MESENCHYMAL STEM CELL DELIVERY, SURVIVAL AND MIGRATION IN A NON-INVASIVE SYSTEM FOR CENTRAL NERVOUS SYSTEM THERAPEUTICS

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Kayla C. Horton

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Department of Biological Sciences
Abstract

of

MESENCHYMAL STEM CELL DELIVERY, SURVIVAL AND MIGRATION IN A NON-INVASIVE SYSTEM FOR CENTRAL NERVOUS SYSTEM THERAPEUTICS

by

Kayla C. Horton

The central nervous system (CNS), consisting of the brain and spinal cord, is a complex network of neuronal and non-neuronal cells that are responsible for sending, receiving and interpreting information from the entire body. Neurodegenerative disease is a general term used for a wide range of acute and chronic conditions that involve neuronal cell death in the CNS. Current treatments are limited to conventional approaches that primarily treat symptoms. The potential for therapy of end-stage CNS degradation is limited, but previous studies have revealed extensive therapeutic windows, prior to the chronic injury phase, that would allow for potential manipulation by exogenous interventions.

Human mesenchymal stem cells (hMSCs) have ideal characteristics that grant them clinical potential for cell therapies. They are easily harvested, from healthy bone marrow donors, and manipulated in culture. MSCs have been shown to provide trophic support, lack immunogenicity, and are capable of self-renewal. They also possess the innate ability to be recruited to a tissue lesion by way of chemotactic machinery. The receptor-ligand binding that occurs during this “homing” process initiates inner cellular processes that lead to enhanced production and secretion of growth factors, cytokines,
and chemokines. Chemokine stromal cell-derived factor-1 (SDF-1), which is expressed at a wound, interacts with its cognate receptor CXC chemokine receptor 4 (CXCR4), expressed by MSCs, and is an important factor in MSC chemotaxis. The MSC secretome, most notable, brain-derived neurotrophic factor (BDNF), has the potential to contribute to the minimization of cell death and reduction of lesion expansion.

Cell-based and gene-based therapy ideology must converge for an efficacious therapy due to the low regenerative capacity of the CNS. The challenges of the mode, timing and location of cell delivery must be further considered. There is potential for an intranasal administration (INA) method due to the unique olfactory region that provides interface between the CNS and the external environment. The current modes of cell delivery are either invasive or inefficient. INA ideology provides a relatively non-invasive method that remains to be controversial by several groups interested in its clinical potential. This study advances upon the knowledge from previous INA studies by (1) the development of a novel device for nasal delivery efficiency, (2) tracking the location and viability of the transplanted hMSCs by bioluminescence (in vivo), as well as, by fluorescence (in situ), and (3) transfecting the hMSCs with a CXCR4 construct to enhance migration to the target tissue. The future steps of this study will be to double transfect hMSCs with a CXCR4-BDNF construct to exploit chemotaxis and enhance the therapeutic potential of the cells for neurodegenerative diseases and injuries.

____________________________________, Committee Chair
Rosalee Sprowls, Ph.D.

____________________________________
Date
DEDICATION

To my brother Ben; I will forever be inspired by your relentless defiance of the odds and your tenacity to live life with great purpose. The impact you have made in my life is immeasurable.

To my brother Mike; you are a brave advocate for all of those who fight against the story written by their genetics. Your participation and dedication to the “Fight for a Cure” knows no bounds, and I am proud of the changes you are making in the world of cell therapy.

To my mom and dad; I have always felt unconditionally loved and understood, even when I didn’t deserve to be. Thank you for all of your support and sacrifice on this long journey to find my passion. I will forever be grateful to you both for the lessons you have taught me about the importance of knowledge.

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To the love of my life; I found my hearts home when I found you. Thank you for selflessly supporting me in my crazy endeavors. You are the yang to my yin.
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LIST OF ACRONYMS AND ABBREVIATIONS
(IN ALPHABETICAL ORDER)

6OHDA – 6-hydroxydopamine (Oxidopamine)
aCSF – Artificial Cerebrospinal Fluid
BBB – Blood Brain Barrier
CSF – Cerebrospinal Fluid
CX3CR1 – C-X3-C Motif Chemokine Receptor 1 (Fractalkine receptor)
CX3CL1 – C-X3-C Motif Chemokine Ligand 1 (Fractalkine)
CXCL12 – C-X-C Motif Chemokine Ligand 12 (Stromal Cell-Derived Factor 1, SDF-1)
CXCR4 – C-X-C Motif Chemokine Receptor Type 4 (Fusin or CD184)
DAPI – 4, 6-diamidino-2-phenylindole
DI – Direct Injection
DPI – Days Post-injury
GFP – Green Fluorescent Protein
hMSC – Human Mesenchymal Stem Cell
IA – Intra-arterial
INA – Intranasal Administration
IP – Intrapleural
IV – Intravenous
IVIS – In Vivo Imaging System
LUCI – Luciferase
NSG – Non-obese diabetic (NOD) Severe Combined Immunodeficient (scid) gamma
PBS – Phosphate Buffered Saline
PD – Parkinson’s Disease
RMS – Rostral Migratory Stream
SCI – Spinal Cord Injury
SDF-1 – Stromal Derived Factor 1
SQ – Subcutaneous
TBI – Traumatic Brain Injury
1. INTRODUCTION

Neurodegenerative disease is a general term used for a wide range of acute and chronic conditions that involve neuronal cell death in the central nervous system (CNS, brain and spinal cord). Death of neurons and glia cause a variety of symptoms and pathologies that are unique to the specific disease, but all result in some type of functional loss, such as, decline in movement or cognition. Some of these conditions are caused by the inheritance of gene mutations, for example Alzheimer’s disease, amyotrophic lateral sclerosis (ALS) and Parkinson’s disease (PD), but can also occur sporadically. Others, such as Huntington’s disease, are acquired in an entirely genetic manner. Neurodegeneration also occurs as a consequence of an external physical insult to the CNS resulting in a traumatic brain injury (TBI) and/or a traumatic spinal cord injury (SCI).

There is a complex interplay between innate and environmental factors, which may regulate the outcome of acute CNS injury and progression of chronic neurodegeneration (53). Resident CNS cells generate inflammatory mediators, in response to disease or injury, that play an important role in acute and chronic CNS disorders. Proinflammatory cytokines, prostaglandins and free radicals are secreted throughout the damaged CNS tissue and initiate an influx of immune cells, activation of resident glial cells and induce chemokines and adhesion molecules (3, 53). The inflammatory response is an immediate and critical innate response to biological system impairments, but chronic, or prolonged inflammation, can alone result in damage to the tissue.
It is estimated that a minimum of 1 million people in the United States and 6 million people worldwide suffer from Parkinson’s disease (PD) (51). It is a chronic and progressive neurological disease that results in the gradual loss of dopamine producing brain cells, particularly within the substantia nigra. Dopamine is the chemical messenger of the brain that is responsible for modulating movement by assisting in the transmission of electrochemical signals from neuron to neuron (57). The cell bodies of dopaminergic neurons reside in the substantia nigra and project their fibers to the striatum by way of the nigrostriatal pathway. As these neurons begin to die the communication between the two brain centers is broken and results in symptoms such as tremors (trembling in hands, arms, legs and face), rigidity (stiffness), bradykinesia (slowness of movement), and impaired balance (52). Due to the unfortunate fact that the cause of the neural degeneration in PD is unknown, there have been serious efforts made to curb the onset and progression of PD (56).

Studies of parkinsonian brains have revealed that there is a significant decrease in important nervous system growth factors (neurotrophins) in the substantia nigra (specifically in the nigrostriatal dopamine regions), when compared to that of control brains (57). The concentration of important neurotrophins, brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), were most notable and could play an important role in the pathogenesis of PD (51, 57). BDNF and NGF are important to survival and differentiation of neuronal cells, so this deficiency seen in parkinsonian brains has lead to the ideology that dopamine neurons are not being protected from degeneration by endogenous neurotrophins (57).
Neurodegeneration can also occur by an external insult that causes an injury to CNS tissue, such as a spinal cord injury (SCI), which affects approximately 2.5 million people worldwide with an estimated 130,000 new cases reported annually (21). People who suffer from a SCI struggle with additional lifelong medical complications, such as blood clots, kidney failure, pressure sores, and breathing problems (23). With medical advancements such as mobility equipment, antibiotics and aggressive rehabilitation, the quality of life and life expectancy have slightly improved, but the lifetime health maintenance costs for a single United States SCI patient, $1.1 – $4.3 million, calls for a more innovative therapy (23).

Spinal cord damage consists of both primary injury mechanisms (the initial mechanical insult) and secondary injury mechanisms, such as the vascular and biochemical responses to injury (22). Physical trauma to the spine, such as a fracture or dislocation, subjects the spinal cord to harmful pressure (20). The immediate repercussions to the site of origin, includes severed neurons, acute cell death and hemorrhaging, leading to the initiation of the secondary injury mechanisms (22). The secondary (acute, sub-acute, and chronic) injury can be distinguished by an extended lesion due to the vascular and biochemical effects of the self-propagating cycle of the inflammatory response (22, 24). The acute damage to the cord causes the innate wound-healing cascade to commence by hemostasis. Degranulation of platelets and activation of resident macrophages generates a cytokine-rich wound site, promoting the recruitment and chemotaxis of immune cells (20, 22).

While inflammation is critical to remove cellular debris that is capable of causing damage to surviving neurons, a prolonged immune system is capable of exacerbating the injury (22).
The recruitment neutrophils, monocytes (macrophages), microglia, and T-lymphocytes starts just minutes following the primary SCI and usually last up to several months (25). These cells secrete pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β, as well as generate free radicals by metabolic activities (25). The excessive existence of a wound environment mediates cell death and inhibits axonal growth in the disseminating area of compromised spinal tissue. Continuous apoptosis, specifically of oligodendrocytes and glial cells, produces myelin and glial scars extending far outside of the original location of trauma (25). The consequence of the prolonged activation of the immune system, during the secondary SCI, is ultimately a complete lesion or chronic injury. The chronic phase of the injury can continue for months, to years, after the primary SCI (22).

Current treatments of such CNS impairments, such as PD and SCI, are limited to conventional approaches that primarily treat symptoms; therefore, in order to develop a more targeted treatment, an understanding of the processes involved in the degradation of CNS tissue is essential. Pharmaceuticals, such as steroids, analgesic, and antibiotics, along with physical therapy techniques are of the most commonly used tactics for reducing symptoms of injury and disease. History has indicated that these pharmaceuticals and therapy techniques, must be started at early stages of CNS degradation in order to be efficacious, and will be continued throughout the lifetime of the patient (15, 20, 45, 46).

The potential for therapy of end-stage CNS degradation is limited, but previous studies have revealed extensive therapeutic windows, prior to the chronic injury phase, that would allow for potential manipulation by exogenous interventions (20, 21). The
further understanding of the CNS wound environment has led to a massive movement of
research to develop cell therapies to achieve neuroprotection and neuroregeneration
(neurorepair) to degenerating or damaged CNS tissue. Neuroprotection refers to the
inhibition of CNS parenchymal cell death, as well as the secondary neuronal cell death
that occurs in a chronically injured or degenerative CNS (15). Neuroregeneration
(neurorepair) refers to the stimulation of endogenous neural progenitor cells, regeneration
of severed axons or sprouting of intact axons to injured sites, and the replacement of lost
neuronal cells (15).

Human mesenchymal stem cells (hMSCs), also known as multipotent
mesenchymal stromal cells, have ideal characteristics that grant them clinical potential
for cell therapies necessary to encourage neuronal protection and repair. MSCs were first
discovered to co-exist with hematopoietic stem cells (HSCs) in the bone marrow, but they
have since been identified in other tissue sources, such as, umbilical cord blood,
periosteum, synovial fluid and adipose tissue (7). These cells maintain the ability to self-
renew and the potential to differentiate towards lineages of mesenchymal origin, for
example, adipose, bone, cartilage, connective tissue, and smooth muscle (6).

For therapeutic and research purposes, MSCs are harvested from healthy human
bone marrow donors, and are easily isolated, expanded and manipulated in culture. An
established MSC culture may express a variety of cell surface antigens (ie; CD73, CD90,
and CD105) that can vary due to the source of isolation and culture conditions (6). An
important characteristic, from a therapeutic perspective, of MSCs is their innate ability to
evade the immune system, or their lack of immunogenicity (4). This is due to the low to
undetectable MSC expression of histocompatibility complex (MHC) class I and class II surface molecules (4). This results in MSCs having a low immunophenotype that protects them from detection and subsequent rejection by the host immune system (55). The low immunogenicity of MSCs is a powerful quality to consider for therapeutic purposes because it permits allogeneic, as well as autologous, transplantation of large numbers of cells without the need of extensive tissue matching or use of immunosuppressive medications (4).

MSCs also possess the innate ability to be recruited to a lesioned tissue by way of chemotactic machinery. Chemokines are small peptides that have the basic function of inducing the recruitment and chemotaxis of cells with the corresponding chemokine receptors. Chemokine receptors including CXCR4, CX3CR1, CXCR5, CXCR6, CCR1, CCR7 and CCR9 are expressed by MSCs and are responsible for the directional migration that occurs by the cell when the receptors come into contact with their cognate ligands (14). Injured CNS tissue has been reported to express monocyte chemoattractant protein-1 (MCP-1), stromal cell-derived factor-1 (SDF-1) and fractalkine (CXCL1) that generates a gradient radiating from the epicenter of the degenerating tissue (4, 10, 14). The receptor-ligand binding that occurs during this “homing” process initiates inner cellular processes that lead to enhanced production and secretion of growth factors, metabolites, cytokines, chemokines and bioactive lipids (7).

Stromal cell-derived factor-1 (SDF-1), which is expressed at a wound, interacts with its cognate receptor CXC-motif chemokine receptor 4 (CXCR4), expressed by MSCs, and is an important factor in MSC chemotaxis (26). It has been observed that
ischemic and hypoxic tissues have an enhanced production of SDF-1, and will create a significant gradient of this chemoattractant molecule for the recruitment of CXCR4-positive cells (28). Endogenous bone marrow MSCs will home to the site of injury by CXCR4 receptor – SDF-1 ligand binding, which has been shown in bone fractures, kidney ischemia and neurodegenerative diseases of animal models (26). CXCR4 is a G-protein coupled receptor that, once bound by SDF-1, can activate the Phosphatidylinositol 3 Kinases (PI3K) pathway. All of the PI3K isoforms in the pathway can actuate Ras-related C3 botulinum toxin substrate 1 (Rac1), which is a GTP binding protein that is important for cytoskeletal reorganization for motility (27). CXCR4/SDF-1 axis is predominately at the leading edge of the migrating MSC, allowing for the stabilization of lamellipodium (cell projections), and encouraging MSC production and secretion of microvesicles that will deliver proteins to neighboring cells (28). The MSCs release of microvesicles containing proteins, such as CXCR4, plays a significant role in recruiting other MSCs for migration and tissue repair (26).

Extensive animal model and MSC transplantation studies have shown that a MSC graft does not incorporate into the damaged CNS tissue by differentiation, but rather offers innate trophic support (8, 9). This alternative view of the use of stem cells in regenerative neurology is known as the paracrine hypothesis, and compelling evidence such as enhanced myelin repair, reduced lesion volume, decreased number of activated microglia, and improved motor function impairments, have proved to be dependent on the MSC secretome (7). Key components of the MSC secretome, that are thought to be responsible for these previously observed therapeutic effects, are hepatocyte growth
factor (HGF), fibroblast growth factor (FGF)-II, platelet-derived growth factor (PDGF)-A/B, as well as brain-derived neurotrophic factor (BDNF) (7). While they all have the potential to contribute to the minimization of cell death and reduction of lesion expansion, much attention is focused on BDNF for CNS disease, such as PD, and injury, such as that of SCI.

BDNF is apart of a family of small proteins, known as neurotrophins that elicit cell survival, differentiation and growth, and it is abundantly expressed in the central nervous system, specifically in the hippocampal formation, cerebral cortex and amygdaloidal complex (32, 33). Neurotrophins, or nervous system growth factors, bind selectively to their tyrosine kinase receptor (TrK) and non-selectively to a 75 kDa neurotrophin receptor (p75NTR), resulting in neuronal responses (21). BDNF endogenously modulates neuronal differentiation and survival, as well as synapse plasticity and transmission in various regions of the central nervous system (31).

There is an established understanding of the mechanisms of BDNF expression, secretion and signaling. BDNF mRNA expression is regulated by several neurotransmitters and hormones, including acetylcholine, serotonin, nitric oxide, thyroxine, glucocorticoids and sexual steroids (33). Mature BDNF is released from neurons and glial cells both constitutively and in a regulated manner (34). The immature form of BDNF protein can be stored in intracellular vesicles localized in the cells appendages, and can be cleaved to the mature protein in an activity-dependent manner based upon calcium-influx (35). Once BDNF is in its mature form it can be secreted from the cell into the extracellular matrix (35).
A vast array of studies have focused on the therapeutic effects BDNF has on the progression of PD and the severity of SCI, as well as many other CNS neurodegenerative models, such as HD, ALS and stroke (4, 5, 7, 8, 11, 13, 16, 17, 32). A study by McCoy et al used histology, electrophysiology and gene expression techniques to determine that MSC secretion of BDNF facilitated the rescue of nigrostriatal function in a 6-OHDA PD mouse model (7, 37). The secretion of BDNF indirectly modulated the oxidative stress caused by the development of the 6-OHDA experimental PD (7,37). Additionally a study, by Lu et al., injected MSCs transduced to over-express BDNF directly into an injured spinal cord in a rat SCI model and compared the effects to a group of SCI rats that was injected with unaltered MSCs (38, 39). The work with BDNF-MSCs was successful in showing that the transduction markedly increased the production and secretion of BDNF, as well as significantly increasing such therapeutic effects as axonal growth (38, 39, 40).

In order to achieve an effective regenerative treatment for complex CNS degradation, it is thought that cell-based and gene-based therapy ideology must converge, primarily due to the low regenerative capacity of the CNS (18). Genetically engineered MSCs have been used extensively in animal models of tissue repair and there is sufficient evidence of their safety and efficacy (2). The integration of a viral vector, along with the appropriate inducible promoters, allows for controlled production and secretion a vast array of therapeutic molecules (ie; drugs, proteins, RNA) at supraphysiological levels, as well as, the ability to program the MSC to increase the expression of specific chemokine receptors to enhance chemotaxis (4, 12, 14). This programming of the cell to produce and
secretes a therapeutic, as well as the cell’s ability to home to a site of injured, became increasingly intriguing to researchers when it was observed that MSCs are able to cross the blood brain barrier (BBB), without adverse effects (13). This is a powerful quality that presents the potential to overcome the BBB, which is the current clinical hurdle of delivering drugs to the CNS. As such, MSCs provide a safe and effective platform for the delivery and sustained production of therapeutics across the BBB and into the CNS, which is thought to have a far greater effect than that of direct administration of therapeutics, which are readily degraded in the wound environment (19).

Two decades of animal studies and clinical trials provide insight into the safety and efficacy of using MSCs as therapy (4, 16, 17, 40). A clinical trial, conducted by Mazzini et al, for the treatment of ALS using MSCs, resulted in the knowledge that the infusion of MSCs into cerebrospinal fluid was tolerated well and did not result in any adverse effects (4, 16). Multiple clinical studies have also been completed, with success, and without adverse events, by the systemic infusion as well as by the direct injection (into the brain/spinal cord) of MSCs (4, 17). Direct delivery of genetically modified hMSCs, for the therapeutic delivery of neurotrophins, to animal model acute spinal cord injuries has resulted in promising improvements in axonal regeneration, lesion size and functional outcome (40).

Insults to the microenvironment, such as hypoxia (low oxygen) or ischemia (low blood supply), both being characteristic of a CNS wound, are capable of modifying MSC expression patterns and levels of proteins, such as chemokine receptors (CXCR4) and therapeutic proteins (BDNF) (29, 36, 41). Differences in expression have been reported
in numerous studies, and could be a possible explanation as to the variance in migratory
tendencies of MSCs transplanted after *ex vivo* expansion (10, 29 36, 41). The common
laboratory conditions for MSC cultures involves ambient oxygen concentration, of about
20% (normoxia), however MSCs natural niche is in environments
with oxygen ranging from 2%-9% (hypoxia) (29, 41). When the cell is in a hypoxic
environment Hypoxia-Inducible factors (HIFs) will respond to the change in oxygen by
regulated differential gene expression (29). Previous studies have shown that the biology
of MSCs, notably chemokine receptor (ie: CXCR4) transcription and expression, is up-
regulated by hypoxia-inducible factor-1 (HIF-1) during *in vitro* hypoxic preconditioning
(REF 29, 41). Therefore, it is thought that if hMSCs are preconditioned, *in vitro* by
hypoxic culturing, following genetic modifications (ie: lentiviral transduction) to enhance
CXCR4 expression, it will result in optimized MSC homing to the injured tissue in vivo,
as well as optimize MSC viability (29, 30, 41).

While the combinatory approach of cell and gene therapy appears to be
promising, additional challenges of the mode, timing and location of delivery of a stem
cell-based treatment remains the challenge (18, 20). Systemic delivery and local (direct)
delivery are the two methods of introducing cells to the body (42). It is important that the
MSC delivery method is optimal for the type of injury/disease of study in order to
achieve optimal therapeutic effects (42). Intravascular injections, intravenous (IV) and
intra-arterial (IA), are the systemic methods of delivering cells and they are advantageous
because they are minimally invasive, with little to no risk to the patient (42). There are
significant efficacious downfalls to these methods, as they expose the entire body to the
treatment and result in very low cell engraftment at the targeted site. IV administration requires the cells to pass through the lungs prior to being distributed throughout the body, and a phenomenon known as the pulmonary “first-pass” effect (cells trapped in lungs) has been repeatedly observed (42, 44). Administration of cells by way of IA administration allows the cells to bypass the lungs, at least once, resulting in a slightly larger number of cells capable of homing to the target tissue (REF 42, 44).

Local delivery techniques into CNS tissue include, direct injection into the tissue (brain/spinal cord) or an intrathecal (most commonly the arachnoid membrane of the brain or spinal cord) injection (42). Intrathecal injections are currently the easiest and safest method of local delivery, mostly because it does not require the invasive traversing of nervous tissue, that direct injection requires, thus minimizing the associated risks that arise from needle placement and administration of the drug/cell therapy (42). These risks occur at low incidence rates and include, but are not limited to, infection, hematomas, nerve trauma, dural punctures and air embolisms (42). Although intrathecal injections are commonly used for administration of drugs the risks associated should not be ignored (45, 46).

The unique innate ability of MSCs to home to inflamed, ischemic and hypoxic tissues, could elicit a relatively non-invasive delivery method of MSC therapeutics for neurodegenerative diseases (4, 30, 45, 46, 47, 48). There is potential for an intranasal administration (INA) method of hMSC therapeutics for CNS therapy due to the unique olfactory region that provides interface between the CNS and the eternal environment. With the risks and associated off-target effects of the current modes of delivery, the INA
ideology provides a relatively non-invasive, nontoxic delivery method, which must be further explored to optimize cell migration and engraftment (45-50).

The INA philosophy has been effective in stirring up controversy over experimental design, data analysis and conclusions drawn from the original INA study done by Danielyan et al, published in 2009 (45-50). The Danielyan group hypothesized that INA MSCs could migrate through the BBB by way of the nasal mucosa, through the cribiform palate along the olfactory neural pathway (45, 46, 50). To facilitate MSC migration through the BBB, they adapted the use of hyaluronidase, an enzyme that cleaves hyaluronic acid (a component of the BBB extracellular matrix), because it has been shown to transiently permeabilize the BBB in a pneumococcal meningitis study (45, 46, 49, 50). They used fluorescently labeled rat MSCs in a mouse and concluded that there was successful migration of cells to the brain. The study was repeated, in 2011 by Danielyan et al, in a unilateral 6-OHDA experimental PD rat model using enhanced green fluorescent protein (EGFP)-MSCs isolated from a transgenic rat (46, 50). The data from this study resulted in the conclusion that EGFP-MSCs successfully migrated into the CNS and were detected by immunofluorescence in the olfactory bulb, cortex, hippocampus, striatum, cerebellum, brainstem and spinal cord (50). Furthermore, the group reported that INA of EGFP MSCs reduced the 6-OHDA-mediated motor impairments (50). The study called for further testing of INA of MSCs for neurodegenerative disease by chronic therapy in order to increase cell numbers making it to the CNS, therefore enhancing the therapeutic effect on diseased tissue (50).
Chartoff et al acquired data that conflicted with that of the Danielyan group findings; in a study using EGFP-MSCs isolated from transgenic mice that were subsequently delivered via INA to mice with brain lesions (REF 48). While the group agreed with the mission to develop a safe and effective cell delivery method, avoiding risks associated with current methods, they were unable to report that INA was successful at delivering cells to the CNS (48, 50). The EGFP-MSCs directly injected into the striatum of the control mice were detected 1 and 7 days following transplantation, but the INA EGFP-MSCs were undetectable by GFP expression 3 hours to 2 months following the delivery (50). The Chartoff group provided several reasons as to why the findings of the two studies may have differed, but the conclusions seem to account the lack of appropriate controls and lack of consideration of tissue auto fluorescence in the Danielyan study (50).

The current study addresses the discrepancies between the Danielyan group and Chartoff group findings, by delivering human mesenchymal stem cells, by INA, to both PD and SCI mouse models, and studying the delivery method, cell survival and cell migration in these models. This study advances upon the knowledge from previous INA studies by (1) the development of a novel device for nasal delivery efficiency, (2) tracking the location and viability of the transplanted hMSCs by bioluminescence (in vivo), as well as, by fluorescence (in situ), and (3) transfecting the hMSCs with a CXCR4 construct to enhance migration to the target tissue. The future steps of this study will be to double transfect hMSCs with a CXCR4-BDNF construct to exploit chemotaxis and enhance the therapeutic potential of the cells for neurodegenerative diseases and injuries.
2. MATERIALS AND METHODS

This work took place at the Institute for Pediatric Regenerative Cures (IPRM, Sacramento, CA) under the supervision of Dr. Veronica Martinez Cerdeno, as well as, at the UC Davis Clinical and Translational Science Center (CTSC, Sacramento, CA) in collaboration with Dr. Fernando Fierro. Bioluminescent imaging using the IVIS Spectrum (Perkin Elmer) was done at the UC Davis Center for Molecular and Genomic Imaging (CMGI, Davis, CA). The project was funded by California Institute for Regenerative Medicine (CIRM) from June 2014 to December 2014.

hMSC harvest and culture

Stem cells for this project were generously provided by Dr. Fernando Fierro (CTSC, Sacramento, CA). Bone marrow aspirate was collected from healthy human donors (Lonza, Allendale, NJ, www.lonza.com) (1). It was subsequently filtered, passaged and cultured according to protocol for MSC isolation and expansion (1). The cultures were passaged a minimum of 2 – 6 times to insure the lack of hematopoietic stem cells and monocytes, prior to use in experimentation (1, 2).

The stem cells that were harvested and cultured for the in vivo comparison of migration of hypoxic preconditioned (ex vivo) hMSCs versus that of normoxic hMSCs, were divided into two separate groups following genetic modifications (see hMSC Genetic Engineering section below). The normoxic-hMSCs were cultured in an incubator with about 21% O₂ exposures for three days prior to animal administration.
hMSCs were kept in a hypoxia chamber with exposure to about 1-9% O₂ exposure for three days (1).

**hMSC genetic engineering**

Careful thought was put into the design of the lentiviral vector used in this study. Due to the controversy (Danielyan versus Chartoff) it was important for this study to improve upon the hMSC tracking by advanced viral design. The goal is to develop a vector that will integrate CXCR4 into the hMSC genome, to allow for the study of the effects that up-regulation of CXCR4 has on migration. Along with CXCR4, Luciferase (LUCI), for the in vivo monitoring of migration by optical imaging was incorporated into the vector, as well as GFP, for the in situ monitoring of the CXCR4-hMSC migration (Figure 1).

Expanded (passaged 2-6 times) hMSCs were transduced with third-generation lentiviral vectors (general cassette pCCLc-MNDU3-CXCR4-PGK-LUC-P2A-EGFP), in order to genetically integrate CXCR4, luciferase (LUCI), and green fluorescent protein (GFP) gene, along with all of the requisite promoters (Figure 1). The cells were transduced in 2mg/mL protamine sulfate and the previously determined volume of the lentivirus to yield 80-95% GFP positive hMSCs after 3 days in culture (1). Following the 3-day post-transduction culture period, the transduced hMSCs were plated in 12-well plates (20,000 MSCs per well) (1). Every other day following this reseed of the cells, they were detached by trypsin and counted by hemacytometer and trypan blue exclusion dye method (1).
Control hMSCs, with the LUCI-GFP construct (Figure 2), were used for the entirety of this study for the proper tracking of unmodified hMSCs. The LUCI transgene was added as a tool to track the in vivo survival of the hMSCs and the GFP transgene was added to the construct for the identification of viable hMSCs in the CNS tissue of sacrificed animals. All of the subsequent experiments with the control hMSCs (LUCI-GFP-hMSCs (Figure 2)) will be repeated with experimental hMSCs (CXCR4-LUCI-GFP-hMSCs (Figure 1)) following this preliminary study. The CXCR4 viral construct and transduction studies were done by another Martinez Lab student (Angela Monterrubio) in parallel with the following preliminary studies of cell delivery and survival using LUCI-GFP-hMSCs.
Figure 1. Schematic of the CXCR4-LUCI-GFP vector designed for the enhancement of cell tracking and migration. This is an ideal vector design because it allows for both in vivo and in situ tracking of the cells, as well as the upregulation of the chosen chemotaxis machinery (CXCR4). Also it is an effective method for future studies because it could be modified by inserting a therapeutic (ie: BDNF) and the cells CXCR4-(X)-hMSCs could be tracked by GFP and LUCI separately.
Figure 2. Schematic of the LUCI-GFP vector used to transfect control hMSCs. While the experimental CXCR4 vector was being tested in vitro to determine transduction efficacy, the LUCI-GFP vector was used to generate control cells to observe the tracking abilities of the dual-reporter construct.
Generating the 6-OHDA experimental PD murine model

The neurodegenerative mouse models that were generated and investigated in this study are that of the experimental 6-hydroxydopamine (6-OHDA) PD mouse and compression SCI mouse. The PD, SCI and control mice were female Swiss Webster mice (CFW, Charles River, www.criver.com) approximately 3 months old at the start time of experimentation.

The PD injury mouse model was achieved by 6-OHDA striatal injection rodent recovery surgeries (Figure 3). 6-OHDA is an analog of dopamine, which is effectively toxic due to its ability to be selectively transported into healthy dopamine neurons via the dopamine transporter (30, 54). The uptake of 6-OHDA results in the retrograde degeneration of the neurons at the injection site through the development of reactive oxygen species (30, 54) The 6-OHDA is specifically introduced unilaterally into the nigrostriatal bundle (Figure 3), which is the area of the brain that is formed by the dopaminergic fibers that run from the substantia nigra to the striatum (30). The injection was limited to one hemisphere (unilateral), in order to have an appropriate control of healthy, unaltered brain tissue for the specific animal, as well as unilateral movement impairments associated with the desired PD injury. For an appropriate surgical control, some mice were subjected to a PBS injection in the nigrostriatal bundle. The Paxinos Brain Atlas was used to determine the correct coordinates (x, y, z plane) for the nigrostriatal bundle (30). We used the Stoelting quintessential stereotaxic unit (model # 53311), which is capable of being programmed to deposit an exact volume at a constant rate in an exact location (Figure 4). 6-OHDA PD mice were assessed 3-4 weeks post
injections, which is the established time required for the PD phenotype to result from the 6-OHDA death of the dopaminergic neurons in the basal ganglia motor loop, to determine injury success (Figure 3)(30).
Figure 3. Schematic of the surgical approach used to generate a 6-OHDA experimental PD mouse model. The 6-OHDA is injected by way of the quintessential stereotaxic unit (Figure 4) into the exact (x, y, z) coordinates of the murine brain to strike the fibers of the nigrostriatal pathway. The fibers will uptake the dopamine analog and reactive oxygen species will be generated from within the neuron. Through retrograde degeneration the dopaminergic neurons of the substantia nigra will die within a few days, leading to the distinct disruption of interaction between the striatum and the substantia nigra. The PD phenotype will not be observed in the mouse for 3-4 weeks.
Figure 4. Stoelting quintessential stereotaxic unit (model # 53311) used for injections in mouse brain and spinal cord. To the left of the stage, the nose unit and ear bars, are placed to assure the mouse is set in a fixed position. The digital component can be programmed to deliver the volume at a specific volume per second. The coordinates on the micro-injector handle provide a point of reference for placing the needle in the desired location of the brain based on the x, y and z planes. This unit was also used for the INA tubing method to deliver the cells at a fixed rate.
Generating the compression SCI murine model

The calibrated forceps compression method was optimized for the development of the SCI murine model of this study (59, Figure 6). The thoracic vertebra at the tenth position (T10) was targeted and the cord was exposed by a dorsal laminectomy, or removal of the vertebral bone (lamina) (Figure 7). Once the spinal cord was exposed and the forceps could reach the floor of the vertebral canal, calibrated forceps (0.35mm) were used to compress the cord for a specific length of time (15 seconds) (Figure 8). Several other compressions were tested prior to determining the width of 0.35mm. The animals that received a 0.25mm injury had too severe of an injury and did not survive long after surgery. The animals that were subjected to 0.55mm width compression did not have a severe enough injury and recovered quickly. The 0.35mm compression SCI model was used for the purposes of this study. For an appropriate surgical control, some mice were subjected to a laminectomy at the same position, but the spinal cord was not damaged. When the compression was complete the muscle was stitched and the skin was stapled shut (Figure 6). The animal was then slowly brought off of anesthesia and placed in recovery. The animals were watched closely following the procedure and monitored through a lengthy post-operative care period.
Figure 5. Schematic of the target region of the mouse vertebral column for the generation of the SCI mouse model. The thoracic region was the general region of focus for the development of the SCI mouse model because we were interested in paralyzing the hind limbs of the mouse. The T12 is highlighted red because it was determined to be critical to stay above this point. It was determined that T10 is the appropriate target for the compression injury because the fibers critical for hind limb movement radiate from this vertebrae position. If the compression is too rostral (R) or too close to the nose, or too caudal (C) too close to the tail then the model was too variable to compare the cohort of mice. The consistency with the compression injury was dependent on the position of the target vertebrae at position T10. (D) = Dorsal, (L) = Lateral, (R) = Rostral, (C) = Caudal
Figure 6. Schematic summarizing the surgical process to develop the calibrated forceps method of generating compression SCI in a mouse from the dorsal view. (A) Following proper cleaning and disinfecting, the thoracic (dorsal) portion of the mouse is shaved and a small incision is made. (B) Thoracic vertebrae 10 (T10) is identified and a dorsal laminectomy is performed by the removal of the lamina. The target tissue of the spinal cord is completely exposed. (C) Calibrated forceps (0.35mm) are used to completely compress the cord for 15 seconds (D) The bleeding is controlled and the development of the tissue bruise is observed (E) The muscle is stitched over the exposed portion of the cord and the skin is stapled. Throughout the extent of the procedure the animal was anesthetized with inhaled isoflurane (1-4%).
Figure 7. Schematic of the dorsal laminectomy of thoracic vertebrae 10 (T10). The most dorsal portion of the lamina or vertebral bone is cut with surgical tools (rompers). The bone is entirely removed from the side of the spinal cord, being careful to remove any bone debris or sharp edges that may cause further damage or transection of the cord.
Figure 8. Schematic of spinal cord compression by calibrated forceps. It was determined through previous observations that the most effective width of spinal cord compression is 0.35mm (for the purposes of this study). The forceps were specially made to ensure that each SCI murine model generated had the same severity of compression.
Animal husbandry and post-operative care

Proper animal husbandry procedures were practiced and all expectations of the Institutional Animal Care and Use Committee (IACUC) were met and accounted for in the protocol and animal care procedures (Martinez Cerdeno, UC Davis #17087) (30). For three days following the PD and SCI procedures, all mice were administered post-operative analgesics (0.0006 mg/kg Bupronex) and sterile saline twice a day to manage pain and fluid loss. The SCI mice needed bladder expression to avoid the development of a bladder infection, due to the loss of function caused by the compression injury. Observations were made in regards to the appetite, energy level and incision condition of each of the mice. Temperature and weight were monitored throughout the post-operative period to insure the animals were free of pain. All surgical and postoperative notes were kept on file for the extent of the animal’s life. Post-operative care was completed twice a day, morning and afternoon, for two weeks (or until the animals regained bladder function).

Intranasal administration (INA) of hMSCs

Two methods were used in the preliminary studies of INA of either vehicle (artificial cerebrospinal fluid, aCSF), or hMSCs; (1) the pipette method and (2) the tubing and injector method (Figure 9). The pipette method is simply the use of a pipette to deliver small volumes of the fluid to the tip of the mouse nostril, which is subsequently inhaled by the breathing of the anesthetized mouse. The tubing and injector method is a novel method developed by our lab, which involves the use of a small catheter (measured to be...
the appropriate length of the mouse nasal cavity) and a micro-injector. This method was developed to try to improve the proximity of cell transplantation to the nasal mucosa.

Once it was observed that the particular injury of study had been developed, PD mice (3-4 weeks following 6-OHDA surgery) and SCI mice (immediately following compression surgery), the nasopharyngeal mucosa of the mice was transiently permeabilized by hyaluronidase. This enzyme has been shown to assist the “loosening” of the nasopharyngeal mucosa and facilitate migration through the cribriform plate (REF 45-50). The mice were temporarily anesthetized by 2-4% isoflurane and administered 100U hyaluronidase (Sigma) in 24uL sterile PBS, intranasally. The hyaluronidase was administered in the same INA method that was being tested as the therapy. For example, the animals that were to receive the therapy (hMSCs) by way of the pipette INA method, also received the hyaluronidase by the pipette method. The hMSCs were lifted by trypsinization and kept on ice prior to INA. The therapy (~5.0x10^5 – 3.5x10^5 cells in aCSF per animal) or the vehicle alone (aCSF) was delivered by INA by splitting the total volume to be administered equally between the left and right nostril.

For the pipette method the animals were held in supine position and the cells were deposited in small volumes on the tip of the nostril. Once the entire volume had been inhaled by the natural breathing of the animal, the next portion was deposited on the alternate nostril. This was repeated until the entire therapy was administered. The animal was taken off of anesthesia and monitored.

The tubing method was done with the use of a microscope, micro-injector and a small cannula. The cannula was placed into one of the nostrils of an anesthetized mouse
laying in supine position. This placement is crucial and must be done with the use of a microscope to ensure there is no harm done to the nasal cavity of the mouse. We measured the appropriate length required to deposit the cells as close to the cribriform plate as possible, and marked the cannula for reference during the administration. The syringe with a needle (small enough to fit into the opposite end of the cannula) was loaded with the cells (in aCSF) to be delivered to the mouse and placed in the stereotaxic unit (micro-injector). We used the Stoelting quintessential stereotaxic unit (model # 53311) (Figure 4), which is capable depositing an exact volume at a constant (pre-programmed) rate. Once the first volume was pumped through the cannula and into the nasal mucosa of the first nostril, the cannula was removed and placed into the alternate nostril. This was repeated until the entire therapy was administered. The mice were then taken off of anesthesia and monitored.
**Figure 9. Schematic of the INA methods tested for delivery efficiency: The tubing method and the pipette method.** The pipette method was making use of a pipette and the natural breathing of the animal. The animal was lightly anesthetized and held in supine position as the volume was deposited at the tip of the nostril with a pipette. The natural breathing of the animal then allowed for the inhalation of the volume. The tubing and pump injection method is a novel method I developed by making use of a very small polyethylene cannula that was measured to the depth of the mouse nasal passage. One end of the cannula was placed into the nasal passage of the mouse when it was anesthetized and placed in supine position, while the other end was attached to a small needle loaded into the stereotaxic unit. The volume was then pumped into the olfactory region at a controlled rate and then moved to the subsequent nostril for deposition of the remaining therapy.
Bioluminescence imaging for in vivo cell tracking

The visualization of hMSC migration in vivo was possible by the luciferase transgene, which allows for a bioluminescent signal to be detected by the advanced optical imaging technology of the *In Vivo* Imaging System (IVIS) Spectrum (Perkin Elmer) at the Center for Molecular and Genomic Imaging, UC Davis. The LUCI-GFP-hMSCs express the LUCI transgene and produce the oxidative enzyme, luciferase, which is capable of catalyzing a bioluminescence pathway. When the substrate D-Luciferin is introduced into an environment with luciferase, the enzyme (along with ATP and magnesium) will catalyze an adenylation reaction that produces oxyluciferin (60). This reaction product is in an electronically excited state and a photon of light will be emitted as it returns to the ground state (60). The light sensitive IVIS Spectrum optical imager with relatively zero background noise can detect this photon emission. To exploit this bioluminescent pathway the luciferase substrate, luciferin, was injected (intrapeural (IP)) into the animals 15 minutes prior to imaging.

This *in vivo* imaging system uses a back-thinned charge coupled device (CCD) camera that is thermoelectrically cooled to -90°C to achieve maximum sensitivity (61). The system measures dark charge during downtime and runs a self-calibration during the initialization. The lightproof stage chamber consists of a gas anesthesia manifold that can maintain up to 5 animals at a time, as well as a heated stage of 37°C to maintain animal body temperature. IVIS software takes an image of the animal under white light and overlays it with the quantitative bioluminescent or fluorescent signal captured (61). Along with the color intensity map resulting from acquisition, luminescence from the
cells can be measured and quantified at specific locations in the animal by using a region of interest tool (ROI), which will provide radiance units (photons/second).

The ROI tool component of the Living Image Software (Perkin Elmer) was used to give insight into cell foci formation and densities, as well as cell survivability over time. This was used to determine photon flux, the number of photons per area per time, which is proportionate to the number of live cells expressing luciferase. Therefore, bioluminescence correlates directly to cell survival in the INA and direct injection transplantations. The software was calibrated to compensate for the device settings to keep consistent reads of photon emissions at different time points throughout the extent of the experiment. Imaging was done at different time points (0 days post-injury (dpi), 3 dpi, 5 dpi, 7 dpi) and photon flux was determined by ROIs for each animal at each time point.

**Tissue harvesting, embedding and sectioning**

Following the completion of in vivo studies, the animals were sacrificed by an intracardial perfusion (REF 58). Animals were anesthetized and the depth of anesthesia was assessed by toe pinching and monitoring of the corneal blink reflexes. When it was assured that the animal was unresponsive to stimuli, the chest cavity was exposed and a needle (30 gauge) was inserted into the left ventricle. A release vessel was clipped in order to drain the blood from the animal and flush out all existing fluid with 20 -30 ml of ice-cold phosphate buffered saline (PBS). When the fluid draining ran clear, the syringe
was changed out and 25 ml of ice-cold 4% paraformaldehyde (PFA) was flushed throughout the animal to fix the tissues.

The brain and spinal cord were dissected from the cranium and spinal column. Small sections of the spinal column were displaced with vanna scissors and forceps in a caudal-to-rostral direction (Figure 10). Special care was taken for the animals with a SCI, as to not alter the injured state of the cord during this sensitive dissection. The use of a stereoscope was essential to visualize the tissue as to not clip the spinal cord. Once the CNS tissue was freed from the protective bone and connective tissue, they were placed in conicals of 4% PFA overnight at 4C. Following the 24 hours in fixative, the tissues were cryoprotected by incubating them in 30% sucrose for 24 hours in 4C.

The tissue was then embedded in optimum cutting temperature (OCT) medium (Tissue-Tek CRYO-OCT compound). This provides convenient specimen matrix for cryostat sectioning at temperatures of -10C and below. The tissue was quickly removed from the 30% sucrose solution and all excess solution was gently removed with a Kim Wipe. The tissue was placed in a cryomold that was previously filled and chilled with OCT. The tissue was incubated in the cryomold for 1 hr. at 4C. Following the incubation the directional orientation of the tissue was confirmed and then the mold was placed in a shallow dish of 2-methylbutane that was previously chilled on dry ice for 1 hour. The OCT was allowed to completely solidify in the 2-methylbutane and then stored in -80C until sectioning. Tissue sectioning was done on a Leica CM 3050S cryostat (Figure 10). The OCT tissue blocks were transferred to the cryostat and cut in 20um sections and mounted on slides. The slides were then stored at -20C until further use.
Fluorescence and immunohistochemistry for in situ cell tracking

Tissue was stored at -20°C until the time of in situ studies. The tissue was imaged on the Nikon Eclipse TE 2000-E inverted microscope for detection of GFP signal. Three channels were utilized to determine auto-fluorescence and artifact in the samples. The blue channel (408 nm) was for nuclei detection, the green channel (488 nm) for GFP expression and the red channel (568 nm) for artifact. Human nuclei staining were also performed to observe the transplanted cells among the mouse endogenous cells.

The human nuclei immunohistochemistry protocol is a two-day process. It starts with antigen retrieval (95°C for 15 minutes) in citrate buffer. Once the tissue has thoroughly cooled throughout multiple PBS 1X washes, the tissue was placed in 2% Triton for 20 minutes to permeabilize the tissue for antigen recognition. The tissue was then blocked for 1 hour in goat serum and subsequently incubated in the primary antibody (1:500 mouse) overnight at 4°C in a dark box to protect the tissue from light. The next day the primary was thoroughly washed off of the tissue with PBS 1X and incubated in the secondary antibody (1:500 goat-mouse Alexa 568), which is labeled with a red fluorophore and incubate at room temperature for 1 hour. The secondary was washed with PBS 1X and the slides were mounted with moiwol and DAPI.
Figure 10. Schematic of the process taken to assess tissue following a compression injury. The CNS tissue was dissected from the brain and spinal cord with caution under visualization by a dissecting scope, following the sacrifice of the animal by intracardial perfusion. The tissue was further fixed and embedded prior to sectioning on a cryostat in OCT molds. The brains were sliced in coronal sections and the spinal cords were sliced in sagittal sections, both at a 0.20um thickness. The tissue was mounted on microscope slides at the time of sectioning and placed in the freezer (-20C) until further processing. Tissue was then used for fluorescence imaging or immunohistochemistry for analyzing.
3. RESULTS

Characterization of transduced hMSCs

CXCR4 vector failed to integrate into hMSC genome.

The experimental hMSCs transduced with the CXCR4-LUCI-GFP construct were assessed by flow cytometry (Figure 11), qPCR (data not shown) and microscopy (Figure 12) to determine the transduction efficacy. This was the first attempt at the transduction of these cells. Flow cytometry analysis revealed that the cells were producing little, to no, GFP signal, in comparison to the signal achieved by the lab standard vector. Flow cytometry data for CXCR4 surface expression resulted in no signal detection. qPCR for the CXCR4-LUCI-GFP vector confirmed the results from flow cytometry and revealed that a very low number of lentiviral constructs were actually integrated into the cell.

In order to confirm that the problem was related to the vector, rather than to the hMSC, we transduced 293T cells with the CXCR4-LUCI-GFP vector (Figure 12). 293T cells provide an excellent reference point for lentiviral transduction because these cells are easy to manipulate in culture. This test revealed that the lab standard was able to integrate into the 293T cells with high efficacy. A side-by-side assessment of the CXCR4 vector and the lab standard was completed with that of a vector that was identical to the CXCR4 vector, but was lacking CXCR4. This vector was also positive for GFP fluorescence, further confirming that the “empty vector” (without CXCR4) was able to integrate into the hMSC genome. This signal was achieved despite the fact that less vector (1/5 that of the lab standard) was used for the transduction.
Figure 11. Flow cytometry of CXCR4-LUCI-GFP-hMSCs to determine transduction efficacy. The standard shows a strong GFP signal, while the hMSCs that were transduced with the CXCR4-LUCI-GFP construct has hardly any GFP expression. This is an observation that the integration of our CXCR4-LUCI-GFP construct is not efficient.
Figure 12. Microscopy of 293T cells transduced with the CXCR4 vector to determine transduction efficiency by GFP expression.
Characterization of the murine models

*SCI and PD mouse models were generated in a reproducible manner with predictable behavior ramifications and tissue morphology.*

Prior to beginning the studies with the intranasal administration of hMSCs of interest it was important to generate the disease models in mice and characterize the injury.

The following gross and histological images of the control spinal cord compared to that of the spinal cord damaged by way of the calibrated forceps method, confirms that the injury that I was attempting to cause was in fact real. The mice behaved as expected phenotypically, paralyzed in the hind legs and lack of bladder function, so it was important to confirm the tissue had developed the cavitation (Figure 13), or hole, as well as the cytostructure disruption (Figure 14) of the gray and white matter disorganization in the spinal cord. The H&E images of the control cord show a very clear separation and organization of the white (fibers) and gray (cell bodies) matter within a healthy spinal cord (Figure 14). The tissue from 3 days post injury, or spinal cord compression, shows the disorganization, as well as the inflammation of the spinal cord due to the infiltration of microglia to the damaged CNS tissue (Figure 14). When the injury has progressed to 7 days post injury, the inflammation has cleared and the cord has thinned out at the epicenter, where the scarring will begin to take place (Figure 14).

This was also important of the PD model, especially due to the importance of the needle placement in delivering a striatal injection of the toxin. I sliced tissue and
visualized coronal sections to identify that the needle placement was correct (Figure 15). This was done prior to moving forward with the studies for intranasal administration of the cells.
Figure 13. Representative images of the whole spinal cord. (A) The dorsal view of a control spinal cord (B) The dorsal view of a spinal cord following 0.35mm compression with calibrated forceps. Arrows indicate the injury border. *Identifies the epicenter of the injury. D = dorsal, L = lateral. Scale bar: 0.50mm
Figure 14. Representative H & E images of mouse control spinal cords and SCI spinal cords (A) without compression injury (E) 3 days post injury in a 0.35mm compression injury model and (F) 7 days post injury in a 0.35mm compression injury. Asterisk identifies the epicenter of the injury. Scale bar is 1.25 mm
Figure 15. Coronal section of a brain that was injected with 6-OHDA 4 weeks prior to tissue harvesting. The mouse was symptomatic and the image confirms the placement of the 6-OHDA injection in the striatum. I have zoomed in on the injection site, which can be identified by an orange/brownish circle or a tiny hole in the tissue.
Intranasal delivery of hMSCs to SCI and PD mouse models

A novel INA method using tubing and micro-injector is an effective delivery method.

Mice imaged by the IVIS optical imager were injected with luciferin and imaged at 15 – 20 minutes post luciferin injection on day zero of intranasal administration by pipette method only. There was some background, or false signal seen on the image (in red) but there was no detection of photon emission from the animals of study (Figure 16). We concluded that this method of delivering cells did in fact not work.

Our novel method of using the polyethylene tubing, or cannula, connected to a stereotaxic unit for delivery of the cells in the deep portion of the nasal mucosa was then tested in comparison to the pipette method (Figure 17). The two mice that were administered hMSCs by direct injection, in either the striatum or the spinal cord, were positive controls in order to see where we would expect the cells to emit photons if they were in the brain or spinal cord (Figure 17). This also gave us a point of reference as to how many cells were able to survive the process of directly placing them in the CNS interstitum. We were able to detect live cells at Day 0 post INA with the tubing method, as well as Day 3 post INA with the tubing method (Figure 18).
Figure 16. Bioluminescence image (BLI) of INA via Pipette. There is no detection of photon emissions, which would be indicative of live cells in the nasal passage. The two animals in the middle of the image are control animals, meaning they do not have an injury. The animals on either end of the image have a spinal cord injury and the animal farthest to the left is in supine position to eliminate several questions about the imaging sensitivity. We wanted to be sure that the staples along the back of the animal were not causing interference with the optical imager and we were also curious to see if the animal had swallowed cells during the INA and if they would be detected. There are small red signals in two spots on the optical image, which are just noise or background created by the machine. There were no live hMSCs detected in this cohort.
Figure 17. Bioluminescence image (BLI) comparing INA of the pipette method with our novel tubing method at Day 0 post delivery. The controls for this image were the two mice on the left of the image that were directly injected, in either the striatum (brain) or the spinal cord, with LUCI-GFP-hMSCs in order to visualize the signal expected from live cells within the CNS, as well as providing a point of reference for the location of the CNS, relative to that of the nasal passages. The pipette method was once again negative for the detection of photons, or that of a signal of live hMSCs within the nasal mucosa. The INA tubing/injector method did result in detection of live hMSCs within the nasal passage.
Figure 18. Bioluminescence image (BLI) comparing INA of the pipette method with our novel tubing method at Day 3 post delivery. This is an image of the same cohort of mice (Figure 17), with the exception of the mouse with the pipette method, indicating that the cells were still viable at 3 days post INA with the novel method.
Survival of hMSCs following INA in SCI and PD mouse models

*Hypoxic pre-conditioning appears to improve hMSC survival following INA.*

The control mice that were directly injected with hMSCs are shown in the BLI of INA mice with either hypoxic or normoxic hMSCs to provide a point of reference as to what we would anticipate a signal to look like (location and intensity) if the cells were persisting or migrating to the brain. With a small, preliminary group (n=2) we were able to conclude that hypoxic pre-conditioned hMSCs appear to be promising when considering an extended length of cell survival at 7 days (Figure 19). The region of interest data (ROIs) were correlated to cell counts, which were graphed in order to quantify this survival seen in the normoxic population as well as in the hypoxic population (Figure 20). This is promising that the cells are able to live in the nasal cavity but there is still no migration visualized.

The in situ analysis of the hypoxic versus normoxic hMSCs delivered is an interesting observation. The directly injected cells maintain MSC morphology and appear to be viable due to the GFP signal, while the INA had interesting “specs” in the olfactory bulbs (Figure 21). We checked the olfactory bulbs of the animals that did not receive cells by INA and the specs were not there. These bright signals do appear to be GFP positive, leading us to think they are of hMSC-origin, but they also appear in the red channel, or artifact channel. This leads us to conclude that there is something crossing the cribriform plate in our INA method but the identity is not yet known, it could be hMSC related, possibly debri from death in the cribriform plate or olfactory region, but it could
also be debris from damaged caused by the actual method. Further studies will be done to investigate the identity of spotted signals.
Figure 19. Representative bioluminescence images (Day 1, 3, 5, and 7) of the control mice (direct injection) and the 6-OHDA PD mice that were either INA normoxic or hypoxic hMSCs. As the images are taken throughout the week of in vivo visualization, the normoxic hMSCs can be seen to decrease greatly after 5 days in the nasal passage, as the hypoxic hMSCs still have a comparable survival signal that was detected on day 1 when imaged at day 7.
Figure 20. Graphical representation of BLI average cell counts in INA hypoxic versus normoxic hMSCs in 6-OHDA mice. The difference in the day 0 count is due to the variability of cells delivered to the animal, due to the lengthy process of transferring and injecting the cells through the cannula tubing. Both the hypoxic (red) and the normoxic (green) hMSCs are declining in numbers over the time period imaged in vivo, but the hypoxic hMSCs appear to have a longer term of survival than that of the normoxic.
Figure 21. Representative fluorescent images of direct injection hMSCs and INA hMSCs. The direct injection of hMSCs into the striatum was visualized in three different channels (DAPI for counterstain, Green for GFP and red for artifact) to determine the cell morphology of the cells detected in the BLI in vivo. The hMSCs directly injected have a characteristic morphology with long appendages and a bright and distinct GFP signal, with little to no artifact signal. The INA tissue was visualized at the olfactory bulb due to its proximity to the nasal passage. The periphery of the tissue was lined with specs that were bright in the GFP, as well as the artifact channel. These do not have the morphology of hMSCs and although they appear GFP-positive, they are not consistent with live hMSCs.
Migration of hMSCs into the CNS following INA in SCI and PD mouse models

*Hypoxic pre-conditioning and cell number administered do not appear to be effective in facilitating migration of hMSCs in the CNS via the olfactory route.*

There was no migration detected by bioluminescence imaging of animals that received 500,000 or 150,000 hMSCs intranasally (Figure 22). The cell number for surface area was explored because we were concerned about overwhelming the olfactory region with cells, or not delivering enough cells to encourage migration into the CNS. The brain is negative for signal (meaning there was no migration through the cribriform plate visualized with BLI), while we still were able to detect cells in the nasal mucosa (Figure 22).

The tissue of these animals was visualized for GFP expression to see if migration had occurred but was not detected by the optical imaging technology and the specs visualized in the hypoxic INA mice were present again (Figure 23). The specs were detected in all of the olfactory bulbs of the animals that were delivered cells by INA, while the olfactory bulbs of the animals that were directly injected with the cells were negative for the specs (Figure 23). This is a further indication that the specs are a result from the INA procedure, we are just unsure of the identity at this point. We are able to conclude that migration of live hMSCs was not detected but that the specs could be indicating that migration is occurring but the cells are subsequently dying in the olfactory bulbs.
Figure 22. Representative bioluminescence images of mice INA 500k or 150k hMSCs. The detection of photons by the optical imager appears to be comparable between the two different cell counts delivered. With either number of cells delivered they appear to remain in the nasal passage and are not detected by bioluminescent imaging in the CNS.
Figure 23. Representative fluorescent images of the olfactory bulbs of mice INjected 500k hMSCs or 150k hMSCs. All mice that were subjected to the INA of hMSCs were positive for the fluorescent specs in the periphery of the bulbs. The specs were visible in the green and red channel.
4. DISCUSSION

There is no cure or treatment for neurodegeneration and the only current form of care that we have for patients who suffer the ramifications of progressive neuron death is to alleviate symptoms by conventional approaches. This dark reality for patients with CNS disease or injury has inspired a movement of research for the development of a cell and gene therapy to achieve neuroregeneration and neuroprotection.

The spotlight has been on mesenchymal stem cells for almost two decades, with research efforts revealing the therapeutic effects of these cells on CNS tissues through their unique paracrine effects, rather than by cell replacement therapy. The secretion of a therapeutic extracellular matrix, the ability to home to the site of damaged and stressed tissue and the ease of culturing and modifying MSCs has resulted in their attractive potential for CNS disease and injury treatments. A massive foundation of safety and efficacy studies in human clinical trials, as well as animal studies, have taught us additional advantageous of using MSCs for cell therapy. The ability to evade the immune system, as well as cross the blood brain barrier are essential to the success of MSCs as a cell therapy using either autologous or allogeneic sources. MSCs have also been shown to be directly injected or infused into the CNS, either the cerebrospinal fluid or the interstitium, and have been well tolerated by patients and did not cause other adverse effects.

While MSCs appear to be a potential answer to the problem of degrading CNS tissue, there is yet another hurdle to the approach of cell therapeutics for the CNS, and that is the mode of cell delivery. There are many methods that are approved and being
used today, but they are all either invasive or inefficient. Local delivery methods, such as intracerebroventricular, or the direct injection into the brain, spinal cord or ventricles, is highly invasive and requires surgical expertise. This would not be a procedure that could be an easily repeated procedure and it comes with its own risk factors, as well as the development of an additional injured tissue. Systemic delivery methods, such as intravenous or intra-arterial, result in low engraftment, due to the blood brain barrier, as well as the potential off target effects of the cell therapy. The cell numbers that are able to make it to the target tissue following the full first pass of the entire body is entirely too low to be clinically relevant for neurodegenerative purposes.

As such, there are efforts to develop a non-invasive model of delivering hMSC therapeutics for the CNS. Our lab is interested in building upon previous studies that have looked at the delivery of these unique cells by way of the nasal passage. In order to do this I had to develop a consistent model of CNS injury, and I chose to generate a well-established PD mouse model by introduction of 6-OHDA into the nigrostriatal pathway, as well as optimizing a method of developing a spinal cord injury in a mouse model. Once the injuries were characterized and phenotype was determined to be consistent both behaviorally and cellularly for both of the injury models, the development of the INA delivery system was explored. This study resulted in the development of a novel method of delivering hMSCs into the olfactory region of CNS diseased or injured mice models.

The bioluminescent in vivo imaging (BLI), along with the fluorescent in situ imaging, allowed for appropriate tracking of the INA hMSCs, as well as the identification of a true-live hMSC signal due to visualization of cell morphology. While BLI was
negative for migration in all of the various experiments, there were positive GFP specs identified in all of the olfactory bulbs of the animals subjected to our novel INA method. Though these cell-like (GFP positive) structures were able to cross from the nasal passages into the brain using the INA tubing method, the morphology of these structures was not similar to direct-injection hMSCs that were still emitting a bioluminescent signal at the time of intracardial perfusion. These cell-like structures also auto-fluoresced in a manner that was not consistent with direct-injection hMSCs, which fluoresced only in the green channel. Therefore I am unable to conclude that these specs are definitively hMSC-originated. The strong GFP signal leads me to believe that these could either be (1) debris from hMSC death following migration across the cribriform plate, or (2) exosomes being secreted by the hMSCs in the olfactory region into the CNS through the cribriform plate. Exosomes, or microvesicles, are the small membrane vesicles that are released by hMSCs and are largely responsible for the therapeutic trophic effects that the cells have in their microenvironment. While we are unsure of the exact identity of these specs on the periphery of the olfactory bulbs, we are hopeful that they are one of the two proposed options, which could lead to further promising studies towards the development of a non-invasive approach to CNS therapeutics.

To further advance upon the findings of this study, the experimental CXCR4-hMSCs will be used in the same experiments performed in this preliminary study to determine the effects of modifying the hMSC expression of chemokine receptor CXCR4 has on the survival and migration of these cells in the nasal passages. We have begun to move forward with this initiative but have reached some unfortunate hurdles with the
CXCR4 construct and lack of transduction efficiency (Figure 24). The CXCR4-GFP-LUCI triple cassette vector construct was unable to integrate into the host cell genome of two different cell types of cells as designed. The two-cassette vector (CXCR4-GFP) is appearing to transduce more efficiently, although it still requires high amounts of virus. The study will be continued to attempt to facilitate migration with the upregulation of CXCR4 on the hMSC cell surface, once the cell studies are completed to troubleshoot the modification issues with the current design of the construct (Figure 24).

While this is a highly ambitious project, seeking to develop a method of delivering stem cells via the nasal passage, if it is shown to be safe and efficient, this non-invasive method has the ability to perpetuate the forward movement to develop cell and gene therapies for those who suffer neurodegeneration and currently have no other treatment options. This method of delivery is in its infancy of development, but the potential of its clinical application is remarkable.
**Figure 24. Schematic of the current CXCR4 vector efforts.** The flow chart shows the steps taken by the Dr. Martinez Cerdeno lab in collaboration with Dr. Fernando Fierro in attempts to generate the CXCR4 construct for hMSC upregulation of CXCR4 surface expression. This is an ongoing effort and when the cell modifications are show efficacious in culture the CXCR4-hMSCs will be used in the same studies done with the LUCI0GFP-hMSCs in order to see if the survival or migration is effected by the upregulation of CXCR4 by the hMSCs.
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