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Effects of Human ES-Derived Neural Stem Cell Transplantation and Kindling in a Rat Model of Traumatic Brain Injury

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Running header: hNSCs in a kindling/TBI model

Abstract

Traumatic Brain Injury (TBI) is one of the leading causes of death and disability in the population worldwide, with a broad spectrum of symptoms and disabilities. Post-traumatic hyperexcitability is one of the most common neurological disorders that affect people after a head injury. A reliable animal model of post-traumatic hyperexcitability induced by TBI which allows one to test effective treatment strategies has yet to be developed. To address these issues, in the present study, we tested human ES-derived neural stem cell transplantation in an animal model of post-traumatic hyperexcitability in which the brain injury was produced in one hemisphere of immunodeficient athymic nude (ATN) rats by controlled cortical impact (CCI) and spontaneous seizures were produced by repeated electrical stimulation (kindling) in the contralateral hemisphere. At 14 weeks post-transplantation, we report human neural stem cell (hNSC) survival and differentiation into all three neural lineages in both sham and injured animals. We observed twice as many surviving hNSCs in the injured vs sham brain, and worse survival on the kindled side in both groups, indicating that kindling/seizures are detrimental to survival or proliferation of hNSCs. We also replicated our previous finding that hNSCs can ameliorate deficits on the novel place recognition task³³, but such improvements are abolished following kindling. We found no significant differences pre or post kindling on the elevated plus maze. No significant correlations between hNSCs survival and cognitive performance on either task were observed. Together these findings suggest that Shef-6 derived hNSCs may be beneficial as a therapy for TBI, but not in animals or patients with post-traumatic hyper-excitability.

Key words

Traumatic brain injury (TBI), Controlled cortical impact (CCI), Human neural stem cell, Cell transplantation, Post-traumatic hyper-excitability, Kindling model, Spatial-hippocampal memory, Emotional memory.

Introduction

Traumatic brain injury (TBI) is defined as any injury resulting in altered brain function or brain pathology as the result of external mechanical force, with grades ranging from no visible damage, to bruising, to edema and hematoma¹. TBI is the leading cause of death and disability in children and young adults worldwide¹. In the United States more than 1.7 million people experience a traumatic brain injury (TBI) that leads to hospitalization². While mild TBI (mTBI) accounts for 75% of all TBIs³, TBI accounts for 30.5% of all injury-related deaths², and 43% of patients discharged after acute TBI hospitalization develop long-term disability⁴. Thus, an estimated 5.3 million people are living with a long-term TBI related disability⁵. This population costs the US an estimated of 56-221 billion each year^{6,7}.

TBI is a disease with a broad spectrum of symptoms and disabilities. Common events that cause TBI include car accidents, falls, violence and abuse, gunshots, explosive blasts and other combat injuries, sport injuries, and any kind of object that penetrates the skull^{2,8}. As a result of TBI, brain function can be temporarily or permanently impaired. Altered brain function resulting from TBI can have significant chronic effects, including difficulties in concentration and memory, balance and vision changes, sleep disturbance and manifestation of spontaneous seizure^{2,9,10}.

In the present study, we focused on one of the common problems associated with TBI, spontaneous seizures. TBI is often accompanied by the delayed development of post-traumatic epilepsy (PTE), which is manifested as [temporal lobe epilepsy \(TLE\)](#) in as many as 62% of trauma patients¹¹; although others put the risk lower and suggest that risk of PTE is related to injury severity¹²⁻¹⁴. Most seizures happen in the first several days or weeks after a brain injury, although some may occur months or years after the initial injury¹⁵. Post-TBI seizures can be divided into early post-traumatic seizure, when they occur in the first week after injury, and late post-traumatic seizures, more than seven days after injury and epilepsy, more than one seizure¹⁵. About 25% of people who have an early post-traumatic seizure will have another seizure months or years later; about 80% of people who have a late post-traumatic seizure will have another seizure¹⁵.

Experimental animal models and human TLE are characterized by hippocampal cell loss and mossy fiber sprouting in the dentate gyrus^{16,17}; however, the development of an animal model which more closely mimics human PTE is still in progress. Of these models, fluid percussion injury (FPI) and weight drop have been the most widely investigated as models of post-traumatic hyperexcitability^{17,18}, in contrast to the controlled cortical impact (CCI) injury model. However, regardless of the injury model used, the incidence of spontaneous seizures in post-TBI rodents is approximately 20%^{19,20}, and may be related to injury severity²¹, making studies of TBI-induced seizures difficult. At a 20% incidence rate, testing stem cell therapies in a TBI-TLE model would be prohibitively difficult as 80% of animals would be unaffected, yet all animals would have to receive a cell therapy. Since CCI has not been used as a model of injury-induced TLE, in the present study we added a classical kindling component to the initial CCI at 10 weeks post CCI injury, to reliably induce post traumatic hyperexcitability in all animals in the “kindled” groups. After our study was completed, a group published a protocol for chemical or electrical stimulation of epilepsy post-TBI in a CCI model, reporting that a prior TBI accelerated the time to kindling versus control animals²².

Kindling is a costly and time-consuming procedure, but is one of the most used models of electrical stimulation for the study of chronic seizures²³. It may be induced in rodents by repetitive, systemic administration of convulsants^{24,25}, or by repeated electrical stimulation of sensitive brain regions^{24,26}. The kindling protocol we used consists of repetitive electrical stimulation in the perforant path (PP), which promotes a gradual and progressive enhancement of electroencephalographic (EEG) activity and behavioral responses, culminating in generalized seizures²⁷. The progression of the seizure is assessed through a behavioral scale known as “Racine Stages”²⁸, which can be directly correlated with the after discharge (AD) duration obtained from an indwelling electrode in response to an otherwise non-seizure inducing stimulus²⁷.

Currently, no effective pharmaceutical or rehabilitative treatments exist for either TBI or post-traumatic TLE^{29,30}. Many groups are evaluating whether stem cells can offer a therapy that can improve the quality and length of life for those suffering from TBIs³¹⁻³³. Cell therapies for TBI have recently been reviewed by several independent groups³⁴⁻³⁶ (see also table 3 in³⁷). Neural stem cell transplantation has been applied in various

neurological disease models, including stroke, spinal cord injury, and neurodegenerative disease for the ability to replace the lost neural cells and improve functional deficits³⁸⁻⁴¹. Recently, we reported that the transplantation of human embryonic stem cell-derived neural stem cells restores cognition in an immunodeficient rat model of traumatic brain injury³³. In the present study, we first assessed whether kindling alone (after a prior TBI) had an effect on novel place recognition (NPR), elevated plus maze (EPM), AD, and histology; in particular, whether kindling exacerbated the effect of TBI on behavioral endpoints. Second, we tested the hypothesis that the transplantation of human neural stem cells can reduce/prevent the effects of kindling and TBI on NPR, EPM and AD, and in particular, on the post traumatic hyperexcitability. We also assessed the effect of kindling on hNSCs transplantation: does kindling increase or decrease the number of surviving hNSCs after TBI and does kindling change the fate of hNSCs? A better understanding of these outcomes and interactions will be important in planning future therapies in patients with TBI based PTE.

Materials and Methods

Experimental design

All experiments, including animal housing conditions, surgical procedures, and postoperative care were in accordance with the Institutional Animal Care and Use Committee (IAUC) guidelines at the University of California, Irvine. The use of human cells was approved by UCI's human Stem Cell Review Oversight committee (hSCRO). 66 male ATN rats (RNU -/-, Cr: NIH-rnu, homozygous, from the National Cancer Institute (NCI), Frederick, MD, 6 weeks old were randomly divided into 5 experimental groups: sham+electrode+vehicle (n=12) (control group with only craniotomy and implant of electrode for kindling), sham+kindle+vehicle (n=13), sham+kindle+hNSCs (n=13), TBI+kindle+vehicle (n=14), and TBI+kindle+hNSCs (n=14), prior to the CCI or craniotomy procedure. **Fig. 1** depicts to experimental timeline of hits, cell transplants, behavior, and kindling.

Controlled Cortical Impact (CCI)

Traumatic brain injuries were produced using a controlled cortical impact device (CCI) (TBI 0310; Precision Systems and Instrumentation, Fairfax Station, VA, USA) which uses a pneumatic piston to deliver a unilateral cortical contusion via a 5mm flat metal impactor tip, with a 2.25 mm impact depth, 4.5 m/sec velocity, and 500 ms dwell time. Injuries were carried out under 3% isoflurane anesthesia with rats positioned in stereotaxic holder (Leica Microsystem Inc., Buffalo Grove, IL, USA). The stereotaxic coordinates for the craniotomy and/or CCI were anterior/posterior (A/P) -4.5mm, medial/lateral (M/L) -3.6mm in the right hemisphere above the hippocampus. A 6mm hole was cut with a hand held trephine and the dura exposed. All animals received a craniotomy, but only the TBI groups received a CCI. All animals received lactated ringers (50 ml/kg) subcutaneously, as well as buprenorphine (Hospira Inc., Lake Forest, IL, USA) (0.5 mg/kg), immediately after surgery and for 2 days thereafter. Due to the immunodeficient nature of ATN rats, an antibiotic (Baytril, 2.5 mg/kg, Western Medical Supply, Arcadia, CA, USA) was administered immediately after surgery, and daily for 5 days thereafter.

Human Neural stem cell (hNSC) preparation and transplantation

Before transplantation, animals were randomly divided into the five experimental groups (see Fig. 1). 13 sham and 14 injured animals were transplanted via bilateral injection with xeno-free CD133+/CD34- Shef6 hNSCs at passage 27 (p27), which were prepared via magnetic-activated cell sorting (MACS) for CD133 and against CD34, as previously reported⁴².

hNSCs and vehicle were prepared fresh the same day as transplantation: 9 days post-TBI. ATN rats were anesthetized with 3% isoflurane and positioned in a stereotaxic holder. A total of six injections, three per hemisphere, of either hNSCs or vehicle, were made. In the ipsilateral side, the three injection sites were made inside the craniotomy hole. On the contralateral side, additional small holes were drilled. A Dremel® rotary tool (Racine, WI, USA), set at 5,000 rpm, was used to create the three contralateral injection burr holes. A 5 µl Hamilton syringe (Cat#87930, Hamilton Company, Reno, NV, USA) with a 1" 30G blunt needle was mounted into an UMP-3 (World Precision Instruments,

Sarasota, FL, USA) injector connected to a SYS-Micro4 controller. The six injections were made at the following coordinates: injection #1 M/L 2.50, A/P -2.50, deep -3.40; injection #2 M/L -4.50, A/P -4.20, deep -4.00; injection #3 M/L -5.40, A/P -5.28, deep -5.40. Cells were first triturated 5 times with a 10 μ l pipette, then pulled up into syringe over approximately 10 seconds. For each injection, the needle was initially lowered an additional 0.15mm to create a pocket in the brain. For each of the six injections, a dose of 7.5×10^4 cells/ μ l (1.11 μ l per site) over 2 minutes with a dwell time after injection of 4 minutes. The total dose was 5×10^5 hNSCs. Prior to penetrating tissue, the syringe was tested each time to make sure it was not clogged by ejecting some of the solution into the air manually through the Micro4 controller. Vehicle injection animals received the same procedure but received six injections of 1.11 μ l of cell culture media. Bone wax was applied to seal each of the burr holes. Animals received lactated ringers (50 ml/kg) subcutaneously immediately after surgery, and buprenorphine (0.5 mg/kg) immediately after surgery and daily for 5 days thereafter.

Kindling

59 days post-TBI, and 50 days post transplantation, all rats were surgically implanted with electrodes under 3% isoflurane (see Fig. 1). The electrode (3.5 mm length and 0.3 mm strip) was stereotaxically positioned contralateral to the injury in the angular bundle of the PP. Implant coordinates were: A/P -7.78 mm, L/M 4.59 mm and dorsal/ventral D/V -2.6 mm from dura and -3.5 mm from the skull surface. Animals are allowed to recover after-surgery for 10 days before the kindling regimen. For the kindling protocol, rats were stimulated for 20 days, beginning on day 71 (D71). Rats were stimulated twice a day, morning and afternoon, with a threshold of 400 μ A, until they reached stage 5 on the Racine scale – kindling was stopped early if they reach stage 5 three times in a row. As some animals kindled faster than others, 10 days after the last day of the kindling protocol (D91), the animals were stimulated once more (D101) in order to analyze the EEG in response to recent stimulation. All EEG recordings were done using LabChart v8 software (AD Instruments, Colorado Springs CO) for 5 minutes. For each stimulation, animals were also evaluated on the Racine scale. The animals were considered kindled if they reached stage 5 three times in a row, which is characterized by rearing and

falling²⁸. For quantitative analyses, we evaluated the duration of the AD, number of spikes in one series, and duration of each spike series (data not shown) on the last day of stimulation. A spike series was defined as a series when there are more than three straight uniform spikes.

Behavioral analysis

In order to see whether kindling and the treatment with hNSCs caused an improvement or impairment of cognitive function, both behavioral tests were performed before (D66-D70) and after kindling (D96-D100), 2 or 3 months post injury respectively, with an interval of one month from each other. All testing was performed by experimenters blind to treatment group.

Novel Place Recognition

A novel place recognition (NPR) task was used to evaluate spatial-hippocampal mediated memory. The NPR task consisted of two open field plastic arenas, each measuring 45x70x70 cm, which were placed next to each other on the floor in a dedicated behavioral testing room with a video camera set above the arenas. Live tracking of the animals was obtained using EthoVision XT software (version 8, Noldus Information Technology, Leesburg, VA, USA). The discrimination index (DI) was used as the main outcome measurement. DI was calculated as the ratio of total time spent exploring the object in the novel location in relation to the object in the familiar location $\{(t.novel-t.familiar/t.novel+t.familiar)*100\}$. We first analyzed DI minute by minute for 5 minutes. However, during NPR analysis, we observed that the animals in the control group (sham+electrode+vehicle) lost interest after the first few minutes, as previously reported^{33,43}. Thus, we calculated DI for the first minute of the task only.

For the NPR task, we tested two animals at the same time, one animal for each arena. The same test was done before and after kindling. First, animals were habituated to the testers by five minutes of handling over five days, and habituated to the testing environment and open field arena by exploring the arena freely for 30 minutes (15 min/day) prior to running the NPR task. The task took place over two days. The first day

was familiarization testing, in which two identical objects were placed in opposing corners of the open field and the animals were allowed 5 minutes to explore both objects. 24 hours later, for the testing phase, the animals were placed back in the same arenas with the two identical objects, but one was moved to a different location (novel place, to an open corner), while the other object remained at its former spatial location (familiar place). For both the familiarization phase and testing phase, rats were placed in the arenas and allowed to explore freely for 5 minutes. For both phases, in order to track the exploration of the objects, we used the “head direction to zone” function in EthoVision XT, whereby a rat was considered to be exploring an object when its head was oriented toward it. Arena and objects were cleaned with 70% ethanol (EtOH) between each trial in order to minimize odor cues.

Elevated Plus Maze

The EPM is a task designed to test anxiety responses in animals. Previous literature has suggested that the amygdala and ventral hippocampus both contribute to anxiety response⁴⁴. The EPM consisted of a cross-shaped arena, with two open arms and two closed black plastic arms (Med Associates Inc., Fairfax, VT, USA). To encourage exploratory behavior, the experiment was performed in a dimly lit room with infrared lighting on the EPM using infrared beam detection, and an infrared video camera suspended above the plus maze. Each rat was placed in the center of the maze facing one of the open arms and tracked for 5 minutes using EthoVision XT software. The software recorded total time spent in the open and closed arms, as well open and closed arm entries (counted when the center point of the rat crossed into either the open and closed arms). The plus maze was cleaned with 70% EtOH between each animal.

Histology Analyses

At 14 weeks post-transplantation, animals were euthanized and brain tissue collection and immunohistochemistry was performed as previously described⁴⁵, with minor modifications. Rats were anesthetized with a lethal dose of euthasol (100 mg/kg, intraperitoneally (i.p.)) and transcardially perfused with 50 ml of phosphate buffered

saline (PBS), followed by 300-400 ml of 4% paraformaldehyde (PFA). Brains were dissected and post-fixed overnight in a solution of 4% PFA and 20% sucrose in PBS at 4°C, then frozen at -65°C in isopentane (2-methyl butane), and stored at -80°C.

Cryosection and Immunohistochemistry

Brains were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek USA, Inc, Torrance, CA, USA). Brains were cut in coronal sections, 30 µm thick, using a cryostat and mounted using a CryoJane tape transfer system (Leica Biosystems, Inc., Buffalo Grove, IL, USA). Slides were stored in sealed slide boxes at -80 or -20°C until use. All the immunostaining procedures were conducted at room temperature.

For 3,3'-diaminobenzidine (DAB) (Vector Laboratories, Burlingame, CA, USA) immunohistochemistry, sections were removed from the freezer and air dried at room temperature for 30 minutes, then sections were washed 3 times (at 5 minutes each) in Tris 1X (Supelco, Bellefonte, PA, USA) followed by a 20 minute incubation in 0.3% hydrogen peroxide/methanol. After one Tris 1X wash for 5 minutes, sections were lightly permeabilized in 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 15 minutes, then washed for 30 minutes with bovine serum albumin (BSA) (20g per 1L) and normal serum donkey (Vector Labs, Burlingame, CA, USA) (150µl per 10 mL) in Tris 1X. Sections were then incubated overnight with the primary antibody. The primary antibody used for DAB staining was an anti-human cytoplasmic monoclonal mouse anti-SC121 (1:8,000; Y40410, Takara, Kusatsu City, Japan). The next day, after two 5 minute washes with 0.1% Triton X-100, and 15 minutes with BSA and normal serum donkey in Tris 1X, sections were incubated with the secondary antibody biotin-SP-conjugated F(ab)₂ purified immunoglobulin G (IgG) secondary antibody (1:500; 715-066-151, Jackson ImmunoResearch, West Grove, PA, USA) pre-absorbed against rat mouse donkey goat. After 2 washes (5 minutes each) in 0.1% Triton X-100 and a wash with BSA in Tris 1X for 15 minutes, sections were incubated for 1 hr with an avidin-biotinylated peroxidase complex (ABC) using Vectastain Elite ABC kit (PK-6100, Vector Laboratories, Burlingame, CA, USA) prepared according to the manufacturer's

recommendations. ABC incubation was followed by 3 washes in Tris 1X (5 minutes each), and a 5 minute incubation with DAB (SK-4100, Vector Laboratories). Sections were counterstained with cresyl violet (Sigma-Aldrich, St. Louis, MO, USA) and cover slipped using Depex (Sigma-Aldrich) mounting medium and allowed to dry overnight at room temperature. Quantification of the survival of SC121-positive human stem cell was performed using MicroBrightfield Stereo Investigator (version 11.09, MBF Bioscience, Williston, VT, USA).

For fluorescent staining, sections were removed from the freezer and left to dry at room temperature for 30 minutes, then sections were wash 4x (5 min each) in PBS, then 1x (5 minutes) in 0.1% Triton X-100 in PBS, followed by a 1 hour wash with BSA and donkey serum in PBS. Sections were then incubated overnight in primary antibody. The primary antibodies used were: monoclonal mouse anti-SC121 (1:500) (AB-121-U-050, Takara), polyclonal rabbit anti-Nestin (1:1000) (ABD69, Millipore, Darmstadt, Germany), polyclonal rabbit anti-Olig2 (1:500) (AB9610, Millipore), polyclonal rabbit anti-neuronal nuclei (NeuN) (1:1000)(ABN78, Millipore) and polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) (1:1000) (Z0334, Agilent Technologies/DAKO, Santa Clara, CA, USA). The next day, sections were washed 3x (5 min each) in PBS, followed by 1 hour in BSA and donkey serum in PBS. Sections were then incubated with fluorescent purified IgG secondary antibody cyanine (Cy)3-conjugated F(ab')₂ fragment donkey anti-mouse (1:500; 715-166-151, Jackson ImmunoResearch) or Alexa Fluor 488-conjugated F(ab')₂ fragment donkey anti-rabbit (1:500, 711-546-152, Jackson ImmunoResearch) pre-absorbed against the species in which the primary antibody was raised, and counterstained with Hoechst (1:500, Life Technologies/ThermoFisher, Waltham, MA, USA) to reveal cell nuclei. After a 5 minute wash with PBS, sections were cover slipped using Fluoromount-G® (SouthernBiotech, Birmingham, AL, USA) mounting medium and allowed to dry overnight. Imaging was performed using a Zeiss Observer.Z1 spinning disk system (Axiovision 4.8.2, Carl Zeiss, Thornwood, NY, USA).

Quantification of Histology

Transplanted hNSCs Survival Analysis

To estimate the total number of human stem cell survival, we used stereology. An Optical Fractionator probe was used for unbiased stereological analysis. Histological sections, which contained visible cortex and hippocampus, comprised anterior and posterior landmarks of approximately A/P -1.80mm to A/P -5.28. A section interval of 1/12 was utilized, placing adjacent sections analyzed at 360 μm apart. In order to identify transplanted human cells, sections with DAB anti-SC121 (human cytoplasm) antibody, with cresyl violet counterstain for histological structure identification, were used. Contours were drawn at 2.5x magnification, outlining areas of human cell engraftment, and human cell identification and counting was performed at 63x magnification. The counting frame utilized was 75x75 μm with grid size of 125x125 μm . The dissector height was set at 12 μm with an upper and lower guard zone of 1 μm . We quantified the estimated population using mean section thickness (only using sites with counts). The Gundersen coefficient of error (CE, $m=1$) for all hNSCs transplanted sham animals ($n=13$) averaged 0.1 and for all injured animals ($n=14$) averaged 0.07.

Transplanted hNSCs Fate Analysis

To determine the percentage of transplanted human cells differentiating along neural fate pathways, and whether differentiation was effected by kindling, we conducted a proportional analysis of human cell fate. Imaging was performed using a Zeiss Observer.Z1 spinning disk system. An average of 4 images per section across an average of 6 sections were randomly collected and cell fate was counted using Imaris Scientific 3D/4D Image processing and Analysis (version 7.6.5, Bitplane AG, Belfast, Ireland). A section interval of 1/12 was utilized, placing adjacent sections analyzed at 360 μm apart from each other. Co-localization was performed using either antibodies specific for neuronal fate (NeuN), astrocyte fate (GFAP), oligodendrocyte fate (Olig2), or undifferentiated cells (Nestin) along with an anti-SC121 (human cytoplasm) antibody. Hoechst 33342 was used as a nuclear counterstain. Each fate group consisted of 8-10 randomly selected animals; quantification was performed blind to treatment group. An average of 24 images per animal were analyzed (e.g. for SC121/GFAP).

Statistical Analysis

All behavioral, histological, and stereological analyses were performed blindly by experimenters. Statistical analyses were performed using Prism (GraphPad Software, Version 7.0b, La Jolla, CA, USA). Behavioral data (NPR and EPM) were analyzed either by one-way or two-way analysis of variance (ANOVA). One-way ANOVA was used when comparing between injury groups. Two-way ANOVA was used when comparing the same group, pre and post kindling (of the latter, data not shown). Stereological quantifications were analyzed via unpaired two-tailed t-tests (surviving hNSCs). Errors are standard error of the mean (SEM) of averaged results and significance was defined as $p < 0.05$ for all statistical analyses. T-test results were reported as the t-ratio (t), degrees of freedom (df), and the p value. Correlation between hNSCs survival and behavior (NPR, EPM) scores was analyzed by Pearson Correlation analysis.

Results

Sham and injured ATN rats exhibit long-term survival of hNSCs Shef6 p27, with higher survival in the hemisphere ipsilateral to the injury

9 days post-TBI, sham and injured ATN rats were transplanted with hNSCs Shef6 (p27) in the injury hemisphere (TBI for injury animals and craniotomy for sham animals) and contralateral hemisphere (side that was kindled). 250,000 cells were transplanted in each hemisphere for a total dose of 500,000 cells. At 14 weeks post-transplantation (wpt) (and 4 days after final AD recording), hNSCs were detected in the hippocampus on both injured and contralateral hemispheres (**Fig. 2**) of both TBI (Fig. 2A-C) and sham (Fig. 2D-F) animals. On the injured side of TBI animals, the hNSCs were found concentrated along the injury site. Of the initial 500,000 hNSCs transplanted, just $8,337 \pm 1,812$ for sham and $19,344 \pm 3,816$ for TBI animals survived long-term (Fig. 2G). Since the kindling was done on the contralateral hemisphere compared to the injury, we quantified the survival of the transplanted hNSCs in each hemisphere and found that, for both groups, the “injury” hemisphere contained a higher number of hNSCs that survived long-term compared to the contralateral “kindled” hemisphere (Fig. 2H).

Shef6 p27 hNSCs differentiated into all three neural lineages

Proportional cell fate analyses showed that, in both sham and injured animals, the transplanted hNSCs differentiate into cells of all three neuronal lineages, and that cell fate was not significantly affected by the injury (**Fig. 3**). The predominant terminal fate was neuronal (NeuN⁺) (sham 13.3% ± 3.6%; injured 13.3% ± 2.2%) (Fig.3 A-B), followed by astroglial (GFAP⁺) (sham 11.2% ± 1.8%; injured 11.5 % ± 1.7 %) (Fig.3 C-D). A smaller percentage of oligodendroglial cells/progenitors (Olig2⁺) (sham 5.5% ± 1.7%; injured 6.4% ± 2.1%) (Fig.3 E-F) was observed and a small percentage remained in an undifferentiated state (e.g. Nestin⁺) (sham 3.9 % ± 1.1%; injured 8.3% ± 1.9%) (Fig.3 G-H). The proportional cell fates observed here, at 14 weeks post-transplantation and following kindling, were similar to proportional cell fates observed in our previous study using these same cells (**Table 1**)³³.

hNSCs transplantation improved long-term cognitive function on the NPR task; this effect was abolished after kindling

In this study, one goal was to examine the behavioral effect of kindling on sham animals and injured animals in the context of hNSC transplantation. An aim was to test whether hNSCs can reduce the deleterious effects of TBI and kindling on cognitive and emotional tasks. Cognitive and emotional effects of TBI were evaluated using NPR and EPM tasks, respectively. Animals were behaviorally assessed before and after kindling (2 months and 3 months post injury, respectively). We predicted that non-transplanted injured and kindled animals would exhibit worse cognitive and emotional deficits compared to hNSC transplanted injured and kindled animals. Comparison of the three sham groups on both the NPR and EPM tasks, both pre-kindling and post kindling via one-way ANOVAs, revealed no significant differences between the control groups, while one outlier was identified via a ROUT test and excluded. The three sham/control groups were then pooled as controls for further comparisons to the respective treatment groups (see **Table 2**).

Comparison of the pooled control/sham groups to TBI+Vehicle and TBI+hNSCs groups performance on the NPR task via one-way ANOVA revealed a significant difference between the controls and the TBI + Vehicle group on the DI ($F(2,59)=3.28$, $p=0.047$)

pre-kindling (**Fig. 4**). A Holm-Sidak correction for multiple comparisons showed a significant difference between controls and TBI+Vehicle treated animals ($p=0.028$), but no difference between TBI+Vehicle and TBI+hNSCs treated animals ($p=0.144$) nor controls and TBI+hNSCs treated animals ($p=0.710$). Thus, TBI animals performed worse on the NPR task in comparison to controls (as reported previously in Haus et al.)³³; however, hNSC treatment returned TBI injured animals to cognitive performance statistically indistinguishable from control levels (**Fig. 4A**). This negative effect on the NPR task following TBI, and restoration of function following hNSC transplantation, was lost following kindling (**Fig. 4B**), where a one-way ANOVA indicated no significant differences ($F(2,55)=1.11$, $p=0.336$) between either treatment group to controls.

In contrast to the significant difference in performance on the more cognitive NPR task, comparison of the TBI groups (with Vehicle or with hNSCs) on a more emotion focused task, the EPM, revealed no significant differences between any groups via one-way ANOVA either pre-kindling ($F(2,62)=2.40$, $p=0.099$) or post-kindling ($F(2,62)=2.35$, $p=0.103$) (**Fig 4C-D**). The lack of significant group effects on the EPM may have been the result of differing levels of cell engraftment within animals in the same group. However, correlations of transplanted hNSC survival number with performance on either cognitive (NPR) or emotional tasks (EPM) were not statistically significant (**Fig 5 A-D**).

hNSC transplantation in ATN rats doesn't have an effect on kindling

In order to evaluate whether kindling can enhance the development of spontaneous seizures in a CCI TBI animal model, or whether kindling effects the survival of transplanted hNSCs, and whether hNSCs can prevent or reduce the development of kindling induced seizures at 10 weeks post-TBI (9 weeks post-transplantation), animals were stimulated for 20 days, twice daily, morning and afternoon, and again 10 days after the last day of kindling (D91). Animals were stimulated once more, at D101, to record their final AD duration. For analyses, we assessed the duration of the AD for all the kindled groups (**Fig. 6**), days of stimulation required to induce kindling, and number of spikes in a spike series post-stimulation. A representative EEG recording showing start of stimulation, start of AD, end of AD, start and end of spike series is shown in Fig. 6A.

We predicted that hNSCs in transplanted animals, both sham or injured, would reduce the duration of AD compared with the vehicle group, but our results show that AD duration is not significantly different among the different groups. In particular, the presence of hNSCs does not effect the AD duration between the different groups (Fig. 6B). Correlations of transplanted hNSCs survival with the duration of an animal's respective AD (at D101) were not statistically significant (Fig. 6C). No differences were observed in the other parameters either.

Surviving hNSC number does not correlate with performance on either the EPM or NPR task

Pearson correlation analyses showed no significant correlations pre-kindling or post-kindling between surviving transplanted hNSC number and either the time in open arms on the EPM or the DI score on the NPR in uninjured animals (**Figure 7**). While the correlation between cell number and NPR post-kindling is “significant”, due to a threshold effect on the DI and lack of spread in cell survival (see Fig. 7D), little can be interpreted from these results.

Discussion

Traumatic brain injuries are known to cause disabilities in cognitive processes, sensation, movement, memory and emotion in both rats and humans alike^{37,46}, with a high incidence in the population. Further, TBIs account for 20% of symptomatic epilepsy in the general civilian population²⁰, and 10% of individuals with severe TBI exhibit PTE⁴⁷. Several laboratories have reported acute seizures, and hippocampal hyperexcitability to stimulation or proconvulsant drug exposure in TBI models²¹, but just a few previous studies have investigated an animal model of spontaneous chronic seizures^{17,19-22} after experimental head injury. Fluid percussion injury (FPI) has been the most widely investigated as a model of post-traumatic hyperexcitability^{17,19,21}. In contrast, until 2016, the CCI model of TBI had not been used in a model of induced post-traumatic epilepsy^{17,22}. In the present study, we tested whether CCI and hippocampal kindling (a model of experimental epilepsy) can induce hippocampal hyper-excitability. We then

assessed the effect of human ES-derived neural stem cell transplantation in this animal model on cognitive and emotional tasks, and a battery of kindling parameters.

At 14 weeks post-transplantation, we found that sham and injured ATN rats maintained long-term survival of hNSCs. Paradoxically, we observed two-fold greater survival in injured animals than sham animals. While it is often presumed that the injured microenvironment is hostile to the survival of transplanted cells⁴⁸, our experience is that the time post-transplant in spinal cord injury models does not effect cell survival^{45,49}. We have historical data suggesting that fetal-derived hNSC survival is slightly better in uninjured vs injured central nervous system (CNS) tissue at 14 weeks post-transplant, and that this difference is decreasing over time post-transplant as cells proliferate (i.e. the number of 5-bromo-20-deoxyuridine (BrdU) positive hNSCs is higher in uninjured tissue at 7dpi and 14dpi, but not at 28dpi)⁵⁰. We also have an “historical” comparison using Shef6 derived hNSCs that can be made from the Haus et al. study³³. In Haus et al.³³, ATN rats of the same age and from the same breeder were given the same injury or sham procedure, but none were subjected to kindling. Animals in Haus 2016 received Shef6 hNSCs at p27, as in the present study, however Haus animals received a dose of 250,000 cells vs 500,000 cells in the present study. At 20 weeks post transplantation in the contralateral hemisphere, Haus et al.³³ reported that 21,846 ±10,408 hNSCs survived (or 8.7%) long term. We observed a survival of just 2,487 cells in the contralateral/kindled hemisphere of the initial 250,000 dose received on that side. The overall average of 19,344 cells per animal following a dose of 500,000 cells is less than 3 percent survival. However, on the non-kindled side, 7.7% of the 250,000 cells survived, comparable to that observed in Haus et al.³³. If we were to extrapolate equivocal cell survival in both studies (e.g. simply “double” the number of surviving cells from Haus et al.³³), we can see that kindling most likely had a significant detrimental effect on the survival of hNSCs. This presumption is also supported by the hemispheric differences in survival of cells on the kindled vs non-kindled side (Fig. 2H).

Why did kindling decrease the number of surviving hNSCs in this TBI model? Little is known about the effect of kindling or seizures on exogenous stem cells. However, others have reported that seizures can increase endogenous neurogenesis, leading to aberrant network reorganization, in adult rats⁵¹. Functional electrical stimulation, at a

sub-seizure threshold, also facilitates an increase in nestin positive precursors in animals with a prior stroke⁵². One might predict that the kindling received in the present study would have facilitated survival and proliferation of exogenously administered stem cells, however the opposite effect was observed. Because we observed less than half the number of surviving cells in the kindled hemisphere vs the non-kindled hemisphere, it is likely that the kindling was directly toxic to the hNSCs or reduced their proliferation. We might postulate that kindling accelerated their differentiation⁵³ and thus less cells remained in a proliferative state once kindling began at Day 71, thus reducing the overall numbers of engrafted hNSCs when sacrificed at later time-points.

We also report that hNSCs differentiate into all three neural lineages (Fig. 3) and that this differentiation is unaffected by whether the animal received a TBI or sham surgery (although both groups were kindled) (Fig 3.). Again, without a hNSC transplanted, uninjured, non-kindled control, it is impossible to demonstrate if kindling altered the fate in the present experiment. But comparing to fate in the present kindling experiment to the Haus et al.³³ study, which used identical late passage P27 Shef-6 hNSCs and assessed fate at 20 weeks post-transplant, we observed 13% of hNSCs differentiating into NeuN+ neurons in the present study versus 19% in Haus et al.; 12% GFAP+ in the present study versus 13% in Haus et al., 6% Olig2+ versus 11% in Haus et al., and 8% Nestin+ versus 10% in Haus et al. (Fig. 7) (see **Table 1**). This suggests that exposure to kindling 2 months post-transplant did not significantly alter hNSC fate in the injured microenvironment. Alternatively, perhaps 2 months post-transplant is too late to alter the dynamics of Shef-6 hNSC fate choice in this injury paradigm.

We have previously reported that ATN TBI rats exhibit long term cognitive impairments in a hippocampal-dependent spatial memory task (NPR) compared to sham animals at 2 months post injury. Additionally, after transplantation of hNSCs, ATN TBI animals show improvement in the NPR task at 8 weeks compared to ATN TBI animals that received the vehicle³³. In the present study, we replicated the beneficial effect of Shef-6 hNSCs on NPR performance. However, this improvement in comparison to TBI animals that received a vehicle injection, was abolished after exposure of animals to kindling (Fig. 4). We did not observe improvement in emotional function following hNSC transplantation as assessed via EPM tasks. Nor did we observe significant correlations between

transplanted hNSC survival number and NPR nor EPM scores in injured animals. It is likely that these tasks were underpowered and would benefit from additional animals should future studies be conducted using this model.

Given the moderate improvement on the NPR task we observed both here, and previously with Shef-6 hNSCs versus the relatively low level of long-term engraftment, one can speculate on the mechanism of action of these cells. It is possible that the hNSC used here integrate with the host tissue and either remyelinate dis-myelinated axons and/or form synaptic connections with the host. Alternatively, the human cells could be providing trophic support of the injured host, or modulating the host inflammatory response. These mechanisms are not mutually exclusive. However, given the low level of final engraftment observed here (3%), and our prior data demonstrating that ablation of human fetal NSCs via diphtheria toxin in a contusion model of SCI abolished the behavioral efficacy of the cells in a model with ~200% engraftment³⁹, it seems more likely that the efficacy observed in TBI was not via integration with the host. However, this possibility should be tested more directly via a diphtheria experiment in TBI animals receiving hNSCs. Diphtheria toxin is 100,000 more toxic to human cells than rodent cells; thus diphtheria can be given to animals after human stem cell engraftment and the human cells can be selectively ablated while the rodent remains unharmed. If the improvements on the NPR task in the hNSC treated group were retained post-diphtheria treatment, this would suggest that the hNSCs exerted their effect by restoring host circuitry and not by functionally integrating with host cells.

Finally, our results show that the AD duration is not significantly different among the different kindled groups, whether they received hNSCs or vehicle. The lack of an effect on AD could indicate that transplanted hNSCs do not integrate with host circuits in a manner that effects AD duration, or conversely, that there aren't sufficient surviving cells to alter neural circuits. Taken together, these data suggest that, while hNSCs have no effect on post-traumatic injury hyper-excitability, they can improve performance on a hippocampal based memory task, NPR; this is a replication of the Haus et al. study³³.

Additional work in a TBI model is necessary to improve post-transplant cell survival (perhaps via co-administration of an immunosuppressant such as Asilo-GM1) to

optimize cell administration and timing of transplantation. For example, would a great delay in transplantation lead to great cell survival and still improve cognitive function? Further, it would be beneficial to compare the efficacy of these Shef-6 hNSCs to other cell populations (iPS, fetal or ES-derived) before an expensive clinical trial is undertaken with less than optimal cells, timing, or delivery methods.

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FIGURE LEGENDS

Figure 1. Experimental groups and experimental design. 66 ATN rats were randomly assigned to one of five groups (Sham+electrode+vehicle, 12; sham+kindle+vehicle, 13; sham+kindle+hNSCs, 13; TBI+kindle +vehicle, 14; TBI+kindle+hNSCs, 14). The sham animals received a craniotomy, while the TBI animals received a CCI injury at D0. 9 days post surgery, animals were re-anesthetized and received either vehicle or 500,000 hNSCs. Animals were allowed to recover for 50 days before being implanted with an electrode. 1 week later, animals were tested for the first time on NPR and EPM tasks. Testing was followed by up to 20 days of kindling and then a second NPR and EPM assessment. After discharge (AD) duration was recorded following behavioral tests, and the animals were sacrificed on day 105 (14 weeks post-transplant). All testing was conducted by persons blind to treatment condition. ATN: immunodeficient athymic nude, TBI: traumatic brain injury, CCI: controlled cortical impact, hNSC: human neural stem cell, NPR: novel place recognition, EPM: elevated plus maze, Sac: sacrifice.

Figure 2. Long-term survival (14wpt) of hNSCs is significantly greater in TBI animals than shams; survival was also better in the non-kindled hemisphere. At 14 weeks post-transplantation and 1 week after stimulating to measure AD, transplanted hNSCs show long-term survival in the brains of both sham and TBI ATN rats. **(A-C)** Immunohistochemistry of transplanted hNSCs with the human cytoplasmic protein marker, anti-SC121, showing survival and engraftment in both injured **(A)** and sham **(D)** brains in photomontage collected at 2.5x (scale bar 1 mm). Boxed areas in A and D shown at higher magnification in B,C and E,F, respectively, taken at 20x (scale bar 100 μ m). **(G)** Stereological quantification of transplanted hNSCs in sham (n=13, 8,337 \pm 1,812) and in TBI animals (n=14, 19,344 \pm 3,816) shows a significant difference in cell survival between experimental groups (two tailed t test $t_{25}=2.54$, $p = 0.0177$). **(H)** Stereological quantification of transplanted hNSCs shows a significant difference between the kindled side vs the contralateral side in both groups (Sham - two tailed t test $t_{24}=2.56$, $p=0.017$; TBI - two tailed t test $t_{26}=3.65$ $p=0.001$). Data are presented as mean \pm standard error of the mean (SEM), with TBI animals demonstrating significantly higher survival. Note: animals were kindled on the contralateral side of the injury (CCI

injured for TBI animals and craniotomy for sham). WPT: weeks post-transplant, hNSC: human neural stem cell, TBI: traumatic brain injury, AD: after-discharge, ATN: immunodeficient athymic nude, anti-SC121: human cytoplasmic marker, CCI: controlled cortical impact. K: kindled.

Figure 3. Human neural stem cell transplantation into either sham or TBI rats' brain show neuronal, astrocytes and oligodendrocytes differentiation or remain undifferentiated. hNSCs Shef6 P27 via bilateral injection (9 day post injury for TBI animals) at 13 weeks post-transplantation and 4 weeks post kindling differentiate into either **(A-B)** mature neurons (NeuN+) (sham, n=10, 13.3% ± 3.6%; injured, n=10, 13.3% ± 2.2%; two tailed t test $t_{18}=0.01$, $P=0.99$), **(C-D)** astrocytes (GFAP+) (sham, n=10 11.2% ± 1.8%; injured, n=10, 11.5% ± 1.7%; two tailed t test $t_{18}=0.087$, $P=0.93$), and **(E-F)** oligodendrocytes (Olig2+) (sham, n=8, 5.5% ± 1.7%; injured rats, n=8, 6.4% ± 2.1%; two tailed t test $t_{14}=0.317$, $P=0.75$) or remain in an undifferentiated state **(G-H)** (Nestin+) (sham, n=8, 3.9% ± 1.1%; injured, n=8, 8.3% ± 1.9%, two tailed t test $t_{14}=1.90$, $P=0.07$). In red, human stem cells and in green, the various lineage cell types. White arrows show co-localized cells. No significant differences between sham and injured groups after kindling in terms of differentiation into any of the three lineage cell types, or undifferentiated cells, were observed. Data are presented as mean ± standard error of the mean (SEM). TBI: traumatic brain injury, hNSC: human neural stem cell, GFAP: glial acidic fibrillary protein.

Figure 4. TBI results in a significant deficit on the novel place recognition (NPR) task in comparison to controls; this deficit is reversed following hNSC transplantation. At 2-3 months post injury, before and after kindling respectively, rats were assessed via NPR and EPM tasks by experimenters blind to treatment. **(A-B)** Comparison of the pooled sham/control groups to TBI+Vehicle and TBI+hNSCS group performance on the NPR task via one way ANOVA revealed a significant difference between the controls and the TBI + Vehicle group on the DI ($F(2,59)=3.28$, $p=0.047$)

pre-kindling. A Holm-Sidak correction for multiple comparisons showed a significant difference between controls and TBI+Vehicle treated animals ($p=0.028$), but no difference between TBI+Vehicle and TBI+hNSC treated animals ($p=0.144$) nor controls and TBI+hNSCs treated animals ($p=0.710$). **(A)** TBI animals performed worse on the NPR task in comparison to controls pre-kindling; however, hNSC treatment returned TBI injured animals to cognitive performance statistically indistinguishable from control levels (as reported previously in Haus³³). **(B)** This negative effect on the NPR task following TBI, and restoration of function following hNSC transplantation, was lost following kindling, where a one way ANOVA indicated no significant differences ($F(2,55)=1.11$, $p=0.336$) between either treatment group and controls. **(C-D)** On the EPM, no significant differences between any groups via one way ANOVA either pre-kindling ($F(2,62)=2.40$, $p=0.099$) or post-kindling ($F(2,62)=2.35$, $p=0.103$) were detected. TBI: traumatic brain injury, hNSC: human neural stem cell, EPM: elevated plus maze, ANOVA: analysis of variance, DI: discrimination index, Veh: vehicle.

Figure 5. There were no significant correlations between the number of surviving transplanted hNSC and performance on either the EPM or NPR in TBI animals. (A-D) A Pearson correlation analysis showed no significant correlations pre-kindling **(A, C)** or post-kindling **(B, D)** between surviving transplanted hNSC quantity and either the time in open arms on the EPM **(A-B)** or the discrimination index score on the NPR **(C-D)** in TBI injured animals. (EPM pre-kindling, $n=14$, Pearson $r = 0.33$, $p = 0.12$; EPM post-kindling $n=14$, Pearson $r = 0.12$, $p = 0.34$; NPR pre-kindling, $n=14$, Pearson $r = 0.25$, $p = 0.20$; NPR post-kindling, $n=13$, Pearson $r = 0.3$, $p = 0.16$, one-tailed t test). Similar correlation analysis showed no significant correlations in sham/control, with the exception on post-kindling NPR performance (see Fig. 7D). TBI: traumatic brain injury, hNSC: human neural stem cell, EPM: elevated plus maze, NPR: novel place recognition.

Fig. 6. hNSCs transplantation doesn't affect the after-discharge (AD) duration in kindled animals. (A) Representative EEG recording showing start of stimulation, start of AD, end of AD, start and end of spike series. AD duration was calculated using

LabChart software blind to treatment group. **(B)** A two-way repeated measure ANOVA shows no significant difference in duration of AD between the different groups. **(C)** Pearson correlation analysis showed no significant correlations between duration of the AD and number of surviving hNSCs (injured, Pearson $r = 0.18$, $P = 0.54$; one-tailed t test). Similarly linear regression analysis presented no significant correlations in sham (data not shown). hNSC: human neural stem cell, EEG: electroencephalogram, ANOVA: analysis of variance, Stim.: stimulation, veh: vehicle.

Figure 7. There were no significant correlations between the number of surviving transplanted hNSCs and performance on either the EPM or NPR in Sham/Control animals. (A-D) A Pearson correlation analysis showed no significant correlations pre-kindling **(A,C)** or post-kindling **(B,D)** between surviving transplanted hNSC quantity and either the time in open arms on the EPM **(A-B)** or the discrimination index score on the NPR **(C)** in uninjured animals. (EPM pre-kindling, $n=134$, Pearson $r = 0.068$, $p = 0.413$; EPM post-kindling $n=12$, Pearson $r = 0.007$, $p = 0.492$; NPR pre-kindling, $n=12$, Pearson $r = 0.20$, $p = 0.261$; NPR post-kindling, $n=11$, Pearson $r = 0.53$, $p = 0.046$, one-tailed t test). **(D)** While this last correlation is significant, due to a threshold effect on the discrimination index and lack of spread in cell survival, little can be interpreted from these results. hNSC: human neural stem cell, EPM: elevated plus maze, NPR: novel place recognition.