Research Article

Alteration of Hippocampal Cytokines and Astrocyte Morphology Observed in Rats 24 Hour after Fluid Percussion Injury

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Abstract

Traumatic Brain Injury (TBI) is one of the few known etiological factors contributing to the development of Post-Traumatic Epilepsy (PTE). An understanding of the mechanisms involved in the development of PTE is vital because PTEs are amongst the most difficult to treat, and are often resistant to first and second line anti-epileptic treatment regimens. TBI-induced inflammation, neuroplasticity and neuropathology in the hippocampus have been observed in animal models of TBI. Components of these alterations have been implicated in TBI-pathogenesis and the epileptogenic development of PTE. However, the early time course of these changes is not fully elucidated. This study was designed to examine inflammation and neuropathology in the hippocampus in a rat Fluid Percussion Injury (FPI) model of PTE. Cytokine analysis in the hippocampus, as well as astrocyte morphology was assessed to determine early inflammatory changes after TBI. To examine early seizure-promoting neuropathology, we performed stereological analysis of parvalbumin-labeled interneurons in the hippocampus at 24 hrs after TBI. The results demonstrate that FPI in rats results in early hippocampal inflammation, but not a loss of parvalbumin-labeled interneurons.

ABBREVIATIONS

TBI: Traumatic Brain Injury; FPI: Fluid Percussion Injury; CCI: Controlled Cortical Impact; C-X-C motif: Chemokine CXCL: Ligand; C-C motif: Chemokine CCL: ligand; IL: Interleukin

INTRODUCTION

Post-Traumatic Epilepsy (PTE) can result from a Traumatic Brain Injury (TBI). In fact, TBI is one of the few known etiological factors contributing to the development of epilepsy. PTE accounts for approximately 5% of all epileptic cases and 20% of symptomatic epilepsies [1-3]. Although it is not known who will develop PTE after a TBI, increased injury severity is associated with an increased incidence of PTE [4-6]. However, even relatively mild TBIs can lead to the development of PTE [2].

Considering that there are approximately 1.7 million reported TBIs in the United States each year, understanding the mechanisms involved in the development of PTE is vital to provide optimal treatment. This is especially important because PTEs are amongst the most difficult types of epilepsy to treat, and are often resistant to first and second line anti-epileptic treatment regimens [7-9].

There are various neuropathological changes that may underlie the development of PTE. Among these, TBI-induced inflammation and neuronal plasticity in the hippocampus is frequently observed. Hilar interneuron loss and dysfunction was reported at 7 days after TBI in rodents [10] and humans [11-13]. TBI-induced dysfunction and/or loss of hippocampal interneurons may alter inhibition within the hippocampus, resulting in hyperexcitability and a pro-epileptogenic state [14,15]. Thus, determining the precise times when neuroplastic changes begin to affect this population of neurons is vital to the mechanistic understanding of TBI and the post-traumatic consequences and developing optimal treatments at the appropriate time points.

In addition to interneuron loss, mounting evidence shows that neuroinflammatory cascades are initiated in the hippocampus following TBI [16,17], and these effects may not be entirely unrelated. For example, Raghupathi and colleagues observed increased IL-1ù and TNFα in the hippocampus and cortex following TBI [18]. Moreover, others have reported elevated levels of IL1α, IL-6, IL1ù and TNFα in the hippocampus following TBI [19] and such inflammation may contribute to neuronal dysfunction and/or loss. It has been suggested that blocking inflammation can improve post-traumatic outcomes [20]. For example, treatment with a TNFα protein synthesis inhibitor prevents cognitive impairment after TBI [21]. Such a finding was supported in a clinical TBI study, where cohorts carrying the homozygous TNFα-308 single nucleotide polymorphism had significantly worse Glasgow outcomes Score [22]. Other studies also support the idea that some components of the neuroinflammatory response may be detrimental outcomes of TBI, because inhibiting neuroinflammation can improve outcome measures [19,23]. However, other studies that inhibited the action of particular cytokine(s) either failed, or provided limited protection from TBI [24,25]. Considering these somewhat conflicting data, it is likely that the positive and negative effects of the neuroinflammatory response are dependent on spatial and temporal release, of the cytokine milieu, as well as the interacting and opposing, post-TBI cytokine milieu. Thus, understanding the full spatial and temporal extent of TBI-induced neuroinflammation is vital to understanding the mechanisms involved in increased seizure susceptibility, the development of PTE and treating the many symptomology associated with brain trauma.

Therefore, the present study was designed to determine the alterations to 23 different cytokines in the hippocampus at 24 hrs after a fluid percussion TBI. To further define the cellular components of the TBI-induced inflammatory response in the hippocampus, astrocyte morphology was assessed in the hilus.

Astrocytosis occurs rapidly in the neocortex after an FPI [26], but the early effects of TBI on astrocytes in the hippocampus has not been defined in this model. Considering that astrogliosis can be indicative of neuronal degeneration, it is possible that early hippocampal inflammation is associated with hilar interneuron loss. The observations that hilar interneuron loss is implicated in the development of epilepsy in other injury models [27-29] suggest a potential avenue for neuroprotection after TBI. Thus, this study also examined the number of parvalbumin-expressing interneurons in the hilus at 24 hrs after a fluid percussion TBI.

MATERIALS AND METHODS

All experimental protocols were approved by the Institutional Animal Care Committee (IACUC) of Texas A&M University, Health Science Center and Scott &White hospital. Animals were housed singly after FPI with a 12-h light–dark cycle (light on 6:00 and light off 18:00). All animals had continuous access to food and water. Twenty male Sprague-Dawley rats (Charles River), 9-10 weeks of age and weighing between 275-310 g were used in this study for cytokine estimation in the hippocampus. Rats were randomly assigned to either the experimental group or the sham group, so that each group had 10 rats. For immunohistochemistry, we used an additional 12 male Sprague-Dawley rats that were the same ages and weights as those used for cytokine analysis.

Fluid percussion injury

Rats were initially anesthetized with 4% isoflurane and oxygen for anesthesia induction and later to 2% isoflurane for maintenance. Once under anesthesia, the heads of the animals were shaved. Strict sterile technique was maintained during surgical procedures. Animals were placed in a stereotaxic instrument (Stoelting, Illinois). A 2 mm hole was drilled, with dura intact, in the skull over the left parietal cortex at 2 mm posterior and 3 mm lateral from the bregma, as previously described, 10. The animals were connected to the fluid percussion instrument (custom design and fabrication from the radiology department from VCU, Model 01-B) via the female Luer-Lok. A single 4–4.25 atm pressure pulse was delivered, with a duration of 18–20 ms, as measured by a digital Oscilloscope (Tenma model # 728395), connected to a signal transducer hooked in-line between the fluid percussion cylinder and the syringe Sham animals were anesthetized and manipulated in an identical manner, but no pressure pulse from the fluid percussion instrument was delivered to their brain. After 24 hrs, animals used for the cytokine assay (N = 10 sham and 10 FPI) were euthanized under deep anesthesia and hippocampal tissue was collected and flash frozen. For immunohistochemistry, animals (N = 6 sham and 6 FPI) were perfused first by normal saline and then with 4% paraformaldehyde under deep anesthesia. After removing the brain and post-fixing for 24 hrs, the tissue was cut into 50 μm sections using a vibratome (Pelco 102, Ted Pella INC., Redding, CA).

Multiplex assay: The assay was performed as previously described (Mukherjee et al [30].) The following 23 different cytokines (See Table 1) were assayed: TNFα, CCL2, CCL3, CCL5, IL 1α, IL 1 ù, IL 2, IL 4, IL 5, IL 6, IL10, IL12, IL 13, IL17, IL 18, IFNÛ, interferon inducible protein 10 (IP 10), Growth-related oncogene (GRO KC), Leptin, Eotaxin, Granulocyte Macrophage Colony Stimulating Factor (GMCSF), Granulocyte Colony Stimulating Factor (GCSF), Vascular Endothelial Growth Factor (VEGF). Frozen tissue was homogenized, as previously described [30] and following the manufacturer's instructions (Milliplex MAP kit, Millipore). 25 μl of undiluted homogenate was added to 25 μl of assay buffer. The samples were analyzed using the Bio-Rad LS200 (Bio-Rad Instruments). Assays were run in triplicate and analytes were normalized to total protein concentration, estimated using a Bradford assay.

Immunohistochemistry: For GFAP labeling of astrocytes, sections were reacted free-floating as previously described 15,16. Briefly, sections were incubated overnight in GFAP antibody (1:1000; Sigma G9269), rinsed and incubated with biotinylated goat anti-rabbit IgG antibody (1:200; Vector Labs) for 1.5-2 h. Tissue was again rinsed and incubated in Vector Elite ABC (Vector Labs) for 1 hr, after which tissue was rinsed and reacted in DAB. For parvalbumin immunohistochemistry, the same protocol was used as above, except that the primary antibody was anti-parvalbumin (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Tracing of astrocytes for Sholl analysis: Astrocytes were traced and reconstructed using the Neurolucida (Microbrightfield Inc. V. 6.0) system. After tracing the hilus, a grid was superimposed

Table 1: Effect of FPI on the analyte concentration in the hippocampus.

Analytes	Sham ipsi		FPI ipsi		Sham contra		FPI contra	
IFN Û:	1.9	± 0.3	1.5	± 0.4	2.2	± 0.3	1.3	± 0.1
IL 13:	8.3	± 1.2	6.6	± 1.5	6.9	± 0.7	5.2	± 0.4
IL 1α:	3.8	± 0.5	6.8	± 1.6	3.9	± 0.4	3.2	± 0.6
IL 2:	10.0	± 1.5	7.9	± 1.4	9.6	± 0.9	7.4	± 0.8
IL 4:	4.4	± 0.7	3.3	± 0.7	3.2	± 0.4	2.5	± 0.4
IL 5:	2.4	± 0.3	2.0	± 0.3	1.8	± 0.2	1.0	± 0.1
TNF α:	0.8	± 0.3	2.5	± 0.6	0.8	± 0.2	1.1	± 0.3
GMCSF:	1.3	± 0.2	1.0	± 0.07	1.2	± 0.15	0.5	± 0.04
IP 10:	3.6	± 0.6	2.7	± 0.7	3.1	± 0.4	2.1	± 0.17
VEGF:	1.1	± 0.3	2.2	± 0.56	1.3	± 0.17	0.7	± 0.1
GCSF:	0.5	± 0.09	0.6	± 0.07	0.5	± 0.06	0.3	± 0.04
GRO KC:	37.5	± 8.6	149.4	± 40.1	20.9	± 3.0	54.5	± 17.1
IL 18:	76.9	± 6.4	88.8	± 10.4	74.1	± 5.2	62.7	± 5.3
CCL2:	12.2	± 2.9	92.64	± 26.7	21.1	± 1.8	17.8	± 2.7
CCL3	3.8	± 2.1	17.4	± 4.6	0.3	± 0.05	6.6	± 4.8
Leptiin:	15.2	± 4.6	13.3	± 3.0	12.4	± 2.1	8.7	± 0.8
IL 17:	31.5	± 125	23.3	± 6.2	30.7	± 3.1	21.2	± 2.1
IL 6:	18.8	± 2.4	35.3	± 12.9	21.1	± 1.9	2.7	± 0.3
CCL 5:	10.1	± 0.9	11.4	± 0.8	7.6	± 1.1	6.8	± 0.9
IL12:	26.3	± 4.0	20.1	± 4.3	21.8	± 2.1	16.1	± 1.4
IL 1ù:	11.2	± 1.3	13.3	± 2.0	11.9	± 0.6	9.2	± 0.7
IL 10:	14.1	± 2.3	11.7	± 2.8	18.6	± 2.6	10.6	± 1.0
Eotaxin:	4.8	± 0.6	3.5	± 0.7	6.1	± 0.6	4.7	± 0.4

Analyte concentration is expressed as pico gram per microgram of hippocampal tissue. For each hemisphere, data was analyzed using Ttest with p<0.05.

and random areas were selected. A higher magnification was then used to zoom in on this location and cells that were within this area were selected for tracing. Only cells located in the hilus and showing clear and relatively complete process in the X,Y and Z plane, were chosen for tracing. After tracing, a Sholl analysis was used to examine various parameters of cell morphology using concentric spheres of a specified distance. For the purposes of our study we chose spheres with 10 μm increment diameter (Figure 1). Within each concentric sphere, overall process length, amount of bifurcations, amount of process endings, and amount of intersections through that sphere are analyzed.

Stereological analysis of parvalbumin-labeled interneurons in the hippocampus: Sections of tissue were stained with parvalbumin to allow for stereological quantification of labeled interneurons using Streoinvestigator (microbrightfield inc. V. 6.0). As part of this technique, hippocampal volume was also stereologically estimated. We performed Stereological estimation in the: pyramidal layer of CA1 and CA3, as well as in the dentate gyrus granule cell layer and hilus. The infra- and suprapyramidal blades were quantified separately and we also provided combined interneuron data.

Data analyzed with T-test and 1 way ANOVA using SPSS software 9.0. A post-hoc Bonferroni multiple comparison was used to compare the groups.

## RESULTS AND DISCUSSION

Multiplex cytokine assay

The entirety of the results from the cytokine analysis is shown in Table 1 and Figure 2. In the ipsilateral hemisphere, the following cytokines were significantly increased at 24 hrs after FPI: GRO KC (P = 0.014), CCL2 (P = 0.008), CCL3 (P = 0.02), TNF-α (P = 0.01). In addition, a trend towards increase was observed for IL 1α (p = 0.09) and VEGF (p = 0.08) (Table 1; Figure 2 A,B). In the contralateral hemisphere, the results demonstrated a significant decrease in the following cytokines at 24 hrs after FPI: IL17 (P = 0.028), IL2 (P = 0.045), IL1ù (P = 0.011), IL10 (P = 0.015), IFNÛ (P = 0.018), IL13 (P = 0.05), IL5 (P = 0.043), GMCSF (P = 0.0006), IP10 (P = 0.022), VEGF (P = 0.021), and GCSF (P = 0.005). Although not significant, a trend was observed where Eotaxin appeared to decrease (p = 0.06) and GRO KC appeared to increase (P = 0.069) (Figure 2 C and D, and Table 1).

Astrocyte morphology

Sholl analysis revealed that at the 20 μm distance from the cell body, a significant increase in the length of ipsi and contra lateral processes of astrocytes from FPI rats was observed (P < .001 and respectively; Figure 3A). In addition, at this distance, astrocytes in the ipsilateral hippocampus had significantly
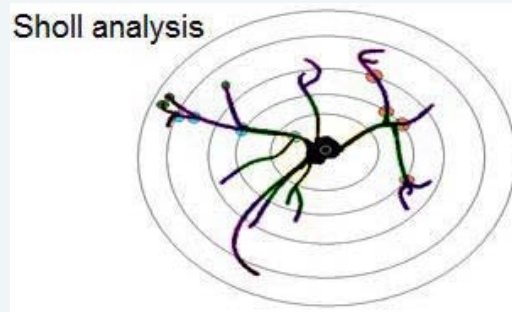
Figure 1 Schematic diagram illustrating the method of sholl analysis. Processes traced directly from cell body are considered first order process and those arising from the first order processes are second order process, and so on. Spheres with a diameter of 10 μm increments were chosen for this study. The total amount of bifurcations, process endings and intersections through each sphere are shown.
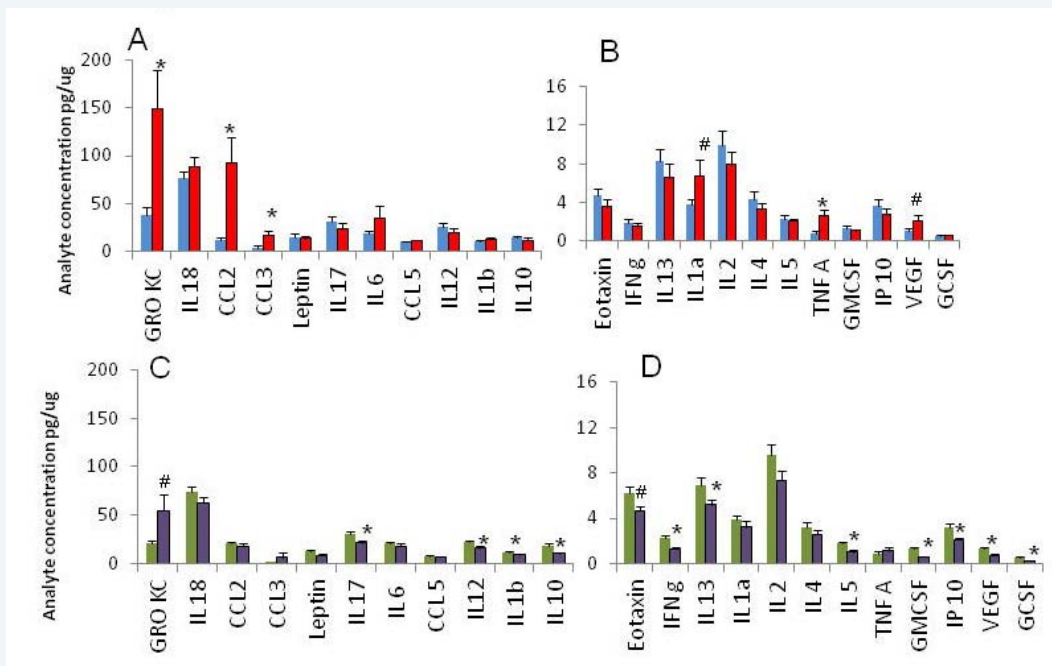
Figure 2 Analysis of cytokine concentrations after sham or FPI in the ipsilateral cortex (A and B) and contralateral cortex (C and D) at 24 hrs after FPI. A number of cytokines were found to be significantly increased in the ipsilateral hippocampus, whereas in the contralateral hippocampi, there were numerous decreases in cytokine concentration. In addition, a trend toward significance was also observed for various cytokines in the ipsilateral and contralateral hippocampi. The raw data for this analysis can be found in table 1. Taken together, the data illustrate a complex inflammatory response in the hippocampus that greatly varies from one hemisphere to the other.
* indicates significant alteration (p < 0.05). # indicates a trend of significant change (p < 0.09 but > 0.05).

more nodes (P < 0.03) and intersections (p < .002) compared to shams, whereas astrocytes in the contralateral hemisphere had significantly more endings compared to shams (P < .02). At the 30 μm distance, astrocytes from the ipsilateral cortex of FPI rats had significantly more endings (P < .002) and greater length (P < .005) compared to shams (Figure 3 B). The data show that the astrocytic processes from FPI animals have increased length, and branch points compared to sham rats, indicative of relatively rapid astrocyte activation in the hippocampus 24 hrs after FPI. Such morphological data is consistent with our data showing increased expression of many pro-inflammatory proteins at this time point and further demonstrate an early post-traumatic hippocampal inflammatory response.

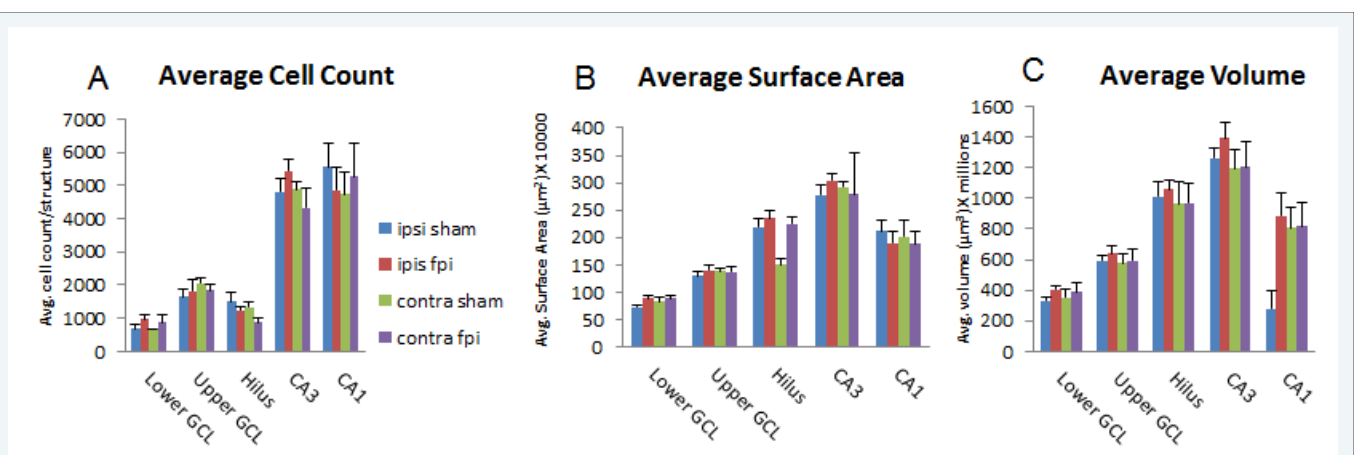**Parvalbumin-labeled interneurons quantification**

Stereological analysis of parvalbumin-labeled interneurons revealed no significant differences in the ipsilateral or contralateral hippocampus following TBI, in any of the regions examined (Figure 4).

DISCUSSION

The results from the current study revealed an increase in several different cytokines in the ipsilateral hippocampus and a concomitant decrease in various cytokines in the contralateral hippocampus at 24 hrs after a FPI. In addition, the data revealed rapid post-traumatic astrocyte activation in hippocampal CA4 (hilus), that is more robust in the ipsilateral hemisphere at the 24 hr post-TBI time point. Despite the rapid appearance of

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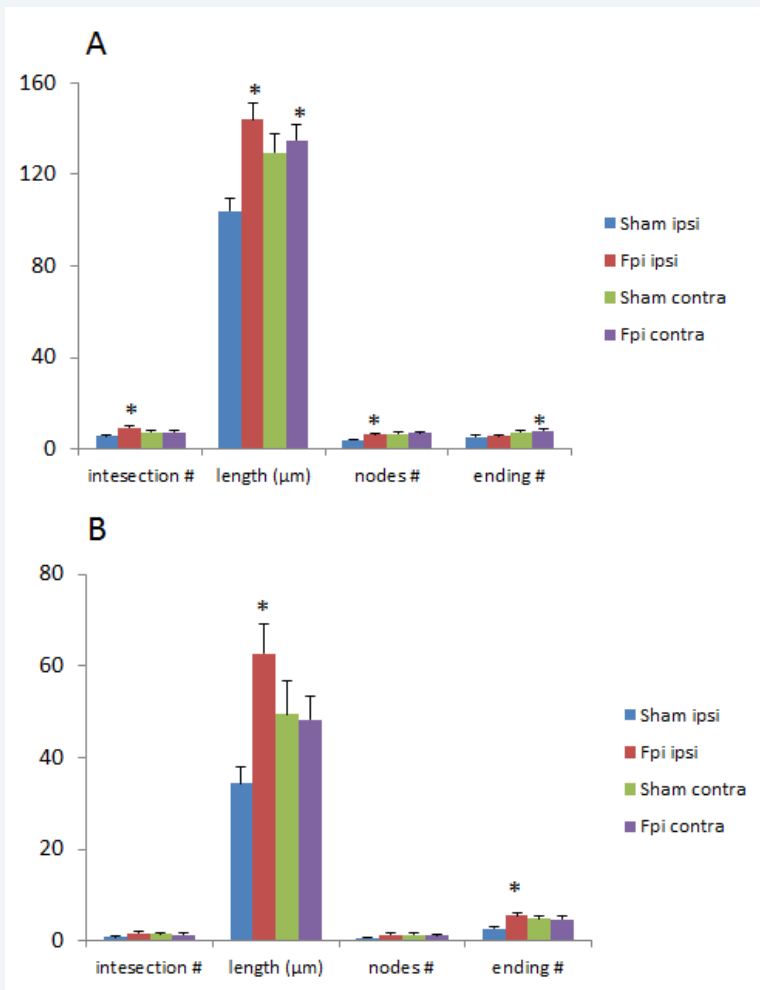
Figure 3 Analysis of astrocyte morphology at 20 μm from the cell body (A) and 30 μm from the cell body (B) at 24 hour after FPI. In A, a significant increase is observed in the number of intersections and nodes at the 20 μm sphere, in the ipsilateral hippocampus following FPI. At this same distance from the cell body, bilateral increase in astrocyte process length was observed. The number of astrocytic endings was significantly increased in the contralateral hemisphere at this distance from the cell body. In B, at 30 μm from the cell body, no significant differences were observed for the total number of intersections or nodes. However, a significant increase in astrocyte length and endings was observed in the ipsilateral hippocampus at this distance from the cell body. Taken together, these data indicate bilateral astrocyte activation at 24 hrs after TBI that is more pronounced in the ipsilateral hippocampi.

Figure 4 Sterological analysis of parvalbumin-labeled cells in the hippocampus. Several parameters are illustrated: number of parvalbumin-labeled cells (A), surface area or regions of interest (B) and volume or region of interest (C). No significant differences were observed for any of the parameters examined.

molecular and anatomical inflammation in the hippocampus, no loss of parvalbumin-labeled interneurons in the hippocampus was observed at 24 hrs after FPI.

The findings from the current study showing a rapid inflammatory response in the archicortex, following an FPI with a neocortical focus, suggests that the TBI-induced neuroinflammatory response extends into the hippocampus within 24 hrs after injury. It is possible that early inflammation can promote a pro-epileptogenic environment because inflammation has been implicated in the development of PTE [31,32]. Moreover, previous studies using chemoconvusant-induced epilepsy have linked neuroinflammation to pro-epileptigenic changes in the hippocampus [33,34,28]. The hippocampal cytokine data from the current study are consistent with, and expand upon, previous studies. For example, Fan and colleagues observed increased IL-1ù mRNA and TNFα mRNA in the hippocampus in rats at 6 hrs after FPI [35,36]. Another study showed acute elevation of IL1α, IL- 6, IL-1ù and TNF-α in the hippocampus of rats following TBI [19]. Therefore, several lines of evidence demonstrate a rapid increase in hippocampal cytokine expression following FPI. However, the present study expands on these data by providing analysis of many more cytokines and chemokines than those previously reported (Table 1) and adds the dynamic of bilateral astrocyte activation in the hippocampus that is more robust in the ipsilateral hippocampi.

Studies have examined the neuropathological and behavioral consequences of altering neuroinflammation after injury. Administration of IL18 binding protein, a specific inhibitor of IL18, promotes an improved neurological score on measures of alertness, motor and physiological behavior after TBI [37]. The administration of IL10, an anti-inflammatory cytokine, promotes enhanced motor outcome after FPI [38].

Studies manipulating TNFα have yielded mixed outcomes. One study showed that increased expression of TNFα exacerbates pathology after TBI [39], whereas other studies have observed a protective role for TNFα after TBI [40,41]. These latter studies demonstrated that TNFα and TNFα-receptor knockout mice have greater deficiencies after TBI [40,41]. These conflicting results suggest both pro- and anti-inflammatory effects of TNFα [42] that are likely related to when, where and in what concentrations TNFα is expressed following injury. Therefore, the spatial and temporal gradient of this (and all) cytokine(s) after TBI needs further elucidation.

In addition to elevated cytokines, the current study also found an alteration to various chemokines (Table 1) in the hippocampus at 24 hrs after FPI. It is pertinent to note that one of the most robust increases was observed in the ipsilateral hippocampus for CCL2 protein, also known as monocyte chemoattractant protein-1 (MCP-1). This finding is consistent with a clinical study where increased MCP-1was observed in the cerebral spinal fluid of TBI patients on days 0, 1 and 2 of their hospital admission [43]. In the same study, researchers observed reduced astrogliosis and neuronal death as well as improved behavioral outcome after TBI in CCL2-/- mice [43]. This finding suggests that increased CCL2 may contribute to anatomical, physiological and behavioral pathology of TBI. Therefore, altering specific chemokines might provide novel therapeutic targets.

Chemokine and cytokine alterations are not the only indices of the neuroinflammatory response following TBI. GFAP-labeled astrocyte activation and its related elevation of GFAP protein is also a hallmark of TBI. Studies in adults and children have found that injury severity predicted the extent of elevated levels GFAP in serum [44-47]. Considering that GFAP is expressed exclusively in astrocytes, astrocyte activation and dysfunction may mediate disease pathogenesis, including seizure disorders [48-52].

For example, ablation of astrocytes resulted in an increased tissue loss after Controlled Cortical Impact (CCI) TBI [53]. Moreover, astrocyte activation in the hippocampus may contribute to a hyperexcitable circuit.

Shapiro and Ribak [54], and Shapiro et al. [55], showed that following pilocarpine-induced seizures, newly born dentate granule cells extend hilar basal dendrites along the processes of hypertrophied GFAP-expressing astrocytes [54,55]. These hilar basal dendrites were targeted for aberrant synaptogenesis by mossy fibers, leading to the development of a so-called, "recurrent excitatory circuitry" [55,56]. Thus, there are several mechanisms whereby TBI-induced changes to hippocampal astrocytes may promote a pro-epileptogenic environment.

In a previous study, Mukherjee et al., showed the spatial and temporal astrocytic response to FPI in mouse cortex [26]. They showed that at 1 day after injury, GFAP-labeled astrocytes were migrating towards the injury site and that by 3 days after injury, a robust astrocyte reaction was observed that was concentrated in the peri-injury area. In the current study, the data show that as early as 1 day after FPI in the rat, astrocyte activation in the hippocampus is observed. Thus, it would seem as though the signal for astrocyte activation in cortex, including archicortex is signaled within 1 day of an FPI.

Considering that the pro-inflammatory proteins are more robustly increased in the ipsilateral hippocampus at 24 hrs after injury, it is not surprising that a more robust activated morphology was observed in the ipsilateral hippocampus relative to the contralateral hemisphere. Interestingly, a previous study reported a significant decrease in the number of astrocytes in the hippocampus at 24 h after FPI with minimal changes in morphology [57]. However, that study did not examine astrocytes in the hilus, which is where astrocytes were examined in the current study. Moreover, Zhao et al. [57] used a more lateral placement for their injury (4.8 mm diameter hole on the right parietal cortex at 4.5 mm behind and 3.0 mm lateral to the bregma) relative to the coordinates used in the current study, which could potentially influence the pattern of astrocytosis.

Astrocyte activation can be indicative of neuronal damage and loss [58-60]. Interneuron loss in the hippocampus has been implicated in epileptogenesis in numerous models [61-63]. Because interneuron loss is known to occur within 1 week following TBI [63-68], we hypothesized that the astrocyte activation observed in the hippocampus by 1 day post-TBI would be indicative of a loss of hilar interneurons.

Such a finding has not been previously reported at such an early timepoint after TBI and evidence was lacking from the present study. In support of this null finding, Toth and colleagues [66] showed that Parvalbumin- positive interneurons are

relatively resilient following TBI. Thus, it is possible that other populations of interneurons are effected following TBI. It is also possible that despite the fact that no changes to the number of Parvalbumin-labeled interneurons was observed in the current study, plasticity of their connectome cannot be ruled out with regards to the contributions of these cells to hyper-excitability following TBI [69]. It is also possible that, as has been previously reported, this population of hilar interneurons become affected at later timepoints after FPI. More studies are needed to better define the timing of interneuron loss and plasticity in different models of TBI.

CONCLUSION

This study demonstrates a rapid inflammatory and astrocytic response in the ispi and contralateral hippocampus at 24 hrs after FPI in the rat, but no change in parvalbumin-expressing neuronal numbers. It is possible that inflammation, as well as neuroplasticity to hippocampal interneurons may contribute to subsequent pro- epileptogenic alterations to hippocampal circuitry function following TBI. Future studies are needed to fully define the epileptogenic contributions of the inflammatory and neuropathological changes in the hippocampus following TBI.

ACKNOWLEDGMENT

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