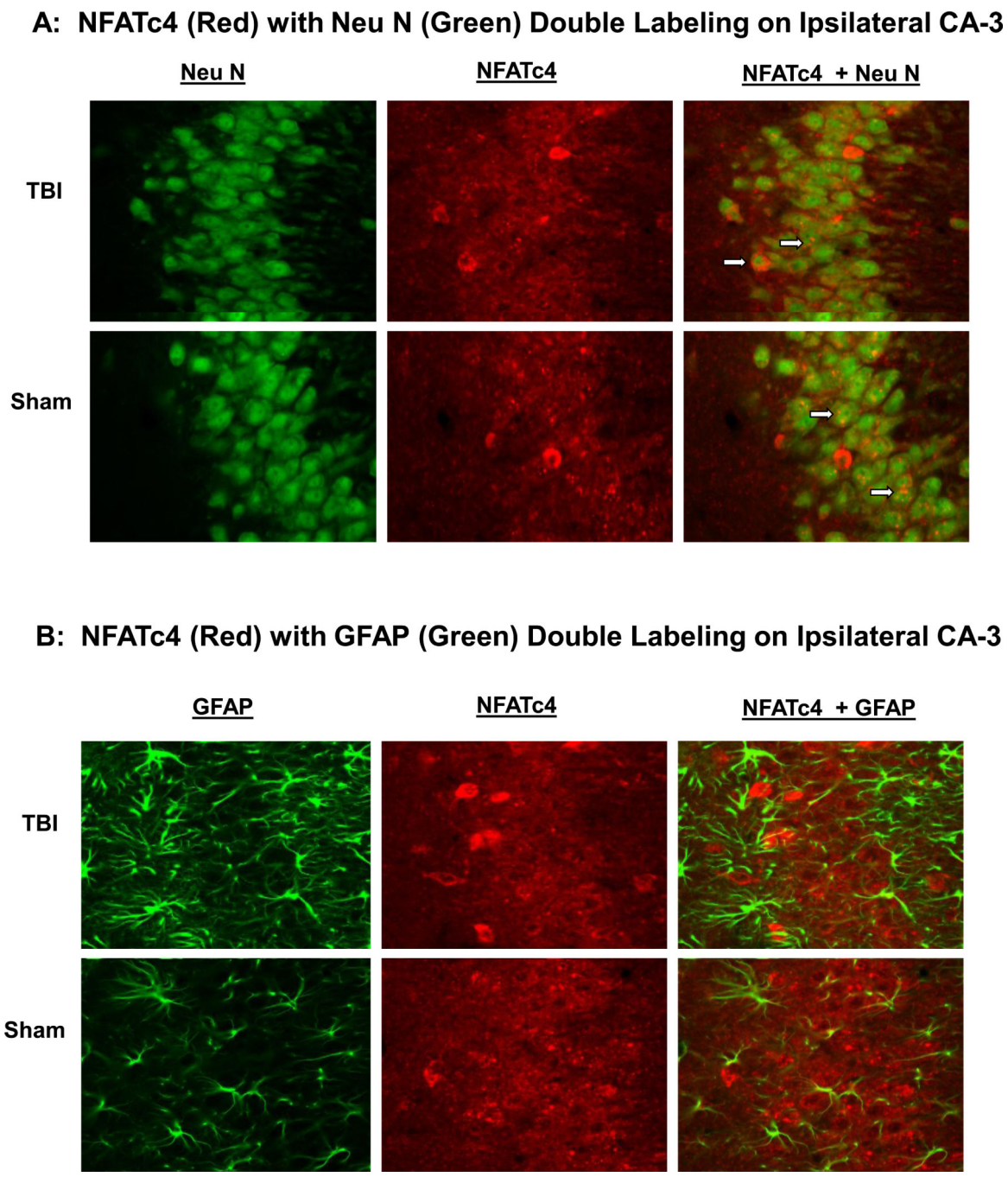

Fig. 6.

Representative microscopic photos of double-labeled immunofluorescent staining from rat ipsilateral hippocampal CA3 region for neuronal marker NeuN (A, green, Alexa Fluor 488) with NFATc4 (red, Alexa Fluor 594) illustrate the localization of NFATc4 to neurons. The bottom panels show double-labeled immunofluorescent staining for astrocyte marker GFAP (B, green, Alexa Fluor 488) with NFATc4 (red, Alexa Fluor 594) showing lack of NFATc4

in astrocytes. All panels were injured tissues at 1 week after TBI or sham operation. The arrows indicate double-labeled NFATc4 positive cells.