Transplantation of Neurospheres Derived from Genetically Modified Adult Bone Marrow Stromal Cells Following a Controlled Cortical Impact (CCI): Effects on Transplant Survival and Behavioral Recovery

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Transplantation of Neurospheres Derived from
Genetically Modified Adult Bone Marrow Stromal Cells
Following a Controlled Cortical Impact (CCI): Effects
on Transplant Survival and Behavioral Recovery

A Thesis Presented in Fulfillment
of the Requirements for
the Degree of Master of Science

December 2013

By
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Abstract

Traumatic brain injury (TBI) affects thousands of individuals annually who have limited options for treatment. The use of mesenchymal stem cells derived from bone marrow (BMSC) has therapeutic potential, free from ethical ramifications. Previous transplant studies have grafted naive BMSCs into injured brain tissue or infused them intraventricularly (i.v.) with varying results (Bonilla 2012, Osani 2012, Bakhitany 2010, Li 2009). In this study, we transplanted Notch-induced rat BMSCs (BMSC-NICD) with properties of neuroprogenitor-like cells, into the rat controlled cortical impact (CCI) model of TBI. Adult rat BMSC were harvested from femur and tibia of adult Fischer 344 rats. These cells were further transfected with the intracellular domain of the Notch1 (NICD) gene and the neomycin resistance gene to generate BMSC-NICD cells. Cells effective transfected with the NICD were selected for resistance to G418. Following selection, BMSC-NICD were infected with lentivirus harboring green fluorescent protein (GFP) and grown under non-adherent conditions to promote formation of Nestin-expressing neurospheres. Adult male Fisher 344 rats (n=8) were injected with BMSC-NICD-derived neurospheres on day 7 following unilateral CCI of the forelimb sensorimotor cortex. Two injections were made posterior to the injured cortex or within the dorsolateral striatum (25,000 cells/μl at 2 μl/injection totaling 100,000 cells/rat). Transplanted BMSC-NICD neurospheres were examined for: 1) survival, 2) effect on contusion size, and 3) effect on behavioral functions. Limb use and foot fault behavioral tests were performed to examine deficits in and possible recovery of forelimb function
following CCI in transplanted rats compared to CCI only rats (n=4). A baseline measurement was taken at day 0 before CCI.

Following, CCI, behavioral tests were performed at day 5 (pre-transplant), and day 12 (5 days post-transplant). Rats were euthanized on day 14 and brains were sectioned and examined for surviving GFP+ cells and for cortical volume. All rats showed marked impairment of the forelimb contralateral to the injury on day 5.

Both cortical and striatal transplants enhanced behavioral recovery of forelimb function compared to CCI only rats by day 12. Survival of transplanted cells was seen in both areas; however, rats with cortical transplants showed decreased numbers of GFP+ cells compared to rats with striatal transplants. Transplantation of cells did not affect lesion size as inferred from total cortical volume. This pilot study suggests that transplantation of neurospheres derived from BMSC-NICD neurospheres at the site of injury and/or in subcortical targets decreases behavioral impairments. Also, subcortical targets such as the striatum, rather than the site of cortical contusion, appear to be a better site for transplant survival.
INTRODUCTION

Traumatic Brain Injury (TBI) affects 1.7 million people annually and is a persistent clinical concern with detrimental neurological and behavioral effects (CDC 2008). TBI typically occurs due to a harsh blow to the head, which can result in different degrees of cell death, and several secondary factors that ultimately produce behavioral dysfunctions. Secondary factors include stroke, seizures, and swelling and may lead to a cascade of neuronal cell death that can persist for months, or even years (Opydo-Chanek 2007). Theoretically, there are three modes of treatment for TBI: 1) the use of behavioral rehabilitation, 2) the reduction of secondary traumatic factors (i.e., cell death) or 3) the restoration of lost tissue. Currently, TBI patients mainly receive rehabilitation therapy (Opydo-Chanek 2007). There are no neuroprotective/neurorestorative drugs or therapies available. Previous studies that examine these therapeutic strategies in isolation resulted in controversial and inconclusive findings. Stem cells are promising candidates for a novel TBI therapy. Transplantation of different cell types (naïve and genetically modified) has been tried in several disease conditions, including Parkinson's disease (PD), stroke and TBI, but progress towards stem cell therapy has been hindered by cell survival and integration into host tissues (Opydo-Chanek 2007, Hoane 2007, Riess 2007, Hoane 2004). Bone marrow stromal cells (BMSCs), stem cells derived from adult bone marrow have shown numerous beneficial effects in different animal models of neurodegenerative disorders mainly through paracrine actions (Sadan et al., 2009). Recent work has shown that these BMSCs can be turned into immature neuronal precursors destined to adopt a neural cell fate after transfection with the intracellular domain of the Notch1 gene (NICD) (Dezawa et al 2004). Moreover, when transplanted in a rat, they can ameliorate loss of neurons in both a rat model of stroke and Parkinson’s disease (PD) (Yasuhara 2009, Hayase 2009,

However, BMSC-NICD transplantation has not been tested in an animal model of TBI. Therefore, the first question to ask is how well BMSC-NICD will survive in the environment of TBI, which has considerable secondary degenerative events. This study investigated the use of BMSC-NICD-derived neurospheres as a therapeutic transplant for TBI. BMSC-NICD neurospheres were injected into two different areas of the brain, the injured cortex and the ipsilateral striatum. The survival, differentiation, and neuroprotective potential of these transplants were examined as well as their initial effect on behavioral recovery.

**BACKGROUND & SIGNIFICANCE**

**Traumatic Brain Injury**

TBI is a prevalent persistent clinical concern that affects over 1 million people each year. Of those 1.7 million; 50,000 die, 235,000 are hospitalized and 1.1 million are treated and released from emergency care (CDC 2013). Nearly 475,000 TBIs occur among children age 0-14 years and this particular age group accounts for 90% of emergency care visits, with falls as the leading cause (CDC 2013). A new demographic has recently emerged due to the current wartime events, with nearly 360,000 troops returning from Iraq and Afghanistan afflicted with a TBI (Zoroya 2009).

There are two types of TBI- a concussion and a contusion. A concussion is closed head injury produced by a blow to the head that does not break or penetrate the skull and typically does not result in any significant cell death. A contusion is an open head injury caused specifically by an impact that cracks the cranium or penetrates the brain. The contusion itself
produces a focal area of cell loss that appears as a cavity in the brain, and is designated into two physical zones, the core and the penumbra (Dixon et. al. 1991). Two commonly used animal models explore the mechanisms and potential therapies for traumatic brain injury (Dixon 1991). The fluid percussion model rapidly injects small amounts of saline into the closed cranial cavity. This process increases cranial pressure and creates neural tissue deformations that produce neurological deficits similar to that of human brain injury (Dixon 1991). In contrast, the controlled cortical impact (CCI) is a precise and measurable impact to the neural tissue following a craniotomy. It is designed to resemble a closed-head injury in humans. Deformation over time can be measured in relation to the size of the injury (Dixon 1991).

Cell death is inevitable and irreversible following a CCI. Initial cell death occurs due to necrosis because of the damage caused by the initial impact. Necrosis is the initial mass cell loss that occurs within 24 hours of the impact and is concentrated in the core of the injury. Apoptosis, or programmed cell death, occurs in the surrounding penumbra. Apoptosis peaks within three days of the impact, but can last for years (Raghupathi 2000). The factors that trigger this secondary cascade of cell death are fairly well understood. Excess release of glutamate, a predominately excitatory neurotransmitter is believed to be responsible for cerebral mitochondrial dysfunction (Fiskum 2000). Another cause of mitochondrial dysfunction is the metabolic mismatch caused by increased glucose metabolism in the brain following TBI, in conjunction with a decreased flow of blood to the area (Fiskum 2000). As the mitochondria begin to function abnormally, a calcium overload is produced in response to excitatory amino acids and free radicals (Fiskum 2000, Hall 1993). Calcium is an essential component of neuronal regulation, and overload of calcium triggers apoptosis as well as necrosis (Hall 1993).

TBI is also associated with the formation of a glial scar following injury, in which glial
cells are recruited to the injury site and form a patch of scar tissue (Bahr 1994, Lynch 1994). The inflammatory response is a key component of all injuries, and the recruitment of immune cells likewise contributes to the formation of scar tissue at the injury site (Raghupathi 2000). Formation of this scar prevents the ability of axons to regenerate and is theorized to also recruit free radicals to the brain (Lynch 1994). The resulting secondary cell death is a certainty following TBI and the detrimental effects are vast. Currently there is no cure or therapy that directly counteracts the necrosis or apoptosis following TBI, although much research is focused on finding one.

**Stem Cells**

The term stem cell is used to describe any cell that meets two criteria: 1) has the ability to divide and reproduce itself, and 2) when it divides, it produces one cell that can go on and differentiate into a specific cell type, and another cell that remains immature and undifferentiated (Brodhun 2004). Stem cells can be derived from all embryonic tissues, but can also be found in certain tissues of the adult, including: blood vessels, brain, bone marrow, skin, liver and skeletal muscle (Opydo-Chanek 2007).

Stem cell transplantation can be performed using embryonic, fetal adult and induced pluripotent (iPS) stem cells, which exhibit differences in potential. Embryonic & fetal stem cells are pluripotent, meaning that they can differentiate into cells from all three germ layers and divide rapidly whereas, adult stem cells are more predetermined to develop into a specific type of cell (multipotent) such as neuronal or cardiac, and don’t divide as readily. Adult stem cells can differentiate into cells from some, but not all, of the three germ layers (Hemmat 2010).

Social and ethical issues concerning the destruction of human blastocysts have hindered
the use of embryonic and fetal stem cells for transplantation for several years, including neuronal transplantation. Many oppose the use of these cells because they are derived from human embryos and fetuses. The lack of readily available embryonic and fetal stem cells, and potential of these cells to form tumors after grafting combined with the social and ethical issues have all forced researchers to examine alternative sources of stem cells. Use of the adult stem cells eliminates much of the controversy. Moreover, adult stem cells are a readily available and easily accessible therapeutic alternative. Adult stem cells can be harvested directly from a patient to eliminate issues with rejection. Theoretically, taking an patients’ own stem cells (autologous stem cells) and injecting them into his/her own TBI would lower the chance for transplant rejection, which is possible when transplanting cells from another person. The use of stem cells attempts to replace the damaged cells and produce a viable and renewed cellular environment (Opydo-Chanek 2007, Peruzzaro 2013). Alternatively, stem cells may provide a source of growth factors or other neuroprotective agents to damaged cells and evoke their recovery (Opydo-Chanek 2007, Peruzzaro 2013).

**Bone Marrow Stromal Cells**

In this study, we used genetically modified BMSCs. BMSCs typically differentiate into cells of mesodermal lineage, including adipocytes, chondrocytes, osteoblasts and myocytes (Gnecchi 2009). BMSCs have been shown to differentiate into neurons and glial cells *in vivo* and *in vitro* under certain experimental conditions (Opydo-Chanek 2007, Kuroda 2011, Dezawa 2004, Ding 2011). Several studies using different neurodegenerative models such as stroke and Parkinson’s disease have seen considerable success in transplanting BMSCs into the degenerating brain. BMSCs injected into the damaged brain tissue of a rat model of ischemic stroke showed significant regression of neurological deficits as well as migration of stem cells
towards the zone of ischemia. This research also showed differentiation of BMSCs into neurons and glia with limited cell death (Skvortsova 2008). Similar results were seen by transplanting BMSCs into the cerebrum following a temporary occlusion of the middle cerebral artery (Chen 2008). This research showed differentiation of stem cells into astrocytes and oligodendrocytes, and also demonstrated that the cells migrated to the damaged site, which significantly reduced artery occlusion-induced cortical loss and had a neuroprotective effect. A significant increase in behavioral functioning was also shown based on several tests of fine motor skills (Chen 2008).

BMSCs in rat models of PD have had similar success with neuroprotection and behavioral improvement. Transplanted BMSCs harvested from adult donor mice were injected into the brains of PD mice and tested for cell survival and functional recovery (Li 2001). Survival was seen at 4 weeks, and fine motor tests demonstrated functional recovery (Li 2001). BMSCs cultured from mouse marrow have also demonstrated differentiation into dopaminergic-like cells after transplantation, and improvement in behavioral function. Differentiation into dopaminergic-like cells is important in PD because of the notable decrease in dopamine following onset (Offen 2007). Based on the previous findings, BMSCs can be beneficial for the treatment of neurodegenerative diseases due to their ability to differentiate into neurons and glial cells. However, in vivo studies have shown their ability to differentiate into neuronal cells is limited. The improved behavioral function is likely a result of trophic factors following transplantation that are able to avoid the inflammatory response (Li 2001).

**Stem Cell Transplantation in TBI**

Transplantation in a traumatic brain injury is not a novel practice and different stem cell types have been explored with varying success. Murine embryonic stem cells, for example, have
been pre-differentiated into GABAergic neurons and transplanted into a rat model of TBI with marked sensorimotor improvement in behavioral tasks (Hoane 2007). Previous studies have also examined genetically modified murine embryonic stem cells as neuronal and glial precursors in traumatic brain injury with similar effects and demonstrated behavioral improvement as soon as 2 days following transplant (Hoane 2004). The therapeutic potential of naive murine embryonic stem cells in rat TBI has been examined, as well. While behavioral improvements were seen as early as 3 days following transplantation and cell survival was seen in 100% of animals one week post transplant, survival was seen in only one animal 7 weeks post-transplant with tumor formation in two of the animals (Riess 2007).

Although behavioral improvement was seen in these studies of embryonic stem cell transplantation, the results beg for more promising and clinically adequate transplant sources that will not lead to tumor formation.

**BMSCs in TBI**

There is a growing body of evidence that transplantation of BMSC in experimental models of TBI can promote neural regeneration and behavioral recovery (Mahmood 2001, Mahmood 2005). The majority of previous studies have injected undifferentiated BMSCs into the brain and expected these cells to integrate as neurons from the blank cell state. Michael Chopp has performed several transplantation studies in TBI models and demonstrated a significant amount of behavioral improvement in almost all studies. However, transplants did not survive for an extended period of time as functional neurons in TBI models. Injection of BMSCs together with Simvastatin, a known neuroprotective agent that is capable of crossing the blood-brain barrier, produced neurorestorative function (Mahmood 2008). Regardless of the concurrent presence of Simvastatin, the MSC number had declined dramatically over the period of three
months. However, the neurorestorative function still persisted at 3 months even after the MSC number had depleted, indicating that restoration is achieved through MSC trophic effects (Mahmood 2008). Several other studies also suggested that behavioral recovery after MSC transplantation in TBI model is achieved through MSC trophic effect since only a limited number of cells survived after transplantation (Mahmood 2005, Mahmood 2007). These studies have utilized intracerebral and intravenous methods of administering MSC to rat models of TBI with success shown in cell survival for a short period of time, cell migration, increased vascularity and limited immune rejection (Mahmood 2005, Mahmood 2007, Mahmood 2008). Cell survival was shown at day 35, for example, with 35.2± 5.58 cells per square millimeter. Unfortunately, this number decreased over time with no significant decrease in lesion volume or differentiation into neural cells, indicating paracrine action (Mahmood 2005, Mahmood 2007, Mahmood 2008).

**Neurospheres Derived from BMSC-NICD Cells**

The Notch pathway is important for maintaining neural progenitor cells (Bohn 2009, Mahmood 2006). The signaling pathway is known to be involved in cell differentiation processes via gene regulation, as well as neuronal function and development (Gaiano 2002). Binding of the ligands to the extracellular domain of notch transmembrane receptors results in proteolytic cleavage and release of the intracellular domain, which enters the cell nucleus to activate different transcription factors (Gaiano 2002). Studies from Dr. Dezawa’s laboratory demonstrated that BMSC genetically modified with NICD have decreased expression of MSC markers and increased expression of neuroprogenitor markers (Dezawa et al 2004). Moreover, these BMSC-NICD cells demonstrate improvement in motor performance correlated with
increasing neuronal differentiation when grafted in a complete 6-hydroxydopamine (6-OHDA) lesion model of PD. Transplantation of human BMSC-NICD in a progressive partial 6-OHDA lesion rat model evoked rejuvenation of host dopaminergic neurons (Glavaski-Joksimovic et al, 2009) which was augmented in the presence of glial cell-derived neurotrophic factor (GDNF) (Glavaski-Joksimovic et al, 2010); Rejuvenating effect of human BMSC-NICD was observed, although, there were almost no surviving cells at few weeks post-graft, suggesting an early protective effect of transplanted cells (Glavaski-Joksimovic et al 2009, 2010). However, when clustered rat BMSC-NICD cells (neurospheres) were transplanted in a rat stroke model, transplanted cells survived well and evoked functional recovery (Hayase et al, 2009). In the same study transplantation of naïve MSCs evoked functional recovery as well, but naïve MSCs began dying shortly after transplantation while BMSC-NICD cells were still viable at 100 days following transplant (Hayase et al, 2009). Also, some of the transplanted BMSC-NICD exhibited markers of neuronal differentiation 100 days following transplantation (Hayase et al, 2009).

In this study we examined whether BMSC-NICD neurospheres can also have therapeutic effect in a rat model of TBI. As opposed to studies from the other groups that have used naïve BMSCs (Mahmood 2006, Mahmood 2005, Mahmood 2007, Mahmood 2008), we used neurospheres derived from BMSC-NICD cells that have been driven into a neuronal precursor fate by the expression of Notch1. Due to their neuroprogenitor characteristics we believe that BMSC-NICD can demonstrate better survival and integration into the host tissue and more prominent therapeutic effect after transplantation in a TBI model.
Hypothesis

We propose that the injection of neural precursors derived from adult rat BMSCs into a TBI will result in survival of the transplant and regeneration of host injured brain tissue. This will decrease the neural loss and behavioral dysfunction.
METHODS

Experiment 1 – Creation Of Neurospheres From Adult Bone Marrow Stromal Cells

Figure 1. Schematic demonstrating the formation of Neurospheres from adult bone marrow stem cells. (Glavaski-Joksimovic et al 2009). Neurosphere creation and staining was conducted at Children’s Memorial Research Center/ Northwestern Medical School in the laboratory of Dr. Bohn under the guidance of Dr. Glavaski-Joksimovic.

Mesenchymal Stem Cells

Mesenchymal stem cells were harvested for downstream transfection to ultimately induce cells to a neuronal fate (BMSC-NICD). Eight-week-old F344 rats’ tibiae and femurae were used for extraction of MSCs under sterile conditions. MSC were incubated at 37°C in 5% CO2 in a solution containing α-MEM medium supplemented with 15% fetal bovine serum (FBS), 2mM L-glutamine, and 0.1 mg/ml kanamycin. (See Figure 1).

Transfection of MSC with Intracellular Domain of Notch-1 Gene (NICD)
MSCs were passaged four times before they were transiently transfected with a plasmid containing NICD and neomycin-resistance gene. Lipofection was performed to insert the plasmid using Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA). Following transfection, the cells underwent selection using G418 for 7 days. Once selection of MSC-NICD was completed, cells were passaged twice (See Figure 1).

**Lentivirus infection**

Lentiviral infection with GFP allowed us to examine survival post-mortem. A lentivirus harboring enhanced green fluorescent protein (EGFP) was used to infect the BMSC-NICD cells. Infected cells expressed GFP, which enabled tracking in the host tissue following transplantation. An HIV-based replication incompetent, self–inactivating lentivirus that was under control of the ubiquitin promoter and contained EGFP was used (Lenti-UbPr-EGFP). The MSC-NICD cells were transduced with the lentivirus at multiplicity of infection (MOI) 5 at the second passage after transfection with the NICD. (See Figure 1).

**Neurosphere Formation**

Four days after lentivirus infection, cells were washed with α-MEM medium and trypsinized (0.25% trypsin and 0.38g/L EDTA tetrasodium salt) for 5 minutes at 37°C. After spinning at 800 rpm, cells were resuspended in a neurobasal medium containing B27 supplement, 20 ng/ml basic fibroblast growth factor (bFGF) and 20ng/ml epidermal growth factor (EGF). Cells were then plated at a density of 1x10^5 cells/ml on poly-HEMA (poly-2-hydroxyethyl methacrilate) coated dishes so that these cells would not adhere to the plate surface and neurospheres would form (See Figure 1)
**Immunocytochemistry to Detect Neurosphere Cell Fate**

At 5 days in culture, BMSC-NICD neurospheres were fixed in 4% paraformaldehyde for 20 minutes and cryoprotected in 20% sucrose and 30% sucrose for 24 hours. Neurospheres were sectioned into 12 μm sections using a cryostat, blocked for one hour, and stained for immunoreactivity for neuroprogenitor marker nestin (Chemicon, 1:100 Temecula, CA). Hoechst nuclear staining was performed on the paraformaldehyde-fixed sections by treating them with Hoechst 33342 dye (Invitrogen; 5 μg/ml in PBS) for 5 minutes. Immunostained spheres were examined using fluorescent microscopy to determine cell phenotype.

**Experiment 2 – Transplantation Of BMSC-NICD Neurospheres Following a Rodent TBI**

A total of three experimental groups (Table 1) of male Fischer-344 rats received a controlled cortical impact (CCI) model of TBI. Two of the groups received BMSC-NICD neurospheres one week following CCI in either 2 sites surrounding the injury itself, or in the dorsolateral striatum, which receives projections from the injured forelimb sensorimotor cortex. The third group of rats received only CCI. Behavioral testing was administered on day zero for baseline, day five to test behavior following CCI prior to transplantation and day 12 following BMSC transplantation and CCI. The rats were sacrificed by perfusion on day 14 and their brains were removed for further analysis. The brains were sliced into sections and mounted in order to analyze contusion size, neuronal number, and transplant survival.

**Animals**

This study used adult Fisher rats aged 2-3 months who were group housed in the DePaul Animal Facility under 12:12 h light/dark cycle. The experiments were conducted using NIH and
Institutional guidelines regarding the use of animals. Animals were divided into the following groups: CCI+ BMSC transplantation into the striatum (CCI+Striatum) CCI+BMSC transplantation into the cortex (CCI+Cortex) and CCI only (See table 1).

**Treatment Groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose</th>
<th>N</th>
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<tbody>
<tr>
<td>CCI Only</td>
<td>N/A</td>
<td>4</td>
</tr>
<tr>
<td>CCI+Cortex</td>
<td>25,000 cells/ul</td>
<td>4</td>
</tr>
<tr>
<td>CCI+Striatum</td>
<td>25,000 cells/ul</td>
<td>4</td>
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Table 1: Three groups of rats were used in the study to test the optimal transplant location

**Surgeries**

**Controlled Cortical Impact (CCI) Injury**

The CCI was administered to all experimental groups and mimicked a contusion. The controlled cortical impact was administered using modified procedures developed previously (Sutton, Lescaudron et al. 1993). Rats were anesthetized with Equithesin / chlortal hydrate-sodium pentobarbital (149 mg/100g chlortal hydrate, 31mg/100g sodium pentobarbital i.p.) for a period of 60 minutes. A toe pinch response test was administered to test the level of anesthesia. The rats were also monitored for respiration rates and heart rate every few minutes. Once deeply anesthetized, the rats’ heads were shaved and the animal was placed in a Kopf stereotaxic apparatus (Kopf,Tujunga, CA). The skull was exposed and the bite bar for the jaw was adjusted to level bregma and lambda in the horizontal plane. The dura and cortex were exposed by a 4-5 mm diameter craniotomy that was placed at 0.5mm anterior and 4.0 mm lateral to bregma, directly over the forelimb sensorimotor cortex (FL-SMC) (See Figure 2). A CCI
device known as the Benchmark Impactor delivered the cortical impact. The injury was produced by a flat and circular impactor tip (3mm diameter). The impactor was angled 18 degrees away from vertical, which enabled the flat impactor tip to be perpendicular to the surface of the brain at the site of injury. The impactor tip depressed the brain tissue at 3.0 m/s at a depth of 2.5mm below the cortical surface for 300 ms. after the impact, the bone cutout was replaced or Gelfoam covered the exposed injury site. The rats were then sutured with monofilament nylon sutures using a continuous suture. Topical analgesia cream was applied to the wound post-op along with a triple-antibiotic. Body temperature was maintained at 37°C during the surgery with a heating pad. Following the surgery, the rats were returned to their cages in the surgery room. During the recovery phases, rats were housed 2 to a cage and monitored daily.

![Figure 2. Placement of the CCI over the FL-SMC](image)

**Injection of Neurospheres**

Neurospheres were injected in two of the experimental groups as a potential therapy to TBI models. One week post-CCI, Rats were anesthetized with Equithesin /chloral hydrate-sodium pentobarbital (149 mg/100g chloral hydrate, 31mg/100g sodium pentobarbital i.p.) for a period of 60 minutes. A toe pinch response test was administered to test the level of anesthesia. The rats were also monitored for respiration rates and heart rate every few minutes.
Sutures were removed and the injury was exposed.

Non-dissociated BMSC-NICD neurospheres received from Dr. Glavaski-Joksimovic were suspended in PBS at a concentration of 25,000 cells/µl. Two stereotaxic deposits, each of 2 µl neurospheres suspension, were injected in the damaged side of the brain, totaling 100,000 cells/animal. The injections into the cortical injury attempted to replace damaged cells, while injections into the striatum tested for connection to the cortex and the ability to produce trophic factors to the injury (See Figure 3). The striatum transplants went into the dorsolateral striatum, which connects to the FL-SMC (injured cortex). Cortical injections were made using the following coordinates: -0.2 A/P, +4.6 M/L, and -3.0 and -2.0 D/V, whereas the striatal transplants were made using the following coordinates: +0.5 A/P, +3.8 M/L and -5.0 and -4.2 D/V (See Figure 3). Neurospheres were injected using a 10µl Hamilton syringe at 0.5µl/min using a Stoelting automatic stereotaxic injector (Stoelting, Wood Dale, IL). The needle was left in place for 2 minutes and then withdrawn at 1 mm/min. After the injection, the rats were sutured with monofilament nylon sutures using a continuous suture. Post-operative analgesia was applied to the wound along with a triple-antibiotic to prevent infections. Body temperature was maintained at 37°C using a heating pad. Rats were returned to their home cage in the surgery room and monitored daily.

Injection Sites
Figure 3. Injections were administered in two deposits surrounding the injury (cortical injections) or in the striatum (striatal injections). Two deposits, each of 2 µl neurosphere suspension at concentration of 25,000 cells/µl were injected per rat (100,000 cells per animal).

Behavior

Two behavioral tests (Foot Fault and Limb Use) were performed to test for forelimb deficits related to the injury and potential improvements from BMSC-NICD neurosphere transplantation. Both tests were administered on days 0 (baseline), 5 (post-CCI, pre-transplant) and 12 (5 days post-transplant). Foot fault test and limb use analysis were performed concurrently.

Foot Fault

The foot fault test was administered to all experimental groups to test the effect of BMSC-NICD neurospheres over a short period of time on motor coordination. It utilizes a grid made of test tube racks with an area of 33.02 x 25.40 x 7.62 cm with openings of 2.54 square cm and tests the number of times the left and right forelimbs fall through each opening after taking 50 steps on the grid. A step is the successful completion of sequential forward movement of the left and right forelimbs. A “fault” is determined by the animal’s forelimb completely falling into
the space between the grid bars without any grip of the paw on the grid itself. Foot Fault Data
was calculated by contrasting ipsilateral and contralateral (to injury) limb faults. Ipsilateral faults
were subtracted from contralateral faults and divided by the total number of steps and multiplied
by 100 to obtain the percent contralateral foot fault ((C-I/50)100).

Limb Use

The limb use test was administered to all three experimental groups to test the
effect of BMSC-NICD neurospheres on weight bearing on the injured forelimb. This test was
performed in a 22.86 x 37.46 x 24.13 cm Plexiglas cylinder, in which the rat was placed in and
videotaped until 10 rears before a wall were executed. A rear before wall occurs when the animal
would rear himself upward onto his hind limbs and brace himself with his forelimbs against the
Plexiglas. Observational analysis of the videotapes allowed for determination of weight-bearing
activity and limb use preference. The animal was observed exploring his surroundings/Plexiglas
in horizontal or vertical movements and its preference in landing was recorded. Data was
calculated for: rears before wall exploration, left wall vertical, right wall vertical, right wall
horizontal, left wall horizontal and landings. Percent Ipsilateral forelimb use was calculated
using the formula (((I-C)/(I+C))100).

Euthanasia and Histological Preparation

Brain samples were fixed for histological analysis in order to examine surviving cells and
their effect on the contusion. Rats were euthanized one week following transplantation (2 weeks
post- CCI) by cardiac perfusion with saline followed by 4% buffered paraformaldehyde, pH 7.6
at 4°C while under sodium pentobarbital (3.5 mg/100g)/chloral hydrate (17mg/100g) anesthesia
for histological preparations.

Brains were removed, post-fixed overnight, stored at 4°C, and cryoprotected in 30%
sucrose/PBS. Serial coronal sections (40µm) were cut and stored in cryoprotective solution at -20°C until stained. Serial sections were grouped in sets of 6 – each section 240µm from the next.

**Cresyl Violet Stain**

Cresyl violet staining was performed to analyze remaining cortical volume of experimental groups. One set of brain sections were mounted onto subbed slides and allowed to dry. The slides were then submerged in 70% EtOH and 95% EtOH for 5 minutes each to dehydrate the sections. Lipid removal was performed by treating the slides with chloroform for 10 minutes and further dehydrated by submerging in 95% EtOH and 100% EtOH for 1 minute each. The slides were dipped in xylene for 2.5 minutes for lipid removal and embedding of the medium. Another sequence of EtOH submersion was performed to rehydrate the sections (100% and 95% at one and two minutes, respectively). The slides were then washed in distilled water and stained with Cresyl Violet (Sigma Chemical, St. Louis, MO) for one minute. The sections were washed in distilled water and placed in acetic formalin for 2.5 minutes before another sequence of alcohol submersion in 95%, EtOH and 100% EtOH for 10 and 15 minutes, respectively. The sections were placed in Xylene for another 20 minutes to further remove any lipids and make unstained portions transparent. The slides were then coverslipped with Cytoseal XYL mounting media (Richard-Allan Scientific, Kalamazoo, MI) to prevent brains from degradation.

**Contusion Size Analysis**

Contusion size was examined using cresyl violet stained cells to determine if the cortical lesion had been reduced in size, or replaced with regenerative tissue as a result of transplantation. Cresyl Violet stained sections were observed under a light microscope to determine the severity of the injury. Unstained sections that were used to examine surviving GFP+ cells were also used
to fill in gaps where Cresyl Violet stained sections did not produce useful tissue. To determine the size of the cortical contusion, remaining cortex was analyzed and quantified using the image-analysis computer program Neurolucida (Microbrightfield; Colchester, VT). For each animal, 6 individual sections that contained contusions were outlined and a total area was calculated by summing the sampled sections. To obtain a volume of remaining cortex, the total cortical area was multiplied by the space between sampled sections, i.e. 240µm.

**Analysis of surviving GFP+ cells**

Transplanted neurospheres were previously infected with a lentivirus harboring GFP that would cause BMSC-NICD cells to fluoresce green (See Experiment 1). One set of brain sections were mounted, dried and coverslipped with Flourosave (Calbiochem, La Jolla, CA) to prevent loss of fluorescence and degradation of tissue. GFP expressing cells were then visualized under a fluorescent microscope. GFP+ cells were analyzed by examining their fluorescence. If cells fluoresced green, the cells were considered viable GFP+ cells and counted using StereoInvestigator Software. GFP+ cells were counted in either cortex or striatum, depending on injection location. Additionally, the area of the transplant was measured using Neurolucida software by outlining the two-dimensional contour of the area that contained fluorescent cells in either the injured cortex or striatum. The area of the transplanted site measured in square millimeters.

**Analysis of neuronal loss in contusion area**

NeuN staining analysis was a measure of neuronal loss/neuronal number within the injured cortex that examined the effect of BMSC-NICD neurospheres on the injured cortex, and if they were capable of regenerating neurons. Neuron number was examined in the contusion area of the FL-SMC following transplantation of BMSC-NICD neurospheres Brain
sections were rinsed in Tris-Buffered Saline (TBS) and incubated for 10 minutes in 50 mM glycine in TBS and another 15 minutes in 0.3% H₂O₂. Sections were then rinsed in TBS+0.1%TX in between incubation periods and blocked in TBS+10% for one hour and incubated overnight at room temperature in primary antibody (monoclonal mouse anti-NeuN antibody (1:500, Vector Laboratories, Burlingame, CA). The following day, sections were incubated for 2 hours in secondary antibody (biotinylated goat anti-mouse IgG (1:250, Vector Laboratories, Burlingame, CA). Cells were visualized using fluorescent light microscopy following incubation in streptavidin-conjugated AlexaFluor 610 (1:500, Vector Laboratories, Burlingame, CA). Sections were rinsed and mounted with FluroSave.

Quantitation of NeuN+ cells was done using unbiased stereology, with the Optical Fractionator Method (Gundersen 1986) on a Leica fluorescent microscope equipped with a camera, motorized stage, and the StereoInvestigator program (Microbrightfield, Williston, VT). NeuN+ cells were counted in 6 layers of cortex containing the FL-SMC in CCI animals. Only cells that contained a defined nucleus and a well-defined cellular NeuN+ border were included in the cell counting. Fragments of NeuN+ cells were not counted. Six sections were measured between approximately A/P +2.2 and -1.5mm from bregma. The medial aspect of the counting area in each section began at the cingulum of the corpus callosum and extended laterally to approximately M/L +/-4.5. Using StereoInvestigator software an estimated total number of NeuN+ cells in the FL-SMC was obtained for each animal (counting frame size = 75µm X 75µm; section thickness 20-25µm; guard zones 3-5µm; CE <0.1).

**Statistical Analysis**
Behavioral data was analyzed with a repeated measures two factor ANOVA for Group and time post-injury. A single factor ANOVA was performed for the contusion size analysis, and for the NeuN analysis. All data are presented as mean±SEM.

RESULTS

Experiment 1 – Generation of Neurospheres from BMSC-NICD cells

Neurospheres Contain Neuroprogenitors

Bone marrow stromal cells isolated from adult rat were transfected with the gene for the Notch-1 intracellular domain and cultured in a free-floating culture system in the presence of growth factors (EGF and bFGF) to form neurospheres. Immunocytochemical staining and qualitative analysis demonstrated that BMSC-NICD-derived neurospheres consist of Nestin expressing neuroprogenitor cells. (See Figure 4) These neurospheres were used for transplantation in Experiment 2. As part of the thesis project, I spent time with Dr. Glavaski in the laboratory of Dr. Bohn learning how to prepare cells after conducting Experiment 2. When preparing neurospheres on my own, they still contained nestin+ progenitor cells, however the lentiviral infection was not optimal and the cells did not fluoresce green (See Figure 5). These cells were not used in Experiment 2.
Figure 4. Neurospheres Used for Experiment 2 Contain Neuroprogenitor Cells

Lentivirus harboring eGFP under control of a ubiquitin promoter was used to transduce adult rat BMSC-NICD cells that were subsequently used for the generation of the neurospheres. At 5 days in culture neurospheres were fixed and sectioned. Nestin staining was performed to examine whether cells within the neurospheres have neuroprogenitor properties. The majority of the cells within the neurospheres express neuroprogenitor cell marker nestin (red; A) and GFP (green; B). All cells in the neurosphere were counterstained with Hoechst (blue; C). Some of the cells within the neurospheres co-express nestin and GFP, and appear orange (E, F). (Images provided by Dr. Glavaski-Joksimovic).
Figure 5. Neurospheres Prepared After Experiment 2.

Neurospheres prepared by myself following Experiment 2 were not used for transplantation. Cells within neurospheres very weakly expressed GFP (B) due to the low level of lentivirus infection. The cells did contain healthy cell nuclei (Blue - C, D, & F) and expressed neuroprogenitor marker nestin (Red - A, D-F) (Images provided by Dr. Glavaski-Joksimovic).
Experiment 2 – Transplantation of BMSC following a Rodent TBI

Behavioral Analysis

Limb Use

The limb-use test is a measurement of weight-bearing movement while the animal explores its environment in a plexiglas cylinder. This test allows us to view any preferences in limb use following a unilateral injury to the FL-SMC, in which the animal typically prefers the use of its ipsilateral (uninjured) forelimb. Forelimb use preference was measured by examining with which forelimb the rat initiated and completed a rear and which forelimb the rat used to land following rear. Exploration of its environment was examined by noting which forelimb the rat preferred to use while moving vertically or horizontally in the plexiglas cylinder. Total number of ipsilateral forelimb use behaviors was compared against contralateral forelimb use. Preference was scored and analyzed by counting total instances of both ipsilateral and contralateral use and a percent was determined \((\frac{I-C}{I+C})*100\).

Total percent ipsilateral forelimb use was measured for all treatment groups and examined statistically using a two-factor repeated-measure ANOVA. The analysis revealed a significant effect of group \((F(2,8)=15.9 \ p<0.001)\); as well as a significant difference in total percent ipsilateral forelimb use over days \((F(2,16)=99.1 \ p<0.0001)\), and a significant day by group interaction \((F(4,16)=4.795 \ p<0.009)\). A post-hoc analysis using one-way ANOVA between days 5 and 12 post-injury revealed a significant difference by day 12 post-transplant \((p<0.05)\) indicating that the CCI produced a deficit and began to show recovery over time. Additionally a post-hoc analysis of group differences at day 12 showed that animals that received
BMSC-NICD neurospheres in either the striatum or cortex had a smaller preference for the ipsilateral forelimb than CCI only rats (p<0.05) (See Figure 6).

**Foot Fault**

The foot fault test was implemented in order to examine deficits in motor coordination following unilateral injury to the FL-SMC. Behavioral testing was performed on Day 0 to achieve baseline pre-injury behavioral analysis, which would typically show no motor coordination deficits between both forelimbs. Behavioral data was then collected on Days 5 (post-injury, pre-transplant) and 12 (post-injury and transplant). The rats’ coordination was analyzed by subtracting total ipsilateral (uninjured) foot faults from contralateral (injured) foot faults then dividing by total steps taken and determining a percentage to obtain total percent contralateral foot fault ([C-I]/total steps (50)]*100) (See Figure 6).

A two-factor repeated-measure ANOVA of all treatment groups was performed. The analysis revealed that there was no significant difference between treatment groups (p=0.10) and no significant group by day interaction was found (p=0.07). A significant difference was shown in motor coordination over days (F(2,16)=62.7 p<0.0001). A one-way ANOVA post-hoc analysis showed a significant difference between days 5 and 12 post-injury (p<0.05) indicating that the CCI produced an initial deficit followed by recovery. Additionally a post-hoc analysis of group differences at day 12 showed that animals that received transplants in either the striatum or cortex post-CCI had a smaller foot fault deficit than control (CCI only) animals at that time point (p<0.05).
Figure 6 - Improvement in Behavioral Deficits Post Transplant

Behavioral deficits in forelimb use for weight bearing movement (A) and forelimb coordination (B) was decreased post transplant (between days 5 and 12). At day 12 in both behavioral tests, rats that received transplants in either location had significantly smaller deficits compared to CCI only rats. *p<0.05.
Transplant Survival

To analyze survival of transplanted BMSC-NICD neurospheres, unstained sections were examined for GFP+ cells. Rats that received neurospheres in the striatum showed greater survival of transplanted cells (See Figure 7 A&C). BMSC-NICD neurospheres transplanted in the cortex were observed around the cortical contusion area. The majority of cells transplanted into the cortex at the contusion site appeared to be non-viable, fragmented cells with only one animal showing a cluster of healthy GFP+ cells (See Figure 7 B&D). Viable GFP+ cells were counted and transplant area was determined using Neurolucida software (See Table 2).

Table 2. Cell Count in Animals with surviving Transplant

<table>
<thead>
<tr>
<th>Animal #</th>
<th>Area (mm²)</th>
<th>Cell #</th>
</tr>
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<tbody>
<tr>
<td>09-27</td>
<td>48.5</td>
<td>24</td>
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<tr>
<td>09-28</td>
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<td>62</td>
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<tr>
<td>09-35</td>
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<td>123</td>
</tr>
</tbody>
</table>
Figure 7 - Transplanted cells survive one week post-transplantation.

Transplant survival was seen in both the striatum (A&C) and cortex (B&D) surrounding the injury in only a few animals. In one animal, two visible striatal injection sites can be seen in
panel C. Most cortical transplants surrounding the injury appeared as fragmented, non-viable cells (D).

**Cortical Volume**

Several 40 µm sections stained with Cresyl violet and unstained sections containing GFP+ cells were examined and analyzed using Neurolucida software to get an overall volume of remaining cortical tissue as a measure of contusion size. A one-way ANOVA revealed that the striatum and cortex groups did not demonstrate significant differences in contusion size as compared to the CCI control group (p=0.74). This suggests that the transplants did not affect the size of the cortical contusion. (See Figure 8).

![Remaining Cortical Volume](image)

**Figure 8 – Cortical Volume not Affected by Transplantation**  All 3 treatment groups had similar amounts of remaining cortex (i.e. similar sized contusions) at the end of the experiment.
Neuron Survival

The effect of contusion and transplant on neuronal survival was examined using stereological counting of NeuN stained cells in all treatment groups. Stereology software was used to quantify the number of NeuN positive cells within the Fl-SMC using 6, 40 μm sections per animal, generating a total estimated number of NeuN+ cells surrounding the injury site. A one-way ANOVA analysis was performed and showed that there was no significant difference between groups for NeuN positive cells \((F(2,7)=4.74 \ p=0.58)\) See Table 3.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell Total</th>
<th>Animal #</th>
</tr>
</thead>
<tbody>
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<td>09-25</td>
</tr>
<tr>
<td>Cortex</td>
<td>52877.19</td>
<td>09-27</td>
</tr>
<tr>
<td>Cortex</td>
<td>68701.75</td>
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<td>Cortex</td>
<td>64842.11</td>
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<td>Striatum</td>
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<td>Striatum</td>
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<tr>
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<tr>
<td>Control</td>
<td>33964.91</td>
<td>09-36</td>
</tr>
</tbody>
</table>

Table 3. Neuron Survival in Each Group by Animal Number
DISCUSSION

The purpose of this study was to examine the efficacy of transplanted neural precursors derived from adult rat BMSCs in a rodent model of TBI. The aim of the study was to examine the transplant survival in the injured brain, and whether transplant would provide therapeutic effect. This is the first study to transplant Notch-induced BMSC in a traumatic brain injury. The therapeutic potential of these cells has been examined in animal models of stroke and PD, but this project was at the forefront of demonstrating the therapeutic potential of these cells in a TBI. The results of the current study demonstrate the successful generation of neurospheres from BMSC-NICD. When transplanted, these neurospheres survive in a rodent model of TBI, with greater initial survival seen in striatally injected rats, as opposed to cortically transplanted rats. Transplanting neurospheres also resulted in initial marked improvement in behavioral deficits. However, transplantation of neurospheres did not have an effect on the contusion size and cortical volume or the amount of surviving neurons in the cortex.

Transplant Creation & Survival

Therapeutic effect of stem cells transplantation following TBI has been extensively studied for several years, with varying levels of success depending on type of transplant. Previous studies have examined hBMSCs, naive and genetically modified embryonic murine stem cells in rat TBI (Mahmood 2005, Kim 2010, Riess 2007, Hoane 2007). Naive and genetically modified adult BMSCs (Mahmood 2001, Lu 2002, Opydo-Chanek 2007); and combination therapy with adult BMSCs and chemical/drug treatments (Mahmood 2008, 2007) have also been examined as a therapeutic approach,
Kim et. al. examined the effects of undifferentiated human MSCs (hMSCs) in a rat model of TBI because of the MSCs’ ability to support formation of neuronal cells, and to secrete different growth factors. Functional improvement was seen as early as 14 days, but hMSC differentiation to neuronal cells was minimal, indicating an environmental change induced a neurorestorative effect. Similarly, a study from Riess et al (Riess 2007) has shown that undifferentiated murine ES cells transplanted in a rat model of evoked behavioral recovery at one week following transplantation. Moreover, at one week post-grafting, a significant number of surviving cells were detectable in 100% of the injured animals (Riess 2007). However, at 7 weeks post-transplantation, cells were seen in only one injured animal and two of the injured animals had tumor formation (Riess 2007). Cells did not differentiate and many were engulfed by macrophages at the lesion site due to the inflammatory response, leading to the decrease in viability and survival (Riess 2007).

Although the transplant type has varied over the years, the results remained similar. Tumor formation, inability to differentiate into neurons, inability to integrate into host tissue and decreased transplant survival over time remain a significant challenge of many of the transplants studied. However, some trends have emerged through the years, with pre-differentiated neurons or neuronal precursors derived from stem cells, whether embryonic or adult, demonstrating greater survival and integration into host tissue than undifferentiated cells (Hoane 2004). When examining the therapeutic potential of naive BMSCs in a traumatic brain injury, there was evidence that, in some cases, less than 10% of the cells survived (GFP+) and only 1-2% of these cells differentiated to neuronal lineage (Chen 2006). However, a novel approach using neurospheres generated from bone marrow derived neuroprogenitor cells (BMSC-NICD) gave very promising results in a
rodent model of stroke (Hayase 2009). These BMSC-NICD neurospheres had the unique ability to survive, distribute and integrate into the host brain at 100 days post transplant with evidence of behavioral recovery and positive neuronal markers (Hayase 2009, Glavaski-Joksimovic 2009). These bone marrow neuronal progenitor cells (BMSC-NICD) showed differentiation into neurons in vitro (Dezawa et al 2004) and expressed markers for neurotransmitter release in an in vivo stroke model (Hayase 2009). We hypothesized that similar results would occur in a model of TBI, and that these bone marrow neural progenitor cells would have better survival rates than previously tested naive BMSCs, which have little evidence of differentiation and survival post-transplantation in TBI (Vaquero 2010). In our study, we demonstrated that we could generate neurospheres from BMSC-NICD and that these neurospheres contained neural progenitor cells. When transplanted into either the cortex or striatum seven days following a CCI, we were able to show transplant survival. However, survival of transplanted BMSC-NICD was not as robust as when these neurospheres were transplanted in a stroke model (Hayase 2009). Survival was visualized in 3 transplanted animals with GFP+ cells expressed at day 14 following the injury (one week post-transplantation). In a study from Hayase et al (Hayase 2009), when neurospheres were transplanted into the cortex and striatum in a rat stroke model, surviving cells were seen at 100 days following transplantation. In addition, surviving cells expressed neuronal and neurotransmitter markers and had projections that synapsed with host neurons (Hayase 2009). In the same study, the neurosphere transplant group had a significantly greater survival rate than the naive MSC group. While we did not assess synaptic activity with neurotransmitter markers, nor determine what our stem cells differentiated into, we did demonstrate that in an environment of TBI, neurospheres can
survive at least out to one week post-transplantation. With established initial survival of neurospheres following TBI, neuronal differentiation of surviving BMSC-NICD cells and their ability to release neurotransmitters can be determined in future, long-term studies.

In addition to demonstrating transplant survival, our study sought to examine whether transplants survived better when injected around the contusion/injured area or into the subcortical striatal target. Transplanting at the site of the lesion is a challenge due to the pathophysiology of the injured region of the brain and secondary traumatic effects associated with the inflammatory response, damage to the blood-brain barrier, excess in free radicals and glutamate, and ionic disproportions due to an influx of sodium and calcium into host neurons (Park 2008). This has driven researchers to evaluate different locations for transplantation, and was the reasoning behind two transplantation sites in our study. Our findings showed better survival rates when neurospheres were injected into the striatum rather than the injury site at the cortical region. In previous transplant studies, transplants were typically localized to either the injured cortex (Hoane 2004, Riess 2007), or placed in both the cortex and striatum in the same animal (Mahmood 2001). In Hoane's study, the transplanted neuronal and glial precursors were qualitatively assed by examining GFP-positive cells in the injured cortex (Hoane 2004). In this study, cells survived and were viable at day 14, while animals injected only with media showed signs of necrosis (Hoane 2004). Moreover, this same study demonstrated the migration of cells injected at the periphery of the lesion to subcortical targets such as the striatum, where cells were able to survive. This suggests survival in subcortical targets may be preferential, and aligns with our study in which we saw a qualitatively better cell survival rate in the striatum as opposed to the cortex. Perhaps injecting into the striatum is the preferential
transplant site if cell migration from the cortex to the striatum is seen and previous studies have shown improved cognitive recovery when injecting into the striatum (Watson 2003). In Hayase’s study (Hayase 2009), transplants were put in both cortex and striatum in a rat stroke model. Cell survival was observed in both the cortex and striatum in the stroke model. At day 100, 21x10^4 cells survived with proliferation, differentiation and migration between the ipsilateral cortex and striatum. A study from Mahmood et al also has shown survival of transplanted cells in the striatum and cortex with cells migrating between the lesion and subcortical sites. However, lesion/cortex versus striatal survival was not examined. Our study demonstrated evidence of better survival and differentiation in the striatum, as opposed to the cortex. This differs from Hayase’s study, which is not surprising since traumatic brain injury has detrimental secondary effects and inflammatory responses following brain injury at the lesion site. Our findings are reinforced by previous research that suggests the striatum supports neuronal differentiation of donor precursor cells (Catapano 1999). Hoane’s study also demonstrated the migration of cells injected at the periphery of the lesion to subcortical targets such as the striatum, where cells were able to survive, indicating that the striatum is a significant area for transplant survival studies (Hoane 2004).

Hoane et. al. also demonstrated significant decrease in lesion size when compared to media-only injected animals in his study that examined the therapeutic potential of murine embryonic stem cells (neuronal and glial precursors) (Hoane 2004). This is not consistent with our studies that showed no statistically significant difference between groups.
Behavioral Effects of Neurosphere Transplantation

In addition to examining whether BMSC-NICD neurospheres would survive in the brain following TBI, the current pilot study sought to examine whether these neurospheres could produce some initial benefit to behavioral recovery. Our study showed marked behavioral improvement in the first week following transplantation. This improvement was seen in both measures of forelimb behavior (foot fault & limb use) and it was similar regardless of the site of transplantation. Similarly, transplantation of BMSC-NICD neurospheres in a rat stroke model (Hayase 2009) evoked behavioral recovery as early as 14 days following transplant. This evidence aligns with our findings of functional and behavioral recovery occurring as early as 12 days following injury (5 days post-transplant). Hoane’s research has shown a functional sensorimotor improvement following his transplant studies, but did not show a cognitive improvement, which is a trend seen in transplant studies following TBI (Hoane 2004, Mahmood 2008). Riess’ study in 2007 also examined functional recovery, and observed an improvement that was seen over a 6 week period (Riess 2007). These papers did not explore the correlation of functional recovery to transplant site since they transplanted either at the lesion site or in both the cortex and striatum.

The exact mechanisms of effects of transplants on early behavioral recovery are not entirely understood and were not fully explored in this pilot study. The current study examined behavioral recovery using the limb-use and foot fault assays, and functional recovery was observed regardless of transplant site and number of surviving GFP+ cells. This study also examined whether transplantation affected the size of the contusion and
the survival of neurons surrounding the contusion site. Transplantation in either site did not significantly change the size of the injury nor did it alter neuronal survival post-injury.

We did not observe a statistically significant difference between groups in NeuN number or volume of remaining cortex that would indicate increased cell survival at the lesion site. The contusion area was devoid of cells regardless of transplant and qualitatively shows a lack of neuronal cells at the contusion site at the time of sacrifice in all 3 groups. With our data not supporting a neuroprotective mechanism, we agree with previous researchers’ conclusion of trophic factors playing a role in behavioral recovery following stem cell transplantation. The current pilot study supports the influx of trophic factors leading to behavioral recovery by the animals’ behavioral tests results showing paralleled improvement regardless of transplant site and survival (cortex vs. striatum), and both of these groups had significantly better recovery at day 12 than injured animals with no transplant at day 12. The fact that the transplant does not replace neural tissue at the time of functional recovery reinforces the trophic factor postulate. The nonlinear relationship between a decrease in contusion size and increased functional recovery supports the fact that the transplant has not yet integrated into the existing circuitry and tissue has not been regenerated, rather trophic effects may be halting further degeneration and restoring functionality. Hawryluk et al demonstrated an influx in neurotrophic factors following BMSC transplantation in a spinal cord injury model. BMSC transplantation upregulated nerve growth factor (NGF), leukemia inhibitory factor (LIF), and insulin-like growth factor 1 (IGF-1) with a significant reduction in platelet derived growth factor A (PDGF-A) (Hawryluk et al 2012). These findings are novel, as this is one of the first studies to examine trophin expression in vivo following BMSC transplantation. While it is not specific
to TBI, it is a representation of trophins expressed following BMSC transplantation in a CNS injury model. It is suggested that these trophic factors may mediate the inflammatory response following injury. Since these trophins are present following injury alone and upregulated by BMSCs, they may be further dampening the immune response as compared to the normal immune response in a CNS injury (Hawryluk 2012).

As noted in Hayase’s study, initial behavioral recovery was also seen as early as within 14 days following transplant, but this was long before cell differentiation and integration into existing neural structure occurred (Hayase 2009). This is strong evidence that neurotrophic factors have played a role in functional recovery and perhaps play a role in neuroprotection. Previous studies that identified neurotrophic factors such as nerve growth factor, brain-derived neurotrophic factor and neurotrophin-3 as early as 2 days following transplantation of hMSCs (Kim 2010). The rapid influx of these trophic factors may create an initial recovery effect that reduces apoptosis and necrosis and leads to almost immediate behavioral recovery. This notion is further supported by the fact that integration of transplants into host circuitry was not seen until 100 days following transplantation, but recovery was seen long before in Hayase’s stroke model.

In addition to trophic factors, it is likely that the BMSC-NICD neurospheres dampened the immune response associated with TBI. In a study by Dao et al, human MSCs were transfected with a plasmid containing the Notch intracellular domain gene and examined for their immunosuppressive potency. The findings concluded that the MSCs transfected with NICD attenuated activation of CD4+ T cells, and this was evident in the reduction of CD69 and HLA-DR, two T cell activation markers. The efficiencies of the MSCs transfected
with NICD were comparable to that of parental MSCs, which are known to have a more immunosuppressive effect than naïve MSCs (Dao et al 2011).

Future, long-term studies will better address the role of trophic factors in BMSC-NICD neurospheres-induced recovery following TBI.

**Conclusions**

In our pilot studies, neurospheres generated from adult rat BMSC-NICD cells were transplanted into the traumatically injured brain. Transplanting in subcortical targets appears to be a better site for cell survival based on our findings. Behavioral improvements, however, were seen regardless of transplant site. Therefore, transplanting in the striatum may ultimately produce long-lasting results. Future studies should examine longer time points in both the cortical and striatal transplant sites to determine how long these cells can survive in the TBI climate. Use of different controls should also be examined, such as transplanting vehicles and dead cells, and utilizing sham animals. Future studies should also examine the potential of restoring cognitive function through the use of memory tests. Through this pilot study and future studies, we will attempt to offer a better alternative to controversial embryonic stem cell transplantation. Our aim is to provide a practical means of obtaining autologous BMSCs that can be harvested and genetically modified before transplantation into the traumatically injured brain, and ultimately improve behavioral function.
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