FTY720 does not protect from traumatic brain injury in mice despite reducing posttraumatic inflammation

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Inflammation is a pathological hallmark of traumatic brain injury (TBI). Recent evidence suggests that immune cells such as lymphocytes are of particular relevance for lesion development after TBI. FTY720, a sphingosine-1-phosphate (S1P) receptor modulator, sequesters T lymphocytes in lymphoid organs and has been shown to improve outcome in a variety of neurological disease models. We investigated the mode of FTY720 action in models of TBI. Focal cortical cryolesion was induced in C57BL/6 mice treated with FTY720 (1 mg/kg) or vehicle immediately before injury. Lesion size was assessed 24 h later. Immune cells in the blood and brain were counted by flow cytometry and immunocytochemistry. The integrity of the blood–brain barrier was analyzed using Evans Blue dye. To validate the findings in a diffuse brain trauma model, FTY720-treated mice and controls were subjected to weight drop contusion injury and neurological deficits were assessed until day 7. As expected, FTY720 significantly lowered the numbers of circulating lymphocytes and attenuated the invasion of immune cells into the damaged brain parenchyma. However, FTY720 was unable to improve lesion size or functional outcome in both trauma models at either stage, i.e. acute vs chronic. Accordingly, the extent of blood–brain barrier disruption and neuronal apoptosis was similar between FTY720-treated mice and controls. We conclude that pharmacological S1P receptor modulation is an unfavorable strategy to combat TBI. Moreover, our findings put into perspective the pathophysiologic relevance of inflammatory cells in traumatic neurodegeneration.

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1. Introduction

There is increasing evidence that the extent of tissue damage after traumatic brain injury (TBI) depends not only on the initial impact but also on secondary inflammatory circuits which can further enhance lesion growth and cause the deterioration of neurological deficits (Morganti-Kossman et al., 2002; Yatsiv et al., 2002; Cedergren and Siesjö, 2010; Xiong et al., 2013). While previous research mainly focused on the role of soluble immune mediators or innate immune cells (Clark et al., 1994; Engel et al., 1996; Morganti-Kossman et al., 1997), more recent studies suggest that T lymphocytes, which belong to the adaptive immune system, are also critically involved in the pathophysiology of TBI (Fee et al., 2003; Clausen et al., 2007; Czigner et al., 2007). Depending on the animal model T lymphocytes can be found in the posttraumatic brain already within hours (Czigner et al., 2007) and it has been shown that lymphocyte-deficient Rag1−/− mice are profoundly protected from stab wound injury of the cortex (Fee et al., 2003). Hence, the prevention of immune cell trafficking, in particular T lymphocytes, might be a promising strategy to combat trauma-related neurodegeneration.

FTY720 is the first of a new class of immunomodulatory agents, sphingosine-1-phosphate (S1P) receptor agonists (Brinkmann et al., 2010). After phosphorylation by the sphenosine-kinase isoform 2 (SK2) FTY720 acts on four of the five S1P receptor subtypes. S1P receptors are widely expressed in the body including the central nervous system (CNS) (Dev et al., 2008) and therefore, exert pleiotropic functions. The most prominent effect of FTY720 is the reduction of peripheral lymphocyte counts by blocking the egress of lymphocytes from lymphoid organs through agonist-induced receptor internalization (Brinkmann et al., 2010). Other potential modes of FTY720 action directly within the CNS include the attenuation of blood–brain barrier disruption (Wei et al., 2011; Campos et al., 2013) or immediate neuroprotection (Czech et al., 2009; Hasegawa et al., 2010). Accordingly, FTY720 has been shown to act beneficial in animal models of multiple...
sclerosis (Kataoka et al., 2005; Balatoni et al., 2007), an indication for which it is already in clinical use, ischemic stroke (Czech et al., 2009; Pfeilschifter et al., 2010; Kraft et al., 2013), or intracerebral hemorrhage (Rolland et al., 2013).

The aim of the present study was to test the efficacy of FTY720 in different models of TBI in mice.

2. Materials and methods

2.1. Mice

A total of 166 C57 BL/6 NCr mice were used in this study. All experiments were approved by institutional (University of Würzburg, Germany) and regulatory (local government of Lower Franconia, Bavaria, Germany) authorities and were conducted according to the EU Directive 2010/63/EU and the ARRIVE criteria (Kilkenny et al., 2012).

2.2. Cortical cryolesion model

Cortical cryolesion was induced as described previously (Raslan et al., 2012). Briefly, mice were anesthetized with intraperitoneal injections of ketamine (0.1 mg/g) and xylazine (0.005 mg/g). Surgery was performed on the right parietal cortex after exposing the skull through a scalp incision. A copper cylinder with a tip diameter of 2.5 mm was filled with liquid nitrogen (−196 °C) and placed stereotactically on the right parietal cortex (coordinates from the bregma: 1.5 mm posterior, 1.5 mm lateral) for 90 s. Sham-operated animals underwent the same surgical procedure but without cooling the copper cylinder. All operations were performed by the same operator and the animals were randomly assigned to the treatment groups by an independent person not involved in data acquisition and analysis. We performed the evaluation of all read-out parameters while being blinded to the experimental groups.

2.3. Weight drop model

Experimental closed head injury was performed as previously described (Flierl et al., 2009; Albert-Weissenberger et al., 2012a, b). Briefly, after the induction of isoflurane anesthesia, spontaneously breathing mice were placed in a stereotactic frame and a midline longitudinal scalp incision was made and the skull was exposed. After the identification of the impact area, i.e. the right anterior frontal area 1 mm lateral to the midline in the mid-coronal plane, a weight with a diameter of 2 mm was dropped with a final impact of 0.01 J, resulting in diffuse brain injury. After trauma, mice received 95% oxygen for 2 min and were returned to their cages. Sham-operated animals underwent the same surgical procedure but without cooling the copper cylinder. All operations were performed by the same operator and the animals were randomly assigned to the treatment groups by an independent person not involved in data acquisition and analysis. We performed the evaluation of all read-out parameters while being blinded to the experimental groups.

2.4. FTY720 treatment

Immediately before the induction of focal cryolesion or diffuse weight drop injury, mice received a single intravenous injection of FTY720 (dissolved in 0.9% sodium chloride, Cayman Chemical, 10006292) at a dose of 1 mg/kg body weight (Kraft et al., 2013). Control animals received equal volumes of 0.9% sodium chloride (vehicle).

2.5. Determination of lesion size after cortical cryolesion

Twenty-four hours after cryolesion mice were sacrificed and the brains were quickly removed and cut in five 2 mm thick coronal sections using a mouse brain slice matrix (Harvard Apparatus). The slices were stained for 20 min at 37 °C with 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich) in PBS to visualize the lesion. Lesion volumes were calculated by volumetry (ImageJ software, National Institutes of Health, USA) (Raslan et al., 2010).

2.6. Determination of brain edema and blood–brain barrier leakage

Brain edema formation after cryolesion in the ipsilesional or contralesional hemisphere was determined using the wet–dry weight technique (Langhauser et al., 2012) according to the following formula: 

\[
\text{Free water content (FWC)} = \left( \frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}} \right) \times 100.
\]

Blood–brain barrier leakage following TBI was determined according to Langhauser et al. (2012) with minor modifications. Briefly, 100 µl of 2% Evans Blue dye (Sigma-Aldrich) diluted in 0.9% NaCl was injected intravenously 23 h after the induction of cortical cryolesion and the brains were removed 1 h later. Then, the brain tissue was immersed in 300 µl formamide (Sigma-Aldrich) at 55 °C overnight to extract the dye. The concentration of extracted dye was quantified by photometry with an optical absorbance at 610 nm against Evans Blue/formamide standards.

2.7. Immunocytochemistry

Immunocytochemistry was performed as previously described (Albert-Weissenberger et al., 2012a). For the detection of macrophages, granulocytes, and neurons cryo-embedded brain slices were stained with antibodies against CD11b (activated microglia/macrophages, rat, diluted 1:100, Serotec MCA711), Ly-6B.2 (neutrophils, rabbit, diluted 1:500; Serotec MCA771G) or NeuN (neurons, mouse, diluted 1:1000, Millipore MAB377), respectively. Neuronal apoptosis was assessed using a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) in situ Cell Death Detection Kit (Roche, Basel, Switzerland) according to the manufacturer’s instructions.

2.8. Real-time PCR

Tissue homogenization, RNA isolation, and real-time PCR were performed as described previously (Albert-Weissenberger et al., 2012a). Briefly, total RNA was prepared with a MicrAc D–8 power homogenizer (ART, Mühlheim, Germany) using the TRizol reagent (Invitrogen, Karlsruhe, Germany). Then, 250 µg of total RNA was reversely transcribed with the TaqMan Reverse Transcription Reagents (Applied Biosystems, Darmstadt, Germany) according to the manufacturer’s protocol using random hexamers. Relative mRNA levels of claudin–5 and occludin were quantified with the fluorescent TaqMan technology. PCR primers and probes specific for claudin–5 (assay ID: MM00727012_s1) and occludin (assay ID: MM00509012_m1) were obtained as TaqMan Gene Expression Array (Applied Biosystems). Glycereraldehyde 3-phosphate dehydrogenase (GAPDH) and β-Actin (TaqMan Predeveloped Assay Reagents for gene expression, part number: 4352339E and 4352341E; Applied Biosystems) were used as endogenous controls to normalize the amount of sample RNA. The PCR was performed with equal amounts of cDNA in the GeneAmp 7700 sequence detection system (Applied Biosystems) using the TaqMan Universal PCR Master Mix (Applied Biosystems). Reactions were incubated at 50 °C for 2 min, at 95 °C for 10 min followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The ΔΔCt method was used for the relative quantification of gene expression (Livak and Schmittgen, 2001).
2.9. Flow cytometry

For flow cytometry analysis of peripheral immune cells, 100 μl blood was collected in heparin-coated tubes and red blood cells were lysed using RBC lysis buffer (Biolegend) following the manufacturer’s description. Lymphocytes were stained with monoclonal antibodies anti-CD4 FITC (Clone GK1.5, Biolegend) and anti-CD8 PE (Clone 53-6.7, BD Biosciences) following the standard protocols. To determine absolute cell numbers, calibration beads (BD Biosciences) were added. Flow cytometry was performed using a FACS Calibur (BD Biosciences).

2.10. Statistics

All results were expressed as mean ± standard error of mean except for the NSS scale which is depicted as scatter plots including median with the 25% percentile and the 75% percentile given in brackets in the text. Numbers of animals necessary to detect a standardized effect size on lesion volumes ≥0.2 on day 1 after cortical cryoesthesia or NSS ≥0.2 on day 1 after weight drop injury, respectively, were determined via a priori sample size calculation with the following assumptions: α = 0.05, β = 0.2, mean, and 20% SEM of the mean (GraphPad Stat Mate 2.0; GraphPad Software). For statistical analysis, PrismGraph 5.0 software package (GraphPad Software) was used. Data were tested for Gaussian distribution with the Kolmogorov–Smirnov test and in the case of measuring the effects of 2 factors simultaneously analyzed by 2-way ANOVA with post hoc Bonferroni correction. If only two groups were compared, unpaired, two-tailed Student’s t-test was applied. P values <0.05 were considered statistically significant.

3. Results

3.1. FTY720 induces rapid and sustained lymphocytopenia

FTY720 sequesters circulating lymphocytes into their lymphoid organs through agonist-induced receptor internalization (Brinkmann et al., 2010). Therefore, we assessed the number of CD4+ and CD8+ lymphocytes in the peripheral blood of mice, 3 h and 24 h after the administration of vehicle or FTY720 (1 mg/kg body weight i.p.). The number of peripheral blood CD4+ and CD8+ lymphocytes after FTY720 changed significantly: compared with vehicle treated mice the baseline cell count of CD4+ and CD8+ cells decreased from 67780 ± 3048 ± 1006 CD4+ cells/100 μl and 48970 ± 4660 CD8+ cells/100 μl to 3048 ± 1006 CD4+ cells/100 μl (P < 0.0001) and 4616 ± 637 CD8+ cells/100 μl (P < 0.0001) after 3 h (Fig. 1). No further changes in lymphocyte fractions were observed between 3 h and 24 h.

These findings indicate that FTY720 in the given dose exerts rapid and pronounced biological effects on peripheral lymphocytes.

3.2. FTY720 does not protect from focal or diffuse brain trauma

Next, we investigated whether FTY720 protects from focal brain trauma. C57BL/6 mice received 1 mg/kg FTY720 and were subjected to cortical cryoesthesia immediately afterwards. Lesion volumes did not differ between FTY720-treated mice and vehicle-treated controls on day 1 after trauma (lesion area: 12.9 ± 2.4 mm³ [vehicle] vs 12.1 ± 2.1 mm³ [FTY720], respectively; P > 0.05) (Fig. 2A).

The cryoesthesia model only causes focal injury of circumscribed cortical areas. Therefore, we also investigated the efficacy of FTY720 in a weight drop model which induces diffuse brain trauma and which better mimics the clinical situation (Albert-Weissenberger et al., 2012b; Xiong et al., 2013). FTY720 did not improve functional outcome on day 1 after diffuse TBI (median NSS: 1.5 [1.0, 2.3] [vehicle] vs 3.0 [1.0, 6.3] [FTY720], respectively; P > 0.05) (Fig. 2B). In order to exclude the possibility that FTY720 mediates neuroprotection only at more advanced stages of TBI we in addition determined the neurological severity score (NSS) on day 3 and day 7 after weight drop (Fig. 2B). Application of FTY720 immediately before the induction of trauma was ineffective also at later time points (day 3: median NSS: 2.0 [1.0, 3.0] [vehicle] vs 2.0 [1.0, 3.3] [FTY720]; P > 0.05; day 7: median NSS: 1.0 [0.0, 1.0] [vehicle] vs 1.0 [0.0, 2.0] [FTY720]; P > 0.05) (Fig. 2B).

Modulation of sphingolipid signaling might exert direct neuroprotective effects because S1P receptors are also expressed in neurons and FTY720, due to its lipophilic structure, can easily cross the blood–brain barrier (Dev et al., 2008; Miron et al., 2008). However, TUNEL assay did not reveal any difference in neuronal apoptosis between FTY720-treated mice and vehicle-treated controls on day 1 after cortical cryoesthesia (253.5 ± 48.1 TUNEL/NeuN positive cells per slice in the ipsilateral hemisphere for vehicle-treated mice vs 263.5 ± 55.1 cells for FTY720-treated mice, P > 0.05) (Fig. 3).

3.3. FTY720 does not stabilize the blood–brain barrier after brain trauma

Blood–brain barrier disruption and subsequent edema formation can cause the deterioration of neurological symptoms in TBI patients. For this reason as well as due to the fact that S1P receptors are expressed at the cerebral endothelium (Weiss et al., 2009; van Doorn et al., 2012), we analyzed the consequences of FTY720 on blood–brain barrier structure and function following focal head trauma. The extent of blood–brain barrier damage as assessed by the concentration of the vascular tracer Evans Blue leaking into the brain parenchyma was similar in FTY720-treated mice and vehicle-treated controls on day 1 after cortical cryoesthesia (Evans Blue extravasation: 20.4 ± 3.1 ng/mg brain tissue [vehicle] vs 23.0 ± 2.4 ng/mg brain tissue [FTY720], respectively; P > 0.05) (Fig. 4A). In line with this finding, the overall brain water content, a surrogate of cerebraledema, did not differ between the two groups (water content: 81.7 ± 0.5%, [vehicle] vs 82.1 ± 0.4% [FTY720], respectively; P > 0.05) (Fig. 4B). Moreover, cerebral mRNA expression of the tight junction proteins claudin-5 (mean: 2.4 ± 0.6-fold induction [vehicle] vs 2.3 ± 0.4-fold [FTY720]-treated; P > 0.05) and occludin (mean: 0.8 ± 0.2-fold induction [vehicle] vs 1.3 ± 0.4-fold [FTY720]-treated; P > 0.05) (Fig. 4C) was similar between FTY720-treated mice and controls after TBI.

3.4. FTY720 reduces the number of infiltrating immune cells after brain trauma

Inflammatory processes have recently been suggested to enhance secondary lesion growth in models of TBI (Morganti-Kossmann et al., 2002; Cederberg and Siesjö, 2010; Xiong et al., 2013). Thus, we next...
analyzed whether FTY720, which induces profound lymphocytopenia in the peripheral blood (Fig. 1), also prevents immune cell invasion into the traumatic brain after cortical cryolesion. Indeed, FTY720 treatment significantly reduced the numbers of brain infiltrating granulocytes (253.5 ± 87.3 cells per slice in the ipsilateral [injured] hemisphere for vehicle-treated mice vs 76.2 ± 13 cells for FTY720-treated mice, P < 0.05) (Fig. 5) and macrophages/microglia cells (197.2 ± 49.5 cells per slice in the ipsilateral [injured] hemisphere for vehicle-treated mice vs 87.0 ± 15.4 cells for FTY720-treated mice, P < 0.05) (Fig. 5) on day 1 after cryolesion as assessed by immunocytochemistry. In contrast, only very few T lymphocytes could be detected by histological means in the traumatic brain tissue on day 1 after cortical cryolesion in both groups making any quantification impossible (not shown). Altered immune cell counts in the traumatic brain following FTY720 treatment strongly suggest that the substance was indeed biologically active in vivo thereby excluding insufficient drug dosing or misapplication as the reason for the lack of FTY720 efficacy observed here.

4. Discussion

Our study failed to demonstrate a protective effect of FTY720 in TBI. Prophylactic application of FTY720 did not affect lesion size or neurological deficits in models of focal head trauma and diffuse head trauma both, during the acute or during the chronic stage. In line with this neutral finding, FTY720 was unable to stabilize the blood–brain barrier or to prevent neuronal apoptosis.

There are currently two other studies which analyzed the mechanisms of FTY720-induced immunomodulation in experimental TBI (Zhang et al., 2007, 2008). Both studies were published by the same group and used the open skull weight drop model in rats. In good accordance with our observations after cortical cryolesion in mice, one study reported a reduction of MHC-II positive macrophages/microglia in the injured brain after FTY720 treatment (Zhang et al., 2007) while in the other study, FTY720 downregulated interleukin-16 expression derived from CD68+ T lymphocytes (Zhang et al., 2008). Unfortunately however, lesion volumes or neurological deficits had not been determined in these previous investigations.

We found an unexpected dissociation between reduced immune cell trafficking and unaltered trauma outcome both, histologically and on a functional level after FTY720 treatment in our model. This casts some doubt on the pathophysiological relevance of inflammatory cells in TBI in general. Indeed, albeit neutrophils are usually ranked among the cell types that can promote traumatic brain damage for instance by producing reactive oxygen species or matrix metalloproteinases (Clark et al., 1994) others failed to confirm this detrimental role of neutrophils (Soares et al., 1995; Hartl et al., 1997a; Whalen et al., 1999; Schwarzmaier et al., 2013). Similarly, Rag1−/− mice, which lack functional T lymphocytes, were fully susceptible to weight drop contusion injury (Weckbach et al., 2012) although T lymphocytes have been shown to invade neuronal tissue in different models of head trauma (Czigler et al., 2007; Jin et al., 2012; Timaru-Kast et al., 2012). Nevertheless, the true function of T lymphocytes in TBI is still under discussion. In contrast to the situation after diffuse brain trauma (weight drop) (Weckbach et al., 2012) Rag1−/− mice displayed reduced lesion size compared with wild-type controls after focal stab wound injury of the neocortex (Clausen et al., 2007). Also, we cannot rule out from our studies that distinct immune cell subsets were unaffected by FTY720 and, therefore, might have mediated most of the inflammatory damage. Moreover, we only investigated the consequences of FTY720 treatment on lesion volume and immune cell invasion on day 1 after cortical cryolesion. At this very early stage of trauma the number of blood-borne immune cells, in particular T lymphocytes, present in the brain parenchyma is relatively low and we cannot exclude that FTY720-mediated effects on the composition of the cellular infiltrate influence lesion development at later stages after trauma (Zhang et al., 2008).

Of note, there is also an ongoing controversy about the efficacy and the mode of action of FTY720 in a neurological disorder related to TBI, i.e. acute ischemic stroke (Liesz et al., 2011; Kraft et al., 2013). S1P1, the primary target of FTY720, shows a widespread expression in the brain including neurons (Dev et al., 2008). This, together with the fact, that FTY720 can readily cross the blood–brain barrier (Miron et al., 2008) points toward a potential neuroprotective potential of FTY720. Indeed, FTY720 preserved the structural integrity of dendritic spines and as a consequence restored synaptic transmission in cortico-striatal brain slice preparations from experimental autoimmune encephalomyelitis (EAE) mice (Gillingwater, 2012). In line with these in vitro findings, mice which are deficient for the S1P1 receptor only in neurons and astrocytes are protected from EAE (Choi et al., 2011). While pharmacological S1P receptor modulation might mediate direct neuroprotection in models of autoimmune CNS inflammation, spinal cord injury (Norimatsu et al., 2012) or certain neurodevelopmental disorders

Fig. 2. FTY720 does not protect from brain trauma in two distinct mouse models. (A) Lesion volumes did not differ between FTY720-treated mice and vehicle-treated controls on day 1 after cryolesion (n = 11–12 per group; P > 0.05, unpaired, two-tailed Student’s t-test). (B) Neurological severity score (NSS) on day 1 (d1), day 3 (d3) and day 7 (d7) after weight drop trauma did not differ between FTY720-treated mice and vehicle-treated controls (n = 10 per group; P > 0.05, ns: not significant, Kruskal–Wallis test with post hoc Dunns correction).

Fig. 3. FTY720 does not reduce neuronal apoptosis after focal cryolesion. Shown are the number of TUNEL-positive neurons per brain slice in the injured hemispheres of both groups on day 1 (n = 8 per group; P > 0.05, ns: not significant, unpaired, two-tailed Student’s t-test).
such as Rett syndrome (Deogracias et al., 2012), the situation in TBI appears to be different. Here, FTY720 did not prevent against neuronal apoptosis (our study) and this goes congruent with the situation in acute ischemic stroke (Kraft et al., 2013).

FTY720 failed to maintain the blood–brain barrier upon TBI in our hands although S1P receptors have been shown to be induced at the blood–brain barrier under different pathological conditions (Zhu et al., 2010; van Doorn et al., 2012). These findings are somewhat discrepant to previous investigations describing blood–brain barrier stabilization and reduced transendothelial trafficking of immune cells by FTY720 in cerebral ischemia and intracranial hemorrhage (Liesz et al., 2011; Wei et al., 2011; Rolland et al., 2013). The exact reasons for these contrary findings are unclear but differences in the disease models and animal species certainly play a role. Also, other doses of FTY720 than the one used here might produce different effects on blood–brain barrier function (Hasegawa et al., 2010; Wei et al., 2011).

**Fig. 4.** FTY720 does not stabilize the blood–brain barrier after brain trauma (A) Evans Blue extravasation into the brain parenchyma of the ipsilateral (ipsi), i.e. injured, or contralateral (contra) hemisphere was similar in FTY720-treated mice and vehicle-treated controls on day 1 after cryolesion (n = 9 per group; P > 0.05, ns: not significant, 2-way ANOVA with post hoc Bonferroni correction). (B) Edema formation as measured by the brain water content in the ipsilateral (ipsi), i.e. injured, or contralateral (contra) hemisphere was similar in FTY720-treated mice and vehicle-treated controls on day 1 after cryolesion (n = 9 per group; P > 0.05, ns: not significant, 2-way ANOVA with post hoc Bonferroni correction). (C) Relative gene expression of claudin-5 and occludin in the lesioned hemispheres was similar in FTY720-treated mice and vehicle-treated controls on day 1 after cryolesion (n = 4–5 per group, P > 0.05, ns: not significant, unpaired, two-tailed Student’s t-test).

**Fig. 5.** FTY720 reduces immune cell invasion after brain trauma. Upper panel shows representative immunohistochemistry for granulocytes (left) and macrophages/activated microglia (right). Lower panel shows the quantification of invaded granulocytes (n = 10–11 per group; *P < 0.05, unpaired, two-tailed Student’s t-test), and macrophages/microglia (n = 10 per group; *P < 0.05, unpaired, two-tailed Student’s t-test). Bar = 50 μm.
5. Conclusions

Taken together, FTY720 failed to prevent trauma-induced neurodegeneration in different models of TBI which is in contrast to other neurological conditions associated with inflammation. Our findings put into perspective the recently claimed fundamental role of immune cells in TBI.

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