Title: Combining Enriched Environment, Progesterone, and Embryonic Neural Stem Cell Therapy Improves Recovery Following Brain Injury

Running Title: Combining EE, PROG, and eNSCs Improves Recovery

Authors: Evan T. Nudi, Justin Jacqmain, Kelsey Dubbs, Katalin Geeck, Garrick Salois, Madeleine A. Searles, and Jeffrey S. Smith*

Email addresses (in order): etnudi@svsu.edu, jmjacqma@svsu.edu, kadubbs@svsu.edu, kjgeeck@svsu.edu, gjsalois@svsu.edu, masearle@svsu.edu, jsmith12@svsu.edu

Affiliations:

The Brain Research Laboratory, Saginaw Valley State University, 7400 Bay Road, University Center, MI, 48710, USA

*__Correspondence:__ Jeffrey S. Smith, Ph.D., The Malcolm and Louis Field Endowed Chair in Health Sciences, Department of Health Sciences, Crystal M. Lange College of Health & Human Services, Saginaw Valley State University, 7400 Bay Road, University Center, MI 48710, USA. E-mail: jsmith12@svsu.edu

Abstract

Millions of people every year are affected by traumatic brain injury (TBI) and currently no therapies have shown efficacy in improving outcomes clinically. Recent research has suggested that enriched environments (EE), embryonic neural stem cells (eNSC), and progesterone (PROG) improve functional outcomes following TBI and furthermore, several investigators have suggested that a polytherapuetic approach may have greater efficacy than a single therapy. The purpose of the current study was to determine if varying combinations of post-injury EE, progesterone therapy, or eNSC transplantation would improve functional outcomes over just a single therapy. A controlled cortical impact was performed to create a lesion in the medial frontal

cortex. The subjects were then placed in either EE or standard environments and administered 10mg/kg progesterone or vehicle injections four hours post-injury and every 12h for 72h following the initial injection. Seven days following the surgery rats were transplanted with either eNSCs or media. Rats were then tested on the open field test, Barnes maze, Morris water maze (MWM), and rotor-rod tasks (RR). Improved functional outcomes were shown on a majority of the behavioral tasks in animals that received a combination of therapies. This effect was especially prominent with therapies that were combined with EE. Immunohistochemistry showed that the transplanted eNSCs survived, migrated, and displayed neural phenotypes. These data suggest that a poly-therapeutic approach following TBI improves functional recovery to a greater magnitude. Moreover, when poly-therapies are combined with EE the effects on recovery are enhanced leading to greater recovery of function.

Keywords:

TBI, enriched environment, progesterone, eNSC

Introduction

An estimated 1.7 million people in the United States suffer from traumatic brain injury (TBI) every year.[1] TBI is one of the leading causes of death and disability within the United States, contributing to nearly one third of injury-related deaths, and is one of the leading causes of hospitalization.[1] Every year millions of dollars are spent on the treatment of those suffering from TBI.[1] High morbidity and mortality rates that accompany TBI make it a major area of concern.[1-3] With car accidents and, most recently, the conflicts in the Middle East, TBI is becoming a more prevalent issue than it has been in the past.[1, 2, 4] One of the most common types of TBI is associated with car accidents and is usually characterized by a lesion to the medial frontal cortex.[4] This injury can produce cognitive deficits associated with learning and memory that can effect educational and employment status of those that survive.[5-7] Yet, very few therapies have been shown to be effective in the treatment of TBI when applied clinically but none have been shown to be effective.[8-12]

These same deficits can be produced in rats in a research setting using a medial frontal cortex (mFC) contusion model.[13] In a rat model, a mFC injury can produce deficits in visual-spatial learning, reversal learning, and motor functioning.[14, 15] Futhermore, the mFC is associated with sequential learning, decision making, and other executive functions.[15, 16] A controlled cortical impact model (CCI) is often utilized as a way to induce mFC TBI in a rodent model. This injury is produced using an impactor tip to damage the cortex[17, 18], which produces deficits in motor functioning[19] as well as impairing cognitive abilities.[20, 21] Though the CCI model does not produce a very diffuse injury, it does produce an easily replicable focal lesion allowing more controllability between animals.[22, 23]

One therapy that is currently being examined preclinically are enriched environments. These environments allow for social interaction and exercise which has been shown to improve functional outcome after traumatic events.[24, 25] The behavioral deficits that are accompanied by TBI have been shown to be significantly reduced in enriched environments.[24-29] Research has also shown that enriched environments can lead to improvements in the plasticity of neural networks as well as contributing to synaptogenesis within the neuron and increasing total volume of the brain.[24, 25, 29, 30] These environments are shown to increase stem cell survival, migration, and proliferation following transplantation in TBI and stroke models.[31-34] It is because of these contributions to neural plasticity, synaptogenesis, and stem cell survival, that enriched environments are used as a treatment post-TBI.[24, 25, 27, 28, 31-34]

Another therapy that is being examined preclinically is progesterone. Progesterone has been shown to have positive effects contributing to functional recovery post-TBI as well as in stroke models. Progesterone has exhibited edema reducing qualities following TBI that greater improve functional outcomes.[35-43] In addition, progesterone exhibits neuroprotective qualities and when used as a treatment leads to less apoptosis within the damaged tissue and other structures of the brain.[35-43] Progesterone has also been shown to improve functional recovery in both male and female rats.[35, 36, 44] The could mean that progesterone could be an effective treatment regardless of the differences present between sexes. Progesterone treatment has been accepted for clinical trials with the first two phases showing better that chance recovery, however the trial was terminated in phase III since data did not indicate a difference between the placebo and progesterone treatment groups.[10, 45]

Embryonic neural stem cells (eNSCs) are another potential therapeutic approach currently being examined preclinically that has been displaying efficacy in improving functional

outcomes following TBI. This treatment, however, can cause an inflammatory response which can hinder eNSC survival and differentiation.[46] Stem cell survival has been shown to be greatly impacted by the time of transplantation following injury.[47] However, when transplanted a week or more following injury eNSCs have shown increased survival rates.[47] When eNSCs do survive, they have the ability to differentiate into neurons and glia when placed into the host and may lessen many of the functional losses that occur following TBI.[48-50] Transplanted eNSCs have also been shown to improve gross-motor functioning following insult.[32, 51, 52] Embryonic neural stem cells have been shown to migrate from the transplantation site as far as 8 mm and form functional synapses in diverse regions.[53]

It has been suggested as of recent that using multiple therapies in combination can improve outcomes over using just a single therapy. In ischemic animals, a combination of enriched environments and stem cells has been shown to increase functional outcome.[54] Research has also suggested that when stem cells and enriched environments are administered concurrently there is an increased survival and greater migration of the stem cells leading to functional improvement following TBI or stroke.[32, 54] Combinational therapies using amphetamines concurrently with rehabilitation have shown to result in better functional outcomes after TBI.[55] Furthermore, enriched environments have been shown to increase the efficacy of drug treatments when these therapies are conducted concurrently.[56, 57]

The goal of the current study is to assess the effects of a combinational approach to treating TBI. Progesterone, eNSCs and enriched environments will be employed concurrently and in varying combinations as a therapy post-TBI. With progesterone, eNSC, and EE affecting different aspects of TBI, a combination of the three could lead to greater recovery of functions after TBI.

Methods

Subjects

Subjects consisted of 80 male Long-Evans rats (Charles River, Portage, Michigan) received at post-natal day 90 and weighing an average of 275-300 grams. Animals were kept on a 12:12 hour reverse day/night cycle. Rats were handled for 5 mins each day in order to familiarize them with human contact before behavioral testing. All subjects were given access to food and water *ad libitum.* Animals were handled and cared for under the guidelines set by the Institutional Animal Care and Use Committee of Saginaw Valley State University.

Surgical procedure

Al surgeries were conducted under aseptic surgical conditions. Each animals scalp was shaved and betadine was applied prior to surgery. Subjects were anesthetized using a 5% isoflurane/O_2 mixture for induction. The animals were then placed in a stereotaxic device (Kopf, Tujunga, California) and a 1-3% isoflurane/O_2 mixture was administered via a nose cone for maintenance. An incision was made along the midline of the scalp and the tissue was held apart using forceps to allow for ease of access during surgery. A 6.0mm craniotomy was made on the midline of the cranium over the prefrontal cortex 3.0mm anterior to bregma. Once the craniotomy is made, the plate of the skull was removed and discarded. A pneumatic piston (Kopf, Tujunga, California) with a 5.0mm tip was placed over the craniotomy and the frontal cortex was impacted 2.0mm at a speed of 2.25m/s for the duration of 0.5s. After the injury, the incision was closed using surgical staples and the subjects were moved to recovery cages with heating pads in order to maintain normal body temperature. Sham animals were anesthetized using 5% isoflurane/O_2 mixture, mounted in a stereotaxic device with a 1-3% isoflurane/O_2 mixture for maintenance and an incision was made along the midline of the scalp. After the

incision was made, it was immediately closed using surgical staples and the subject was placed in a recovery cage. Following surgery, rats were randomly assigned to treatment groups which were Sham/EE (uninjured and assigned to enriched environments, *n* = 8), Sham/SE (uninjured and assigned to standard environments, *n* = 8), TBI/EE/PROG (injury group, assigned to enriched environments, and received progesterone, *n* = 8), TBI/SE/PROG (injury group assigned to standard environments and received progesterone, *n* = 8), TBI/EE/VEH (injury group, assigned to standard environments and received vehicle, *n* = 8), TBI/SE/VEH (injury group, assigned to standard environments, and received vehicle, *n* = 8), TBI/eNSC/EE/PROG (injury group, received embryonic neural stem cell transplantation, assigned to enriched environments, and received progesterone, *n* = 8), TBI/eNSC/SE/PROG (injury group, received embryonic neural stem cell transplantation, assigned to standard environments, and received progesterone, *n* = 8), TBI/eNSC/EE/VEH (injury group, received embryonic neural stem cell transplantation, assigned to enriched environments, and received vehicle, *n* = 8), and TBI/eNSC/SE/VEH (injury group, received embryonic neural stem cell transplantation, assigned to standard environments, and received vehicle, *n* = 8).

Enriched environments

The subjects were either assigned to standard laboratory environments (SE) or enriched environments (EE) following injury or sham preparation. SE consisted of a standard plastic cage (26.0cm(w), 47.0 cm(d), and 20.3cm(h), Alternative Design, Siloam Springs, Arkansas) with food and water given *ad libitum*. One animal was housed per cage in the standard environments. The EE consisted of a 116.0cm(w), 69.0cm(d), and 22.0cm(h) drawer (Freedom Breeders, Turlock, California) with food and water given *ad libitum*. The enriched environments housed six subjects and were supplemented with igloo housing, chew blocks, tunnels, animal toys, and

climbing apparatus. There were 14 of these environmental stimuli, which were adjusted daily and changed twice a week.

Progesterone administration

A dose of 10 mg/kg, i.p. was selected for use during this study.[58] Progesterone (P3972, Sigma-Aldrich, St. Louis, Missouri) was diluted in a vehicle of Lou Ana® peanut oil (Ventura Foods, LLC, Brea, California). Animals in the treatment group (PROG) received the progesterone solution while animals in the vehicle group received the peanut oil alone. Subjects were administered an initial injection of progesterone or the vehicle 4h post-CCI and every 12h for 72h after the initial injection.[58]

Neurosphere dissociation

Embryonic neural stem cells (eNSC) arrived as mouse cortical neurospheres (Stem Cell Technologies, Vancouver, British Columbia, Canada). Neurosphere dissociation was performed using a chemical dissociation technique (per package instructions; NeuroCult Chemical Dissociation Kit (Mouse), Catalog# 05707, Stem Cell Technologies, Vancouver, British Columbia, Canada) that provided an increased rate of survival as compared to traditional dissociation techniques. First, the neurospheres were centrifuged at 400rpm for five minutes and as much supernatant was removed as possible. Then, 0.50ml of Neurocult chemical dissociation solution A was added. Using a micropipette the neurospheres were then gently resuspended. After eight minutes, 0.125mL of Neurocult chemical dissociation solution B was added. The solution was gently agitated at three and seven minutes with a micropipette following the addition of Neurocult chemical dissociation solution B. At eight minutes 0.04mL of Neurocult chemical dissociation solution C was added and then mixed using a micropipette. Finally, eNSCs were suspended in NeuroCult™ Basal Medium (Mouse, STEMCELL Technologies, inc.,

Vancouver, British Columbia, Canada). The stem cell concentration and viability was determined with a hemacytometer from a small sample of the eNSCs suspension stained with Trypan blue.

Stem cell transplantation

Embryonic stem cell transplantation occurred seven days following the first surgical procedure. The animals were prepped and anesthetized prior to transplantation. Sham animals were placed in the stereotaxic device (KOPF, Tujunga, California) and had their incisions reopened. The incisions were then immediately closed and the sham animals were removed from the stereotaxic device and placed in a recovery cage. Animals that had received a contusion were anesthetized, had their incisions reopened and then received injections of either eNSCs or media. A 10-μL Hamilton syringe and a microdrive (Stoelting Instruments, Wood Dale, Illinois) attached to the stereotaxic device was used for the delivery of the eNSCs or the media. The stem cell transplantation was done through the pre-existing craniotomies.[59] A syringe was loaded with the eNSCs (20K/ μL) or media (NeuroCult™ Basal Medium (mouse, STEMCELL Technologies, inc, Vancouver, British Columbia, Canada) (two separate syringes were used to prevent cross contamination) and then the tip of the syringe was centered over bregma. The syringe was moved to two locations (A/P +1.0, M/L ±1.5, D/V-2.0) and 2.5 μL of eNSCs (~100K eNSCs) or 2.5 μL of media was deposited in the periphery of the injured cortex in each of the two locations. The eNSCs or media were deposited at a rate of 0.5 μL/min and the needle remained in the tissue in place for two minutes before being raised slowly. Following the transplantation, the incisions were closed as previously mentioned in the surgical methods and the animals were placed in a recovery cage.

Behavioral testing

Open field test

The open field test was conducted on days 1, 3 and 22 post-stem cell transplantation and was used as an assessment of activity following brain injury. Subjects were tested in 10min trials once per day on these days. At the beginning of the trial, Animals were placed in the center of a 40.64cm(w) x 40.64cm(d) x 38.1cm(h) clear Plexiglas box (San Diego Instruments, San Diego, California) facing away from the center of the room. Each animal was given 10mins to explore within the box before they were removed. A total of five animals were placed in five separate boxes with a delay of approximately 10secs between each one. Using the PAS Open Field software (San Diego Instruments, San Diego, California), the mean distance traversed and mean number of rears were measured.

Barnes maze

The Barnes maze (Kinder Scientific, Poway, California) was conducted on days 2 through 7 post-stem cell transplantation [60]. The Barnes maze consists of a single, white circular platform 122cm in diameter situated on a stand that is 95cm off the ground. Around the edge of the circular platform are 18 evenly spaced black circles (9.5cm in diameter). An escape box measuring 10.5cm wide, 20.5cm long, and 9cm deep was put in place of one of the black circles with a small cup with 5ml of chocolate milk (TruMoo®, El Paso, Texas), used as a reward for finding the box, placed in the corner opposite the entrance. The Barnes maze uses rat's natural aversion to bright light to motivate them into finding the escape box.[60] Two trials were conducted each day over the course of six days. A ceiling of four minutes was implemented for each trial. The first trial on day one was the acquisition trial. Animals were placed directly into the escape box and remained there for the full four minute duration. During the acquisition trial 5ml of chocolate milk was placed in the escape box at the one minute mark. For every trial

following the acquisition trial, each animal was placed in the center of the maze in the starting cylinder (16.5cm diameter, 24cm height) where they remained for 30secs. Following the 30secs, the starting cylinder is lifted straight up, beginning the trial. Latency was recorded when the subject was completely in the escape box or if the four minute ceiling was reached. On trials 1 through 7 the escape remained in the same position and on trials 8 through 11 the escape box was rotated ~135 °and the subjects had to relocate it. Latency was recorded using a stopwatch and the trial was terminated when the animal was completely in the escape box (both hind paws in the escape box) or the ceiling of four minutes was reached. If the subject failed to locate the escape box, they were guided into it. All subjects stayed in the box for one minute to allow them to consume the reinforcer. Following that one minute, the escape box was removed with the subject still inside and was transported to that subject's home cage.

Morris water maze

Following the Barnes maze, the rats underwent the Morris water maze (MWM) on days 8 through 17 post-stem cell transplantation.[61] A circular fiberglass tank (180.0cm diameter, 75.0cm height) was filled with water (32.5cm deep, 21 °±2 ℃). The water was made opaque using two bottles of white non-toxic tempura paint. A clear platform was submerged 2cm under the water in the northwest quadrant of the MWM. The MWM was conducted over the course of 10 days with two trials per day. Animals were placed facing the wall at either the north, south, east, or west sides at the beginning of each trial. Entry locations were randomly assigned in order to keep consistency between groups and switched for each trial. Subjects were given 90secs to locate the escape platform. If subjects were unable to locate the escape platform within the 90secs allotted, they were guided to it. Each subject was given 30secs on the escape platform once it was reached before being placed in a heated cage. A 15min inter trial interval (ITI) was given to each subject.

Using ANY-maze (San Diego Instruments, San Diego, California) computer software, data on latency, path length, and pattern were recorded.

Rotor-rod

The Rotor-rod (San Diego Instruments, San Diego, California) was conducted over the course of five days following the MWM as an assessment of gross locomotor performance on days 18-22 post-stem cell transplantation.[62] The rotor rod consists of an elevated rotating cylinder that gradually accelerates from 0-30 rpm over the course of one minute. The 7.0cm(d) cylinder is located 1m above a foam pad, which was used to cushion the rats when they fell. Animals are placed facing the back of the rotor rod as the cylinder begins to rotate. The latency was recorded until the trial was terminated. The trial was terminated after the rat broke the infrared beam by falling, or after the ceiling of 180secs was reached. The subjects were given four trials per day over the course of five days with an ITI of approximately 10mins.

Histology

On day 24 post stem cell transplantation, the animals were anesthetized using Euthasol® and a continuous dose of a 5% isoflorane and were transcardially perfused using 500ml 10% phosphate buffered solution (PBS) followed by 10% formalin solution. Brains were extracted and sectioned in half at the longitudinal fissure. The left hemispheres were placed in formalin (25ml) overnight. On the following day, the formalin was drained and tissue was then placed in 25ml of 10% PBS solution until immunofluorscence labeling and optical clearing was conducted. Further processing of the right hemispheres was conducted following extraction using the Tissue-Tek vacuum infiltration processor (IMEB Inc., San Marco, California, USA) and were then embedded in paraffin wax using a tissue embedding console system (Miles Scientific, Fergus Falls, Minnesota, USA). Using a rotary microtome the tissue was sectioned on the sagittal

plane at a thickness of 30 µm. The tissue was mounted using Tru-Bond 380 adhesive slides (Tru Scientific, Bellingham, Washington).

Hematoxylin and eosin staining

A hematoxylin and eosin staining procedure was chosen in order to stain cell bodies allowing for stereological analysis. Slides were put through a sequence of washes to remove paraffin, rehydrate, stain, dehydrate and coverslip. For paraffin removal, the slides were placed in xylene (3 x 5 min), then followed by 100% EtOH (2 x 5 min), then 95% EtOH (2 x 5 min), then 70% EtOH (1 x 5 min), followed by distilled water (1 x 5 min) to rehydrate. Staining consisted of hematoxylin (1 x 2 min), rinsed with distilled water until clear, a brief immersion in bluing solution (1 x 5 s), a distilled water rinse (1 x 5 min), then 70% EtOH (1 x 5 min), and finally eosin (1 x 1 min). Slides were then dehydratedin 70% EtOH (2 x 5 min) and cleared in xylene (3 x 3 min). Following the clearing slides were coverslipped to be used for light microscopy.

Stereological analysis

Five sections each at 30 µm thick were selected from the right hemisphere for lesion analysis and cell counts. All analysis was done using the Olympus BX61 light microscope with the Visiopharm Deployed Stereology software (Visiopharm, Broomfield, Colorado) utilizing the Newcast module. Stereological analysis was conducted to measure volume of the remaining cortex and estimate cell counts within the CA1, CA2 and CA3 regions of the hippocampus. Five depths (+0.3mm, +0.6mm, +0.9mm, +1.2mm, and +1.5mm) were chosen for use during stereological analysis. Images were acquired of one slice on each of the five slides with a 1.25x magnification lens and two regions of interest (ROI) were selected on each brain (the cortex and CA1, CA2, CA3 of the hippocampus). Cortical volume was assessed with the Cavalieri estimator

using a point grid and sampling 100% of the cortex at a 10x magnification.[63] The starting point was selected at random by the computer and points were chosen if the fell within the ROI and on the tissue. The software calculated the total volume (V) estimate of the cortex = $T\frac{a}{p}\sum P$, where *T* is the distance between sections, $\frac{a}{p}$ is the area per point, and $\sum P$ is the summation of the selected points on the grid.

Cell counts were conducted using the optical disector method of total number estimation using the global probe and sampling 5% of the CA1, CA2, and CA3 regions of the hippocampus using a 100x magnification oil iris lens. Neurons were marked only if they fell within the counting probe and were in the selected ROI. The guard heights were established by determining the upper and lower bounds of each of the sampling areas with a guard height of 2.5μm on both the upper and lower bounds of the tissue. The computer randomly selected a starting point and neurons that fell within the counting frame were excluded if they fell outside of the ROI, outside of the counting frame, or were on the exclusion line. The volume weighted number density (*Nv*) was calculated by the software = $\frac{1}{af\ hd} \bullet \frac{\sum Q-}{\sum Pf}$, where *af* is the area of the sampling field, *hd* is the disector height established before counting, $\sum Q -$ is the total number of neurons counted, and $\sum Pf$ is the number of counting frames sampled (denoted by the corner points marked on counting frames).

Immunofluorescence and SeeDB

The left hemispheres were labeled using immunofluorescent labels and optically cleared for analysis on the Fluoview FV-10i (Olympus, Tokyo, Japan) confocal microscope. Tissue was labeled with anti-NeuN (Anti-Rabbit, 1:100; Abcam, Cambridge, Massachusetts, USA) and M2/M6 (Anti-Rat, Supernatant, Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, Iowa, USA) labels in order to locate differentiated stem cells.[32] The tissue was

first sliced into three 500 μm thick sections using the Vibratome 1000 semi-automatic tissue sectioning system. The tissue was then placed on a 25 x 25mm no.1 gold seal cover glass and placed in a tissue cassette. A dehydration process than occurred in increasing concentrations of EtOH solution in the Tissue-Tek vacuum infiltration processor (IMEB, inc., San Marcos, California). The brain slices were then placed in conical tubes and placed in the -80 refrigeration system. A freeze-thaw method was implemented with the tissue being kept in the -80 refrigeration system for one hour and then removed and kept at room temperature for one hour over the course of four cycles.[64] Rehydration occurred in the Tissue-Tek vacuum infiltration processor following the freeze-thaw cycle. When the rehydration was completed the tissue was placed in a 50ml conical tube completely filled with a 10% PBS (IMEB, inc., San Marcos, California) and 1% Triton X-100 (VWR, Radnor, Pennsylvania); PBS-T solution and placed on a rotator (VWR, Radnor, Pennsylvania) for 12h. Following the PBS-T wash, tissue was placed in a 12-well plate with 2.5 ml of DaVinci Green (Biocare Medical, Concord, California)+1% Triton X-100 solution and incubated at 37˚C for 24h. The tissue was then washed in PBS-T for 15mins and placed back in the 12-well plate with Rodent Block and incubated for 24h. Following the Rodent Block Stage, the tissue was washed in PBS-T in conical tubes for 15mins and placed back in a 12-well plate filled with a 2.5 ml solution of M2/M6 (Supernatant, Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, Iowa) and incubated for 24 hours. Following the incubation period in M2/M6 solution another 15min PBS-T wash occurred and the tissue was then placed back in the 12-well plate filled with a 2.5 ml solution of DaVinci Green (Biocare Medical, Concord, California) and anti-NeuN (Anti-Rabbit, 1:100; Abcam, Cambridge, Massachusetts, USA) and was incubated at 37˚C for 24h. Following the primary antibody incubation the tissue was rinsed three times in PBS-T solution for 30mins each then placed back

in a 12-well plate in a 2.5 ml solution of Fluorescence Antibody Diluent (Biocare Medical, Concord, California), Alexafluor 594 (1:200 Donkey Anti-Rabbit; Life Technologies, Grand Island, New York, USA), and Alexafluor 350 (1:200 Goat Anti-Rat; Life Technologies, Grand Island, New York, USA) and incubated for 24h. Tissue was then washed in 10% PBS three times for 30mins each time and prepared for the SeeDB process.

The left hemispheres of each brain were then put through an optical clearing method known as SeeDB.[65] The SeeDB optical clearing procedure consists of increasing concentrations of D-Fructose solution (Sigma-Aldrich, St. Louis, Missouri) in which the hemisphere will rotate in a 60ml conical tube for an allotted amount of time (8h each for 20% SeeDB, 40% SeeDB, 60% SeeDB, 12h each for 80% SeeDB and 100% SeeDB and 24h for the SeeDB solution).[65] Sixty mililiters of each solution was prepared in advance for each conical tube. These solutions are 20% D-Fructose+10%PBS weight to volume (20% SeeDB), 40% D-Fructose+10% PBS weight to volume (40%SeeDB), 60% D-Fructose+10% PBS weight to volume (60% SeeDB), 80% D-Fructose+10% PBS weight to volume (80% SeeDB), 100% D-Fructose+DI water weight to volume (100% SeeDB), and the final solution consists of 60.75g of D-Fructose dissolved in 15ml of DI water (SeeDB). Each solution was placed in an oven at 60˚C and stirred occasionally until completely dissolved. The SeeDB solution were then removed from the oven and allowed to reach room temperature. Upon reaching room temperature, 300µl of α-thioglycerol (Sigma-Aldrich, St. Louis, Missouri, USA) was added into each 60ml SeeDB solution to prevent browning due to the Maillard reaction. These solutions were then incubated at 37˚C for the remainder of their use. The hemispheres were rotated in each SeeDB solution for an allotted amount of time (8h each for 20% SeeDB, 40% SeeDB, 60% SeeDB, 12h each for 80% SeeDB and 100% SeeDB and 24h for the SeeDB solution) in the incubator at 37˚C. Upon completion of

the 24h rotation, imaging of the tissue was conducted immediately following the optical clearing using the Olympus Fluoview FV10i confocal microscope. The hippocampus, penumbra, and motor cortex were designated as regions of interest in the location of eNSCs. In each of those regions an comprehensive search was implemented to locate cells that expressed both M2/M6 and NeuN. Once these cells were located a 220x220 μm image of that area was captured at 60x magnification.

Statistical Analysis

The appropriate analysis of variance (ANOVA) tests were performed using the procedures for general linear models (SPSS 20 for Windows) with options for repeated measures where necessary. The between-subject factor consisted of the group assignments and the within-subjects factor consisted of the day of testing for each behavioral task. *Post-hoc* analyses were conducted using Fisher's LSD.

Results

Open field test

Total distance traversed

The average distance traversed was analyzed using a repeated measures analysis of variance (RM-ANOVA). A significant effect was shown across days ($F_{2, 140}$=13.597, p<.001, Fig. 1A) but no interaction was shown in the Day x Group data ($F_{18, 140}$=.670, p=.836, Fig. 1A) on average distance traversed. Since there was significant effect found across days the data was further collapsed to assess between-subjects differences ($F_{9, 70}$=5.615, p<.001, Fig. 1B). Due to the significant difference between-subjects, Fisher's LSD *post-hoc* was used to compare the performance from the various groups. Uninjured animals in enriched environments traversed less distance within the open field arena than the animals assigned to the Sham/SE (p=.016),

TBI/SE/PROG (p<.001), TBI/SE/VEH (p=.011) groups and all groups that received eNSC therapy (p's<.001). The Sham/SE group traversed less distance than the TBI/eNSC/SE/PROG (p=.023) and TBI/eNSC/SE/VEH (p=.006) groups. All eNSC groups (TBI/eNSC/EE/PROG (p=.013), TBI/eNSC/SE/PROG (p<.001), TBI/eNSC/EE/VEH (p=.004), and TBI/eNSC/SE/VEH (p=.001)) as well as the TBI/SE/PROG (p=.009) group traversed less distance than the TBI/EE/PROG group within the open field arena. Rats that were assigned to the TBI/EE/VEH group ambulated less than the TBI/SE/PROG (p=.042), TBI/eNSC/SE/PROG (p=.001), TBI/eNSC/EE/VEH (p=.023), and TBI/eNSC/SE/VEH (p=.006) groups. Animals that received a TBI but no additional therapies (TBI/SE/VEH) ambulated significantly less than animals that were assigned to the TBI/eNSC/SE/PROG (p=.009) and TBI/eNSC/SE/VEH (p=.033) groups.

Rearing

The average number of rears was analyzed using a repeated measures analysis of variance (RM-ANOVA). A significant effect was also shown in the data for average number of rears when compared across days ($F_{2, 140}$=3.620, p=.029, Fig. 1C) however, no interaction effect was seen in the Day x Group data ($F_{18, 140}$=1.339, p=.173, Fig. 1B). Since there was a significant effect found across days the data was further collapsed to assess between-subjects effects ($F_{9, 70}$=3.314, p=.002, Fig. 1D). Due to the significant effect found between-subjects the data was further collapsed and Fisher's LSD *post-hoc* test was used to compare differences between groups. The data for rearing behavior shows that sham animals housed in EE displayed significantly more rearing behavior than animals in the TBI/SE/VEH (p=.013), TBI/eNSC/SE/PROG (p=.05) and TBI/eNSC/EE/VEH (p=.011) groups. Animals in the Sham/SE and TBI/EE/PROG groups reared more than animals in the TBI/SE/VEH, TBI/eNSC/SE/PROG, TBI/eNSC/EE/VEH, and TBI/eNSC/SE/VEH groups (p's<.05). The

Sham/SE group also exhibited more rearing behavior than the TBI/SE/PROG (p=.011). Injured animals that received no treatment reared on average less than the animals in the TBI/EE/VEH (p=.046) and TBI/eNSC/EE/PROG (p=.034) groups. Injured animals in EE that received no additional treatments also reared significantly more than the TBI/eNSC/EE/VEH group (p=.039). Lastly, injured animals that received all three treatments reared more on average than the animals in the TBI/eNSC/EE/VEH group (p=.029).

Barnes maze

Pre-box position change

A RM-ANOVA was performed to analyze latency to find the escape box across days and groups. A significant effect was shown in latency pre-box position change both by day ($F_{3, 210}$=24.603, p<.001, Fig. 2A) and there was a significant interaction in the Day x Group data ($F_{27, 210}$=1.647, Fig. 2A). A significant effect was shown between-subjects on the Barnes maze pre-box change ($F_{9, 70}$=6.157, p<.001, Fig. 2B). Data was collapsed and analyzed further using Fisher's LSD *post-hoc* was used to compare the performance across the various groups. Uninjured animals in enriched environments were able to locate the escape box with lower latencies than most of the injury groups excluding the TBI/EE/VEH group (TBI/EE/PROG (p=.009), TBI/SE/PROG (p<.001), TBI/SE/VEH (p<.001), TBI/eNSC/EE/PROG (p=.023), TBI/eNSC/SE/PROG(p=.001), TBI/eNSC/EE/VEH(p=.002) , and TBI/eNSC/SE/VEH (p=.004)). There was no significant difference between the uninjured animals in standard environments and the injury groups assigned to enriched environments with the exclusion of the TBI/eNSC/EE/VEH group (*p's*>.05). The TBI/SE/PROG group was out performed by the TBI/EE/PROG (p=.008), TBI/EE/VEH (p=.001), TBI/eNSC/EE/PROG (p=.003), TBI/eNSC/SE/PROG (p=.050), TBI/eNSC/EE/VEH (p=.027), and TBI/eNSC/SE/VEH (p=.016)

groups. Injured animals that received no treatments were, also, outperformed by the TBI/EE/PROG (p=.006), TBI/EE/VEH (p<.001), TBI/eNSC/EE/PROG (p=.002), TBI/eNSC/SE/PROG (p=.041), TBI/eNSC/EE/VEH (p=.021), and TBI/eNSC/SE/VEH (p=.012) groups.

Post-box position change

A RM-ANOVA was performed to analyze latency to find the escape box across days and groups. A significant effect was shown in latency post-box position change by day ($F_{1, 70}$=10.156, p=.002, Fig. 2A) and an interaction effect was shown in the Day x Group data ($F_{9, 70}$=2.286, p=.026, Fig. 2A). Significant effects were shown between-subjects post-box change ($F_{9, 70}$=3.105, p=.003, Fig. 2C). Fisher's LSD *post-hoc* was used to compare the performance across the various groups. Post-box change injured animals that received no treatments displayed much higher latencies on than all other groups within the study (p's<.05). The Sham/EE (p=.042) and TBI/eNSC/EE/PROG (p=.041) groups outperformed the TBI/eNSC/SE/PROG group.

Morris Water Maze

The latency to find the escape platform across days and groups was analyzed using a RM-ANOVA. A significant effect was shown when comparing latency performance across days ($F_{81, 630}$=3.393, p<.001, Fig. 3A) as well as an interaction effect being shown in the Day x Group data ($F_{9, 630}$=112.595, p<.001, Fig. 3A). When between-subjects performance was assessed and a significant effect was shown ($F_{9, 70}$=112.595, p<.001, Fig. 3B). Fisher's LSD *Post-hoc* analysis was used to compare the performance from the various groups due to the significant difference between-subjects. The uninjured animals in enriched environments showed a significantly better performance when compared to all injury groups excluding the TBI/EE/PROG group

(TBI/SE/PROG (p<.001), TBI/EE/VEH (p<.001), TBI/SE/VEH (p<.001), TBI/eNSC/EE/PROG (p=.017), TBI/eNSC/SE/PROG (p<.001), TBI/eNSC/EE/VEH (p=.003) , and TBI/eNSC/SE/VEH (p<.001)). The Sham/SE group also showed a significantly better performance than the TBI/SE/PROG (p<.001), TBI/EE/VEH (p=.001), TBI/SE/VEH (p<.001), TBI/eNSC/EE/PROG (p=.043), TBI/eNSC/SE/PROG (p=.001), TBI/eNSC/EE/VEH (p=.007) , and TBI/eNSC/SE/VEH (p<.001) group. The TBI/EE/PROG group displayed improved performances over the TBI/SE/PROG (p=.001), TBI/EE/VEH (p=.016), TBI/SE/VEH (p<.001), TBI/eNSC/SE/PROG (p=.024), and TBI/eNSC/SE/VEH (p<.001) groups. The TBI/eNSC/EE/PROG (p=.008) and TBI/eNSC/EE/VEH (p=.046) outperformed the TBI/SE/PROG on latency to reach the escape platform. The injured animals that did not receive any treatment performed worse on this task than the animals in the TBI/EE/VEH (p=.007), TBI/eNSC/EE/PROG (p<.001), TBI/eNSC/SE/PROG (p=.004), and the TBI/eNSC/EE/VEH (p<.001) groups. The injured animals that only received eNSC thearpy displayed an impaired performance when compared to the TBI/eNSC/EE/PROG (p=.003) and TBI/eNSC/EE/VEH (p=.017) groups.

Rotor-rod

A RM-ANOVA was performed to analyze latency to fall off the cylinder across days and groups. A significant effect was found when comparing latency performance across the days variables ($F_{36, 280}$=39.385, p<.001, Fig. 4A) as well as an interaction effect being shown in the Day x Group data ($F_{4, 280}$=2.889, p<.001, Fig. 4A). A between-subjects effect was also shown on the Rotor-rod ($F_{9,70}$=7.490, p<.001, Fig. 4B). Since there was significance between-subjects a Fisher's LSD *post-hoc* was analyzed to compare differences performance by the various groups. Uninjured animals assigned to enriched environments outperformed all the groups that were

assigned to standard environments (Sham/SE (p<.001), TBI/SE/PROG (p=.001), TBI/SE/VEH (p<.001), TBI/eNSC/SE/PROG (p<.001), and TBI/eNSC/SE/VEH (p=.003)). The TBI/EE/PROG group outperformed the Sham/SE (p=.031), TBI/SE/PROG (p=.045), TBI/SE/VEH (p<.001), and TBI/eNSC/SE/PROG (p=.014) groups. The TBI/EE/VEH group exhibited an improved motor function when compared to the Sham/SE (p=.040), TBI/SE/VEH (p<.001), and TBI/eNSC/SE/PROG (p=.019) groups. Greater motor functioning was shown by the animals that received all three treatments when compared to all groups that were assigned to standard environments (Sham/SE (p=.001), TBI/SE/PROG (p=.002), TBI/SE/VEH (p<.001), TBI/eNSC/SE/PROG (p<.001), and TBI/eNSC/SE/VEH (p=.010)). Injured animals that were assigned to enriched environments and received eNSC therapy without progesterone showed improved performance over all groups that were assigned to standard environments (Sham/SE (p=.001), TBI/SE/PROG (p=.002), TBI/SE/VEH (p<.001), TBI/eNSC/SE/PROG (p<.001), and TBI/eNSC/SE/VEH (p=.008)). Conversely, the TBI/SE/VEH group was outperformed by Sham/SE (p=.040), TBI/SE/PROG (p=.027), and TBI/eNSC/SE/VEH (p=.007) groups.

Cortical volume estimates

A one-way RM-ANOVA was conducted to analyze estimates of cortical volume between groups. There was a significant effect when comparing between-subjects measures of cortical volume ($F_{9, 79}$=19.984, p<.001, Fig. 5A). Data for cortical volume were analyzed using Fisher's LSD *post-hoc* for multiple comparisons. A lesion effect was shown in cortical volume with the animals in the sham groups displaying greater cortical volume than the animals in the injury groups ($p's$<.001). Uninjured animals assigned to enriched environments also displayed greater cortical volume than uninjured animals assigned to standard environments (p=.044). Injured animals that received all treatments (TBI/eNSC/EE/PROG, p=.017) and injured animals that

received eNSC therapy and were assigned to enriched environments (TBI/eNSC/EE/VEH, p=.020) showed a significantly greater cortical volume than animals in the injured animals that received progesterone and were assigned to enriched environments. Injured animals that were assigned to standard environments and only received progesterone had significantly less cortical volume than the TBI/EE/VEH (p=.041), TBI/eNSC/EE/PROG (p=.001), TBI/eNSC/SE/PROG (p=.043), TBI/eNSC/EE/VEH (p=.001), and TBI/eNSC/SE/VEH (p=.046) groups. Injured animals that received all three treatments (p=.008) and injured animals that were assigned to enriched environments and received eNSC therapy with vehicle injections (p=.009) groups showed a significantly greater cortical volume then animals in the injury group that received no additional treatments.

Hippocampus total neuron estimates

Total cell estimates of the CA1, CA2 and CA3 regions of the hippocampus were performed using a one-way RM-ANOVA. A significant effect was found between groups on this measure ($F_{9, 79}$=2.056, p=.046, Fig. 5B) and this effect was further decomposed using Fisher's LSD *post-hoc*. Uninjured animals that were assigned to enriched environments displayed a greater average neuronal count than injured animals assigned to standard environments with the exclusion of the TBI/eNSC/SE/PROG group (TBI/SE/PROG (p=.017), TBI/SE/VEH (p=.003), and TBI/eNSC/SE/VEH (p=.014)). Injured animals that received no additional treatments had a marked decrease in the average number of neurons when compared to injured animals that were assigned to enriched environments and only received progesterone (p=.035) and injured animals that received all three treatments (p=.003). Injured animals that received all three treatments displayed a greater average neuron estimate than the TBI/SE/PROG (p=.021) and TBI/eNSC/SE/VEH (p=.018) groups.

eNSC transplant survival

Immunofluorscence labeling techniques using NeuN and M2/M6 (shown in Fig. 6) were implemented in determining stem cell migration and survival. Images of the penumbra region and transplantation site were captured via confocal microscopy. Hippocampal regions (CA field horn and polymorph layer) were also captured as the average number of neurons assessed previously suggested possible changes in the hippocampus. NeuN and M2/M6 positive cells were found within the penumbra region of the TBI however, labeling in this region was sparse. Co-labeling of M2/M6 and NeuN was also present within the polymorph layer of the dentate gyrus as well as within the horn of the CA field approximately 2mm away from the transplantation site. These cells also displayed the morphology of mature neurons with multiple processes extending out from the neuron cell body. This suggests that eNSCs were able to survive, migrate and differentiate into various regions of the brain following transplantation.

Discussion

Currently there are very few treatments that show efficacy preclinically and none have shown efficacy during clinical trials.[8, 12, 66-70] However, these studies often focus on a single treatment and its limited effects on recovery. The current data supports the fact that a polycombinational therapy might show greater efficacy on recovery following traumatic brain injury. There have been examples from different research groups that have shown the efficacy of a combinational approach to therapeutic treatments following TBI.[31, 32, 55-57, 71] These research groups were able to demonstrate increased effectiveness of treatments following TBI when applied in a combinational manner, showing that one treatment could produce some functional recovery but a combination produced greater recovery.[31, 32, 55-57, 71] The current data supports

previous findings that when therapies are applied in a combinational approach they exhibit a greater effect on recovery.

The analysis of the behavioral data revealed that visual-spatial learning and reference memory was improved when a combination of treatments were employed.[32, 56, 57] The data suggests that EE was able to enhance the effects of the combination of progesterone and embryonic neural stem cells on recovery of reversal learning which was shown by the post-box change Barnes maze data. Improvements in visual spatial learning and reference memory of the groups that received the full combination of therapies of a dual combination with EE were elucidated by both the Barnes maze and Morris water maze. This is an effect that has been also been shown by several research groups showing that a combinational treatment leads to a greater recovery or reference memory and learning where a combination of therapies improved recovery over just a single therapy.[32, 54, 56, 57] Other research groups have suggested that progesterone and enriched environment therapies, when administered separately, have been shown to improve cognitive function and our data illustrates that when they are given concurrently there effects are amplified.[28, 29, 40] Exercise alone has been shown to improve cognitive functioning following TBI and could explain the performance of the TBI/EE/VEH group pre-box change.[72, 73] The data clearly suggests that a poly-therapy can produce greater functional recovery than a single therapy in terms of cognitive functioning.

The data also suggest a strong environmental effect on recovery of gross motor function with enriched environments restoring function equivalent to sham levels. Two major components of the enriched environments are exercise and social aspect. When these are combined, they have been shown to improve motor functioning over just a single therapy given at any one time.[26] Progesterone and eNSC therapies when given without EE leads to recovery of function to that of

sham animals in SE conditions which is consistent with previous research conducted into these therapies.[31, 32, 36, 38, 40, 47, 52, 59] However, the data suggests that when animals are housed in enriched environments the effects of the other therapies go mostly unnoticed. This environmental effect also does not appear to be an effect of the SE animals being less active. As the data suggested in the OFT, animals in SE were more active than their EE counterparts.

It is interesting to note that animals that received all three treatments displayed close to sham level performances on the earlier behavioral measures. However, on the later behavioral measures, such as the MWM and the rotor rod, performances from the dual therapy groups or mono therapy groups approached the levels of those animals that had received the full combination of treatments. This could potentially suggest that if behavioral testing were to be conducted 3 or 4 months following injury, recovery might stabilize at around the same levels regardless of treatment group. If this is the case, in order to determine the actual effectiveness of pre-clinical therapies, additional behavioral testing needs to be performed so that later time points are included. These additional testing days/weeks should be sufficient to determine if recovery plateaus regardless of the therapeutic approach. This type of evaluation would also be more analogous to what is performed clinically where the participants recovery is assessed for a much longer period of time and over the course of several months post-injury.[10, 66, 69, 70]

The findings of the current study suggests that a poly-therapeutic approach can lead to less tissue necrosis and neuronal cell loss or recovery. Enriched environments [74, 75] and progesterone therapy [36, 40, 58] have been shown to lead to less tissue necrosis and neuronal cell loss and recovery when administered separately following TBI. When these treatments and eNSCs are combined with EE it was found in the current study to increase neuronal counts in the hippocampus. It has been shown that when other treatments are combined with EE a synergistic

effect is produced and greater number of neuronal counts have been shown following TBI.[56, 57] These effects appear to be translatable with differing therapies and their combinations with EE as shown in the current the study.

Additionally, the data from this investigation suggests that eNSCs were able to survive, migrate, and differentiate following transplantation. These effects seem to be amplified with the addition of enriched environments, which have been shown to increase eNSC viability.[32] Enriched environments have been shown to increase NGF levels within the brain and this increase in NGF could lead to an increased viability following transplantation.[76] NGF has also been suggested to regulate the proliferation and differentiation of eNSCs and the increased levels of NGF in EE could have been a factor in eNSC survival.[34] It has been suggested that eNSC viability is greatly impacted by the inflammatory response following transplantation.[46] Progesterone's anti-inflammatory qualities may have caused this increased cell viability though additional research needs to be conducted in that area.[26, 29, 33] With no negative impact from the inflammatory processes, the eNSCs could have potentially had an increased likelihood for survival.

Conclusion

These data suggest that a single therapy following TBI may not suffice in producing optimal recovery and a combinatorial approach to treating TBI may be a more effective strategy. Moreover, an enriched environment seems to enhance the effects of therapies administered with it concurrently. It has also been suggested that long-term enrichment as well as post-injury enrichment without additional treatments can produce a greater recovery of function following TBI.[77] The use of animals that were not raised in complex and enriched environments may potentially deceases the ecological validity of pre-clinical studies and could hamper the

interpretation of these data.[77] In the future, these therapies should be evaluated in subjects that were reared in pre-injury enrichment environments in order to assess if the strength of the effects reported in the current study are observed. Furthermore, these data also suggest that additional behavioral testing needs to be conducted 2 or 3 months following treatment to assess whether there is a ceiling in recovery that is more readily reached when animals receive a combinational therapy. This type of approach, as well as the later application of therapies, has already been suggested by several investigators and the resistance of investigators to move toward more complex designs and therapeutic approaches could potentially be the one of the reasons why previously successful pre-clinical approaches fail to produce the same effect in clinical trials.[78, 79]

Acknowledgments

This study was funded, in part, by a Dow Student Research and Creativity Institute grant, awarded to E.T.N. and a Field Neuroscience Institute research contract, awarded to J.S.S. The authors would like to thank Jennica Young, Ivy Hiatt, Tim Pionk, and Kimberly Meerschaert for their help with animal care and assistance throughout the course of the study.

Author Disclosure Statement

No competing financial interests exist.

References

1. Coronado, V.G., McGuire, L.C., Sarmiento, K., Bell, J., Lionbarger, M.R., Jones, C.D., Geller, A.I., Khoury, N. and Xu, L. (2012). Trends in Traumatic Brain Injury in the U.S. and the public health response: 1995-2009. Journal Of Safety Research 43, 299-307.

2. Champion, H.R., Holcomb, J.B. and Young, L.A. (2009). Injuries from explosions: physics, biophysics, pathology, and required research focus. Journal of Trauma-Injury, Infection, and Critical Care 66, 1468-1477.

3. Adekoya, N., Webb, K.W. and White, D.D. (2002). Surveillance for traumatic brain injury deaths--United States, 1989-1998. Centers for Disease Control and Prevention.

4. Sutton, R.L., Lescaudron, L. and Stein, D.G. (1993). Unilateral cortical contusion injury in the rat: vascular disruption and temporal development of cortical necrosis. Journal Of Neurotrauma 10, 135-149.

5. Curtiss, G., Vanderploeg, R.D., Spencer, J. and Salazar, A.M. (2001). Patterns of verbal learning and memory in traumatic brain injury. Journal of the International Neuropsychological Society 7, 574-585.

6. Pierce, J., Smith, D., Trojanowski, J. and McIntosh, T. (1998). Enduring cognitive, neurobehavioral and histopathological changes persist for up to one year following severe experimental brain injury in rats. Neuroscience 87, 359-369.

7. Ruttan, L., Martin, K., Liu, A., Colella, B. and Green, R.E. (2008). Long-term cognitive outcome in moderate to severe traumatic brain injury: a meta-analysis examining timed and untimed tests at 1 and 4.5 or more years after injury. Archives of physical medicine and rehabilitation 89, S69-S76.

8. Roberts, I., Schierhout, G. and Alderson, P. (1998). Absence of evidence for the effectiveness of five interventions routinely used in the intensive care management of severe head injury: a systematic review. Journal of Neurology, Neurosurgery & Psychiatry 65, 729-733.

9. Clifton, G.L., Miller, E.R., Choi, S.C., Levin, H.S., McCauley, S., Smith Jr, K.R., Muizelaar, J.P., Wagner Jr, F.C., Marion, D.W. and Luerssen, T.G. (2001). Lack of effect of induction of hypothermia after acute brain injury. New England Journal of Medicine 344, 556-563.

10. Wright, D.W., Kellermann, A.L., Hertzberg, V.S., Clark, P.L., Frankel, M., Goldstein, F.C., Salomone, J.P., Dent, L.L., Harris, O.A. and Ander, D.S. (2007). ProTECT: a randomized clinical trial of progesterone for acute traumatic brain injury. Annals of Emergency Medicine 49, 391-402. e392.

11. Maas, A.I., Roozenbeek, B. and Manley, G.T. (2010). Clinical trials in traumatic brain injury: past experience and current developments. Neurotherapeutics 7, 115-126.

12. Maas, A.I., Murray, G., Henney III, H., Kassem, N., Legrand, V., Mangelus, M., Muizelaar, J.-P., Stocchetti, N. and Knoller, N. (2006). Efficacy and safety of dexanabinol in severe traumatic brain injury: results of a phase III randomised, placebo-controlled, clinical trial. The Lancet Neurology 5, 38-45.

13. Kolb, B. (1984). Functions of the frontal cortex of the rat: a comparative review. Brain Research Reviews 8, 65-98.

14. Hoffman, S.W., Fülöp, Z. and Stein, D.G. (1994). Bilateral Frontal Cortical Contusion in Rats: Behavioral and Anatomie Consequences. Journal of neurotrauma 11, 417-431.

15. Shaw, C.L., Watson, G.D., Hallock, H.L., Cline, K.M. and Griffin, A.L. (2013). The role of the medial prefrontal cortex in the acquisition, retention, and reversal of a tactile visuospatial conditional discrimination task. Behavioural brain research 236, 94-101.

16. Kesner, R.P. and Churchwell, J.C. (2011). An analysis of rat prefrontal cortex in mediating executive function. Neurobiology of learning and memory 96, 417-431.

17. Dixon, C.E., Clifton, G., Lighthall, J., Yaghmai, A. and Hayes, R. (1991). A controlled cortical impact model of traumatic brain injury in the rat. Journal of neuroscience methods 39, 253-262.

18. Lighthall, J.W. (1988). Controlled cortical impact: a new experimental brain injury model. Journal of neurotrauma 5, 1-15.

19. Fox, G.B., Fan, L., Levasseur, R.A. and Faden, A.I. (1998). Sustained sensory/motor and cognitive deficits with neuronal apoptosis following controlled cortical impact brain injury in the mouse. Journal of neurotrauma 15, 599-614.

20. Hamm, R.J., Dixon, C.E., Gbadebo, D.M., Singha, A.K., Jenkins, L.W., Lyeth, B.G. and Hayes, R.L. (1992). Cognitive deficits following traumatic brain injury produced by controlled cortical impact. Journal of neurotrauma 9, 11-20.

21. Scheff, S.W., Baldwin, S.A., Brown, R.W. and Kraemer, P.J. (1997). Morris water maze deficits in rats following traumatic brain injury: lateral controlled cortical impact. Journal of neurotrauma 14, 615-627.

22. Morales, D., Marklund, N., Lebold, D., Thompson, H., Pitkanen, A., Maxwell, W., Longhi, L., Laurer, H., Maegele, M. and Neugebauer, E. (2005). Experimental models of traumatic brain injury: do we really need to build a better mousetrap? Neuroscience 136, 971-989.

23. Gold, E.M., Su, D., López-Velázquez, L., Haus, D.L., Perez, H., Lacuesta, G.A., Anderson, A.J. and Cummings, B.J. (2013). Functional assessment of long-term deficits in rodent models of traumatic brain injury. Regenerative medicine 8, 483-516.

24. Will, B., Galani, R., Kelche, C. and Rosenzweig, M.R. (2004). Recovery from brain injury in animals: relative efficacy of environmental enrichment, physical exercise or formal training (1990-2002). Progress in neurobiology 72, 167-182.

25. Van Praag, H., Kempermann, G. and Gage, F.H. (2000). Neural consequences of enviromental enrichment. Nature Reviews Neuroscience 1, 191-198.

26. Sozda, C.N., Hoffman, A.N., Olsen, A.S., Cheng, J.P., Zafonte, R.D. and Kline, A.E. (2010). Empirical comparison of typical and atypical environmental enrichment paradigms on functional and histological outcome after experimental traumatic brain injury. J Neurotrauma 27, 1047-1057.

27. Risedal, A., Mattsson, B., Dahlqvist, P., Nordborg, C., Olsson, T. and Johansson, B.B. (2002). Environmental influences on functional outcome after a cortical infarct in the rat. Brain research bulletin 58, 315-321.

28. Galani, R., Jarrard, L., Will, B. and Kelche, C. (1997). Effects of postoperative housing conditions on functional recovery in rats with lesions of the hippocampus, subiculum, or entorhinal cortex. Neurobiology of learning and memory 67, 43-56.

29. Kozlowski, D.A., Nahed, B.V., Hovda, D.A. and Lee, S.M. (2004). Paradoxical effects of cortical impact injury on environmentally enriched rats. Journal of neurotrauma 21, 513-519.

30. Jones, T.A., Liput, D.J., Maresh, E.L., Donlan, N., Parikh, T.J., Marlowe, D. and Kozlowski, D.A. (2012). Use-dependent dendritic regrowth is limited after unilateral controlled cortical impact to the forelimb sensorimotor cortex. Journal of neurotrauma 29, 1455-1468.

31. Hicks, A., Hewlett, K., Windle, V., Chernenko, G., Ploughman, M., Jolkkonen, J., Weiss, S. and Corbett, D. (2007). Enriched environment enhances transplanted subventricular zone stem cell migration and functional recovery after stroke. Neuroscience 146, 31-40.

32. Peruzzaro, S.T., Gallagher, J., Dunkerson, J., Fluharty, S., Mudd, D., Hoane, M.R. and Smith, J.S. (2013). The impact of enriched environment and transplantation of murine cortical embryonic stem cells on recovery from controlled cortical contusion injury. Restorative Neurology And Neuroscience 31, 431-450.

33. Komitova, M., Mattsson, B., Johansson, B.B. and Eriksson, P.S. (2005). Enriched environment increases neural stem/progenitor cell proliferation and neurogenesis in the subventricular zone of stroke-lesioned adult rats. Stroke 36, 1278-1282.

34. Cattaneo, E. and McKay, R. (1990). Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor. Nature 347, 762-765.

35. Pettus, E.H., Wright, D.W., Stein, D.G. and Hoffman, S.W. (2005). Progesterone treatment inhibits the inflammatory agents that accompany traumatic brain injury. Brain research 1049, 112-119.

36. Shear, D.A., Galani, R., Hoffman, S.W. and Stein, D.G. (2002). Progesterone protects against necrotic damage and behavioral abnormalities caused by traumatic brain injury. Experimental Neurology 178, 59-67.

37. He, J., Evans, C.O., Hoffman, S.W., Oyesiku, N.M. and Stein, D.G. (2004). Progesterone and allopregnanolone reduce inflammatory cytokines after traumatic brain injury. Exp Neurol 189, 404-412.

38. Guo, Q., Sayeed, I., Baronne, L.M., Hoffman, S.W., Guennoun, R. and Stein, D.G. (2006). Progesterone administration modulates AQP4 expression and edema after traumatic brain injury in male rats. Exp Neurol 198, 469-478.

39. Stein, D.G. (2001). Brain damage, sex hormones and recovery: a new role for progesterone and estrogen? Trends in neurosciences 24, 386-391.

40. Roof, R.L., Duvdevani, R., Braswell, L. and Stein, D.G. (1994). Progesterone facilitates cognitive recovery and reduces secondary neuronal loss caused by cortical contusion injury in male rats. Experimental neurology 129, 64-69.

41. Sayeed, I., Wali, B. and Stein, D.G. (2007). Progesterone inhibits ischemic brain injury in a rat model of permanent middle cerebral artery occlusion. Restorative neurology and neuroscience 25, 151-159.

42. Robertson, C.L., Puskar, A., Hoffman, G.E., Murphy, A.Z., Saraswati, M. and Fiskum, G. (2006). Physiologic progesterone reduces mitochondrial dysfunction and hippocampal cell loss after traumatic brain injury in female rats. Experimental neurology 197, 235-243.

43. Wright, D.W., Bauer, M.E., Hoffman, S.W. and Stein, D.G. (2001). Serum progesterone levels correlate with decreased cerebral edema after traumatic brain injury in male rats. Journal of neurotrauma 18, 901-909.

44. Bramlett, H.M. and Dietrich, W.D. (2001). Neuropathological protection after traumatic brain injury in intact female rats versus males or ovariectomized females. Journal of neurotrauma 18, 891-900.

45. National Institute of Neurological Disorders and Stroke (2014). Traumatic Brain Injury Research (TBI).

46. Molcanyi, M., Riess, P., Bentz, K., Maegele, M., Hescheler, J., Schäfke, B., Trapp, T., Neugebauer, E., Klug, N. and Schäfer, U. (2007). Trauma-associated inflammatory response impairs embryonic stem cell survival and integration after implantation into injured rat brain. Journal of neurotrauma 24, 625-637.

47. Shear, D.A., Tate, C.C., Tate, M.C., Archer, D.R., LaPlaca, M.C., Stein, D.G. and Dunbar, G.L. (2011). Stem cell survival and functional outcome after traumatic brain injury is dependent on transplant timing and location. Restorative neurology and neuroscience 29, 215-225.

48. Itoh, T., Satou, T., Hashimoto, S. and Ito, H. (2005). Isolation of neural stem cells from damaged rat cerebral cortex after traumatic brain injury. Neuroreport 16, 1687-1691.

49. McDonald, J.W., Liu, X.-Z., Qu, Y., Liu, S., Mickey, S.K., Turetsky, D., Gottlieb, D.I. and Choi, D.W. (1999). Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. Nature medicine 5, 1410-1412.

50. Cao, Q., Benton, R.L. and Whittemore, S.R. (2002). Stem cell repair of central nervous system injury. Journal of neuroscience research 68, 501-510.

51. Riess, P., Molcanyi, M., Bentz, K., Maegele, M., Simanski, C., Carlitscheck, C., Schneider, A., Hescheler, J., Bouillon, B. and Schäfer, U. (2007). Embryonic stem cell transplantation after experimental traumatic brain injury dramatically improves neurological outcome, but may cause tumors. Journal of neurotrauma 24, 216-225.

52. Riess, P., Zhang, C., Saatman, K.E., Laurer, H.L., Longhi, L.G., Raghupathi, R., Lenzlinger, P.M., Lifshitz, J., Boockvar, J. and Neugebauer, E. (2002). Transplanted neural stem cells survive, differentiate, and improve neurological motor function after experimental traumatic brain injury. Neurosurgery 51, 1043-1054.

53. Labbe, R., Firl, A., Mufson, E.J. and Stein, D.G. (1983). Fetal brain transplant: reduction of cognitive deficits in rats with frontal cortex lesions. Science 221, 470-472.

54. Hicks, A.U., Hewlett, K., Windle, V., Chernenko, G., Ploughman, M., Jolkkonen, J., Weiss, S. and Corbett, D. (2007). Enriched environment enhances transplanted subventricular zone stem cell migration and functional recovery after stroke. Neuroscience 146, 31-40.

55. Ramic, M., Emerick, A.J., Bollnow, M.R., O'Brien, T.E., Tsai, S.Y. and Kartje, G.L. (2006). Axonal plasticity is associated with motor recovery following amphetamine treatment combined with rehabilitation after brain injury in the adult rat. Brain research 1111, 176-186.

56. Kline, A.E., McAloon, R.L., Henderson, K.A., Bansal, U.K., Ganti, B.M., Ahmed, R.H., Gibbs, R.B. and Sozda, C.N. (2010). Evaluation of a combined therapeutic regimen of 8-OH-DPAT and environmental enrichment after experimental traumatic brain injury. Journal of neurotrauma 27, 2021-2032.

57. Kline, A.E., Olsen, A.S., Sozda, C.N., Hoffman, A.N. and Cheng, J.P. (2012). Evaluation of a combined treatment paradigm consisting of environmental enrichment and the 5-HT1A receptor agonist buspirone after experimental traumatic brain injury. Journal of neurotrauma 29, 1960-1969.

58. Peterson, T.C., Anderson, G.D., Kantor, E.D. and Hoane, M.R. (2012). A comparison of the effects of nicotinamide and progesterone on functional recovery of cognitive behavior following cortical contusion injury in the rat. Journal of neurotrauma 29, 2823-2830.

59. Hoane, M.R., Becerra, G.D., Shank, J.E., Tatko, L., Pak, E.S., Smith, M. and Murashov, A.K. (2004). Transplantation of neuronal and glial precursors dramatically improves sensorimotor function but not cognitive function in the traumatically injured brain. J Neurotrauma 21, 163-174.

60. Barnes, C.A. (1979). Memory deficits associated with senescence: a neurophysiological and behavioral study in the rat. Journal of comparative and physiological psychology 93, 74.

61. Morris, R. (1984). Developments of a water-maze procedure for studying spatial learning in the rat. Journal of neuroscience methods 11, 47-60.

62. Hamm, R.J., Pike, B.R., O'Dell, D.M., Lyeth, B.G. and Jenkins, L.W. (1994). The rotarod test: an evaluation of its effectiveness in assessing motor deficits following traumatic brain injury. Journal of neurotrauma 11, 187-196.

63. Coggeshall, R.E. (1992). A consideration of neural counting methods. Trends in neurosciences 15, 9-13.

64. Gleave, J.A., Lerch, J.P., Henkelman, R.M. and Nieman, B.J. (2013). A method for 3D immunostaining and optical imaging of the mouse brain demonstrated in neural progenitor cells. Plos One 8, e72039.

65. Ke, M.T., Fujimoto, S. and Imai, T. (2013). SeeDB: a simple and morphology-preserving optical clearing agent for neuronal circuit reconstruction. Nature neuroscience 16, 1154-1161.

66. Temkin, N.R., Anderson, G.D., Winn, H.R., Ellenbogen, R.G., Britz, G.W., Schuster, J., Lucas, T., Newell, D.W., Mansfield, P.N. and Machamer, J.E. (2007). Magnesium sulfate for neuroprotection after traumatic brain injury: a randomised controlled trial. The Lancet Neurology 6, 29-38.

67. Bilotta, F., Caramia, R., Cernak, I., Paoloni, F.P., Doronzio, A., Cuzzone, V., Santoro, A. and Rosa, G. (2008). Intensive insulin therapy after severe traumatic brain injury: a randomized clinical trial. Neurocritical care 9, 159-166.

68. Ikonomidou, C. and Turski, L. (2002). Why did NMDA receptor antagonists fail clinical trials for stroke and traumatic brain injury? The Lancet Neurology 1, 383-386.

69. Saul, T.G., Ducker, T.B., Salcman, M. and Carro, E. (1981). Steroids in severe head injury: A prospective randomized clinical trial. Journal of neurosurgery 54, 596-600.

70. Morris, G.F., Bullock, R., Marshall, S.B., Marmarou, A., Maas, A. and Marshall, L.F. (1999). Failure of the competitive N-methyl-D-aspartate antagonist selfotel (CGS 19755) in the

treatment of severe head injury: results of two phase III clinical trials. Journal of neurosurgery 91, 737-743.

71. Maegele, M., Lippert-Gruener, M., Ester-Bode, T., Garbe, J., Bouillon, B., Neugebauer, E., Klug, N., Lefering, R., Neiss, W.F. and Angelov, D.N. (2005). Multimodal early onset stimulation combined with enriched environment is associated with reduced CNS lesion volume and enhanced reversal of neuromotor dysfunction after traumatic brain injury in rats. European Journal of Neuroscience 21, 2406-2418.

72. Griesbach, G.S., Hovda, D.A. and Gomez-Pinilla, F. (2009). Exercise-induced improvement in cognitive performance after traumatic brain injury in rats is dependent on BDNF activation. Brain research 1288, 105-115.

73. Griesbach, G.S., Hovda, D., Molteni, R., Wu, A. and Gomez-Pinilla, F. (2004). Voluntary exercise following traumatic brain injury: brain-derived neurotrophic factor upregulation and recovery of function. Neuroscience 125, 129-139.

74. Passineau, M.J., Green, E.J. and Dietrich, W.D. (2001). Therapeutic effects of environmental enrichment on cognitive function and tissue integrity following severe traumatic brain injury in rats. Experimental neurology 168, 373-384.

75. Monaco, C.M., Mattiola, V.V., Folweiler, K.A., Tay, J.K., Yelleswarapu, N.K., Curatolo, L.M., Matter, A.M., Cheng, J.P. and Kline, A.E. (2013). Environmental enrichment promotes robust functional and histological benefits in female rats after controlled cortical impact injury. Experimental neurology 247, 410-418.

76. Angelucci, F., De Bartolo, P., Gelfo, F., Foti, F., Cutuli, D., Bossù, P., Caltagirone, C. and Petrosini, L. (2009). Increased concentrations of nerve growth factor and brain-derived

neurotrophic factor in the rat cerebellum after exposure to environmental enrichment. The Cerebellum 8, 499-506.

77. Nudi, E.T., Jacqmain, J., Fluharty, S. and Smith, J.S. (2013). Pre- and Post-Injury Environments Effect Functional Recovery Following Traumatic Brain Injury. Journal of Neurotrauma 30, A-157.

78. Diaz-Arrastia, R., Kochanek, P.M., Bergold, P., Kenney, K., Marx, C.E., Grimes, C.J.B., Loh, L.Y., Adam, L.G.E., Oskvig, D. and Curley, K.C. (2014). Pharmacotherapy of Traumatic Brain Injury: State of the Science and the Road Forward: Report of the Department of Defense Neurotrauma Pharmacology Workgroup. Journal of neurotrauma 31, 135-158.

79. Kabadi, S.V. and Faden, A.I. (2014). Neuroprotective strategies for traumatic brain injury: improving clinical translation. International journal of molecular sciences 15, 1216-1236.

FIG. 1. Open field test. (A) Mean difference in distance travelled across the days. (B) Groups differences in total distance travelled. (**p*<.05) represents the significant difference to Sham/EE group. (***p*<.05) represents the significant difference to the Sham/EE and TBI/EE/PROG group. (****p*<.05) represents the significant difference to the Sham/SE group. (#*p*<.05) represents the significant difference to the TBI/EE/VEH group. (##*p*<.05) represents the significant difference to the TBI/SE/VEH group. (C) Mean number of rears across days. (D) Group differences in rearing. (**p*<.05) represents the significant difference to the Sham/EE. (***p*=.011) represents the significant difference to the Sham/SE group. (****p*<.05) represents the significant difference to the Sham/SE and TBI/EE/PROG groups. (#*p*<.05) represents the significant difference to the TBI/EE/VEH and TBI/eNSC/EE/PROG.

FIG. 2. Barnes maze. (A) Mean latency to locate escape box both pre- and post-box switch across days. (B) Groups differences in latency pre-box switch. (**p*<.05) represents the significant difference to the TBI/SE/PROG and TBI/SE/VEH groups. (***p*<.05) represents the significant difference to the Sham/EE group. (****p*<.05) represents the significant difference to the

Sham/SE group. (C) Group differences in latency post-box switch. (*p<.05) represents the significant difference to the TBI/SE/VEH group. (**p<.05) represents the significant difference to the TBI/eNSC/SE/PROG group.

FIG. 3. Morris water maze. (A) Mean latency to locate escape platform across days. (B) Group differences in latency. (*p<.05) represents the difference to the Sham/EE and Sham/SE groups. (**p<.05) represents the difference to the TBI/EE/PROG group. (***p<.05) represents the difference to the TBI/SE/PROG group. (#p=.007) represents the difference to the TBI/EE/VEH group. (##p<.05) represents the difference to the TBI/SE/VEH group. (###p<.05) represents the difference to the TBI/eNSC/EE/PROG and TBI/eNSC/EE/VEH groups.

FIG.4. Rotor rod. (A) Mean latency on the rotor rod across days. (B) Group differences in latency on the rotor rod. (*p<.05) represents differences in latency to all EE groups. (**p<.05) represents differences in latency to all EE groups excluding the TBI/EE/VEH group. (***p<.05) represents differences in latency to all EE groups excluding the TBI/EE/VEH and TBI/EE/PROG groups. (#p<.05) represents differences in latency to the TBI/SE/VEH group.

FIG.5. (A) Mean volume estimates of the cortex. (*p<.001) represents differences in cortex volume to the Sham groups. (**p<.05) represents mean cortex volume differences to the TBI/EE.PROG and TBI/SE/VEH groups. (***p<.05) represents the volume differences between the TBI/SE/PROG group. (#p=.044) represents mean difference in cortical volume to Sham/EE group. (B) Mean neuronal estimates in the CA1, CA2, and CA3 regions of the hippocampus. (*p<.05) represents differences in neuronal estimates to the Sham/EE group. (**p<.05) represents neuronal estimate differences to the TBI/EE/PROG group. (***p<.05) represents mean neuronal estimate differences to the TBI/eNSC/EE/PROG group.

FIG. 6. M2/M6 and NeuN labeled cells in the polymorph layer of the dentate gyrus from an animal in the TBI/eNSC/EE/PROG group. Clear indications of co-labeling of M2/M6 and NeuN positive cells are present in the merged panel.