Brain Region-Specific Histopathological Effects of Varying Trajectories of Controlled Cortical Impact Injury Model of Traumatic Brain Injury

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Summary

Aims: Traumatic brain injury (TBI) occurs when the head is impacted by an external force causing either a closed or penetrating head injury through a direct or accelerating impact. In laboratory research, most of the TBI animal models focus on a specific region to cause brain injury, but traumatic injuries in patients do not always impact the same brain regions. The aim of this study was to examine the histopathological effects of different angles of mechanical injury by manipulating the trajectory of the controlled cortical impact injury (CCI) model in adult Sprague-Dawley rats. Methods: The CCI model was manipulated as follows: conventional targeting of the frontal cortex, farthest right angle targeting the frontal cortex, closest right angle targeting the frontal cortex, olfactory bulb injury, and cerebellar injury. Three days after TBI, brains were harvested to analyze cortical and hippocampal cell loss, neuroinflammatory response, and neurogenesis via immunohistochemistry. Results: Results revealed cell death in the M1 region of the cortex across all groups, and in the CA3 area from olfactory bulb injury group. This observed cell death involved upregulation of inflammation as evidenced by rampant MHCII overexpression in cortex, but largely spared Ki-67/nestin neurogenesis in the hippocampus during this acute phase of TBI. Conclusion: These results indicate a trajectory-dependent injury characterized by exacerbation of inflammation and different levels of impaired cell proliferation and neurogenesis. Such multiple brain areas showing varying levels of cell death after region-specific CCI model may closely mimic the clinical manifestations of TBI.

Introduction

Traumatic brain injury (TBI) affects more than 1.7 million people annually in the USA [1] and a major cause of long-term disability and death. The high financial costs necessary to facilitate the care and rehabilitation of affected individuals [1,2] coupled with the current lack of therapies indicate that TBI represents a significant unmet medical need. TBI has become a signature of wars in Afghanistan and Iraq, with the surge in patient population consisting of soldiers coming back from the war. Blast-induced injury is the most common type of injury among military personnel [3,4]. These people suffer physical and mental dysfunctions ranging from mild headaches, dizziness, and nausea to severe motor abnormalities and cognitive impairments, such as problems with memory, concentration, and decision making [5]. The diagnosis of TBI focuses on the history and physical examination, with continuing observation of the patient for the development of possible worsening symptoms that point toward the progression of physical and cognitive deficits, development of seizures, and overall altered mental status and well-being. TBI can be categorized depending on its level of severity and mechanism of action [6–8]. Replicating in animal models, the exacerbating clinical factors involved in the severity and outcomes of TBI injuries will be critical to our understanding of the disease pathology and treatment. Most of the animal models used to detect the effects left after TBI use a single trajectory of impact toward the brain, but in reality, especially in the battlefield, an impact to the head can come from any direction or angle, consequently affecting different areas of the brain. For this reason, we hypothesized that depending on the angle of the impact and specific brain regions affected, TBI would produce varying histopathological outcomes. In this study, we manipulated the trajectories and the brain regions (cortex, olfactory bulb, and cerebellum) targeted by the controlled cortical impact (CCI) model of TBI. Endpoints involved immunohistochemical analyses combined with unbiased stereology when possible, to assess cell loss and neuroinflammation in the cortex and...
hippocampus, and neurogenesis in the hippocampal CA3 which has been implicated in the manifestation of cognitive impairments in many acute and chronic brain disorders, including TBI [8–10].

Material and Methods

Subjects

Experimental procedures were approved by the University of South Florida Institutional Animal Care and Use Committee (IACUC). All animals were housed under normal conditions (20°C, 50% relative humidity, and a 12-h light/dark cycle). Necessary precautions were taken to reduce pain and suffering of animals throughout the study. All studies were performed by personnel blinded to the injury condition.

Surgeries

Ten–week-old male Sprague-Dawley rats (n = 40) (Harlan Laboratories, Inc., Indianapolis, IN, USA) were subjected to different TBI angular manipulations using a controlled cortical impactor (CCI) (Pittsburgh Precision Instruments, Inc., Pittsburgh, PA, USA) or sham surgery (non-TBI or control) (n = 6). Animals were placed under deep anesthesia using 1–2% isoflurane, and it was maintained using a gas mask. All animals were fixed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). After exposing the skull, the following manipulations of the CCI model were performed: conventional rat model of TBI targeting the frontal cortex (AP = 2.0 mm, ML = 2.00, DV = 1.0 mm) (n = 8), farthest right angle from midline targeting the frontal cortex (FRA) (AP = 2.0 mm, ML = 2.0 mm, DV = 1.0 mm) (n = 8), closest right angle from midline targeting the frontal cortex (FRA) (AP = 2.0 mm, ML = 2.0 mm, DV = 1.0 mm) (n = 8), and cerebellum (AP = −11.6, ML = 0.2 mm, DV = 1 mm) (n = 8). The velocity of the impactor reached 6.0 m/s with a depth of 1.0 mm below the dura matter layer and remained in the brain for 150 milliseconds (ms). The impactor rod was angled 15° vertically to maintain a perpendicular position in reference to the tangential plane of the brain curvature at the impact surface. The impactor was adjusted to 10° for the CRA and 20° for the FRA. A linear variable displacement transducer (Macrosensors, Pennsauken, NJ, USA), which was connected to the impactor, measured the velocity and duration to verify consistency as previously described [8, 10, 11]. Sham surgery consisted of animals exposed to anesthesia, craniectomy, and suturing. An electric drill was used to perform the craniectomy of about 2.5 mm radius with coordinates calculated from the bregma at −0.2 anterior and +0.2 mm lateral right. An automated thermal blanket pad and a rectal thermometer allowed maintenance of body temperature within normal limits. All animals were closely monitored postoperatively with weight and health surveillance recording to minimize any pain or stress associated with the surgery. Rats were kept hydrated at all times, and the analgesic ketoprofen was administered after TBI surgery and as needed thereafter. Pre- and post-TBI, rats were fed regular rodent diet. Animals were euthanized 3 days after CCI, perfused, then brains harvested and processed for immunohistochemical analyses.

Histology

Hematoxylin and Eosin Analysis

Hematoxylin and eosin (H&E) staining was performed to confirm the core impact injury of the different angles of TBI. Coronal brain sections (30 μm thick) covering the M1 region of the cortex and the CA3 region of the hippocampus were selected to reveal secondary cell loss. Starting at coordinates AP 6.2 mm and ending at AP-3.8 mm from bregma, olfactory bulb, and coronal brain sections covering the dorsal hippocampus were selected. Series of 6 sections per rat were processed for staining. Cells presenting with nuclear and cytoplasmic staining (H&E) were manually counted in the M1 and CA3 neurons. CA3 cell counting spanned the whole CA3 area, starting from the end of hilar neurons to the beginning of curvature of the CA2 region in both the ipsilateral and contralateral side. Sections were viewed with an Olympus BX60 microscope and MBF CX 9000 camera. All data are represented as mean values ±SEM, with statistical significance set at P < 0.05.

Immunohistochemistry

Under deep anesthesia, rats were sacrificed 3 days after TBI surgery and perfused through the ascending aorta with 200 ml of ice-cold phosphate-buffered saline (PBS), followed by 200 ml of 4% paraformaldehyde (PFA) in PBS. Brains were removed and post-fixed in the same fixative for 24 h followed by 30% sucrose in phosphate buffer (PB) for 1 week. Coronal sectioning was carried out at a thickness of 30 μm by cryostat. H&E staining was performed on every sixth coronal section spanning the dorsal hippocampus. Staining for major histocompatibility complex II (OX6) was performed on every sixth coronal section throughout the entire olfactory bulb, M1, and dorsal hippocampus. Sixteen free-floating coronal sections (30 μm) were incubated in 0.3% hydrogen peroxide (H2O2) solution followed by 1 h of incubation in blocking solution (0.1 M phosphate-buffered saline (PBS) supplemented with 3% normal goat serum and 0.2% Triton X-100). Sections were then incubated overnight with OX6 (major histocompatibility complex II; 1:750 BD) antibody markers in PBS supplemented with 3% normal goat serum and 0.1% Triton X-100. Sections were then washed and biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA) in PBS supplemented with 3% normal goat serum, and 0.1% Triton X-100 was applied for 1 h. Next, the sections were incubated for 60 min in avidin–biotin substrate (ABC kit; Vector Laboratories). All sections were then incubated for 1 minute in 3,3-diaminobenzidine (DAB) solution (Vector Laboratories). Sections were then mounted onto glass slides, dehydrated in ascending ethanol and xylene, and cover-slipped using mounting medium.

Immunofluorescence Staining

Three coronal sections of every sixth coronal section spanning the olfactory bulb and M1 and dorsal hippocampus were processed for neurogenesis analyses from each trajectory groups. There was only sporadic Ki67 or nestin-positive cells detected in the M1 region of the cortex across all groups; therefore, the analyses focused only on the CA3 region of the hippocampus for this part.
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**Stereology and Image J Analysis**

Unbiased stereology was performed on brain sections immunostained with H&E and OX6. Sets of 1/6 section, about 240 μm apart, were taken from the brain spanning AP 6.2 mm to AP – 3.8 mm in all rats. Positive stains were analyzed with a Nikon Eclipse 600 microscope and quantified using Stereo Investigator software, version 10 (MicroBrightField, Colchester, VT, USA). The estimated volume of H&E and OX6-positive cells was examined using the Cavalieri estimator probe of the unbiased stereological cell technique revealing the volume of H&E and OX6 in the olfactory bulb, cortex, hippocampus, and cerebellum. The sampling was optimized for 30% of tissue sections to reduce variance due to counting error. The sampling was optimized for 30% of tissue sections to reduce variance due to counting error. The percent cell loss when compared to the control at 3 days after TBI (Figure 1) was statistically analyzed using ANOVA, and pairwise comparisons among the rest of the TBI groups did not show any significant effects (P > 0.05). Overall, the data demonstrate that even though both sides had significant cell loss in the M1 region, the ipsilateral side displayed more cell death than the contralateral side.

**H&E Quantification in the CA3 Region of the Hippocampus**

Next, we examined the cell loss in the ipsilateral side of the CA3 area of the hippocampus. ANOVA revealed no significant injury effect at 3 days post-TBI on CA3 cell loss in the ipsilateral side (F₅,2₇ = 0.9058) (Figure 2). There was also no significant injury effect on the CA3 cell loss in the contralateral side (F₅,2₇ = 0.8726). Additionally, when the percent difference from ipsilateral side to contralateral side was compared ANOVA did not detect any significant injury effect (F₅,₂₇ = 0.5327).

**Activated Microglial Expression in the M1 Area of the Cortex**

Using Ox-6 marker to detect activated microglia, ANOVA revealed significant injury effect on microglial expression in the ipsilateral side of the M1 region (F₅,₂₂ = 30.69, P < 0.0001) (Figure 1). The ipsilateral side of the conventional, OB, cerebellum, FRA, and CRA models of TBI showed significant cell loss when compared to control at 3 days after TBI (Figure 1) (P < 0.05), but when all the ipsilateral sides of the different groups were compared among each other, there were no other significant differences between them (P > 0.05). Interestingly, we also detected significant difference in cell loss in the contralateral side of the M1 region of the cortex (F₅,₃₅ = 9.206, P < 0.0001). The contralateral side of the conventional, OB, cerebellum, FRA, and CRA groups showed significant cell loss compared to the control (P < 0.05), but there were no other significant differences between groups when all the contralateral sides were compared among each other (P > 0.05). The percent cell loss in M1 (ipsilateral cell loss/contralateral side × 100) showed overall significant injury effect (F₅,₃₂ = 5.622), with the conventional and the CRA groups displaying significantly more cell loss when compared to the cerebellum (P < 0.05), but pairwise comparisons among the rest of the TBI groups did not show any significant effects (P > 0.05). Overall, the data demonstrate that even though both sides had significant cell loss in the M1 region, the ipsilateral side displayed more cell death than the contralateral side.

**Results**

**Histopathological Analysis in the M1 Region of the Cortex**

ANOVA revealed significant injury effect on cell loss in the ipsilateral side of the M1 region of the cortex (F₅,₃₄ = 68.08, P < 0.0001) (Figure 1). The ipsilateral side of the conventional, OB, cerebellum, FRA, and CRA models of TBI showed significant cell loss when compared to control at 3 days after TBI (Figure 1) (P < 0.05), but when all the ipsilateral sides of the different groups were compared among each other, there were no other significant differences between them (P > 0.05).

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**Statistics**

Contralateral and ipsilateral corresponding brain areas were used as raw data providing 2 sets of data per injury condition (TBI vs. sham surgery); therefore, one-way analysis of variance (ANOVA) was used for group comparisons, followed by subsequent pairwise comparisons via Bonferroni test. All data are represented as mean values with ±SEM. Statistical significance was set at P < 0.05 for all analyses.

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**References**

1. Cavalieri estimator probe of the unbiased stereological cell technique revealed the volume of H&E and OX6 in the olfactory bulb, cortex, hippocampus, and cerebellum. The sampling was optimized to count 30% of cells per animal with error coefficients less than 0.07. Each counting frame (100 × 150 μm) for H&E and OX6 was placed at an intersection of the lines forming a virtual grid (175 × 175 μm), which was randomly generated and placed by the software within the outlined structure. Section thickness was measured in all counting sites [9]. Fluorescent imaging was quantified using Zeiss microscope and ImageJ to determine the percent fluorescent area. The level of fluorescent was determined using the integrated density, and mean gray value for a 5 random regions per tissue section and the corrected total fluorescence (CTCF) was calculated using formula CTCF = integrated density – (area of selected cell × mean fluorescence of background readings).

**Histopathological Analysis in the M1 Region of the Cortex**

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**H&E Quantification in the CA3 Region of the Hippocampus**

Next, we examined the cell loss in the ipsilateral side of the CA3 area of the hippocampus. ANOVA revealed no significant injury effect at 3 days post-TBI on CA3 cell loss in the ipsilateral side (F₅,2₇ = 0.9058) (Figure 2). There was also no significant injury effect on the CA3 cell loss in the contralateral side (F₅,2₇ = 0.8726). Additionally, when the percent difference from ipsilateral side to contralateral side was compared ANOVA did not detect any significant injury effect (F₅,₂₇ = 0.5327).

**Activated Microglial Expression in the M1 Area of the Cortex**

Using Ox-6 marker to detect activated microglia, ANOVA revealed significant injury effect on microglial expression in the ipsilateral side of the M1 region (F₅,₂₂ = 30.69, P < 0.0001) (Figure 3). Pairwise comparisons revealed that the conventional, FRA, and CRA models of TBI displayed a significant increase in microglial expression when compared to the control (P < 0.05). The ipsilateral side of the OB and cerebellum showed a significant decrease when compared to the conventional model of TBI (P < 0.05). The ipsilateral side of the FRA and CRA was significantly increased when compared to the OB and also when compared to the cerebellum (P < 0.05). Finally, the ipsilateral side of the FRA was significantly decreased when compared to the CRA group (P < 0.05). Interestingly, a significant injury effect was also detected in the contralateral side determined by ANOVA (F₅,₂₂ = 4.923, P = 0.0036). The pairwise comparison detected that the contralateral side of the control was significantly decreased when compared to the conventional group (P < 0.05). Moreover, the conventional group was significantly increased when compared to the CRA group (P < 0.05). Pairwise comparisons among the rest of the TBI groups did not show any significant effects (P > 0.05).
When the percent difference of OX-6 expression in the contralateral side was compared to the ipsilateral side, there was a significant injury effect ($F_{4,19} = 15.04$, $P < 0.0001$) as determined by ANOVA. The pairwise comparison detected a significant increase in the conventional group versus the OB. The CRA was significantly higher than the conventional group ($P's < 0.05$). Also, there was a significant increase in OX-6 expression in the FRA and CRA groups when compared to the OB group ($P's < 0.05$).

Figure 1 Variations of the CCI trajectory produce consistent cell loss in the M1 region of the cortex. Data confirm typical cell loss in the M1 cortical region in the conventional TBI group (A) as determined by H&E-positive histological staining and Cavalieri method of unbiased stereology. Interestingly, we also detected significant cell death in M1 region of the animals that received OB (B), cerebellum (C), FRA (D), and CRA (E) types of injury. All the groups displayed comparable cortical damage to the conventional TBI group. The contralateral side of the OB, cerebellum, and CRA demonstrates significant cell loss that is also analogous to the conventional group. Interestingly, there was a significant effect in the ordinary and the FRA when contralateral versus ipsilateral sides where compared (F). When all the groups were compared (G), there was also a notable significant cell loss as compared to the non-TBI group. Asterisks (*) denotes significance. Right panel: H&E images from the contralateral and ipsilateral sides of the M1 region of the cortex. Scale bar = 5 μm.
Finally, the OX-6 expression in the cerebellum was significantly decreased when compared to the FRA and CRA groups (\(P's < 0.05\)). Even though both contralateral side and ipsilateral side displayed positive expression of microglia, the ipsilateral side displayed a higher OX-6 expression than the contralateral side (\(P's < 0.05\)).

Figure 2 Variations of the CCI trajectory fail to yield cell loss in the CA3 region of the hippocampus. Further examination of the H&E-positive expression in the hippocampus revealed no significant cell loss across all of the different types of impact trajectories (A–E). Moreover, there was no apparent difference when the percent difference of contralateral versus ipsilateral (F) and the ipsilateral sides (G) was compared. Right panel: H&E images from the CA3 of the hippocampus. Scale bar = 5 μm.
Active Microglia Expression in CA3 Region of the Hippocampus

There was a significant Ox-6 positive effect seen in the CA3 region of the hippocampus in the ipsilateral side as determined by ANOVA ($F_{5,27} = 3.590, P = 0.0128$) (Figure 4). Interestingly, the pairwise comparison showed a significant increase in the ipsilateral side of the OB when compared to the ipsilateral side of the conventional group ($P < 0.05$). Additionally, the OB also showed a significant increase in OX-6 expression when compared to the conventional group ($P < 0.05$).
Controlled impact injury to the olfactory bulb activates microglial cells in the CA3 region of the hippocampus. Increase in expression of the inflammatory marker OX-6 was detected in the CA3 region of the hippocampus in the OB group when compared to the conventional group (A,B), but no significant inflammatory response was detected in all the other groups (C–E), suggesting a critical role for neuroinflammation in the OB 3 days post-TBI. When the percent difference between contralateral side versus ipsilateral side (F) was compared, there was an increase in expression in the OB group as compared to all the other groups. The same was seen when the ipsilateral side of all the groups were compared (G). Asterisk (*) denotes significance at $P < 0.05$. Right Panel: Images of OX-6-positive expression in the CA3 region of the hippocampus. Scale bar = 5 μm.
ipsilateral side of the CRA ($P's < 0.05$). Subsequently, we examine the OX-6 expression in the contralateral side and ANOVA revealed no significant effect at 3 days post-TBI on CA3 region of the hippocampus ($F_{5,27} = 0.8959$). Furthermore, we analyzed the percent difference of the contralateral side versus the ipsilateral side and notice that there was a significant effect detected ($F_{5,22} = 8.967, P = 0.0002$) as determined by ANOVA. Pairwise comparisons demonstrated that the percent difference in OX-6 expression of the OB impact trajectory group was significantly increased when compared to the conventional, cerebellum, FRA, and CRA groups ($P's < 0.05$). Pairwise comparisons among the rest of the TBI groups did not show any significant differences in OX-6 expression ($P's > 0.05$).

**Ki67/Nestin Expression in the CA3 Region of the Hippocampus**

A significant injury effect was detected in the ipsilateral side of the CA3 region of the hippocampus when the fluorescent intensity of neurogenic marker Ki-67 was analyzed ($F_{5,128} = 9.504, P < 0.0001$) as determined by ANOVA (Figure 5). Pairwise comparisons determined that there was a significant decrease in Ki-67 expression in the ipsilateral side of the conventional, OB, cerebellum, FRA, and CRA groups compared to the control group ($P's < 0.05$). However, pairwise comparisons revealed that there was a significant increase in Ki-67 expression in the FRA when compared to the OB group ($P < 0.05$). ANOVA analysis also revealed there was a significant Injury effect on Ki-67 expression when we examined the contralateral sides ($F_{5,125} = 10.14, P < 0.0001$). Pairwise comparisons of the contralateral side demonstrated that there was a significant decrease in the Ki-67 expression in the conventional, OB, cerebellum, FRA, and CRA groups when compared to the control group ($P's < 0.05$). Pairwise comparisons among the rest of the TBI groups did not show any significant differences in Ki-67 expression ($P's > 0.05$). Subsequently, we decided to compare the percent difference of the contralateral side versus the ipsilateral side, but there was no significant injury effect on Ki-67 expression detected ($F_{4,114} = 1.969$).

When nestin immunofluorescent staining was analyzed in the CA3 region of the brain, a significant injury effect was detected in the ipsilateral side of the brain ($F_{5,127} = 7.896, P < 0.0001$) as determined by ANOVA (Figure 6). Pairwise comparisons determined that nestin expression was significantly increased in the FRA when compared to the OB, the cerebellum, and, CRA groups ($P's < 0.05$). There were no other significant differences detected between the groups ($P's > 0.05$). Analyses of the contralateral side revealed no significant injury effect across groups ($F_{5,125} = 1.760$) as determined by ANOVA. Finally, the percent difference between contralateral side versus ipsilateral sides was compared across groups which also revealed no significant injury effect ($F_{4,114} = 0.2916$).

**Discussion**

This study recognized that TBI in the clinic presents with injuries to different parts of the brain. Our laboratory results showed that manipulating the trajectories of the CCI model of TBI led to varying levels of cell death, microglial activation, and neurogenesis. Cell death in the M1 region of the cortex was observed in all groups, but hippocampal cell death was only detected in OB-exposed rats. This cell death involved upregulation of inflammation and impairment in neurogenesis at such early time point post-TBI (3 days). Interestingly, nestin-positive immature cells in the M1 cortical region appear to be spared during this acute TBI, which may be a target for regenerative therapy. Further studies are warranted to assess long-term secondary cell death effects of specific impacted brain trajectories to provide a better guidance of the management and treatment of TBI patients. Additionally, post-TBI symptoms include cognitive and motor alterations [11]. Therefore, future studies should also evaluate behavioral consequences following different impact trajectories.

Blast injury, which is a clinically relevant TBI model [12–15], is technically challenging to recreate in animal models; thus, most TBI approaches focus on a specific type of injury, that is, targeting the cortex [8,16–20]. The present study offers an alternative model in capturing the clinical scenario of varying injuries in TBI. Manipulating the CCI trajectories allows us to mimic the brain region-specific cell loss, but also mechanisms of cell death, such as increased inflammation and impaired neurogenesis [21–25]. There are several experimental models employed to replicate the clinical pathology of TBI. One of the original models of TBI involves the weight drop technique [26,27]. Over the last decade, the CCI model and the fluid percussion injury have been the two widely used TBI models in the laboratory [15,28,29]. Although large animal models are a useful tool in replicating the morphological and different pathological stages of TBI (albeit white matter injury) [30,31], they have been seldom used largely due to the expensive cost and extensive manpower required for post-operational monitoring [15,32,33]. However, for translating novel therapies from the laboratory to the clinic, there has been an increased demand for large animal models of TBI [34,35]. Equally important for TBI animal modeling, the wars in the Middle East have necessitated the development of TBI models to replicate combat scenarios. The penetrating ballistic-like brain injury [26,27,36,37] and shock wave injury models [38,39] have been established to mimic the warfare-associated TBI symptoms [40,41]. The selection of the ideal TBI model is crucial in the investigation of novel therapies. Rigorous testing is required to validate any positive effects these therapies might have toward treating TBI, and in many occasions, the use of several models, including large animals, is needed for optimal results. As the majority of TBI events observed in humans are unique, the model used to replicate this traumatic event must also mirror these distinctive effects. Using a varying CCI model, where the angle of impact is manipulated yet controlled, allows for the validation of potential novel therapies due to the fact that it accounts for the variability that is inherent in cases of TBI occurring in humans.

A major finding here was the increase in the expression of OX-6 marker for active microglia at the acute TBI phase. We attempted to establish the early inflammatory response after TBI. This inflammatory effect was significant in all of the groups regardless of the angle of impact. We also noted a significant effect in the contralateral side of the different trajectories of CCI. Interestingly, the OB group resulted in the highest level of cortical OX-6-positive expression, with a positive
correlation between the increase in cell loss as evident with H&E staining and the increase in OX-6-positive expression in the same region of the cortex. Such cell loss and inflammation in this might be due to anatomical connections, in that the OB projects to the ventral part of the lateral entorhinal cortex, and eventually sending projections to the CA1 field of the ventral hippocampus and feeding to three different areas: the main olfactory bulb, the anterior olfactory nucleus, and the primary olfactory cortex [42–44]. These anatomical pathways may be candidate therapeutic regions for abrogating the inflammatory response to attenuate the cell loss following TBI [45–50].

TBI has been associated with olfactory dysfunction in humans [51,52]. Olfactory alterations can affect the quality of life of patients and are an early indicator of head trauma [51,52].
Post-TBI olfactory dysfunction has been associated with neuropsychiatric dysfunction and cognitive alterations [52]. However, the mechanisms underlying post-TBI olfactory system pathology and its relationship to neuropsychiatric and cognitive symptoms remain to be understood. We acknowledge that the direct injury to OB as performed in this work is nearly impossible in humans. Consequences of the TBI observed in humans are difficult to replicate using rodent models because of differences in the olfactory system anatomy and cranial structure [51]. Nonetheless, our results provide insights about the complex pathology and variable symptomatology of TBI and their relationship with the direction of the impact.

An equally important observation here is the altered proliferation and differentiation of endogenous cells after varying CCI trajectories. Newly formed cells (e.g., neuroblasts) are known to migrate, to a modest extent, to the injured peri-infarct cortex from the neurogenic subventricular zone [53,54]. Similarly, cell proliferation increases in the other neurogenic niche of the hippocampal dentate gyrus, and these immature cells may rescue the hippocampal cell death after experimental TBI [55,56]. In the

![Nestin Images](image-url)

Figure 6 The ipsilateral side of the FRA group shows nestin expression 3-days post-TBI. Positive nestin immunofluorescent staining was detected in the ipsilateral side of the brain of the FRA (D) group when compared to the OB (B), the cerebellum (C), and CRA (E) groups. There were no other significant effects detected (A, D, F, and G). Asterisk (*) denotes significance at $P < 0.05$. Right panel: Nestin-positive fluorescent images in the CA3 region of the hippocampus. DAPI was used as counterstaining (blue). Scale bar = 5 μm.
In summary, this study showed varying histopathological effects after different CCI angles at 3 days post-TBI. We demonstrated that the initial cell death response in all the different trajectories involved the inflammatory response accompanying cell loss that was prominent in the M1 region of the cortex. However, while inflammation accompanied cortical cell death, inflammation and cell death did not extend yet to the CA3 region of the hippocampus at this acute TBI phase. Interestingly, CCI damage to the OB already resulted in early elevation of microglial activation in the hippocampus, implicating OB’s key role in TBI-induced inflammation. At the same acute TBI time point, a neurogenic response was nondetectable in the cortex, whereas cellular proliferation was impaired but neural differentiation was unaltered in the CA3 region of the hippocampus, which would suggest that neurogenesis is partly intact as the TBI secondary cell death progresses from the primary damaged cortical site toward the hippocampus. Additionally, studies are warranted to determine the extent of the secondary cell death damage in chronic TBI following varying CCI trajectories, as well as to gauge the behavioral effects associated with different angles of mechanical injury. Successful translation of novel therapies from the laboratory to the clinic requires the use of relevant animal models. In the battlefield, for example, an impact to the head can come from any direction. In this study, we have shown that the direction of the impact to the head affects the resulting brain damage. Varying the trajectory of the CCI model of TBI could provide insights about the variable clinical manifestations of TBI. Future studies should consider the effect of the direction of the impact on the TBI pathology, and in the development of new models and research instruments. We also acknowledge that a blast injury may replicate many of the pathological symptoms manifested by combination of trajectory injuries, but also recognize the logistical challenge of pursuing such model, which has relegated the use blast injury model of TBI to a few research teams.

Conclusion

Altogether, these results suggest that varying the trajectory of the CCI model of TBI resulted in different levels and extent of brain damage. Manipulating the angle of impact to the cortical region appears to consistently produce similar core and peri-impact damage to M1 and CA3, respectively, at least for the short 3-day period following TBI. Increased OX-6 expression in the M1 cortical region in all CCI trajectories, and in the CA3, area of OB-injured brain indicates a pivotal role of inflammatory response at this acute phase of TBI. Equally important is the observation of impaired cell proliferation, but intact neural differentiation in the CA3 region of the hippocampus, suggesting partly functional neurogenesis during TBI secondary cell death.

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Conflict of Interest

The authors declare no conflict of interest.

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