Chapter 8
Angiogenesis and Functional Recovery After Traumatic Brain Injury

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Abstract Brain injuries caused by trauma remain a major cause of death and serious long-term disability worldwide, especially in children and young adults. However, nearly all Phase III traumatic brain injury (TBI) clinical trials have failed to provide safe and effective treatment for improving functional recovery after TBI. This review discusses recent promising preclinical and clinical data indicating that TBI promotes angiogenesis (formation of new blood vessels from preexisting endothelial cells), which couples with neurogenesis (generation of new neurons) and oligodendrogenesis (generation of new oligodendrocytes), in concert, contributing to spontaneous functional recovery. Selected cell-based and pharmacological therapies that can amplify these endogenous neurorestorative effects to enhance cognitive and neurological functional recovery after TBI are discussed. Perspectives for further investigation of angiogenesis after TBI and associated therapeutic treatments are provided.

8.1 Introduction

Traumatic brain injury (TBI) is a leading cause of mortality and morbidity worldwide. TBI survivors often suffer cognitive deficits and sensorimotor dysfunctions [1]. Many therapeutic strategies have shown promise in the laboratory setting [2–4] but failed in human clinical trial [5, 6]. Thus, it is imperative to develop therapies for TBI to reduce neurological deficits.

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Emerging data from preclinical TBI studies indicate that angiogenesis plays an important role in mediating brain repair by coupling with neurogenesis and oligodendrogenesis and that cell-based and pharmacological therapies targeting amplification of angiogenesis and white matter remodeling substantially improve sensorimotor functions and reduce cognitive impairments. In this chapter, we will review TBI-induced angiogenesis and the coupling of angiogenesis with neurogenesis, oligodendrogenesis, and white matter remodeling. We will then highlight therapies that amplify these events, leading to improvement in neurological outcomes after TBI.

8.2 Angiogenesis After TBI

The endothelial cells (ECs) of cerebral capillaries, unlike those from non-cerebral capillaries, are linked by complex tight junctions that along with astrocyte end-feet, microglial cells, and pericytes form the blood–brain barrier (BBB) [7]. Under physiological conditions, the cerebral ECs are relatively quiescent with a turnover rate of approximately 3 years in the adult rodent [8]. Angiogenesis is the sprouting of new capillaries from preexisting vessels, involving the proliferation and migration of ECs, formation, branching, and anastomosis of tubes [9, 10]. TBI induces angiogenesis at an early stage after injury. After TBI, using immunohistochemistry with antibodies against bromodeoxyuridine (BrdU) and measurement of capillary density, newly formed vessels are found and capillary density increases [11]. To monitor development of angiogenesis after TBI noninvasively and longitudinally, magnetic resonance imaging (MRI) indices including cerebral blood volume (CBV), cerebral blood flow (CBF), blood-to-brain transfer constant (Ki) marked with extrinsic-contrast agents, such as gadolinium DTPA (diethylene triamine pentaacetic acid), and T1- or T2-weighted imaging have been used. Hyperpermeabilities on the Ki map in the injured brain indicate vascular leakage. New vessels are permeable at the early phase of angiogenesis, and become less leaky as they mature [12–14]. The feature of a transient increase in vascular permeability is used to detect formation of new blood vessels [13]. Newly generated leaky cerebral vessels with immature BBB which are present in the lesion boundary zone 2 days after TBI are detected by Ki maps [15, 16]. Angiogenic areas identified on the Ki map become apparent 3–4 weeks after TBI [15]. These vessels appear less leaky 6 weeks after TBI and may contribute to an increase in CBF [15]. Furthermore, as confirmed with endothelial barrier antigen (EBA) immunoreactivity, the angiogenic area on the Ki map identifies enlarged thin-walled vessels [15]. The correlation between increase of CBF and enhancement of vessel density indicates that TBI induces functional new vessels in the lesion boundary zone. Vessels with BrdU-positive ECs are detected in the ipsilateral dentate gyrus (DG) in the rats after TBI, indicating that TBI induces angiogenesis in the DG [17]. Additionally, elevated CBV is reported starting at day 1 after injury and lasting for 2 weeks in the

ipsilateral DG after TBI [18]. Elevated CBV in the DG after TBI suggests that newly generated vessels by TBI-induced angiogenesis present to be functional.

Vascular endothelial growth factor (VEGF) and its receptors initiate the formation of immature vessels, while angiopoietins (Ang 1, Ang 2) and their receptor Tie2 are essentially involved in maturation, stabilization, and remodeling of vessels [19]. VEGFR1 and VEGFR2 mRNA and protein are present in vessels adjacent to the lesion from 1 day after injury [20]. VEGF and VEGFR2 assessed by Western blot analysis also increase in the ipsilateral hippocampus after TBI [21]. With double immunofluorescent staining of endothelium and VEGFR2, it is revealed that increased VEGFR2 is expressed in the endothelium [11]. Although there is no direct evidence that TBI induces angiogenesis in human brain in response to TBI, serum and intracerebral extracellular fluid levels of angiogenic factors, such as VEGF and Ang-1, peak at 14 days post-trauma and subsequently decline [22, 23]. Furthermore, in a 21-day clinical study, serum VEGF level significantly increases during the entire period while there is no difference of serum Ang-1 level between severe TBI patients and control subjects during the first 4 days after TBI but then Ang-1 increases after 4 days [23]. However, VEGF and Ang-1 are expressed not only within vasculature but also in large numbers of platelets [24–26]. Therefore, in vitro, increased level of VEGF and Ang-1 in serum collected from patients may also be caused by platelet clotting in serum tubes [27]. For both healthy controls and patients in this 21-day clinical study [23], the average level of Ang-1 in serum on day 1 is tenfold higher than the levels reported from other studies [24, 28]. Therefore, the levels of VEGF and Ang-1 could result from an artifact caused by platelet activation after TBI [27]. In uninjured rats, none or weak immunoreactivity with matrix metalloproteinases (MMPs), such as MMP2 and MMP9, is detected in cortical capillaries [29]. As measured using gelatin zymography, MMP9 is elevated from 3 h after TBI, reaches a maximum at 24 h, and persists to 2 weeks, while MMP2 is increased from 1 day and persists to 2 weeks [30–32]. Likewise in humans, as measured by ELISA, serum MMP9 is significantly increased during the follow-up period after TBI [33]. Robust MMP2 and MMP9 immunoreactivities are found to colocalize to the vessels adjacent to the lesion site, and particularly in the immature ECs [29]. In vitro studies show that ECs secrete MMP2 and MMP9 before and after tumor necrosis factor (TNF)-α stimulated injury [34]. Increased expression of VEGFR2 and MMP9 is detected in the basement membrane of the new capillaries 2 days after TBI [29]. These results show that TBI also induces the expression of EC MMPs which are involved in angiogenesis [29]. In vitro, exogenous MMP2 increases EC tube formation while addition of MMP inhibitors or synthetic MMP agonists decreases EC tube formation [35].

Collectively, these data indicate that angiogenic factors and MMPs play important roles in TBI-induced angiogenesis.

8.3 Angiogenesis Couples with Neurogenesis and Oligodendrogenesis

In addition to providing nutritive blood flow, cerebral ECs regulate biological activity of neural progenitor cells [36].

Cerebral ECs activated by cerebral ischemia secrete VEGF that acts on neural stem cells (NSCs) and consequently leads to augmentation of newborn neurons [37]. Blockage of VEGFR2 not only reduces EBA-immunoreactive vascular density (an indicator of angiogenesis) but also reduces the number of newborn neurons in the DG in rats after TBI, which is associated with decreased neurological function recovery [21]. These data indicate that angiogenesis cooperates with neurogenesis and is involved in the recovery of neurological function after brain injury.

Neurogenesis occurs in the subventricular zone of the lateral ventricle and the subgranular zones of the DG in mammalian adult brains under normal conditions [38, 39] and pathological situations including TBI [40–44]. In the normal hippocampus, newborn neurons are detected after 1 h of [^{3}H]thymidine injection and continually generated from the DG subgranular zone, with a significant increase after 2 weeks, and these newborn cells migrate laterally into the granule cell layer, projecting axons to the CA3 region of the hippocampus within a 4-week study [45]. Following TBI, by immunofluorescent double-labeling of the proliferation cell marker BrdU and the mature neuronal marker NeuN or the astrocytic marker GFAP, there is a significant peak period of cell proliferation at 2 days post-injury in the DG both in injured juvenile and adult rats compared to shams [44], and the majority of BrdU+ cells which survive for 10 weeks become dentate granule neurons [46]. Injured animals display significant cognitive deficits at 11–15 days post-moderate injury, while there is no significant difference of cognitive deficits at days 56–60 between injured and sham animals, which shows cognitive recovery over time following TBI [46]. Therefore, injury-induced limited endogenous neurogenesis may partially contribute to spontaneous cognitive functional recovery after TBI.

In the central nervous system (CNS), the neurovascular unit (NVU) comprises ECs, pericytes, neurons and glial cells, as well as growth factors and extracellular matrix proteins close to the endothelium [47, 48]. New blood vessels in peri-infarct cortex are closely associated with new neurons identified by BrdU+/doublecortin+ (DCX, a marker of migrating neuroblasts) after ischemic injury, which indicates that neurogenesis coexists with angiogenesis in peri-infarct cortex [49]. Correlation analysis shows that the cognitive function outcome is significantly correlated with the number of the newborn neurons generated in the DG [50] and also with the increased number of vessels in ipsilateral cortex, DG, and CA3 region examined 35 days after TBI [51]. This evidence indicates that angiogenesis is coupled with neurogenesis and may improve neurological functional outcome after TBI spontaneously or with therapy [15, 16, 52, 53].

Oligodendrocytes (OLGs) are the major cell type in the white matter in the CNS, maintaining the integrity of the white matter in the adult brain. Mature OLGs generate myelin which forms sheaths for axons in the adult mammalian CNS but are unable to proliferate in response to injury [54]. However, there are plentiful oligodendrocyte progenitor cells (OPCs) in the white matter of normal CNS with functions including proliferation and maturation to remyelinate the demyelinated axons [55, 56]. Oligodendrogenesis occurs after injury in the lesion area and corpus callosum where OPCs proliferate, mature, migrate, and are regulated by the factors secreted from ECs such as fibroblast growth factor (FGF), VEGF, brain-derived neurotrophic factor (BDNF), and MMPs. TBI alone significantly increases the number of OPCs in the ipsilateral cortex and hippocampus (CA3, DG) compared to sham controls, suggesting that oligodendrogenesis may partially be responsible for spontaneous functional recovery presumably myelinating axons [57, 58]. Cognitive function recovery is significantly and positively correlated with both angiogenesis and neurogenesis in the hippocampus region after TBI [51]. After TBI, myelin content, as measured by staining with myelin-specific stain Luxol fast blue, is reduced in many white matter regions [59]. Therefore, OPCs may play an important role in remyelination in the injured brain even though the axonal regeneration is limited in adult brain after injury [60].

An interaction between OLGs and cerebral ECs has been investigated. In OLGs culture system derived from 1- to 2-day SD rats, MMP9 is not detected in OLGs under normal conditions but is secreted after stimulation by IL-β [61]. U0126, a MEK inhibitor, is able to block MMP9 secretion in IL-β-treated OLGs culture system, indicating that the MEK/ERK signaling pathway regulates OLGs to secrete MMP9 under stimulated situation [61]. Furthermore, the inhibition of MMP9 decreases newborn ECs and EC density [61]. Therefore, MMP9 released from OLGs after injury plays a critical role in white matter remodeling.

In vitro, coculture of OPCs with cerebral ECs promotes OPC survival and proliferation via the Akt and Scr signaling pathways [62]. In addition, VEGF, involved in angiogenesis, is primarily released by EC and its receptor, VEGFR2, is expressed in OPCs [63]. In endothelial-conditioned media, VEGF promotes OPC migration at 24 h after incubation and its effect can be inhibited by anti-VEGFR2 antibody. Therefore, these findings provide a novel concept of the oligovascular niche, with trophic factors secreted from ECs, activated through the Akt and Src signaling pathways to regulate OPC function including proliferation and migration. Furthermore, sensorimotor function is significantly correlated with the axonal density of ipsilateral hemisphere after TBI [64]. Taken together, these data indicate that angiogenesis coupling with oligodendrogenesis may contribute to white matter remodeling including axons and synapses after brain injury, which improves sensorimotor function recovery.

8.4 Enhancement of TBI-Induced Angiogenesis by Cell-Based Therapies

8.4.1 Bone Marrow Stromal Cells

Marrow stromal cells (MSCs) are extracted from bone marrow and include mesenchymal stem or progenitor cells [65–68], which can replicate and differentiate to other cells including neural cells [69–72]. The restorative therapy of MSCs has been performed intravenously or through direct implantation. Intravenous administration of MSCs after TBI significantly enhanced improvement in functional outcome [73]. In vitro, TBI-conditioned cultured hMSCs increase BDNF, NGF, VEGF, and hepatocyte growth factor (HGF) [74]. Furthermore, in vivo, MSCs also induce intrinsic parenchymal cells to produce the above growth factors after TBI [73]. Rats treated with MSCs cultured with BDNF and NGF have more engrafted cells than the group treated with MSCs cultured without these factors, and more robust motor function recovery is detected in the MSC groups cultured with neurotrophic factors [75]. These data suggest that motor function recovery after TBI is accomplished by transplantation of MSCs and enhanced by additional neurotrophic factors. To investigate the changes of the vascular system after TBI with acute treatment of human MSCs (hMSCs), MRI T2 maps are used to monitor and quantify the volumetric changes in the lesion area, while CBF (measured by perfusion-weighted MRI) and Ki (extrinsic-contrast agents) are used to monitor hemodynamic alteration and the BBB permeability, respectively [15]. After TBI, Ki-detected angiogenesis occurs significantly earlier in the MSC-treated group compared to the control group and the angiogenic area on Ki map is confirmed histologically by enlarged thin-walled vessels [15]. Furthermore, compared to control subjects, this early angiogenesis is not only associated with a significantly higher vessel density in the lesion boundary region of cell-treated animals but also associated with improved behavioral status after MSC treatment [15]. Pre-labeled MSCs can be tracked in the brain using MRI and verified by immunostaining [76–78]. After hMSCs are injected intravenously and detected to migrate into brain around the injury site [79], they promote cell proliferation in the subventricular zone, hippocampus, and boundary zone of injury, and some of these newly generated cells expressed the neuronal markers (Tuj1 for immature neurons, DCX for migrating neuroblasts, NeuN for mature neurons) with improved cognitive function recovery [80]. Compared to hMSCs alone injected intracerebrally or intravenously 1 week after TBI, hMSC-impregnated scaffolds transplanted into the lesion cavity 1 week after TBI significantly increase the number of hMSCs which migrate from lesion cavity to the boundary zone and also increase the vascular density in the boundary zone and hippocampus after TBI, and enhance cognitive and sensorimotor function [81]. Thus, scaffolds impregnated with MSCs provide a promising therapy option to tissue repair and functional recovery after TBI. With enhanced neurological and cognitive function recovery,

MRI shows that white matter reorganization is located in the extended area of the corpus callosum where labeled hMSCs are co-localized [78]. hMSCs secrete angiogenic factors such as VEGFA, FGF1, and MMP9 after TBI associated with enhanced neurologic and cognitive function recovery [82], which may lead to the restructuring of axons and myelin after TBI to reorganize the white matter through oligodendrogenesis.

In summary, MSC treatment amplifies neurogenesis, angiogenesis, and oligodendrogenesis after TBI.

8.4.2 Neural Stem Cells

Stem cells are able to self-renew and differentiate into multiple cell types. NSCs can differentiate into neurons, astrocytes, and OLGs [83, 84]. NSCs with their intrinsic ability of regeneration have been used in the treatment of many neurological diseases in animal models including TBI [85–87]. After transplantation into corpus callosum of brain-injured animals 2 days after TBI, some of NSCs which are derived from the neonatal murine cerebellar external germinal layer express NG2 (marker for OPCs) and migrate to the injury area 2 weeks after transplantation [88]. In a subacute therapy (1 week after injury), NSCs injected in the striatum remain in the brain and improve motor recovery on a rotarod test at 14 days after cell placement [89]. There are two possible strategies for NSC treatment of TBI: transplantation of exogenous NSCs and stimulation of endogenous NSCs [90]. Local or systemic administration of pre-differentiated human fetal neural progenitor cells improves long-term motor and sensory function recovery, decreases trauma lesion size, and increases neuronal survival in the border zone of the lesion, which are likely to be attributable to transiently increased angiogenesis and reduced astrogliosis in the border zone instead of cell replacement from donor cell transplantation [91].

8.5 Augmentation of TBI-Induced Angiogenesis by Pharmacological Therapies

8.5.1 Erythropoietin

Erythropoietin (EPO) and its receptor (EPOR) are essential for erythropoiesis and EPO has been widely used in the clinic for treatment of anemia since it regulates the maturation, differentiation, and survival of hematopoietic progenitor cells [4]. In normal adult brains, low levels of EPO and EPO receptors are detected, while after injury, increased levels of EPO and EPO receptors are found in neurons, NPCs, glial cells, and ECs [92]. EPO treatment (24 h after TBI) increases expression of

VEGF and phosphorylation of VEGFR2 as well as results in a significant increase of newborn neurons and vascular density in the cortex, DG, and CA3. However, after blockage of VEGFR2 with SU5416, newborn neurons and vascular density are all significantly decreased and functional recovery in EPO-treated TBI rats is abolished [21]. Therefore, EPO therapy improves sensorimotor and cognitive functional recovery after TBI by promoting neurogenesis and angiogenesis through upregulating VEGF/VEGFR2 expression in the brain [21, 43, 93]. Previous studies show that cognitive function recovery is mediated by neurogenesis coupled with angiogenesis in the hippocampus [42, 43, 51] while sensorimotor function recovery is associated with brain angiogenesis and spinal cord axon remodeling [94]. However, in animals null for the EPOR gene in neural cells, EPO treatment still significantly reduces cell loss in the hippocampus compared with saline controls, as well as improves sensorimotor and cognitive function after TBI, which suggests that therapeutic benefits of EPO may be mediated through its vascular protection but not via neural EPOR [95]. Carbamylated erythropoietin (CEPO), a modified erythropoietin molecule that does not affect hematocrit, is as effective as EPO in terms of reducing hippocampal cell loss, enhancing angiogenesis and neurogenesis in the injured cortex and hippocampus, and improving sensorimotor functional recovery and spatial learning in rats after TBI [53, 96]. EPO and its derivatives have a potential value in TBI therapy.

8.5.2 Statins

Statins lower cholesterol levels and also have neuroprotective and neurorestorative effects including angiogenesis, neurogenesis, and synaptogenesis and improve function recovery in rats after TBI [97, 98]. To measure the effect of atorvastatin on improvement of microvascular integrity and cognitive function recovery after TBI, animals are perfused with FITC–dextran to track the vascular changes, and the water maze test is performed to investigate the spatial learning on injured rats [97]. Compared with saline treatment group, atorvastatin treatment significantly improves spatial learning, increases the vessel-to-tissue ratio and vascular length on days 8 and 15 after TBI in both hippocampal CA3 region and boundary zone of injured area, and also augments vascular diameter on day 8 after TBI in the boundary zone of contusion [97]. Simvastatin upregulates VEGFR2 expression after TBI, increases the BrdU+ ECs in the lesion boundary zone and hippocampus with improved functional recovery in rats, and enhances in vitro capillary-like tube formation after oxygen glucose deprivation (OGD), indicating that simvastatin-enhanced angiogenesis may be related to activation of the VEGFR-2/Akt/eNOS signaling pathway [16]. A recent study shows that increasing circulating EPCs with atorvastatin treatment may contribute to the observed increase in brain angiogenesis and improved functional outcome after TBI [99].

8.5.3 Thymosin Beta 4

Tβ4 is a multifunctional regenerative small peptide comprising 43 amino acids and its major function is G-actin-sequestering [100]. Tβ4 is involved in many cellular procedures including cell proliferation, mobility, antiapoptosis, anti-inflammation, and promotion of wound healing [101–104]. Tβ4 is a novel therapeutic choice for CNS trauma, which promotes endogenous neurorestorative processes in animal models of TBI [52, 57]. Tβ4 is evaluated to be safe in clinical treatment of acute myocardial infarction [105]. Early treatment (6 h after TBI) shows that Tβ4 significantly improves spatial learning and sensorimotor functional recovery, and promotes neurogenesis in the DG [52]. Late treatment (24 h after TBI) indicates that Tβ4 significantly increases the vascular density in the injured cortex, CA3, and DG of the ipsilateral hemisphere, and enhances neurogenesis in the injured cortex and hippocampus, along with increased generation of mature OLGs in the CA3 region, which are associated with improved sensorimotor and cognitive functional recovery after TBI [57]. The mechanisms underlying the beneficial effects of Tβ4 remain unknown. However, a recent study shows that Tβ4 is able to induce endothelial progenitor cell migration via the phosphatidylinositol 3 kinase/Akt/endothelial nitric oxide synthase (eNOS) signal transduction pathway, which may mediate angiogenesis [106]. Tβ4 treatment induces OLG differentiation by inducing p38MAPK with parallel inactivation of ERK1 and JNK1, thus preventing the accumulation of phosphorylated c-Jun [107]. Therefore, Tβ4 treatment-induced angiogenesis, neurogenesis, and oligodendrogenesis, in concert, may contribute to functional recovery in rats after brain injury.

8.6 Other Growth Factors

VEGF is an important regulator of angiogenesis. VEGF is neuroprotective in several models of experimental brain injury [108–112]. The expression of VEGF and VEGFR2 is increased in rodents subjected to TBI [20, 113], and inhibition of VEGF expression after injury decreases newborn neurons and newly generated vessels with aggravated function outcome [21], suggesting that VEGF-induced angiogenesis and neurogenesis promote neurological and cognitive function recovery [21]. Hepatocyte growth factor (HGF) is an important molecule for tissue repair [114]. Enhancement of vascular pixel intensity and GAP-43-positive cells (a crucial component of the axon and presynaptic terminal) is detected at the ischemic boundary zone with HGF treatment [115], indicating that HGF is involved in angiogenesis and synaptogenesis after injury. HGF is also known to induce angiogenesis in cooperation with VEGF [116]. Basic fibroblast growth factor (FGF2) is a potent angiogenic agent present in neurons and glia, vascular basement membrane of blood vessels, and in the ependymal cells of the ventricles [117]. After TBI, FGF2 treatment significantly decreases lesion size, increases the number of blood

vessels in the cortex around the lesion, and improves sensorimotor function recovery [110]. Granulocyte-colony stimulating factor (G-CSF), a hematopoietic growth factor, significantly increases 3 h after TBI and peaks at 8 h [118]. In the ischemic hemisphere post-stroke, G-CSF treatment increases endothelial proliferation, vascular density, expression of eNOS and angiopoietin-2, and decreases BBB disruption and function deficits [119]. Most of these growth factors, with large molecular-weighted and hydrophilic proteins, have a limited access to the CNS after systemic administration, principally due to poor BBB permeability. Cerebrolysin is a mixture of low-molecular-weight neuropeptides derived from purified brain proteins by standardized enzymatic proteolysis, with proposed neuroprotective and neurotrophic properties similar to naturally occurring growth and neurotrophic factors [120]. Direct and indirect evidences indicate that low-molecular-weight Cerebrolysin, which contains many neurotrophic factor-like peptides, is able to cross the BBB [121]. Early intervention with Cerebrolysin reduces BBB permeability changes, attenuates brain pathology and brain edema, and mitigates functional deficits [120]. Recent data show that Cerebrolysin enhances neurogenesis in the ischemic brain and improves functional outcome after stroke [122]. Taken together, these data suggest that Cerebrolysin has potential therapeutic value in TBI.

8.7 Perspectives

There is evidence for a prominent role of angiogenesis in the recovery of neurological function post-TBI. It is well known that TBI induces angiogenesis, particularly in the injury boundary zone. An angiogenic environment is essential for tissue repair and functional recovery after injury. The contribution of endogenous angiogenesis, however, may not be sufficient to support the degree of neuroplasticity required for functional recovery after TBI. The therapeutic approaches that enhance brain remodeling via angiogenesis are promising. In addition to cell-based therapy including MSCs and NSCs, many promising drugs such as EPO, CEPO, Tβ4, statins, and growth and neurotrophic factors, all of which amplify endogenous angiogenesis, have been evaluated in TBI. Enhanced angiogenesis, coupled with neurogenesis, oligodendrogenesis, and white matter remodeling, contributes to improvement of functional recovery induced by these treatments. The therapeutic window for stimulation of angiogenesis, neurogenesis, and white matter remodeling after TBI has not been ascertained. Further investigation of angiogenesis and its correlation between neurogenesis and white matter remodeling is also warranted to better understand mechanisms underlying functional recovery after TBI and to develop effective therapeutic treatment for improving outcomes in patients with the CNS injury.

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