The Contribution of Stem Cell Factor and Granulocyte-Colony Stimulating Factor in Reducing Neurodegeneration and Promoting Neural Network Reorganization after Traumatic Brain Injury

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Abstract

Background Traumatic brain injury (TBI) is a major cause of death and disability in young adults worldwide. TBI-induced long-term cognitive deficits represent a growing clinical problem. Stem cell factor (SCF) and granulocyte-colony stimulating factor (G-CSF) play a role in neuroprotection and neuronal plasticity. However, the knowledge concerning reparative efficacy of SCF+G-CSF treatment in post-acute TBI recovery remains incomplete. This study aims to determine the effects of stem cell factor (SCF) and granulocyte colony-stimulating factor (G-CSF) on post-TBI recovery in young adult mice. The controlled cortical impact model of TBI was used for inducing a severe damage in the motor cortex of the right hemisphere in 8-week-old male C57BL mice. The combination treatment of SCF and G-CSF (SCF+G-CSF) was initiated 3 weeks after induction of TBI.

Results Severe TBI led to persistent motor functional deficits (Rota-Rod test) and impaired spatial learning and memory (Morris water maze test). SCF+G-CSF treatment significantly improved the severe TBI-impaired spatial learning and memory at 6 weeks post-treatment. TBI also caused significant increases of Fluoro-Jade C positive degenerating neurons in bilateral frontal cortex, striatum and hippocampus, and significant reductions in MAP2+apical dendrites and overgrowth of SMI312+axons in peri-TBI cavity frontal cortex and in the ipsilateral hippocampal CA1 at 24 weeks post-TBI. SCF+G-CSF treatment significantly reduced TBI-induced neurodegeneration in the contralateral frontal cortex and hippocampal CA1, increased MAP2+apical dendrites in the peri-TBI cavity frontal cortex, and prevented TBI-induced axonal overgrowth in both the peri-TBI cavity frontal cortex and ipsilateral hippocampal CA1.

Conclusions These findings reveal a novel pathology of axonal overgrowth after TBI and demonstrate a therapeutic potential of SCF+G-CSF in ameliorating TBI-induced long-term neuronal pathology, neural network malformation, and impairments in spatial learning and memory.

Background

As a growing clinical problem around the world, traumatic brain injury (TBI) remains the leading cause of death and disability in young adults [1]. The period of post-TBI is divided into 3 phases: an acute phase, a subacute phase, and a chronic phase. Generally, the acute phase is the first 7 days after TBI, the subacute phase is between 7 days and 3 weeks after TBI, and the chronic phase begins 3 to 5 weeks after TBI [2–5]. To date, the majority of pre-clinical studies have focused on pharmaceutical interventions in neuroprotection in the acute phase of TBI, such as complement inhibition [6], immunomodulation [7], angiotensin II receptor blockage [8], and cerebral infusion of insulin-like growth factor-1 [9]. However, little research has been conducted in post-acute TBI recovery. As of today, there is a lack of effective therapy to ameliorate neurodegeneration, to repair the impaired neural network, and to improve functional outcomes in the subacute and chronic phases of TBI.

The process of the secondary brain injury is progressive, and secondary neuron loss happens in both the ipsilateral and contralateral hemispheres. A recent study has revealed a long-term and progressive neuropathology in bilateral hemispheres up to one year after a single severe TBI in mice [10]. Moreover, progressive neurodegeneration after TBI has been found in both the ipsilateral and contralateral hemispheres [11, 12]. Widespread neurodegeneration after TBI has been thought to be the results of secondary brain injury and lead to cognitive impairments [13]. In addition to widespread neurodegeneration, reduced functional connectivity in both hemispheres has also been observed in TBI patients [14]. TBI-induced loss of dendrites and axons is also related with cognitive impairments [15, 16]. The pharmacological intervention for inhibiting widespread neurodegeneration and enhancing neural network reorganization may restrict TBI-induced progressive neuropathology and improve functional outcomes after TBI.

Stem cell factor (SCF) and granulocyte-colony stimulating factor (G-CSF) are the key hematopoietic growth factors to regulate bone marrow stem cell proliferation, differentiation and mobilization [17–20]. Accumulating evidence shows that SCF and G-CSF also play a role in neuroprotection and neuronal plasticity. Many studies have demonstrated that administration of SCF [21], G-CSF [21–26], or SCF+G-CSF [21, 27, 28] leads to reduction in infarction size and amelioration of neurological deficits in experimental stroke. In addition to neuroprotection, SCF has been shown to promote neurite outgrowth [29, 30], and lack of SCF impairs spatial and learning memory [31]. G-CSF deficient mice also show cognitive problems, impairments in long-term potentiation, and reductions in dendrites in the hippocampus [32]. SCF and G-CSF have been demonstrated to cross the blood-brain barrier. SCF and G-CSF combined treatment (SCF+G-CSF) has shown synergistic effects in enhancing neurite outgrowth [33] and neural network reorganization in chronic stroke [30]. We have also demonstrated that SCF+G-CSF treatment in the chronic phase of experimental stroke synergistically improves functional recovery [30]. SCF+G-CSF-improved functional recovery in the chronic phase of experimental stroke is dependent on the SCF+G-CSF-enhanced neural network remodeling [34, 35]. However, the knowledge concerning reparative efficacy of SCF+G-CSF treatment in post-acute experimental TBI recovery remains incomplete.

The aim of the present study is to determine the efficacy of SCF+G-CSF treatment at the late subacute stage of TBI in brain repair and functional recovery of mice.

Methods

The animal experiments followed ethical guidelines of the Animal Research: Reporting In Vivo Experiments (ARRIVE). All procedures of animal experiments were approved by the Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animals and Experimental Design

A total of 30 male C57BL/6J mice (8 weeks old) (The Jackson Laboratory Co., Bar Harbor, Maine, US) were used for this study. These mice were housed in a 12-h light/dark cycle with food and water available *ad libitum*.

Three weeks after induction of TBI, mice were randomly divided into two groups: a Vehicle control group (n = 11) and an SCF+G-CSF-treated group (n = 12). Mice without TBI served as sham operative controls (n = 7). SCF (200µg/kg/day, diluted by saline) and G-CSF (50µg/kg/day, diluted by dextrose) or an equal volume of vehicle solution (50% of saline with 50% of dextrose) were subcutaneously injected for 7 consecutive days starting on day 21 post-TBI. The dosage and treatment paradigm were selected according to our earlier studies in chronic stroke [34, 36].

Neurobehavioral tests were performed for determination of functional recovery one week before treatment as well as 2 and 6 weeks after treatment (Additional file 1).

Controlled Cortical Impact Murine Model of TBI

Controlled cortical impact (CCI) model was used to induce TBI. Mice were anesthetized with Avertin (Sigma) (0.4g/kg, i.p.) and placed in a head holder, where the head of the mouse was immobilized with two ear bars and a tooth bar. A midline cranial incision was made, and the skull was exposed. A 4-mm-in-diameter circle with a central point of 2 mm lateral to the bregma was drawn on the right side of the skull (somatosensorimotor cortex of the forelimb and hindlimb in mice). A craniotomy was performed following the circle with a dental drill. The skull was carefully removed without damaging the dura. The mouse was then subjected to the IH-0400 Impactor (Precision Systems and Instrumentation Co.) with a 125kdyne force level through a 3-mm-in-diameter round tip, with force curves recorded for abnormalities in impact using PSI-IH Impactor software. The skull flap was then placed over the craniotomy without adhesive due to concerns of increasing intracranial pressure. The wound was closed with 3-0 prolene sutures. Sham mice were operated in the same procedure except the cortex was not impacted.

Administration of Stem Cell Factor and Granulocyte-Colony Stimulating Factor

Recombinant mouse SCF (PeproTech) was diluted with saline, and recombinant human G-CSF (Amgen) was diluted with 5% dextrose. SCF (200µg/kg/day) and G-CSF (50µg/kg/day) were injected subcutaneously for 7 consecutive days at 24-hour intervals in the TBI-S+G group. The location of the injection was on the backside of the neck. Due to the small volume and difficulty with maintaining the mice in appropriate position during injection, mice were anesthetized with isoflurane to ensure proper subcutaneous injection. An equal volume of vehicle solution (50% of saline with 50% of dextrose) was subcutaneously injected in the TBI-Vehicle group.

Morris Water Maze Test

The Morris water maze testing was performed in a water tank with 4 quadrants and a hidden platform placed in quadrant IV. Mice were tested for a total of 8 trials, 2 in each quadrant every day. Each trial ended with either the maximum time limit of 60 seconds or when the mouse found the platform. The mice were placed in quadrant I at the beginning of each trial and followed a fixed order of I, II, III, IV, and I, II, III, IV. The testing was performed over the course of 5 days. Trials were performed early in the morning within a 1-hour time frame each day, with a 1-hour acclimation period to the testing room before testing would begin. The latency to find the platform and the distance the animal traveled were recorded through ANY-Maze Video Tracking System (Stoelting Co.).

Rota-Rod Test

Mice were placed on Rota-Rod (Coulbourn Instrument) for testing motor function. Once all 5 mice were set, start button was pressed. The speed of the Rota-Rod was set as starting at 4 rpm and ending at 40 rpm with a maximum 5 min duration (by increasing 4 rpm every 30 sec). The test ended at 5 min or when the mouse fell. The testing was repeated 3 times with at least 15 minutes of rest between each trial. The latency to fall from Rota-Rod was recorded.

Brain Section Preparation

At 24 weeks after TBI, mice were anesthetized with overdose of Avertin (Sigma) (0.6g/kg, i.p.) and transcardially perfused with phosphate buffered saline (PBS) (50 ml) followed by 4% formaldehyde (Sigma) (50 ml). The brains were excised and immediately immersed in 4% formaldehyde for 24 h for post-fixation and then cryoprotected in 30% sucrose in PBS for 2 days. The brains were then sectioned into 30-μm-thick sections with a microtome (American Optical Corp.).

Fluoro-Jade C Staining

Brain sections (2–4 sections/brain) at the level 1.10 mm anterior to bregma and 1.10 mm posterior to bregma (Additional file 2A, B and C) were mounted on Superfrost Plus Slides (Fisher Scientific) and allowed to dry overnight at room temperature. The slides were immersed in 100% ethanol then 70% ethanol. Brain sections were then rinsed in distilled water and immersed in 0.06% potassium permanganate ((Sigma). After being rinsed in distilled water, the slides were incubated with 0.001% Fluoro-Jade C (Millipore) for 30 min, dried at room temperature overnight in the dark, cleared by brief immersion in xylene, and mounted with DepeX mounting media (Electron Microscopy Sciences). Images of the cortex at 500 μm away from the TBI cavity, the corresponding area in the contralateral cortex, and bilateral striatum and CA1 (Additional file 2A, B and C) were captured with a 20x objective using a

confocal fluorescence microscope (Zeiss LSM780, Germany). Fluoro-Jade C-positive staining was analyzed by Image J software.

Immunohistochemistry

Free-floating technique was used for immunohistochemistry. Briefly, brain sections (3–4 adjacent sections/brain) at the level 1.00 mm anterior to bregma and 1.10 mm posterior to bregma (Additional file 2A and C) were chosen for immunohistochemistry. The sections were rinsed with PBS, incubated in solution of PBS-diluted 10% normal goat serum (NGS), 1% bovine serum albumin (BSA), and 0.3% Triton X–100 for 1 hour at room temperature to block non-specific binding. The sections were then incubated with the primary antibody polyclonal rabbit anti-microtubule associated protein 2 (MAP2, 1:600, Millipore), or monoclonal mouse anti-SMI312 (1:500, Biolegend) in PBS solution with 1% BSA and 0.3% Triton X–100 overnight at 4°C. In negative control brain sections, the primary antibodies were omitted. The next day the sections were washed with PBS and incubated with the secondary antibody DyLight 488-labeled goat anti-rabbit or DyLight 488-labeled goat anti-mouse in PBS solution with 1% BSA and 0.3% Triton X–100. Sections were rinsed with PBS again and then mounted on Superfrost Plus Slides and coverslipped with Vectashield Antifade Mounting Medium (Vector Laboratories). Confocal images of MAP2 at layer 1 and layer 2 cortex 500 µm away from the TBI cavity as well as the stratum radiatum (RAD) and stratum lacunosum moleculare (LM) of hippocampus CA1 in both the ipsilateral hemisphere and corresponding contralateral sites (Additional file 2A and C) were taken with a confocal fluorescence microscope (Zeiss LSM780, Germany). Confocal images of SMI312 at layer 1–5 cortex 500 µm away from the TBI cavity as well as the hippocampal CA1 in both the ipsilateral hemisphere and corresponding contralateral site were taken with the confocal fluorescence microscope. The Integrated optic densities of MAP2 positive dendrites, the MAP2 positive area (%), and the SMI312 positive area (%) were analyzed by Image J software.

Tissue Loss Volume Determination

Serial coronal brain sections (30 µm thickness at 15 section intervals) were taken and processed for Hematoxylin and Eosin (H&E) staining. Briefly, brain sections were rehydrated, washed with tap water, subjected to hematoxylin stain for 1–2 minutes. The sections were washed with tap water again and dipped in ammonia water until blue. The sections were then washed with tap water and 95% alcohol until the slide cleared, subjected to eosin for 10 seconds, dehydrated, and mounted in xylene based medium. The measurement of the width and the depth of the cavity through cross sections were cumulated to calculate the volume of the cavity. Briefly, the area of tissue loss was calculated by subtracting the ipsilateral remaining hemisphere from the contralateral hemisphere in each section. The volume of tissue loss was calculated by multiplying the mean area of tissue loss by the number of sections and the thickness of each section. The volume of tissue loss was then divided by the volume of contralateral

hemisphere to get the percentage of tissue loss. The final value was normalized through subtracting the tissue loss volume of each mouse by the average of the injury volume of the Sham group.

Statistical Analysis

Statistical analysis was performed using Prism (GraphPad) software. Shapiro-Wilk's normality test was used to assess normality. No data was significantly different than the Gaussian distribution. Student's t-test was used to analyze pretreatment behavioral data and tissue loss volume between two experimental groups. A one-way ANOVA was utilized to analyze Fluoro-Jade C and immunohistochemical data among three experimental groups. A two-way repeated measures ANOVA was used to evaluate time treatment differences for the data collected from Morris water maze and Rota-Rod testing. Tukey's *post hoc* analysis was used to compare the differences between groups. Significance was set at $P < 0.05$.

Results

SCF+G-CSF treatment does not improve TBI-impaired cognitive and motor functions 2 weeks after treatment

To ensure that TBI mice presented equal pathological conditions in the treated and non-treated groups before the SCF+G-CSF treatment initiation, we examined cognitive and motor functional deficits one week before treatment by Morris water maze test and Rota-Rod test, respectively. The latency to find the platform was longer in all TBI mice than in sham control mice ($P < 0.01$) (Additional file 3A). Then TBI mice were randomly divided into two groups: a TBI-vehicle control group and a TBI-SCF+G-CSF-treated group. The escape latency did not show any difference between the two experimental groups (Additional file 3B). A similar findings was found in a 3-day pretesting Rota-Rod (Additional file 3C, D). The findings from demonstrate the equal pathological conditions in the vehicle control group and TBI-SCF+G-CSF treatment group before the treatment initiation.

To determine the efficacy of SCF+G-CSF on cognitive and motor functions, we performed Morris water maze and Rota-Rod tests 2 weeks after the treatment, respectively. In Morris water maze test, the escape latency was longer on day 4 and day 5 in TBI-vehicle-treated mice when compared with sham-operated mice ($P < 0.05$). There was no significant difference in the escape latency between vehicle-treated controls and SCF+G-CSF-treated mice (Figure 1A). In Rota-Rod test, the latency to fall was reduced in vehicle-treated TBI mice as compared to sham mice from day 2 to day 5 ($P < 0.05$). There was no significant difference between the TBI-vehicle group and the TBI-SCF+G-CSF group (Figure 1C). These data indicate that SCF+G-CSF treatment does not improve spatial learning and memory and motor function at 2 weeks post-treatment.

SCF+G-CSF treatment improves TBI-impaired cognitive function but not motor function 6 weeks after treatment

Next, we examined the long-term effects of SCF+G-CSF treatment on cognitive and motor functions after TBI. Morris water maze and Rota-Rod tests were carried out 6 weeks after treatment.

In the Morris water maze, we found that the escape latency to platform was increased in the TBI-vehicle mice on day 1 ($P < 0.05$), day 4 ($P < 0.05$) and day 5 ($P < 0.01$) as compared with sham controls (Figure 1B), indicating that TBI leads to a long-term impairment in spatial learning and memory. The SCF+G-CSF-treated TBI mice, however, showed a reduction in escape latency when compared to the TBI-vehicle control mice on day 5 ($P < 0.01$, Figure 1B). These data suggest that SCF+G-CSF treatment improves TBI-impaired long-term spatial learning and memory.

In Rota-Rod test, TBI-vehicle control mice showed a decrease in latency to fall in comparison with sham controls on days 1, 2, 3 and 5 ($P < 0.05$). The latency to fall in TBI-SCF+G-CSF-treated mice did not differ from TBI-vehicle controls (Figure 1D). These findings suggest that SCF+G-CSF-treatment is insufficient in improving motor function after severe TBI.

SCF+G-CSF treatment does not influence the tissue loss volume after TBI

To examine whether SCF+G-CSF treatment influences the total tissue loss after TBI, we assessed the total tissue loss volume using H&E staining 20 weeks after SCF+G-CSF treatment. Our data showed that there was no significant difference in the volume of brain tissue loss between TBI-Vehicle controls and TBI-SCF+G-CSF-treated mice ($P > 0.05$) (Additional file 4).

SCF+G-CSF treatment prevents TBI-induced neurodegeneration in the contralateral cortex and hippocampal CA1

To investigate the effect of SCF+G-CSF on long-term neurodegeneration after TBI, we performed Fluoro-Jade C staining 20 weeks after SCF+G-CSF treatment. Fluoro-Jade C positive cells were increased in TBI-vehicle mice as compared with sham controls in both ipsilateral ($P < 0.05$) and contralateral cortex ($P < 0.01$), in ipsilateral ($P < 0.01$) and contralateral striatum ($P < 0.05$), and in ipsilateral ($P < 0.05$) and contralateral CA1 ($P < 0.01$) (Figure 2), suggesting that the severe TBI causes a long-term neurodegeneration in both ipsilateral and contralateral hemispheres. SCF+G-CSF treatment reduced the number of Fluoro-Jade C positive cells in the contralateral cortex ($P < 0.05$) and CA1 ($P < 0.05$) as compared with TBI-vehicle control mice (Figure 2A, C, D and H). These findings indicate that SCF+G-CSF treatment ameliorates severe TBI-induced long-term neurodegeneration in the contralateral cortex and hippocampal CA1.

SCF+G-CSF treatment increases apical dendritic density in the ipsilateral cortex after TBI

We then aimed to know the efficacy of SCF+G-CSF treatment in remodeling of dendritic branching after TBI. In the contralateral cortex, we did not find any differences in MAP2 positive dendrites (MAP2 positive dendritic optic density and relative MAP2 positive area) between sham controls, TBI-vehicle control mice, and SCF+G-CSF-treated TBI mice ($P > 0.05$) (Figure 3A, C, D, G and H). In the ipsilateral cortex, however, TBI-vehicle mice showed reductions in MAP2 positive dendritic optic density in layer 1 ($P < 0.01$) and layer 2 ($P < 0.001$) as compared to sham-operated mice. This finding was further validated by analysis of MAP2 immunopositive area (TBI-Vehicle controls vs. sham controls: $P < 0.01$ in both ipsilateral layer 1 and 2). This observation indicates that the severe TBI leads to a long-term dendrites reduction in the ipsilateral cortex. SCF+G-CSF treatment, however, increased MAP2 positive apical dendrites (MAP2 positive dendritic optic density and relative MAP2 positive area) in both layer 1 ($P < 0.05$) and layer 2 ($P < 0.05$) of the ipsilateral cortex in comparison with TBI-vehicle controls (Figure 3B, E, F, I and J), suggesting that SCF+G-CSF treatment ameliorates TBI-reduced dendritic density in the ipsilateral cortex.

In the contralateral hippocampus, we did not observe any differences in MAP2 positive dendrites (MAP2 positive dendritic optic density and relative MAP2 positive area) between sham controls, TBI-vehicle control mice, and SCF+G-CSF-treated TBI mice in both the RAD CA1 and LM CA1 regions ($P > 0.05$) (Figure 4A-C, and F-H). However, in the ipsilateral hippocampus, the MAP2 positive dendritic optic density in TBI-vehicle control mice was decreased in RAD CA1 ($P < 0.05$, Figure 4D) and LM CA1 ($P < 0.01$, Figure 4I) as compared to sham controls. These data were further confirmed by analysis of relative MAP2 positive area (TBI-vehicle controls vs. sham controls: $P < 0.01$ in RAD CA1, Figure 4E; $P < 0.05$ in LM CA1, Figure 4J). These findings indicate a long-term decrease in dendritic density in the ipsilateral hippocampus by severe TBI. We did not see any increases of MAP2 positive dendritic optic density

(Figure 4D and I) and relative MAP2 positive area (Figure 4E and J) in ipsilateral RAD or LM CA1 after SCF+G-CSF treatment, suggesting that SCF+G-CSF does not modify the TBI-induced long-term reduction of dendrites in the ipsilateral hippocampus.

SCF+G-CSF treatment prevents TBI-induced overgrowth of axons in the ipsilateral cortex and hippocampus

To determine whether SCF+G-CSF treatment modifies axonal density after TBI, we analyzed SMI312 immunopositive axons in both the cortex and hippocampus 20 weeks after treatment.

In the contralateral cortex, the SMI312 positive axons in layer 1 were increased in TBI-vehicle mice as compared to the sham controls ($P < 0.05$) (Figure 5A and B). TBI-vehicle mice also showed a trend toward increasing SMI312 positive axons in layer 2-3 ($P = 0.081$, Figure 5C) and layer 4-5 ($P = 0.066$, Figure 5C) in comparison with sham controls. SCF+G-CSF-treated TBI mice displayed the trend toward decreasing SMI312 positive axons in layer 1 ($P = 0.087$, Figure 5B) as compared to the TBI-vehicle control mice.

In the ipsilateral cortex, TBI-vehicle mice showed increases in SMI312 positive axons in layer 1 ($P < 0.05$, Figure 5B), layer 2-3 ($P < 0.05$, Figure 5C), and layer 4-5 ($P < 0.01$, Figure 5D) when compared to sham controls, demonstrating a TBI-induced long-term overgrowth of axons in the ipsilateral cortex. SCF+G-CSF treatment, however, reduced SMI312 positive axons in layer 2-3 ($P < 0.01$, Figure 5C), and layer 4-5 ($P < 0.05$, Figure 5D) as compared to TBI-vehicle control mice. A trend toward decreasing SMI312 positive axons in layer 1 ($P = 0.068$, Figure 5B) was also observed in SCF+G-CSF-treated TBI mice as compared to the TBI-Vehicle control mice. This observation suggests that the TBI-induced long-term overgrowth of axons in the ipsilateral cortex is inhibited by SCF+G-CSF treatment.

In the contralateral hippocampus, differences in SMI312 positive axons in the CA1 region were not seen between sham controls, TBI-vehicle control mice, and SCF+G-CSF-treated TBI mice (Figure 6A and B). In the ipsilateral hippocampus, however, TBI-vehicle mice showed an increase in SMI312 positive axons in CA1 ($P < 0.05$ vs. sham controls, Figure 6C). SCF+G-CSF treatment decreased SMI312 positive axons in the ipsilateral CA1 when compared to TBI-vehicle control mice ($P < 0.05$, Figure 6C). These findings indicate that TBI-induced long-term overgrowth of axons in the ipsilateral hippocampus is prevented by SCF+G-CSF treatment.

Discussion

In the present study, we have demonstrated that the combination treatment of SCF and G-CSF at the late subacute stage of TBI (3 weeks post-TBI) ameliorates a severe TBI-induced long-term impairment in spatial learning and memory, reduces neurodegeneration, and enhances neural network structural reorganization.

SCF+G-CSF treatment improves spatial learning and memory 6 weeks, but not 2 weeks, after treatment in a severe CCI-TBI model. This observation suggests that SCF+G-CSF requires a prolonged period (6 weeks) to repair the brain damaged by a severe TBI. This finding is in line with the data of previous publications. SCF+G-CSF treatment post-acute TBI improves spatial learning and memory in water maze testing 6 weeks after treatment [37]. Using a cortical ischemia model of stroke, Kawada and coworkers have revealed that SCF+G-CSF treatment during 11–20 days after cortical ischemia leads to improvements of spatial learning and memory in water maze test at 4 weeks post-stroke [27]. The question as to why the SCF+G-CSF treatment improves recovery of spatial learning and memory in a delayed time remains to be addressed in future studies. In this study, we have also observed that SCF+G-CSF treatment fails to improve motor functional recovery in the severe CCI-TBI model. This may be due to the severe damage in the motor cortex by the CCI-TBI model. This finding is also consistent with our previous observation that persistent impairments in motor function by a severe CCI-TBI are not improved by SCF+G-CSF treatment [37]. Although SCF+G-CSF treatment at 3 weeks post-TBI has no effect on improving motor function or on reducing lesion size in our severe CCI-TBI model, the findings of SCF+G-CSF-improved spatial learning and memory recovery suggest that the reparative processes may occur in the remaining brain regions remote from or next to the TBI-damaged motor cortex.

We have observed that the TBI-induced neurodegeneration in the contralateral cortex and hippocampal CA1 is prevented by SCF+G-CSF treatment. Accumulating evidence has demonstrated that a moderate or severe CCI-TBI causes widespread, progressive, and long-term neurodegeneration [38, 39]. Progressively increased neuron loss has been found at 5, 12, and 52 weeks post-CCI-TBI as compared to one week after injury [38]. In the present study, we have revealed that widespread degenerating neurons are increased in the cortex, striatum, and hippocampal CA1 in both hemispheres long-term 24 weeks after a severe CCI-TBI. It has been shown that the widespread neurodegeneration after TBI is tightly linked to cognitive impairments [13, 40]. The present study has demonstrated that SCF+G-CSF ameliorates long-term neurodegeneration in the contralateral frontal cortex and hippocampus remote from the injury site. Whether increased dose or prolonged treatment would significantly reduce neurodegeneration in the ipsilateral hemisphere remains to be addressed. Although the precise mechanism underlying the SCF+G-CSF-inhibited post-TBI neurodegeneration remains unclear, a large body of evidence has demonstrated the contribution of SCF and G-CSF in neuroprotection. SCF acts as a neurotrophic factor supporting neuron survival during the development of the peripheral nervous system [29, 41]. SCF [42] or G-CSF [24] protects cultured neurons from excitotoxicity [42] and programmed neuron death [24, 42] through PI3K pathway. Administration of SCF [21], G-CSF [21–26] or SCF+G-CSF [21, 28] in the acute phase of experimental stroke results in infarction size reduction. Neuroprotective effects of G-CSF [43] or SCF+G-

CSF [27] treatment in the subacute phase of experimental stroke have also been demonstrated. These studies suggest that SCF+G-CSF-inhibited post-TBI neurodegeneration may be mediated through the similar process of neuroprotection.

Increasing evidence has shown that neural network remodeling in the cortex next to the brain injury area plays a key role in functional recovery [34, 35, 44]. The biological base for the neural network remodeling is brain plasticity that drives reorganization of neural networks after brain injury in either a positive way (adaptive) or a negative way (maladaptive) [45]. The findings of the present study reveals that TBI causes reductions of MAP2 positive apical dendrites in the ipsilateral cortex next to the TBI-cavity. SCF+G-CSF treatment leads to increases in the apical dendrites of in the peri-cavity cortex. The apical dendrites of pyramidal neurons in layer II, III and V extend to layer I, and these apical dendrites play a vital role in learning and memory [46]. In chronic stroke studies, in addition to SCF+G-CSF-improved somatosensory motor functional recovery [30], the increased MAP2 positive dendrites are also observed in the peri-infarct cortex after SCF+G-CSF treatment [47]. By contrast, the data of the present study also show that SMI312 positive axons in the cortex next to the TBI cavity are *increased* in TBI-vehicle control mice as compared to the sham controls. The TBI-induced overgrowth of SMI312 positive axons in this region are prevented by SCF+G-CSF treatment. These *novel* findings suggest that the severe TBI-induced maladaptive (negative) changes in structural neural networks in the frontal cortex adjacent to the TBI-cavity may be reorganized by SCF+G-CSF treatment in a positive/adaptive way. The frontal cortex, which has functional connections to many brain regions including multiple memory systems such as the hippocampus, has been demonstrated to play a vital role in processing learning, memory, and decision-making [48, 49]. TBI survivors with problems in learning and memory, cognitive function, and decision-making show impaired brain networks in the frontal cortex [50, 51]. Therefore, the positively reorganized neural networks in the ipsilateral frontal cortex by SCF+G-CSF treatment could be beneficial to the improvement of spatial learning and memory after TBI.

The RAD area comprises the apical dendrites of pyramidal neurons, and the LM region comprises the superficial tufts of the apical dendrites in CA1 [52]. The apical dendrites of CA1 pyramidal neurons in the RAD and LM area splay a key role in cognitive function. The thickness of RAD and LM in the CA1 has been found to be related to delayed recall performance in patients with Alzheimer's disease [53]. In our current study, the severe TBI model causes reductions of apical dendrites in the ipsilateral RAD and LM of the CA1, which is associated with impaired spatial learning and memory. Most importantly, we have also observed the TBI-induced long-term overgrowth of axons in the ipsilateral hippocampal CA1. Similar to our observation, increased axon length and enhanced axon sprouting of granule neurons in the hippocampus have been found in epileptic rodents, which may negatively affect functional characteristics of the hippocampus networks [54–56]. Although SCF+G-CSF treatment has no effects on dendritic density in the hippocampal CA1, the TBI-induced overgrowth of axons in the CA1 of the ipsilateral hemisphere is reduced by SCF+G-CSF treatment, and the axonal density in the ipsilateral CA1 of SCF+G-CSF-treated TBI mice does not show differences with the sham-operative controls. The SCF+G-CSF-prevented axonal overgrowth in the ipsilateral CA1 may be involved in the improvement of cognitive function after severe TBI.

Axon pathology induced by TBI has been well recognized in the field of TBI research. Diffuse axonal injury is a direct consequence of mechanical injury and induces neurodegeneration and functional deficits [57, 58]. However, few, if any, research reported the overgrowth of axons after TBI. The present study has discovered that a single severe CCI-TBI causes long-term axonal overgrowth in both the perilesion cavity frontal cortex and ipsilateral hippocampal CA1. This abnormal overgrowth of axons may contribute to the TBI-induced long-term impairments in spatial learning and memory. SCF+G-CSF treatment completely prevents the axonal overgrowth. Further studies are warranted to clarify the mechanisms underlying the severe TBI-induced axonal overgrowth and how SCF+G-CSF treatment prevents this unique axonal pathology after TBI.

Conclusions

A single severe TBI in unilateral motor cortex does not only induce motor and cognitive deficits, but also causes long-term and widespread neurodegeneration in bilateral frontal cortex, striatum and hippocampus, and leads to long-term reductions in apical dendrites and axonal overgrowth in the cortex adjacent to the lesion cavity and in the ipsilateral hippocampal CA1. SCF+G-CSF treatment ameliorates severe neuronal pathology, neural network malformation, and impairments in spatial learning and memory after TBI. These findings advance our knowledge of axonal overgrowth in post-TBI pathology, and indicate the therapeutic potential of SCF+G-CSF in restricting a severe TBI-caused pathological progression.

Additional File

Additional file 1. Flowchart of the experimental procedures. Neurobehavioral testing was performed 2 weeks after TBI. Three weeks after TBI, SCF+G-CSF treatment was given subcutaneously (s.c.) for 7 consecutive days. Neurobehavioral testing was performed again 2 and 6 weeks after the last day of treatment. Mice were euthanized 20 weeks after the last day of treatment. (83K, pdf)

Additional file 2. Diagram of the selected imaging regions in the cortex, striatum and hippocampal CA1. (A) Imaging regions in the ipsilateral cortex 500 μm away from the TBI cavity and the corresponding cortex in the contralateral hemisphere at 1.00 mm or 1.10 mm anterior to the bregma. (B) Imaging regions in the ipsilateral and contralateral striatum at 1.00 mm anterior to the bregma. (C) Imaging regions in the ipsilateral and contralateral hippocampal CA1 at 1.10 mm posterior to the bregma. (96K, pdf)

Additional file 3. Behavioral testing before treatment initiation. (A-B) Quantification of escape latency to platform in the Morris water maze test over the 3 days of trials at 2 weeks post-TBI (1 week before treatment). Combined data of 3-day testing show that the escape latency to find the platform is significantly increased in all TBI mice (A), but it does not show differences between the two TBI groups (B). (C-D) Quantification of latency to fall in the Rota-Rod test one week before treatment. Combined data of 3-day testing indicate that the latency to fall is significantly decreased in all TBI mice (C), but it does not show differences between the two TBI groups (D). * $P < 0.05$, ** $P < 0.01$, Student's t-test. (193K, pdf)

Additional file 4. SCF+G-CSF treatment does not affect brain tissue loss volume after TBI. (A) Representative images of the brain sections from the TBI-Vehicle control mice and TBI-SCF+G-CSF-treated mice. Brain sections were stained by H&E. Scale bar, 1mm. (B) The percentage of brain tissue loss volume that was normalized to the sham group. Student's t-test. (70K, pdf)

Declarations

Ethics approval

The animal experiments followed ethical guidelines of the Animal Research: Reporting In Vivo Experiments (ARRIVE). All procedures of animal experiments were approved by the Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed in this study are available from the corresponding author on reasonable request.

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Competing interests

The authors declare that they have no competing interests. The contents do not represent the views of the U.S. Department of Veterans Affairs or the United States Government.

Authors’ contributions

JH performed immunohistochemistry and Fluoro-Jade C staining, analyzed the data and drafted the manuscript. TR performed TBI, drug injection, and behavioral tests. XQ and FH performed immunohistochemistry. MK performed H&E staining. LC contributed to research project discussion. LZ conceived the study, was in charge of overall direction and planning, and revised the manuscript.

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Not applicable.

Abbreviations

TBI	traumatic brain injury
SCF	Stem cell factor
G-CSF	granulocyte-colony stimulating factor
CCI	Controlled cortical impact
PBS	phosphate buffered saline
NGS	normal goat serum
BSA	bovine serum albumin
MAP2	microtubule associated protein 2
RAD	stratum radiatum
LM	stratum lacunosum moleculare

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Figures

Figure 1

SCF+G-CSF treatment improves TBI-impaired cognitive function but not motor function 6 weeks after treatment. (A) The average escape latency to find the platform in the Morris water maze test at 2 weeks after SCF+G-CSF treatment. (B) The average escape latency to find the platform in the Morris water maze test at 6 weeks after SCF+G-CSF treatment. (C) The average time latency to fall from the Rota-Rod at 2 weeks after SCF+G-CSF treatment. (D) The average time latency to fall from the Rota-Rod at 6 weeks after SCF+G-CSF treatment. * $P < 0.05$, ** $P < 0.01$, TBI-Vehicle mice vs. sham mice. ## $P < 0.01$, TBI-S+G mice vs. TBI-Vehicle mice. Two-way repeated ANOVA

Figure 3

The TBI-reduced apical dendritic density in the ipsilateral cortex adjacent to the TBI cavity is significantly increased by SCF+G-CSF treatment. (A-B) Representative images of MAP2 immunohistochemistry in the cortex adjacent to the TBI cavity (B) and the corresponding area in the contralateral cortex (A). Dashed rectangle: selected area in layer 1 or layer 2 for the magnification images. Scale bar, 30μm. (C-F) Quantification data of MAP2 positive dendritic optic density. (G-J) Quantification data of the percentage of MAP2 positive area. NSP>0.05, *P<0.05, **P<0.01, ***P<0.001, One-way ANOVA

Figure 4

SCF+G-CSF treatment does not affect TBI-reduced apical dendritic density in the ipsilateral hippocampal CA1. (A-E) MAP2 positive apical dendrites in the stratum radiatum (RAD) of CA1. (F-J) MAP2 positive apical dendrites in the stratum lacunosum molecular of CA1. (A and F) Representative images of MAP2 immunohistochemistry in the RAD (A) and LM (F) regions of bilateral CA1. Dashed rectangle: selected RAD or LM region for the magnification images (indicated by arrows). Scale bar, 30μm. (B, D, G, and I) Quantification data of MAP2 positive dendritic optic density in bilateral RAD (B, D) and LM (G, I). (C, E, H and J) Quantification data of the percentage of MAP2 positive area in bilateral RAD (C, E) and LM (H, J). NSP > 0.05, *P < 0.05, **P < 0.01, One-way ANOVA

Figure 5

SCF+G-CSF treatment prevents the TBI-induced overgrowth of axons in the ipsilateral cortex. (A) Left panel: Cresyl violet staining illustrates the layers of the cortex. Right panel: Representative images of SMI312 immunohistochemistry in bilateral cortex containing layer 1-5 of the three experimental groups. Scale bar, 50µm. (B-D) Quantification data of the percentage of SMI312+ area at different layers. * $P < 0.05$, ** $P < 0.01$, One-way ANOVA

Figure 6

SCF+G-CSF treatment prevents the TBI-induced overgrowth of axons in the ipsilateral hippocampal CA1. (A) Representative images of SMI312 immunohistochemistry in bilateral hippocampal CA1. Scale bar, 30µm. (B-C) Quantification data of the percentage of SMI312+ area in the contralateral CA1 (B) and ipsilateral CA1 (C). NSP > 0.05, *P < 0.05, One-way ANOVA

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