NASAL MESENCHYMAL STEM CELL TREATMENT FOR THE REPAIR OF CHEMOTHERAPY-INDUCED NEUROTOXICITIES

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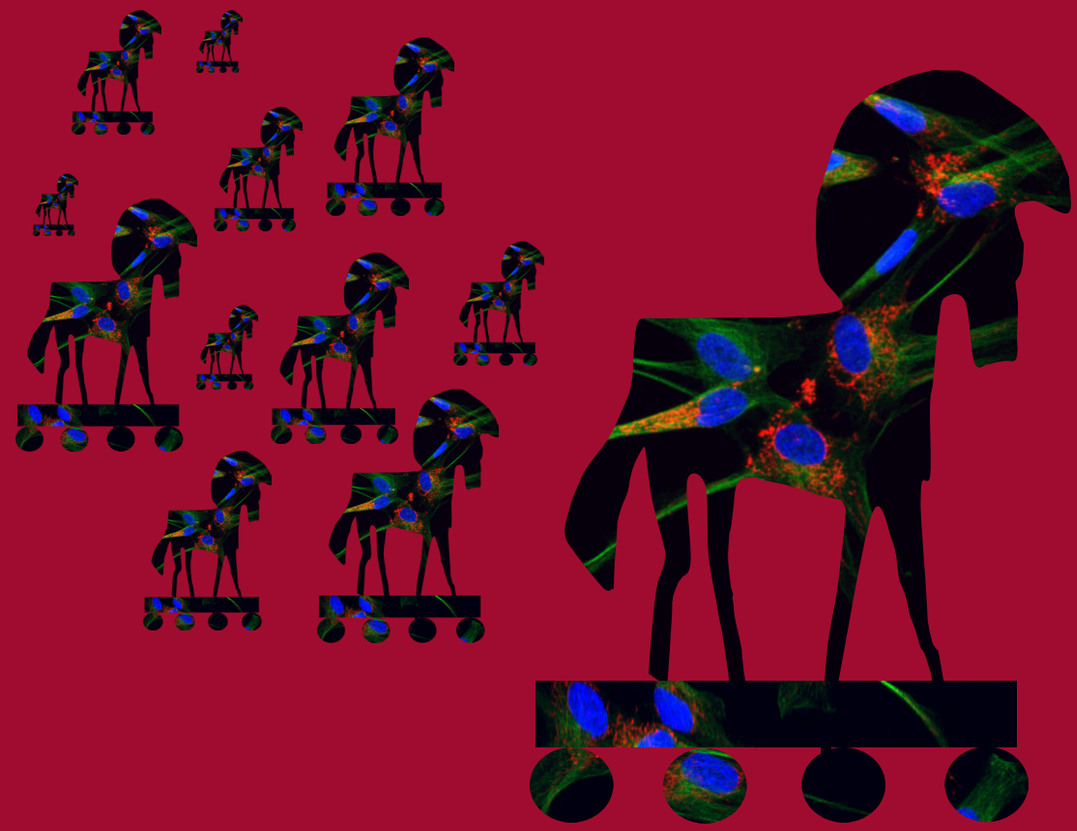
NASAL MESENCHYMAL STEM CELL TREATMENT FOR THE REPAIR OF CHEMOTHERAPY-INDUCED NEUROTOXICITIES: LET THE TROJAN HORSE IN!



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Nasal Mesenchymal Stem Cell Treatment for the Repair of Chemotherapy-Induced Neurotoxicities: Let the Trojan horse in!

Nasale mesenchymale stamcel behandeling voor herstel van neurotoxiciteit veroorzaakt door chemotherapie: Laat het Trojaanse paard binnen!

(met een samenvatting in het Nederlands)

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« *Du combat, seuls les lâches s'écartent* »
Homère; L'Iliade, XVI, 5 - IX^{e} s. av. J.-C.

« *C'est l'idée qui fait le bon bûcheron, ce n'est pas la force* »
Homère; L'Iliade, XXIII, 315 - IX^{e} s. av. J.-C.

To the ones I love

1 Introduction

GENERAL INTRODUCTION

Although chemotherapy is often a life-saving therapy, it represents a double-edged sword since it does not only lead to tumor cell killing but it has at the same time a deleterious effect on healthy tissues including its effect on the central and peripheral nervous system [10, 45, 56, 62, 77, 80]. These neurotoxicities often lead to dose reduction or even termination of treatment. Moreover, cancer treatment can severely hamper quality of life of the patient and side effects often persist long into survivorship. To date, there are no FDA-approved therapeutic approaches to prevent or mitigate the devastating effects of life-saving cancer treatment. Therefore, a better understanding of the mechanisms underlying the development of these neurotoxicities might lead to efficacious therapeutic strategies.

Recently, stem cells have been coined as great candidates for regenerative therapies in several animal models of disease. In this thesis, we focus on the use of Mesenchymal Stem Cells (MSC) administered via the nasal route as a therapeutic approach for the repair of adverse effects of cisplatin treatment on the central and peripheral nervous system. These include cognitive impairments and peripheral neuropathy as they represent two of the most devastating side effects of cancer treatment. Using a mouse model of cisplatin-induced neurotoxicity, we describe here the use of nasally administered MSCs for the treatment of chemotherapy-induced cognitive impairment in chapter 2. Later, in chapter 4, we focused on the use of intranasal MSCs to reverse cisplatin-induced peripheral neuropathy.

Mitochondria are organelles that function as energy producers. Mitochondria convert nutrients into the source of energy adenosine triphosphate (ATP), primarily through oxidative phosphorylation. In neuronal cells, mostly all ATP production through mitochondrial oxidative phosphorylation is required for the maintenance of normal cellular activities. Inadequate synthesis of sufficient amounts of ATP for neuronal activities requiring high energy, including generation of action potential, synaptic transmission and axonal transport, is suggested to be one of the first mechanisms underlying CIPN [9, 68].

Chemotherapeutic agents including platinum drugs and paclitaxel, have been shown to cause mitochondrial dysfunction, despite differences in their

anti-tumor mode of action. For example, cisplatin intercalates mitochondrial DNA, leading to defective protein synthesis and to mitochondrial impairment. Mitochondrial DNA in plasma has therefore been reported as a valid marker for brain mitochondrial defects and associated functional brain deficits [16].

Neurons are very sensitive to chemotherapy and very much dependent on intact mitochondrial function as fast energy providers. One of the functions of MSC with respect to regeneration of cells and tissues is their capacity to transfer healthy mitochondria to cells in need. In chapter 3, we investigated whether MSC can transfer mitochondria to cisplatin-damaged neural stem cells (NSC) in an *in vitro* system as a potential mechanism of MSC repair. Finally, chapter 5 will focus on the fate of nasally applied MSCs, as we describe their arrival in the meninges of the brain and subsequent migration into the meninges of the spinal cord. Chapter 6 will give an extensive review on the importance of mitochondrial transfer in the communication between MSC and neuronal cells.

Chemotherapy-related neurotoxicities

As cancer therapeutics improve, the number of cancer survivors has increased significantly. However, many cancer survivors report a large array of treatment-related toxicities which negatively affect their quality of life after completion of cancer treatment. As reported by the International Agency for Research on Cancer (IARC), 17 million new cancer cases have been reported in 2018, a number predicted to increase to 27.5 million by 2040, due to population growth and aging (American Cancer Society). The debilitating side effects arising from chemotherapy often persist months to years after treatment completion [36, 56, 59, 74, 82]. Moreover, the severity of symptoms can lead to treatment cessation, which in turn negatively influences survival outcomes [36, 56, 59, 82].

Cisplatin is a highly effective platinum-based antineoplastic agent widely used to treat solid tumors. Cisplatin is a cytotoxic drug inducing DNA damage, inhibiting DNA synthesis and causing apoptosis in cancer cells. The molecular mode of action includes production of reactive oxygen species which increase lipid peroxidation, induction of p53-mediated cell cycle arrest, down-regulation of proto-oncogenes, anti-apoptotic proteins and induction of apoptotic pathways [32]. Administration of cisplatin leads to

significant side effects affecting both central and peripheral nervous system, including cognitive impairments and peripheral neuropathy.

Chemotherapy-induced cognitive impairments (CICI)

The central nervous system (CNS) represents the primary target of chemotherapy-induced toxicities. Symptoms associated with damage to the CNS have been clinically described as chemotherapy-induced cognitive impairment. Various reports suggest that cognitive impairments occur in 35-85% of cancer patients and can persist long after treatment cessation [3, 36, 82]. Cross-sectional and longitudinal studies suggest that chemotherapy results in deficits in working memory, executive functioning, attention and processing speed [30, 41, 43, 80, 82]. Studies using brain magnetic resonance imaging (MRI) have shown volumetric changes in gray and white matter. Resting state functional MRI of the brain of cancer patients treated with chemotherapy demonstrated abnormalities in the functional connectome indicating an inefficient information exchange and network integration [41-43]. Multiple mechanisms underlying cognitive impairments have been proposed, including direct neurotoxicity, decreased neurogenesis and neuroinflammation [73].

Mechanisms of CICI

Several mechanisms underlying cognitive dysfunction resulting from chemotherapy have been proposed. By penetrating the blood-brain barrier (BBB), chemotherapeutic agents can directly damage cells of the central nervous system and induce oxidative stress, low grade neuroinflammation, mitochondrial dysfunction, and immune cell activation and infiltration [72]. Although data are conflicting, studies conducted with breast cancer patients show a modest increase in plasma pro-inflammatory cytokine levels after chemotherapy initiation, including interleukin 1β, interleukin-6, and tumor necrosis factor alpha (TNF-α). Further mechanisms underlying the development of CICI include impaired neurogenesis, as evidenced by reduced number of neural progenitors in the dentate gyrus (DG), myelin sheath breakdown in white matter and axonal degeneration [17, 19, 76].

In the hippocampus, dendritic spines, which are the postsynaptic components of most excitatory synapses, are important as the molecular and cellular correlates of learning and memory happen at excitatory synapses. Synapse connectivity and functionality are thought to underlie essentially

all cognitive functions [34, 35]. Studies on CICI show that chemotherapy-induced memory deficits are associated with a loss of spine density and morphology which essentially reflects modifications in the density and strength of excitatory synapses [18, 87]. As a major component of excitatory synapses, spines provide a structural scaffold to act as biochemical and electrical compartments important for plasticity at individual synapses [51].

Our group recently reported that cisplatin induces a loss of synaptic integrity in the hippocampus by showing that cisplatin decreases synaptophysin expression, a presynaptic marker of synaptic integrity. In relation to a decrease in synaptophysin, cisplatin treatment also resulted in a reduced number of dendritic spines and neuronal arborizations in the cingulate cortex, a common morphological alteration that correlates with modified synaptic function and behavioral deficiencies in models of neurodegenerative diseases [12, 17, 18, 85, 87]. Finally, at the structural level, we demonstrated that cisplatin reduces coherency of white matter fibers in the cingulate cortex [17] and the number of neuronal progenitors as defined by doublecortin positive (DCX^+) cells in the subventricular zone (SVZ) and DG of the hippocampus.

An important underlying mechanism of cisplatin-induced neurotoxicities is mitochondrial dysfunction. The brain is a high energy consuming organ that uses about 20% of basal oxygen of the body to function properly [33]. Thus, it is not surprising that disturbances in brain bioenergetics lead to neuronal dysfunction, cognitive decline, accelerated aging and neurodegeneration. We have demonstrated that systemic administration of cisplatin leads to structural changes in brain synaptosomal mitochondria, as characterized by an increase in mitochondrial width and percentage of atypical mitochondria [17]. This aberrant morphology is associated with a decrease in mitochondrial bioenergetic function, represented by a decrease in oxygen consumption and spare respiratory capacities in cerebral synaptosomes of mice treated with cisplatin [17]. Most cells function at basal level which requires only part of their bioenergetic capability. The difference between oxygen consumption of cells at baseline and under maximal stimulation is called spare respiratory capacity. This reflects the bioenergetic capacity of the cell to fulfill the requirements to provide a sudden burst of activity under stressful conditions [15]. Decreased spare respiratory capacity was associated with cognitive impairments in Alzheimer's as well as aging.

Furthermore, Lomeli et al. showed that acute cisplatin treatment impairs mitochondria in hippocampal neurons and neuronal stem cells, inducing cognitive impairments [54]. Moreover, we showed that this mitochondrial dysfunction remains many months after termination of cisplatin treatment. Preventing the initial mitochondrial dysfunction as a result of cisplatin treatment with a small molecule Pifithrin-μ, also prevented the development of synaptosomal mitochondrial dysfunction [18]. These results suggest that mitochondrial dysfunction in the brain is a crucial mechanistic component of cognitive impairments after cisplatin treatment.

Chemotherapy-induced peripheral neuropathy (CIPN)

One of the most dose-limiting neurotoxicities of chemotherapy is the damage caused to the peripheral nervous system which presents itself as sensory, motor and sometimes autonomic dysfunction. CIPN can occur during chemotherapy and can persist long after termination of treatment. CIPN is present in approximately 68% of cancer patients one month following completion of chemotherapy treatment, while 30% of patients still suffer from CIPN six months or more after the end of treatment [61, 69]. In many cases, the persistence of these symptoms leads to a dose reduction and in some cases treatment interruption. CIPN symptoms are often reported in a “glove and stocking” distribution [83]. CIPN is described by patients as pain, numbness, burning, tingling, heat and cold hyperalgesia as well as mechanical allodynia mostly in hands and feet [44]. Another sign of CIPN is the retraction of intraepidermal nerves in the extremities as quantified by the density of nerve fibers penetrating the epidermis [9, 49, 66].These neuropathic symptoms are common to many chemotherapeutic agents, including platinum drugs as well as cytoskeleton disrupting agents like paclitaxel [50]. Current treatments for CIPN are suboptimal and efficacious therapeutic strategies are urgently needed.

Mechanisms of chemotherapy-induced peripheral neuropathy

The molecular/cellular mechanisms of CIPN are complex, ranging from mitochondrial dysfunction to altered ion channel expression and activity in sensory neurons including voltage gated Na^+ and K^+ channels as well as transient receptor potential (TRP) channels such as pain-sensing TRPV1 [14].

Preclinical studies have shown that chemotherapeutic agents causing CIPN induce mitochondrial dysfunction in peripheral sensory neurons, despite

their different modes of action [9]. Mitochondrial dysfunction generates (nitro-) oxidative damage to mitochondrial DNA and proteins which causes energy deficiency in sensory neurons. In rodent models of cisplatin- induced peripheral neuropathy, peripheral nerve and dorsal root ganglion (DRG) cells harbor abnormally swollen and vacuolated mitochondria with a lack of defined cristae structure and a disrupted inner and outer membranes [54, 84]. Deficiencies in mitochondrial bioenergetics in peripheral nerves and DRG neurons were demonstrated to occur along with these abnormalities in mitochondrial morphology [9].

In addition, nerve terminals have been reported to be extremely sensitive to cisplatin. In mice, cisplatin has been demonstrated to reduce the density of intraepidermal nerve fibers (IENFs) crossing the basement membrane in the plantar surface of the paw. Insufficient energy supply has been suggested to result in the failure of IENFs to branch within the epidermis, leading to loss of IENFs. Loss of IENFs has therefore been coined as a marker of CIPN [9, 49, 66].

Another player contributing to CIPN is the immune system. Inflammation is believed to contribute to CIPN and is characterized by activation of glial cells (especially astrocytes), infiltration of immune cells and the production of inflammatory mediators in the nervous system. Chemotherapy has been shown to induce infiltration of macrophages within the DRG through chemoattractants C-C motif chemokine 2 (CCL2) as well as C-X3-C motif chemokine ligand 1 (CX3CL1). Infiltrating macrophages secrete proinflammatory cytokines that reduce the excitability threshold of spinal and DRG neurons leading to hyperexcitability and spontaneous discharges, which contribute to the pain phenotype. In response to these inflammatory stimuli, the phenotype of glial cells changes, leading to higher proinflammatory cytokines production, thus increasing neuronal excitability and pain hypersensitivity. The use of interleukin 1 receptor antagonist (IL-1ra) and antibodies against tumor necrosis factor-α (TNF-α) to block proinflammatory signaling have been shown to lessen CIPN in rodents. Furthermore, depleting macrophages via clodronate administration, or blocking macrophage infiltration by intrathecal administration of CCL2 or CX3CL1 neutralizing antibodies has been shown to prevent CIPN in several pain models [13, 37].

As opposed to the pain-promoting effects of proinflammatory cytokines such as TNF-α and IL-1β, studies demonstrated that anti-inflammatory cytokines such as IL-10 as well as regulatory T cells (T_{reg}) are crucial for resolution of CIPN. T_{reg} cells are a specific subpopulation of immunosuppressive T cells essential for immune homeostasis [25]. Reduced numbers of T_{reg} cells have been shown in models of oxaliplatin-induced and paclitaxel-induced pain [53, 55]. Moreover, multiple studies have reported that promoting the T_{reg} subpopulation via intrathecal adoptive transfer or administration of the T_{reg}-enhancing bee venom-derived phospholipase A2 reduces CIPN in rodents [53].

Oxaliplatin-induced mechanical hypersensitivity is associated with downregulation of anti-inflammatory cytokines (IL-10 and IL-4) in the spinal cord [40]. Moreover, IL4-10 fusion protein was shown to inhibit allodynia in a mouse model of neuropathic pain [26].
We have shown previously that IL-10 signaling is critical for resolution of neuropathy in mice. IL-10 is an important endogenous counter-regulator of pro-inflammatory cytokine function that also acts in the nervous system. Within the central nervous system, a number of studies suggest that IL-10 is neuroprotective, as evidenced by enhanced survival of different cell types in culture *in vitro*, including embryonic or immature cortical neurons and spinal cord neurons [8, 88]. IL-10 is expressed by cells of the innate immune system, including macrophages, dendritic cells, naturel killer (NK) cells, mast cells, eosinophils and neutrophils. It is also expressed by many cells of the adaptive immune system, including T_{reg} cells, $CD8^+$ T cells and B cells [63]. IL-10 anti-inflammatory activity develops following IL-10R binding and activation of the Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) pathway. STAT3 nuclear translocation leads to binding of STAT3 to several promoter elements of genes such as IL-10, generating an anti-inflammatory activity [63]. IL-10 inhibits nuclear factor-kB (NF-kB) activity that leads to decreased production of immune-response genes necessary for proinflammatory cytokine (e.g. IL-1β and TNF-α) and chemokine (CCL2) production by immune cells including macrophages, dendritic cells, natural killer cells, and Th-1 and Th-2 cells [58].

We recently found that endogenous IL-10 signaling is involved in spontaneous resolution of CIPN. Blockade of IL-10 signaling through the intrathecal injection of a neutralizing antibody delayed recovery from CIPN [48].

Moreover, IL-10-deficient mice did not recover from CIPN. These findings further underscore the therapeutic potential of IL-10 therapy to resolve CIPN.

We have shown previously that $CD8^+$ T cells are necessary for resolution of CIPN and that they may act partially through increasing IL-10 receptor expression in DRG neurons [48]. We suggest that $CD8^+$ T cells increase IL-10 receptor expression in DRG. Moreover, IL-10 is involved in the suppression of abnormal paclitaxel-induced spontaneous discharges by DRG neurons resulting in the recovery from CIPN.

Interestingly, IL-10 also is involved in the repair of mitochondrial dysfunction. IL-10 has been shown to promote mitophagy that results in the removal of dysfunctional mitochondria thereby preventing production of ROS, activation of the NLRP3 inflammasome and production of IL-1β [38]. Ip et al. also demonstrated that IL-10 inhibits the inflammation-induced glycolysis and promotes oxidative phosphorylation in macrophages after stimulation with Lipopolysaccharide (LPS). If this would also be a mechanism in the peripheral nociceptor, it would explain the powerful action of IL-10 by restoring mitochondrial health through mitophagy and metabolic reprogramming [38].

Mesenchymal stem cells and their immunomodulatory capacities

Mesenchymal Stem Cells (MSC), also defined as mesenchymal stromal cells, are self-replicating stem cells derived from tissues including bone marrow, adipose tissue, peripheral blood, dental pulp, umbilical cord and placenta. MSCs were initially discovered in the 1970s in the bone marrow and were defined as colony-forming unit fibroblasts in view of their morphology [57]. In the bone marrow, MSC represent an important component of the hematopoietic stem cell niche and can be phenotypically characterized into cells positive for CD146, CD106, CD105, CD73, CD44, CD90, CD29, STRO-1 (Antigen of the bone marrow stromal-1 antigen), and negative for CD45, CD34, CD14 (i.e. hematopoietic markers), CD11b, CD79α/CD19 and HLA-DR [22]. MSC are plastic adherent cells and can be differentiated into at least three mesodermic lineages: chondrogenic, osteogenic and adipogenic cells [75]. Interestingly, when using the right culture media, MSC cannot only differentiate into cells of mesodermic origin but also have the potential to differentiate into cells with neuroectodermal potential [22].

Bone marrow derived MSCs are the most frequently used cells for cellular therapy because of their easy access and powerful regenerative and immunosuppressive capacities.

Therapeutic application of mesenchymal stem cells

MSC have high regenerative, immunosuppressive and trophic capacities which position them as the perfect candidates for use in regenerative medicine and as mitigators of inflammation.

From a regenerative perspective, MSC can migrate to injured sites, therefore, contributing to tissue repair [64]. An important feature of MSC use in tissue repair is their capacity to secrete trophic factors that promote important biological activities. Indeed, MSC secrete growth factors, cytokines, and chemokines. MSC have been shown to be capable of decreasing inflammation by modulating the immune system [2, 21, 27, 46, 47], promoting cell migration, differentiation, angiogenesis and formation of extracellular matrix (ECM) [29].

Mesenchymal stem cells in neurological disorders

MSC have therapeutic properties for the treatment of neurological diseases and injury including traumatic brain injury and ischemic stroke. In these models, MSC exerted neurotrophic effects by releasing molecules promoting endogenous repair, including insulin-like growth factor 1 (IGF-1), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF). These factors can also have neurotrophic and neuroprotective effects on progenitor cells, thereby inducing neurogenesis, oligodendrogenesis, neurite outgrowth, angiogenesis, as well as inhibiting apoptosis of neurons and glial cells. Our group has shown that MSC administration improves functional outcome and lesion size in experimental models of stroke and neonatal hypoxic-ischemic brain injury [23, 24, 79]. Moreover, we reported that MSC reduce white matter loss and regenerate cerebral lesions in a rat model of subarachnoid hemorrhage [65, 78].

Lately, extracellular vesicles (EVs) secreted by MSC have been identified as a potential other paracrine mechanisms of MSC action in tissue repair. EVs are nanometer-sized cytosolic fragments with a spherical morphology released by most cell types, including MSC. EV's include exosomes, microvesicles and

apoptotic bodies. Proteomic analysis of MSC-derived EVs showed that these vesicles carry mediators such as FGF, VEGF, and EGF [29].

Recent studies comparing the effect of MSC and MSC-derived EVs on functional recovery after ischemia reported equal efficacy. Both MSC and EVs improved neurological impairment and stimulated neuroprotection and neurogenesis [20]. It is assumed that MSC achieve their therapeutic effect through secretion of a cocktail of factors present in EVs into the microenvironment to promote repair and neuroprotection [20].

Mesenchymal stem cells and their mitochondrial transfer to target cells

Apart from the crucial function of the MSC secretome mentioned above, another aspect of regeneration of damaged tissue by MSC is their capability to transfer mitochondria [67]. Indeed, the capacity of MSC to transfer healthy mitochondria to cells in need has been the subject of many preclinical studies attempting to find a therapy for various diseases where mitochondrial dysfunction is associated to pathogenesis. These diseases models include brain injury, stroke, lung disorders, ischemic heart disease, cardiomyopathy as well as Parkinson and Alzheimer disease. In these models, MSC-derived mitochondrial donation has been demonstrated to rejuvenate damaged cells such as cardiomyocytes, epithelial cells, endothelial cells as well as neuronal stem cells through restoration of their cellular bioenergetics need [4, 11, 28, 52, 71, 81, 86]. Several mechanisms of mitochondrial transfer from MSC have been identified including de novo formation of tunneling nanotubes (TNTs) [28, 39, 52]. TNTs are temporal filamentous membrane protrusions of 50-1500 nm wide ranging from tens to hundreds of microns and forming cell-to-cell bridges to connect cytoplasm of adjacent or distant cells [5, 60]. TNTs allow trafficking of various cellular cargo including organelles, plasma membrane components as ion channels, mitochondrial DNA (mtDNA), and cytoplasmic molecules between cells. The transport of proteins, mtDNA and cellular organelles relies on intact cytoskeleton fibers, functioning as a backbone of an actin polymer [5]. TNT from MSC have been shown to have the specific capacity to transfer mitochondria to recipient cells including endothelial cells, cardiomyocytes, pulmonary alveolar epithelial cells, renal cells as well as macrophages, thus promoting modification of the functional properties of these cells [1, 4, 39, 52, 70, 81].

The mitochondrial movement along these TNTs depend on the Rho GTPase Miro1 (RhoT1 or Miro1), as was shown for mitochondrial transfer from MSCs to injured alveolar epithelial cells in a mouse models of airway injury as well as neural cells in a rat model of ischemic brain injury [4, 6, 7]. In addition to Miro1, a recent report identified CD38/cyclic ADP-ribose signaling pathway as an important pathway facilitating mitochondrial transfer [31].

Mitochondrial dysfunction leading to decreased ATP production and reactive oxygen species production plays a pivotal role in tissue injury, showing that restoring the function of mitochondria or replacing damaged mitochondria improves cellular survival after injury. The application of mitochondrial transfer as a mechanism of MSC-based therapy is a novel way enabling damaged cells to acquire healthy mitochondria to produce ATP, reduce apoptosis and eventually rescue damaged cells.

Stem cell-based therapy has proven to be a promising treatment strategy for many conditions affecting the central nervous system. Despite multiple delivery methods, nasal administration is emerging as a promising non-invasive, fast and safe route of administration of MSC to the CNS. Given the paucity of available curative treatments for chemotherapy related side effects, we investigated in this thesis the regenerative potential of non-invasive nasal administration of MSC for the repair of chemotherapy-induced central and peripheral neurotoxicities. To this end, we used a mouse model of cisplatin-induced cognitive impairment and peripheral neuropathy. By combining mice behavioral models of chemotherapy-induced neurotoxicities, *in vitro* assays, and imaging techniques, we investigated both the efficacy and safety of use of MSC for treatment of cisplatin related neurotoxicities as well as delineated potential mechanisms through which MSC exert their regenerative action on damaged cells and tissues.

AIM AND OUTLINE OF THE THESIS

The overall aim of this thesis is to explore the regenerative capacity of MSC in the resolution of chemotherapy-induced cognitive deficits and peripheral neuropathy with a focus on the therapeutic benefit and mechanism of action of MSC.

Chapter 2 describes the use of nasally applied MSC for the treatment of cisplatin-induced cognitive impairments and brain damage in mice. We investigated the mechanism of repair through RNA sequencing analysis as well as assessment of mitochondrial function in brain synaptosomes. We also investigated the structural and functional deficits resulting from cisplatin treatment by exploring white matter damage and resting functional MRI respectively. Moreover, we assessed the safety of MSC use by determining their effect on potential tumor growth in the long-term.

Chapter 3 focuses on an *in vitro* approach to investigate whether MSC can protect neuronal stem cells (NSC) in culture from the neurotoxic effects of cisplatin, through donating healthy mitochondria. In addition, we determined the regenerative potential of nasally applied MSC in protecting the loss of neurogenic pools in the brain as a result of cisplatin treatment.

Chapter 4 focuses on the use of nasally administered MSC to resolve cisplatin-induced peripheral neuropathy (CIPN), another debilitating side effect of chemotherapy. We investigated in this chapter MSCs potential in resolving CIPN symptoms, including mechanical allodynia and spontaneous pain. Moreover, we examined whether MSC restore mitochondrial dysfunction in the peripheral sensory system, as it plays a role in CIPN pathogenesis. Finally, in an attempt to look for a mechanism underlying MSC action, we investigated the role of IL-10 in MSC mediated resolution of CIPN symptoms.

In chapter 5, we explored the fate of nasally applied MSC in the meninges of the brain and the spinal cord of cisplatin-treated mice. As many MSCs were found in the meninges, we also explored potential interactions between MSC and immune cells in the meninges of the brain.

Chapter 6 will review the existing literature on the therapeutic potential and mechanism of action of MSC to transfer mitochondria to neurons and the consequences for neuronal and brain health.

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Nasal administration of mesenchymal stem cells restores cisplatin-induced cognitive impairment and brain damage in mice

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ABSTRACT

Cognitive impairments are a common side effect of chemotherapy that often persists long after treatment completion. There are no FDA-approved interventions to treat these cognitive deficits also called 'chemobrain'. We hypothesized that nasal administration of mesenchymal stem cells (MSC) reverses chemobrain. To test this hypothesis, we used a mouse model of cognitive deficits induced by cisplatin that we recently developed. Mice were treated with two cycles of cisplatin followed by nasal administration of MSC. Cisplatin treatment induced deficits in the puzzle box, novel object/place recognition and Y-maze tests, indicating cognitive impairment. Nasal MSC treatment fully reversed these cognitive deficits in males and females. MSC also reversed the cisplatin-induced damage to cortical myelin. Resting state functional MRI and connectome analysis revealed a decrease in characteristic path length after cisplatin, while MSC treatment increased path length in cisplatin-treated mice. MSCs enter the brain but did not survive longer than 12-72 hrs, indicating that they do not replace damaged tissue. RNA-sequencing analysis identified mitochondrial oxidative phosphorylation as a top pathway activated by MSC administration of cisplatin-treated mice. Consistently, MSC treatment restored the cisplatin-induced mitochondrial dysfunction and structural abnormalities in brain synaptosomes. Nasal administration of MSC did not interfere with the peripheral anti-tumor effect of cisplatin. In conclusion, nasal administration of MSC may represent a powerful, non-invasive, and safe regenerative treatment for resolution of chemobrain.

INTRODUCTION

Chemotherapy is still one of the most effective treatments to combat cancer. However, chemotherapy is associated with many negative side effects, including fatigue, pain, numbness and tingling in hands and feet, and cognitive impairments[1–4]. In view of the rapidly growing number of cancer survivors, there is an urgent need for an effective therapeutic intervention to treat these neurotoxicities.

Chemotherapy-induced cognitive impairment, commonly known as "chemobrain", involves impairment in working memory, attention, processing speed, concentration, and executive function[1,3,5–7]. Chemobrain has been observed in 78% of cross-sectional and 69% of prospective longitudinal studies performed between 1995 and 2012 in patients treated for breast cancer[3]. Resting state functional magnetic resonance imaging (rsfMRI) analysis of patterns of brain network connectivity indicate that chemobrain is associated with abnormalities in the functional connectome indicative of inefficient information processing[5–7]. In approximately 30% of patients, symptoms of chemobrain persist long after completion of treatment thereby severely hampering quality of life and limiting the home and occupational activities[4]. There is also a growing concern that chemotherapy may increase the risk for accelerated aging and later neurodegenerative conditions[6].

Cognitive impairments also frequently develop as a result of cisplatin treatment[8,9]. Cisplatin being the most widely used chemotherapeutic, is part of the standard treatment for numerous malignancies including head and neck, testicular, ovarian, and non-small cell lung cancer[10,11]. Cisplatin penetrates into the brain in low concentrations[12,13].

We recently developed a mouse model of cisplatin-induced cognitive impairment. We demonstrated that cisplatin treatment results in long lasting cognitive dysfunction in association with impaired neurogenesis, white matter damage and a loss of neuronal dendritic spines and arborizations[14,15]. In addition, we demonstrated that deficiencies in mitochondrial bioenergetics likely underlies the cisplatin-induced cognitive dysfunction[15].

Recent advances in regenerative medicine identified MSC as a potential treatment for cerebral stroke, traumatic brain injury, hypoxic ischemic

encephalopathy, status epilepticus, cranial radiation damage as well as for neurodegenerative diseases[16–26]. We demonstrated before that nasal MSC administration to neonatal mice with ischemic cerebral damage has life-long efficacy and does not induce malignancies or other pathologic abnormalities[24,27]. Here, we tested the hypothesis that nasal administration of MSC is an efficacious non-invasive strategy for reversing chemobrain and investigated the mechanism of repair via RNA sequencing and assessment of synaptosomal mitochondrial function. In addition, we investigated whether nasal MSC administration after completion of cisplatin treatment interfered tumor recurrence or growth.

RESULTS

Effect of MSC on Cisplatin-Induced Cognitive Impairment.

Male and female mice were treated with two cycles of cisplatin (2.3 mg/kg for 5 days)[15] followed by nasal administration of MSC at 48 and 96 h after the last dose of cisplatin. Behavioral testing started 7-10 days after MSC administration. We used the puzzle box test (PBT) as a measure of executive functioning[28]. The PBT consists of a total of 11 trials at 3 levels of complexity (for details see methods and legend to Fig.1A). The results in Fig.1A demonstrate that in male mice, cisplatin did not affect the time to enter the dark compartment in the easy trials 1-4 (open tunnel) and the intermediate trials 5-7 (bedding-covered tunnel). However, in the difficult trials 8 to 11 (tunnel blocked by lid), cisplatin-treated male mice took significantly more time to enter the dark compartment than control mice, indicating impaired executive function. The heatmap of trial 8 showed that the cisplatin-treated mice spent approximately 60% of the time in the area around the tunnel, indicating that the delay in entering the dark compartment was not due to lack of motivation but rather to a deficient executive function (Fig.S1A-B). In female mice, the effect of cisplatin on performance in the puzzle box was similar to what was observed in males (Fig.S2A). Interestingly, in both male and female mice nasal administration of two doses of $1x10^6$ MSC at 48 and 96 h after completion of cisplatin treatment completely reversed the impaired executive function induced by cisplatin (Fig.1A (males) and Fig. S2A (females)).

The effect of cisplatin and MSC on spatial and working memory was tested using the novel object/place recognition test (NOPRT) that is based on the

innate preference of rodents for novelty (Fig.1B-C and Fig.S2B-C). Cisplatin treatment decreased the preference for the novel object/place in the NOPRT, indicating impaired spatial and/or working memory (Fig.1B (males) and Fig. S2B (females)). Nasal administration of MSC normalized performance of cisplatin-treated male and female mice in the NOPRT. We observed no significant differences between groups in total interaction time with the objects between cisplatin-treated mice vs control (Fig.1C (males) and Fig.S2C (females)), indicating that the effect of cisplatin was not due to decreased interest or motivation.

Cisplatin treatment reduced the number of perfect alternations in the Y-maze test in males and females, without changes in the number of total arm entries (Fig.1D-E (males) and Fig.S2D-E (females)). A reduction in perfect alternations indicates a decrease in spatial memory. Consistent with what we observed in the PBT and the NOPRT, nasal MSC administration resulted in recovery from the cisplatin-induced deficit in the Y-maze in both males and females (Fig.1D and Fig.S2D). Because the effects of cisplatin and MSC on cognitive function was similar in males and females, we focused our further studies on males.

We showed earlier that this regimen of cisplatin treatment does not lead to differences in total locomotor activity in a novel environment nor in immobility in the forced swim test[15] indicating that there are no confounding effects of sickness, anxiety, or depression on performance in the cognitive tasks.

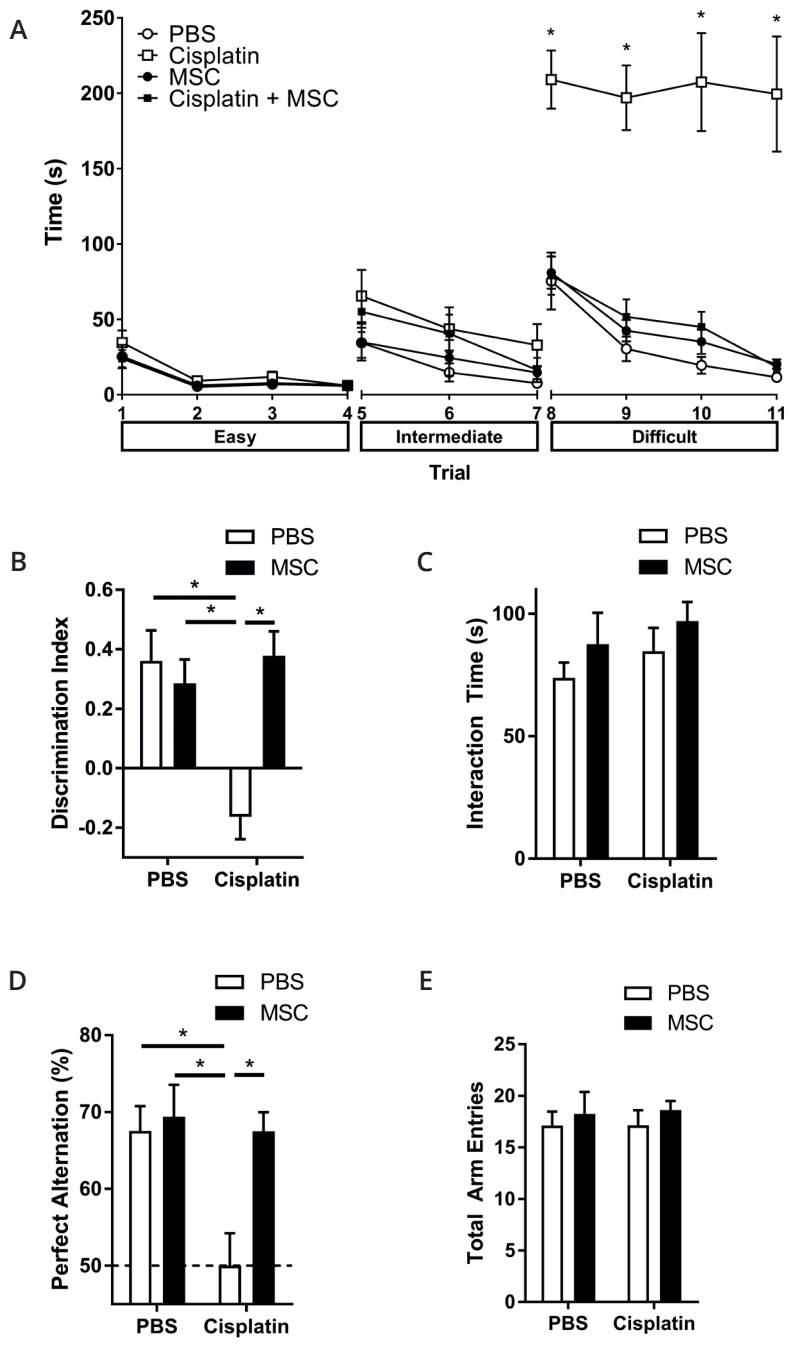

Figure 1. Effect of nasally administered MSC on cisplatin-induced cognitive impairments

Figure 1: Continued
Male mice were treated with cisplatin (2.3 mg/kg/day) for two 5-day cycles with 5 days of rest in between, followed by 2 doses of $1x10^6$ MSC administered nasally at 48- and 96-h post-cisplatin. (A) The PBT was performed 7-10 days after the last dose of MSC. Each animal was given 3 consecutive trials per day for the first 3 days and 2 consecutive trials on day 4. The test is divided into 3 difficulties, easy (trials 1-4), intermediate (trials 5-7), and difficult (trials 8-11). During the easy trials, the animals are allowed free passage between the light and dark compartment via the connecting tunnel. In the intermediate trials, the tunnel is covered with normal bedding and animals must burrow through to reach the dark side. In the difficult trials, the tunnel is blocked by a lid and the animals must learn to remove the blockage before they can escape to the dark compartment. Time until entrance into the dark compartment is recorded. Results are expressed as mean ± SEM; n = 8 in 2 separate cohorts. *Tukey post hoc:* $*P < 0.05$ versus PBS controls. (B) NOPRT was performed 4 days after the completion of the PBT. The discrimination index was calculated as $(T_{Novel} - T_{Familiar})/(T_{Novel} + T_{Familiar})$; 0 represents no preference for the novel object. Results are expressed as means ± SEM; n = 7-8 in 2 separate cohorts. Two-way ANOVA Cisplatin x MSC interaction (F [1, 26] = 12.98, p = 0.0013), *Tukey post hoc:* $*P < 0.05$ versus PBS controls. (C) Total interaction times in the NOPRT were not affected by cisplatin and MSC treatment. Two-way ANOVA Cisplatin x MSC interaction (F [1, 26] = 0.0792, p = 0.7805). (D) The percentage spontaneous alternation in a Y-maze was determined 1 day after the completion of the NOPRT. Dotted line indicates random chance. Results are expressed as means ± SEM; n = 7-8. Two-way ANOVA Cisplatin x MSC interaction (F [1, 26] = 4.786, p = 0.0379), *Tukey post hoc:* $*P < 0.05$. (E) Total arm entries in the Y-maze were not affected by cisplatin and MSC treatment. n = 7-8. Two-way ANOVA Cisplatin x MSC interaction (F [1, 26] = 0.0135, p = 0.9083).

Migration of nasally administered MSC into the brain

MSC were labelled with either PKH-26 (red) or CTGB (green). Labeled MSC were nasally administered 48 h after the last dose of cisplatin. Brains were harvested 12 h later. $PKH\text{-}26^+$ MSC were detected in the hippocampus, cortex, thalamus, and the olfactory bulb (Fig.2A-D, and Fig.S3). MSC were not detected in the hypothalamus. We did not detect any signal in the green channel, confirming that the signal was specific and cannot be attributed to autofluorescence (compare Fig.2E and F). Similar results were obtained with CTGB as the fluorescent tag to trace the MSC (Fig.2G) with no autofluorescence detected in the red channel (Fig.2H). In a separate set of experiments, MSC isolated from GFP-transgenic mice were administered to cisplatin-treated animals as before. To determine the fate of GFP^+ cells, we performed qPCR analysis of the GFP encoding sequence at 12- and 72- hrs after GFP^+ MSC administration. At 12 hours after MSC administration, the GFP transgene was detectable in the hippocampus in all mice (4 out of 4).

We no longer detected the GFP transgene in any of the mice at 72 hours or 7 days after MSC administration. These findings indicate that MSC are present in the brain parenchyma at 12-hrs, but are gone by 72-hrs and did not transdifferentiate into other cell types such as neurons or glia.

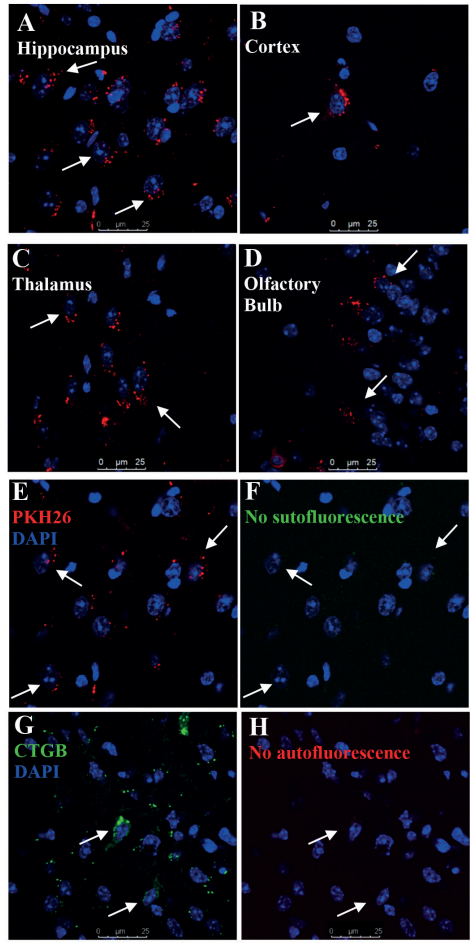

Figure 2. Migration of nasally administered MSC into the brain.
Mice were treated with cisplatin for two 5-day cycles followed by nasal administration of PKH26- or CTGB-labelled MSC at 48 h after cisplatin. Brains were taken 12 h after nasal administration of MSC and analyzed for the presence of $PKH26^+$ or $CTGB^+$ MSC by confocal microscopy. $PKH26^+$ cells were detected in the (A) hippocampus, (B) cortex, (C) thalamus, and (D) olfactory bulb. (E) $PKH26^+$ cells were detected in the hippocampus while (F) no signal was detected in the green channel. (G) $CTGB^+$ cells were also detected in the hippocampus while (H) no signal was detected in the red channel. Therefore we can conclude that the fluorescent signals are specific and cannot be attributed to autofluorescence.

Effect of nasally administered MSC on cisplatin-induced changes in brain functional connectivity

The functional connectome is a map of connections among brain regions that provides insight into the patterns of brain network connectivity that support efficient information processing. To test whether cisplatin induces changes in the functional connectome, mice underwent rsfMRI after completion of behavioral testing (4-5 weeks after MSC administration). A 62x62 correlation matrix was calculated for each mouse using regions identified via a co-registered T2-weighed volume normalized to a C57BL/6J mouse brain template. The results in Fig.3A-C depict the heatmaps for the correlation matrices in each group. Cisplatin-treated mice demonstrated significantly lower characteristic path-length compared to PBS mice ($F = 16.4$, $p = 0.02$, Fig.3). Regionally, cisplatin-treated mice showed significantly lower nodal clustering of left thalamus, left striatum, left cortical subplate, left pallidum and right vermis (all $p < 0.05$, Table.1).

Next, we compared the connectome of cisplatin-treated mice with mice that had received MSC. Nasal administration of MSC increased the characteristic path length when compared to cisplatin-treatment alone ($p = 0.04$). Regionally, none of the above areas that were affected by cisplatin alone, were significantly different from PBS in cisplatin + MSC-treated mice. The right ectorhinal area showed higher clustering in cisplatin + MSC mice ($p < 0.04$) as compared to cisplatin-treated mice (Table 1).

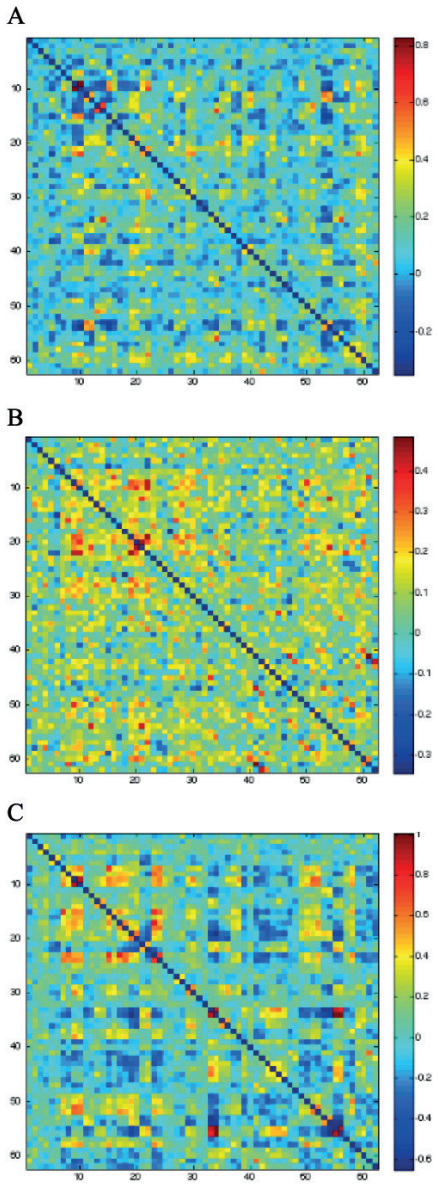

Figure 3. Effect of cisplatin and MSC on brain functional connectivity.
After completion of behavioral analysis, rsfMRI followed by connectome analysis was performed as described in the methods section. Comparing the connectome of control (A) and cisplatin-treated mice reveals that cisplatin treatment results in significantly lower connectome organization (B) which appears more "noisy" than the cisplatin + MSC connectome (C).

Table 1: Regional connectome comparison

PBS vs cisplatin treatment	
Region	**p-value**
Thalamus L	0.01
Striatum L	0.02
Cortical Subplate L	0.04
Pallidum L	0.04
Vermis R	0.05
Cisplatin vs cisplatin + MSC treatment	
Region	**p-value**
Ectorhinal R	0.03

Nasally administered MSC restore white matter integrity

We next examined whether cisplatin induces morphological abnormalities in the white matter using Black Gold staining[29]. Histological assessment of the brain after cisplatin treatment showed a global disruption of myelination (Fig.4A). More specifically, there was a significant loss of myelin density in in the cingulate cortex (Fig.4B-C). These deficits were normalized in response to nasal MSC administration (Fig.4A-C). Cisplatin induced an increase in fiber coherency in the cingulate cortex, indicating reduced myelin arborization. MSC administration also normalized this decrease in coherency in the cingulate cortex (Fig.4D-F).

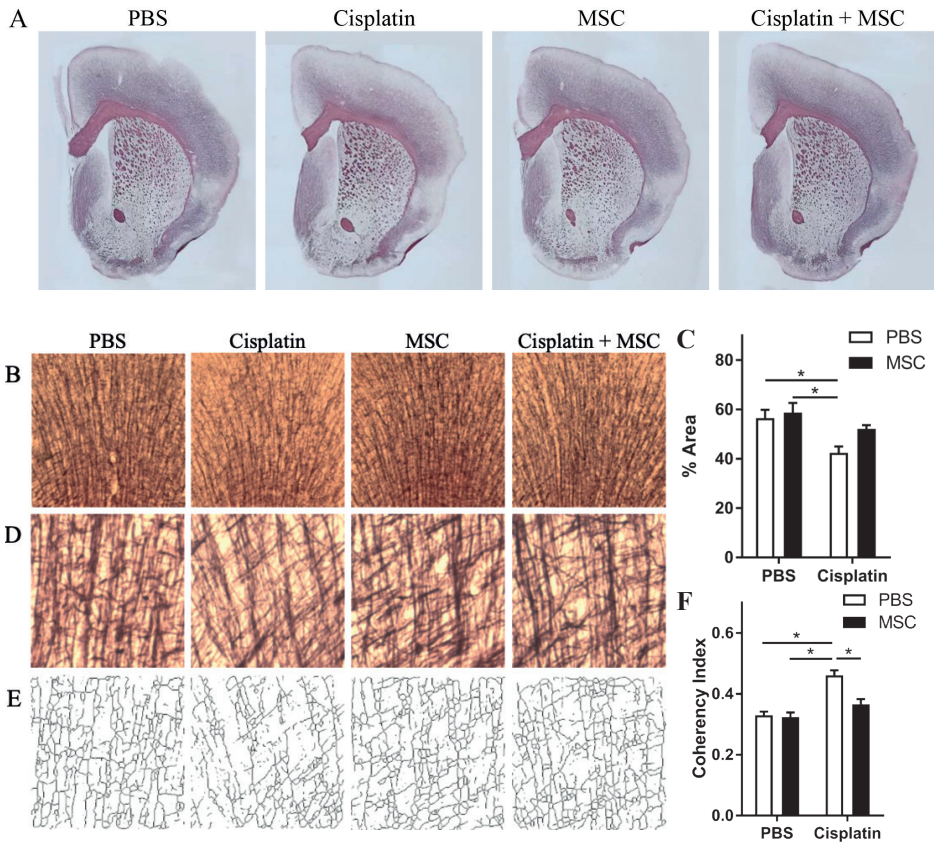

Figure 4. Effects of nasally administered MSC to cisplatin-treated mice on white matter integrity.
(A) Representative images of Black Gold II staining for myelin in brains of mice treated with PBS or cisplatin for two 5-day cycles followed by nasal administration of PBS or MSC as in Fig.1. (B) Black Gold II staining in cingulate cortex in animals treated with PBS, MSC, cisplatin, or cisplatin + MSC. (C) Percent area stained for Black Gold II was measured. (D) Higher magnification (20x) of myelin fibers in the cingulate cortex was (E) skeletonized, and the coherency index was measured (F) to assess the complexity of cortical myelination. Results are expressed as means ± SEM; n = 3 per group. Two-way ANOVA, *Tukey post hoc:* $*P < 0.05$.

Effects of nasally administered MSC on the hippocampal transcriptome

Transcriptional changes induced by cisplatin and MSC in the hippocampus at 7 days after the last dose of cisplatin (3 days after the last dose of MSC) were analyzed using RNA sequencing. Fig.S4A depicts a heat map of the differentially expressed genes (adjusted $p<0.05$). Cisplatin altered expression of 390 genes as compared to the saline control group. In addition, 1335 genes differed in expression when comparing the cisplatin + MSC group to the cisplatin alone group. 105 of the genes that were differentially expressed in response to cisplatin also changed in expression in response to administration of MSC to cisplatin-treated mice (Fig.5A). Out of these 105 overlapping genes, 45 were increased by cisplatin and reduced in response to MSC, while 30 were decreased by cisplatin and increased in response to MSC (Fig.5B and Table.S1 and S2 for a list of these 75 genes). Since MSC were no longer detectable in the brain at 72 hrs after administration (as confirmed by the absence of the GFP transgene in the hippocampus at 72 hrs and 7 days after administration), we propose that these changes in gene expression reflect effects of the administered MSC on gene expression by the host.

We next performed pathway analysis to get more insight in the effect of nasally administered MSC on gene expression profiles in the brain of cisplatin treated mice. Interestingly, two of the top five canonical pathways identified ("Oxidative Phosphorylation" and "Mitochondrial dysfunction") indicated changes in expression of genes involved in bioenergetic pathways (Fig.5C). Details on the specific genes that were differentially expressed in the 5 top pathways are presented in figure S5. A closer look at the two bioenergetic pathways shows that MSC administration to cisplatin-treated mice changed the expression of 29 nuclear encoded genes associated with mitochondrial function (27 upregulated and 2 downregulated). Next, we examined the effects of cisplatin and MSC treatment on the expression of the 13 mitochondrial encoded genes. Changes were detected in expression of mitochondrial genes encoding for complex I (Fig.5D), complex III (Fig.5E), and complex IV (Fig.5F), while expression of the mitochondrial genes encoding ATP synthases was not changed (Fig.5G). In addition, MSC treatment increased expression of one of the genes encoding mitochondrial ribosomal RNA (Fig.5H).

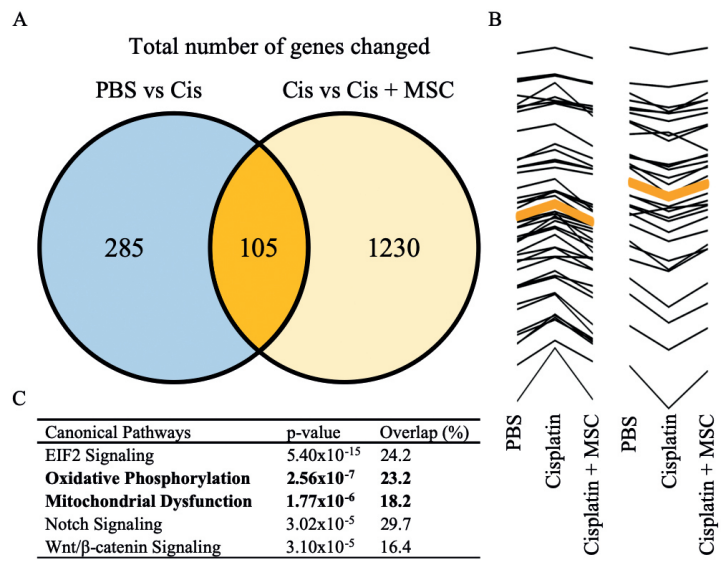

Figure 5. Effects of nasally administered MSC to cisplatin-treated mice on the hippocampal transcriptome.

Figure 5. Continued
Mice were treated with cisplatin for two 5-day cycles followed by nasal administration of MSC at 48- and 96-h post cisplatin. Total hippocampal RNA was collected at 72 h after the last dose of MSC. (A) Expression of a total of 390 genes was changed due to cisplatin treatment when compared to PBS controls. A total of 1335 genes were changed in response to nasal administration of MSC to cisplatin-treated mice when compared to mice treated with cisplatin alone. (B) Of the 105 shared genes that were changed due to cisplatin and cisplatin + MSC, 45 genes increased due to cisplatin treatment and were normalized after nasal MSC treatment. 30 genes decreased due to cisplatin treatment and were normalized after nasal MSC treatment. (C) The top 5 canonical pathways altered by administration of MSC to cisplatin treated mice (comparison of cisplatin alone versus cisplatin + MSC) as analyzed by Ingenuity Pathway Analysis. Bolded are the two pathways related to mitochondrial function. (D-H) Expression of mitochondrially encoded genes. The effect of nasal MSC administration to cisplatin-treated mice on total read numbers is depicted for (D) complex I, (E) complex III, (F) complex IV, (G) ATP Synthase, and (H) rRNA. Results are expressed as means ± SEM; n = 3. One-way ANOVA, *Tukey post hoc:* **P* < 0.05.

Effects of nasally administered MSC on cisplatin-induced changes in mitochondrial respiratory function and morphology

Our RNA sequencing analysis identified changes in the expression of genes involved in mitochondrial functions as top pathways that had changed after administration of MSC to cisplatin-treated mice. In addition, we demonstrated recently that cisplatin-induced cognitive deficits are associated with structural and functional mitochondrial abnormalities in brain synaptosomes[15]. Seahorse analysis of synaptosomes demonstrates that cisplatin-treated mice showed reduced maximal and spare respiratory capacity 3 weeks after the last dose of MSC. Administration of MSC led to a full recovery of the cisplatin-induced impairment in maximal and spare respiratory capacity (Fig.6A).

To determine whether nasal MSC treatment reverses already existing mitochondrial deficiencies or prevents mitochondrial damage, we also tested mitochondrial respiratory function at 48 h after completion of cisplatin treatment (Fig.6B), which is the time point before MSC administration. At 48 h after cisplatin treatment, we already detected a marked decrease in maximal respiration and spare respiratory capacity in the synaptosomes from cisplatin-treated mice, suggesting that nasal MSC treatment truly reverses existing mitochondrial deficiencies.

The results in Fig.6 show that nasal MSC administration also restored mitochondrial morphology (Fig.6C-E); the cisplatin-induced increase in mitochondrial width (Fig.6D) as well as the increase in the percentage of atypical mitochondria (Fig.6E) were reversed by nasal MSC administration to cisplatin-treated mice.

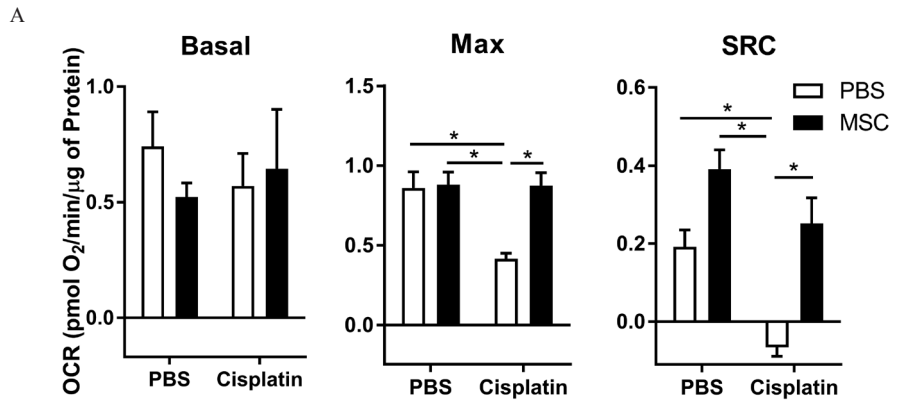

Figure 6. Effects of nasally administered MSC on cisplatin-induced changes in mitochondrial respiratory function and mitochondrial morphology

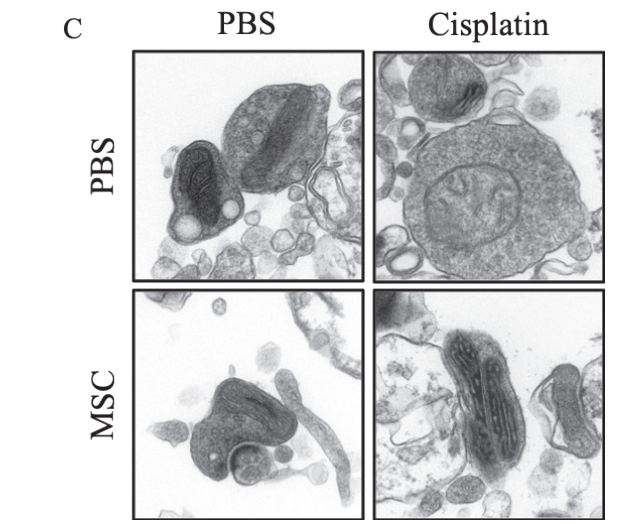

Figure 6. Continued
(A) Mice were treated with cisplatin for two 5-day cycles followed by 2 doses of $1x10^6$ MSC nasally at 48 h and 96 h post cisplatin. Synaptosomes were isolated 3 weeks after the last dose of MSC. Results are expressed as means ± SEM; n = 10. Two-way ANOVA, *Tukey post hoc:* **P* < 0.05 versus PBS controls. Basal, basal respiration; Max, maximal respiratory capacity; SRC, Spare respiratory capacity. (B) At 48 h after completion of cisplatin treatment, synaptosomes were isolated from the brains of animals treated with cisplatin for two 5-day cycles. Oxygen consumption rates were analyzed using the Seahorse XFe 24 Analyzer. Basal, max, and SRC were calculated. Results are expressed as means ± SEM; n = 5. T-test: **P* < 0.05. Basal: p = 0.6394; Max: p = 0.0116; SRC: p = 0.0071. (C) Synaptosomes were isolated from the brains of mice treated with PBS, cisplatin, MSC, or cisplatin + MSC at 3 weeks after the last dose of MSC. (D) Mitochondrial width and (E) and percentage of atypical mitochondria were quantified. Results are expressed as means ± SEM; n = 4. Two-way ANOVA, *Tukey post hoc:* **P* < 0.05 versus PBS controls.

Nasally administered MSC after completion of cisplatin treatment did not promote tumor growth

While unlikely, it is possible that MSC or factors released by MSC could be released into the periphery and interfere with the anti-tumor effects of cisplatin and/or promote tumor growth. Therefore, to assess the potential interference of nasal administration of MSC with cancer treatment, we used a heterotopic syngeneic HPV-related head and neck tumor model. mEER cells (5×10^4 cells) were injected into the hind leg. Seven days after tumor cell implantation, mice were treated with cisplatin or PBS followed by MSC treatment using the same schedule as above. Cisplatin treatment significantly delayed tumor growth as compared to controls. Nasal administration of MSC after completion of cisplatin treatment did not affect tumor (Fig.7).

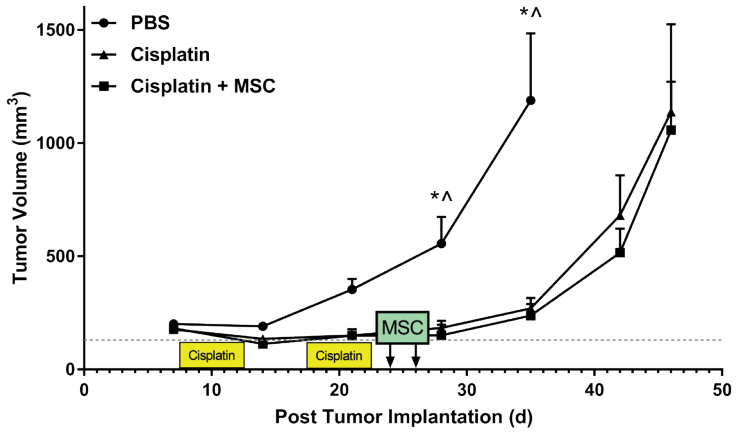

Figure 7. Effects of cisplatin and nasally administered MSC on tumor growth.
Mice were implanted with mEER tumors in the hind leg. 7 days post-implantation animals were treated with cisplatin or PBS for two 5-day cycles followed by nasal administration of PBS or MSC as in all other experiments. Tumor volumes were measured during and after cisplatin and MSC treatment. Results are expressed as means ± SEM; n = 5. Two-way ANOVA, *Tukey post hoc:* **P* < 0.05 versus cisplatin. ^*P* < 0.05 versus cisplatin + MSC.

DISCUSSION

In the present study, we show for the first time that nasal administration of MSC promotes recovery from cisplatin-induced cognitive impairments including deficits in working memory, spatial recognition and executive functioning in both male and female mice. Furthermore, we show for the first time that cisplatin administration induces a decrease in global functional neuronal connectivity in the mouse brain as measured by rsfMRI. Nasal MSC administration reverses the abnormal characteristic path length observed as a result of cisplatin treatment. RNA sequencing analysis of the hippocampus of cisplatin-treated mice revealed that MSC administration increased expression of nuclear and mitochondrially encoded genes involved in mitochondrial respiration. Consistently, nasally administered MSC repaired existing synaptosomal mitochondrial dysfunction and abnormal mitochondrial morphology in cisplatin-treated mice, indicating a true restoration of function by MSC. The cisplatin-induced structural changes in cortical myelination were also completely restored by nasal MSC administration. Finally, we show that nasal MSC administration does not have any effect on tumor growth in cisplatin-treated mice.

Nasal administration of MSC is a non-invasive strategy that could be of great benefit for clinical practice[30]. Apart from the easy route of administration, it is superior to intravenous (i.v.) injections, which require many more MSC and have the disadvantage that MSC will end up for the greater part in peripheral organs like lung and liver[31]. Indeed, preliminary evidence we obtained in a model of cognitive impairment induced by cranial irradiation and temozolamide indicates that nasal administration of MSC improves cognitive function, while intravenous administration of MSC, even when given at twice the dose, is not effective (unpublished data, Chiu et al.)

Nasally administered MSC migrated into the brains of the cisplatin-treated mice where fluorescinating MSC could be found in several brain areas, including the hippocampus, thalamus, and cortex. We did not detect MSC in the brains of PBS-treated mice (data not shown) which may imply that MSC are specifically signaled to migrate into brain areas where there is cisplatin-induced damage. Indeed, cisplatin causes abnormalities in myelin staining in cortical areas (Fig.4 and [14,15]) as well as damage to proliferating neuronal precursors in hippocampus and the subventricular zone[15].

In this respect, it is of interest that after unilateral neonatal hypoxia-ischemia, nasally administered MSC also migrate into the brain but exclusively to lesioned hemisphere even when administered to both nostrils[19,32].

Unbiased RNA sequencing analysis revealed that administration of MSC increased the expression of nuclear encoded genes for components of the four complexes of the electron transport chain. When analyzing mitochondrial RNA, a similar pattern emerged, showing that expression of genes encoding components of complex I, III, and IV of the electron transport chain were upregulated. Functionally, MSC administration normalized the cisplatin-induced mitochondrial damage as measured by oxygen consumption rate and also normalized the mitochondrial morphology. We previously reported that neuronal mitochondrial dysfunction is the mechanism underlying cisplatin-induced cognitive dysfunction and the associated brain damage[15]. Specifically, we showed that the spare respiratory capacity of mitochondria in synaptosomes of cisplatin-treated mice is diminished. However, co-administration of the mitochondrial protectant, pifithrin-μ, which prevents the translocation of p53 to the mitochondria, prevented mitochondrial abnormalities, the cisplatin-induced cognitive dysfunction and the associated brain damage[15]. The latter data suggest that mitochondrial dysfunction is the underlying mechanism of cognitive impairment as a result of cisplatin.

In line with its mechanism of action, pifithrin-μ did not reverse existing cognitive impairment but only prevented the cognitive dysfunction. Here, we show that nasal MSC treatment has the advantage of being capable of reversing already existing damage as the mitochondrial dysfunction had already fully developed at the time of MSC administration 48 h after completion of the full regimen of cisplatin injections. Restoration of synaptosomal mitochondrial function to control levels in response to nasal MSC administration was associated with complete recovery of cognitive abilities. Together, these findings suggest that MSC act by balancing the bioenergetic machinery allowing repair of cognitive functions.

Current research in cancer survivors suggests that the use of chemotherapeutics is associated with decreased functional and structural connectivity in the brain[7–9]. In line with these findings, we here present the first evidence that in mice cisplatin treatment affects functional network

connectivity as measured with rsfMRI. Characteristic path length is a measure of the connection between various regions of the brain, where decreases in path length are associated with lower overall cognitive ability[5–7]. Our data suggest that the brain network is overly integrated in cisplatin-treated mice and more closely resembles that of random, noisy networks, which is consistent with the higher density of connectomes in the cisplatin group. Nasal MSC administration increased the path length in cisplatin-treated mice, indicating normalization of the functional connectome which underlines the powerful action of nasal MSC administration. Only the right ectorhinal area showed higher clustering in cisplatin + MSC mice ($p = 0.03$) as compared to cisplatin-only mice. This hyper-connectivity may reflect some connectome remodeling associated with MSC and/or some over-correction of cisplatin-related injury. The structural changes in myelination including fiber complexity and global density of myelin as a result of cisplatin were also completely restored by MSC indicating that the regenerative capacities of MSC are powerful and may involve repair of a shared mechanism of cisplatin-induced brain damage.

We showed before that MSC administered either via the nose or directly via cranial administration as a treatment for neonatal hypoxic-ischemic brain damage do not integrate into the brain but merely act by boosting endogenous repair mechanisms leading to lesion repair and restoration of cognitive and motoric function[17,20,25]. MSC genetically overexpressing green fluorescent protein could only be found 48-72 h after administration either by immune fluorescence or by genomic DNA analysis. When investigating migration of MSC into cisplatin-treated brains, we could only detect MSC until 24 h after administration (data not shown). Consistently, MSC administered after cisplatin are no longer detectable at 72 hrs after the last dose; MSC isolated from GFP+ animals were detected in the brain by 12-hrs but were undetectable by 72-hrs, suggesting that the long-lasting effects of MSC is due to a promotion of endogenous recovery, and most likely the MSC did not transdifferentiate into another cell type such as neurons. It has been reported that MSC injected intravenously for other clinical purposes such as treatment to reduce the infarct size after a myocardial infarction, have a half-life of only 24 h as well[33]. We therefore propose that MSC do not survive long and do not integrate into the host in the cisplatin-treated brain, which is important from a perspective of safety. Importantly, MSC administration

after cisplatin treatment did not affect tumor growth in cisplatin-treated mice, indicating that it is a safe intervention.

Taken together, our results indicate that nasal administration of MSC is an effective treatment for chemobrain as a result of cisplatin treatment. Nasal administration of MSC restores executive function and memory after cisplatin treatment. This reversal of cognitive deficits is associated with a restoration of cortical myelination and functional connectivity between brain regions. MSC act via repairing synaptic mitochondrial function as shown by a repair of the morphology and oxygen consumption rates of mitochondria. Therefore, nasal MSC administration may be considered as an important therapeutic strategy to treat established cancer-treatment-induced cognitive impairment.

MATERIAL AND METHODS

Animals

C57BL/6J male and female mice (Jackson Laboratory) aged 9 weeks were housed at 22 ± 2°C, on a 12/12 h reverse dark–light cycle (dark 830–2030 h) with water and food *ad libitum*. Video recording of animal behavior was performed under red light using a Bell & Howell Rogue Night Vision Digital Video Camcorder. C57BL/6-Tg (UBC-GFP) 30Scha/J male mice (The Jackson Laboratory) were used as a source for bone marrow-derived GFP+ MSC. All experiments were conducted at MD Anderson Cancer Center in Houston, Texas in accordance with Institutional Animal Care and Use Committee-approved protocols.

Experimental Design

Mice were randomly assigned to experimental groups and behavioral tests were performed by an independent investigator blinded to drug treatments. Videos were scored by investigators blinded to the experimental set up. For all power analyses, we did set the type I error set at 0.05 and the power at 80%. The minimal change to be detected was set at 25%.

Chemotherapy and MSC treatment

Cisplatin (2.3 mg/kg/day; Fresenius Kabi USA, Lake Zurich, IL) or phosphate-buffered saline (PBS) was administered intraperitoneally daily for 5 days, followed by a 5-day rest without injections and another 5-day injection cycle[15]. This dose regimen of cisplatin is similar in the cumulative dose used in human cancer patients. Mouse MSC were purchased from Invitrogen (GIBCO Mouse C57BL/6, Invitrogen, Carlsbad, CA) or isolated from C57BL/6J or C57BL/6-Tg (UBC-GFP) 30Scha/J animals as previously described[19]. Briefly, bone marrow from femur and tibia of 8- to 10-week-old mice were collected to isolate cells. MSC were cultured in Dulbecco's Modified Eagle's medium/F12 medium with GlutaMax-I, supplemented with 10% MSC-qualified fetal Bovine Serum (FBS) and 5 µg/mL gentamycin (GIBCO Life Technology, Invitrogen, Carlsbad, CA).

Thirty minutes before administration of MSC, 3 µl per nostril of hyaluronidase in PBS (total 100 U per mouse, Sigma-Aldrich, St. Louis, MO) was administered to each nostril to increase the permeability of the nasal mucosa[24,25]. Mice received MSC in a volume of 3 µL, twice applied to each nostril with a total volume of 12 µL or PBS. Based on previous studies in neonatal brain damage, administration of MSC around 3 days after injury is optimal for recovery[17]. We first determined the optimal dose of MSC in a range of 0.125-1x10^6 MSC/ mouse/day administered 48 and 96 h after cisplatin treatment. Robust effects were observed at a dose of 1x10^6 MSC/mouse/day in the PBT (not shown). This dose was therefore selected for further study.

For cell tracking studies, MSC were labeled with PKH-26 Red fluorescent cell linker kit (Sigma-Aldrich, St. Louis, MO) or with CTGB (green) (Life technologies, Carlsbad, CA) following manufacturer's instructions. MSC from GFP-transgenic mice were used for assessment of survival of MSC and/or progenitors in the brain by real time PCR analysis of the presence of the transgene.

Behavioral Testing

To assess cognitive function, we tested mice using the puzzle box test (PBT), the novel object/place recognition test (NOPRT), and Y-maze test 7-10 days after MSC administration.

Puzzle box test. The PBT, which consists of 11 trials at three levels of complexity, is used as a measure of executive functioning[28]. Cisplatin-treated and control mice were submitted to the PBT 7–10 days after the last MSC/PBS administration. Mice were transferred to a testing arena (71 cm x 28 cm) containing a lighted (55 cm x 28 cm) and a dark compartment (15 cm x 28 cm) separated by a wall with a connecting tunnel (4 cm x 2.5 cm). Mice were introduced to the lighted side, and in a series of trials with increasing difficulty, the time to enter the preferred dark compartment was recorded. During the "easy" trials the tunnel was freely accessible. During the "intermediate" trials, the tunnel was filled with bedding, requiring the mice to burrow through to enter the dark compartment. During the "difficult" trials, the tunnel was closed with a cardboard lid that the mice were required to remove before they could enter the tunnel. Testing occurred over 4 days; three consecutive easy trials on day one (trials 1-3), one easy trial (trial 4), followed by two intermediate trials on day 2 (trials 5 and 6), one intermediate trial (trial 7), followed by two difficult trials (trials 8, 9) on day 3, and two additional difficult trials (trial 10, 11) on day 4[28].

Novel Object/Place Recognition Test. This test was performed as previously described[14,15]. In brief, mice were introduced to two identical objects for 5 min (training phase) and then returned to their home cages. After 30 min. mice were returned to the arena which contained one now-familiar object in the same location as in the training session, and one novel object placed on the opposite end of the arena. Investigative behavior toward either object during the 5-min testing period was evaluated using EthoVision XT 10.1 video tracking software (Noldus Information Technology Inc., Leesburg, VA). Discrimination index was determined by the equation $(T_{Novel} - T_{Familiar})/(T_{Novel} + T_{Familiar})$.

Y-Maze Test. This test was performed as previously described[15,34]. Mice were randomly placed in one of the arms of a symmetrical three-arm, gray plastic Y-maze with external spatial room cues. Movement was recorded for 5 min., and the percentage of perfect alternations defined as exploration of all three arms sequentially before reentering a previously visited arm was calculated.

Resting-state fMRI. To test whether cisplatin induces changes in the functional connectome, mice underwent rsfMRI using a 7 Tesla Bruker BioSpec small animal scanner while mice were anesthetized using isoflurane. Isoflurane was administered at 1% (mixed with O_2) to keep the respiration rate between 80 and 120 beats per minute[35]. Mice were secured into the head coil with a bite bar and the head was taped down to minimize motion. We first acquired a single-shot gradient, axial echo planar imaging (EPI) functional sequence [slice thickness = 0.5 mm, gap = 0.0 mm, repetition time (TR) = 2000 ms, echo time (TE) = 12 ms, matrix = 80x64x32, field of view (FOV) = 20x16 mm, flip angle = 75°, number of volumes = 450, averages = 1, scan time = 15 min] followed by a T2-weighted, turbo spin echo, rapid acquisition with refocused echoes (Turbo RARE) sequence (slice thickness: 0.5 mm, gap = 0.0 mm, TR = 4000 ms, TE = 40.00 ms, matrix = 256x180, FOV = 26.600x18.000 mm, flip angle = 90°, number of images = 32, scan time = 4 mins. and 24 secs).

RsfMRI volumes were preprocessed to reduce noise (motion, signal and physiological artifacts)[36] and restrict the signal to the low frequency range (< 0.1 Hz) associated with intrinsic functional networks[37]. Preprocessing included spatial normalization of rsfMRI volumes via a co-registered T2-weighted volume to the Allen Mouse Brain atlas[38]. Using regions from this template, we computed a 62x62 correlation matrix for each mouse.
We then applied graph theoretical analysis using the Brain Connectivity Toolbox[39] and in-house software (https://github.com/srkesler/bnets) to measure connectome properties. Connectomes are graphs that model the brain network as a system of nodes (regions) and edges (connections)[40]. Connectomics provides unique insight regarding the brain's topological organization[40]. We measured the normalized clustering coefficient, characteristic path length, small-worldness index, global/local efficiency, and density of each mouse connectome[39,40].

Functional connectomes were constructed for each subject with N = 62 nodes, network degree of E = number of edges and a network density of $D = E/[(N \times (N-1))/2]$ representing the fraction of present connections to all possible connections. Negative functional edges were zeroed given evidence that properties of negative correlation networks are different than those of positive correlation networks[41]. We evaluated weighted networks without any thresholding. Because individual variation in network density can affect connectome properties[42], connectome properties were compared between

groups using the general linear model with network density as a covariate[43]. We also examined regional effects by measuring and comparing clustering coefficient of all nodes in the networks at minimum connection density using Wilcoxon ranksum test. Data from one mouse in the cisplatin-MSC group and one in the PBS-MSC group had to be excluded from analyses due to lack of small-worldness organization (i.e. small-worldness index was less than one)[44]. This may have been due to the effects of anesthesia which can affect individual mice differently and is known to affect functional connectivity, although we did not observe it in the control mice[45] .

RNA Sequencing

Transcriptional changes induced by cisplatin and MSC in the hippocampus were investigated using whole-genome RNA sequencing. RNA was collected 72 h after the last dose of MSC. Total RNA was isolated using the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany) and analyzed by the RNA Sequencing Core Lab personnel at MD Anderson Cancer Center. The sample libraries were generated using the Stranded mRNA-Seq kit (Kapa Biosystems, Wilmington, MA) following the manufacturer's guidelines. One lane in a 75-nt paired-end run format was performed using a HiSeq 4000 Sequencer. Raw data were analyzed in the Bioinformatics and Computational Biology Department.

We analyzed the genes that were differentially expressed (adjusted $p<0.05$) in the hippocampus of cisplatin treated mice who did and did not receive MSC using Ingenuity Pathway Analysis (IPA; Qiagen Inc., https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis). Expression of mitochondrial encoded genes is presented as average total mitochondrial RNA read ± SEM and analyzed using a One-Way ANOVA.

Tissue processing and Black Gold II staining

Brains were fixed in 4% PFA and frozen sections were taken at 25 µm. Myelin staining was performed using Black Gold II (AG105, Millipore) according to manufacturer's instructions[29]. Free floating sections were mounted onto slides and dried overnight at room temperature. Slides were rehydrated in miliQ water and immersed in Black Gold II at 60°C for 16 minutes. Following washes in water, slides were transferred to prewarmed 1% sodium thiosulfate for 3 minutes at 60°C.

Slides were then rinsed and dehydrated through a series of ethanol and xylene and cover slipped with Permount. Bright field images of myelin were taken and quantified using ImageJ.

Mitochondrial function and morphology

Mitochondrial function and morphology were analyzed in synaptosomes isolated from total brain[15]. Synaptosomes were resuspended in base media (Seahorse Biosciences/Agilent Technologies, Santa Clara, CA) supplemented with 11 mM glucose, 2 mM glutamine, and 1 mM pyruvate. 75 µg of total protein was plated in a Seahorse XFe 24 microplate (Seahorse Biosciences) pre-coated with GelTrex (1 h in 37°C; Life Technologies/Thermo Fisher Scientific, Waltham, MA). Oxygen consumption rate at baseline and the response to 4 µM oligomycin, 6 µM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and 2 µM of rotenone and 2 µM of antimycin A were determined. All values were corrected for non-mitochondrial oxygen consumption.

For TEM analysis of mitochondrial morphology, synaptosomes were fixed in 2% glutaraldehyde plus 2% PFA in PBS and processed[15,46]. Quantification of mitochondrial morphology was performed by two independent researchers blinded to treatment using ImageJ. Mitochondrial width was defined as the measurement perpendicular to the orientation of the cristae. Atypical mitochondria were defined as those with a mitochondrial width greater than 300 nm and opacity less than 50%.

MSC Migration into Brain

MSC labelled with PKH-26 (red) or CTGB (green), were administered 48 h after the last dose of cisplatin/PBS. Brains were harvested 12 h later. Frozen brain sections were examined by confocal microscopy for presence of labelled MSC. In a separate experiment, MSC were isolated from GFP transgenic mice and administered to cisplatin treated animals as before. The presence of GFP transgenic transcript was measured using qPCR with the forward Primer (AGT GCT TCA GCC GCT ACC), reverse primer (GAA GAT GGT GCG CTC CTG), and transgenic probe (TTC AAG TCC GCC ATG CCC GAA).

Effect of nasal MSC administration after completion of cisplatin treatment on tumor growth

Mice were injected with a heterotopic syngeneic murine model of human papilloma virus (HPV)-related head and neck cancer into the hind leg (5 x 10^4 cells) as we described before[9]. Tumor cells are derived from C57BL/6 oropharyngeal epithelial cells transfected with oncogenes E6/7 of HPV 16 and hRAS (mEER)[47,48]. Seven days after tumor cell implantation, animals were treated with cisplatin or PBS and MSC using the same schedule as for the behavioral experiments (2.3 mg/kg) for two 5-day cycles followed by nasal MSC administration at 48 and 96 hours after completion of cisplatin treatment. Tumor volume was measured using a Vernier calipers from three mutually orthogonal tumor diameters, where volume = (π/6)(d1*d2*d3)[47,48].

Statistical analysis of behavioral, white matter staining and mitochondrial data

Data are presented as mean ± standard error of the mean (SEM). Data were analyzed using GraphPad Prism 6 (GraphPad). One-way or two-way analysis of variance (ANOVA) was used with or without repeated measure to test for statistical significance where applicable, with alpha = 0.05. Post hoc pair-wise, multiple-comparisons were performed using the two-tailed Tukey's test. Differences were considered statistically significant at $P < 0.05$.

Abbreviations

CTGB	cell tracker green BODIPY
MSC	mesenchymal stem cells
NOPRT	novel object/place recognition test
rsfMRI	resting-state functional MRI
PBS	phosphate-buffered saline
PBT	puzzle box test
SEM	standard error of the mean

Author Contributions

All authors contributed to the design and conception of the experiments and the final draft of the paper.

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Conflicts of Interest

The authors report no conflicts of interest related to this work.

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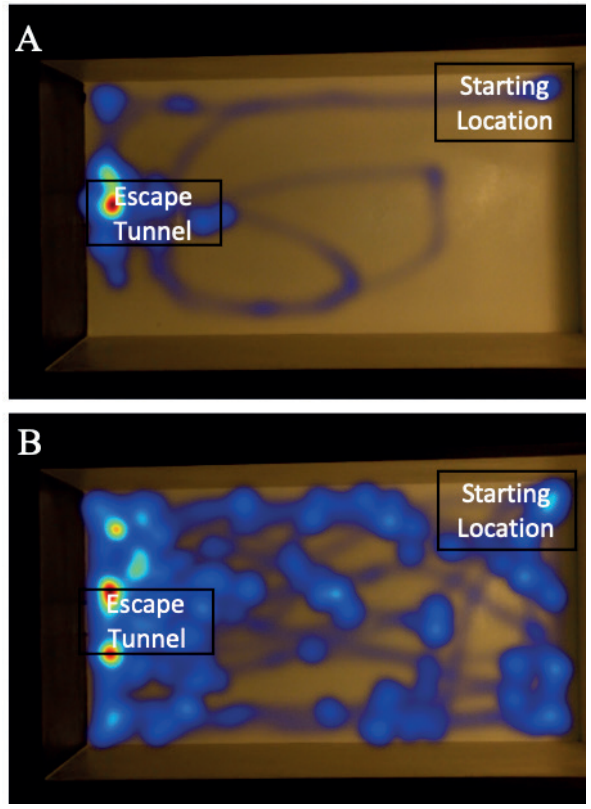
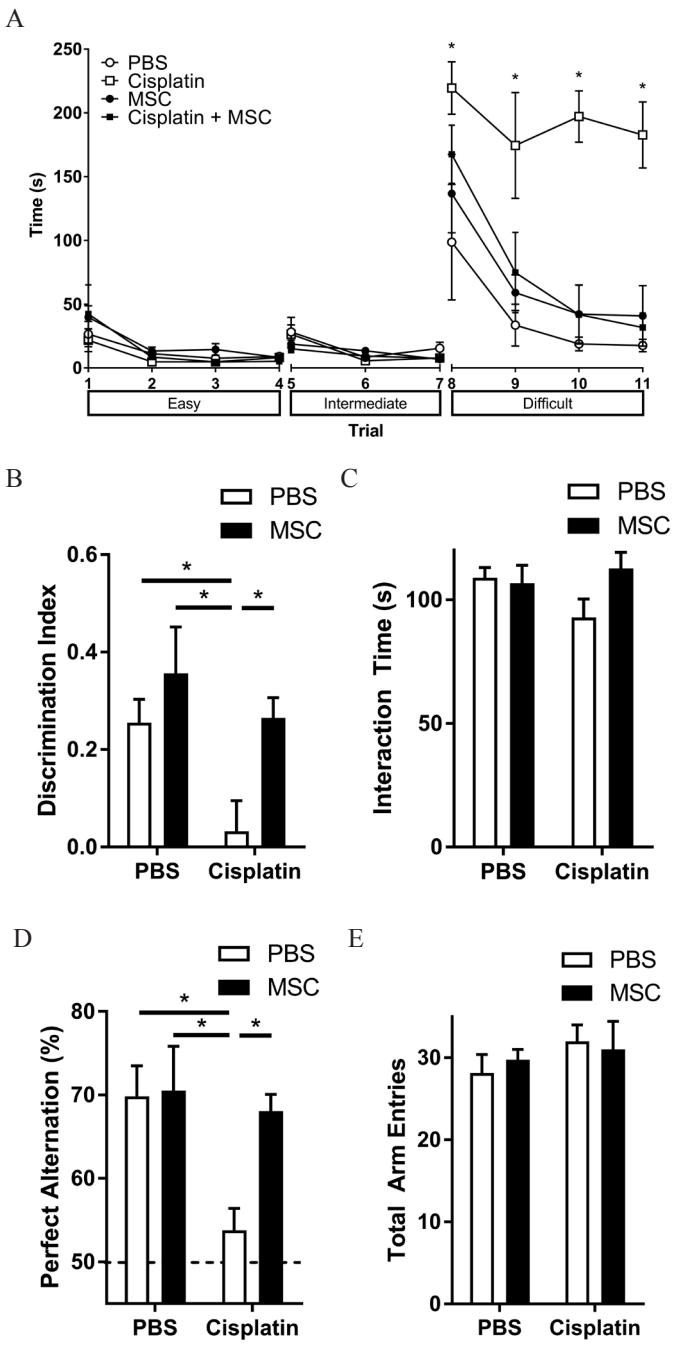
SUPPLEMENTARY MATERIALS

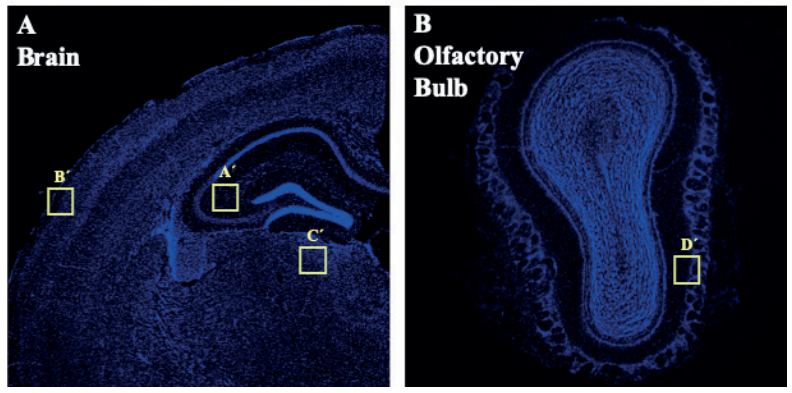


Supplementary Figure 1. Representative heat map of time spent in the light compartment of the puzzle box
Male mice were treated with cisplatin and MSC, and the PBT was performed as in Fig.1A. Representative heat map of time spent in the light compartment of the puzzle box of PBS (A) and cisplatin–treated (B) male mice in trial 8 (first difficult trial).

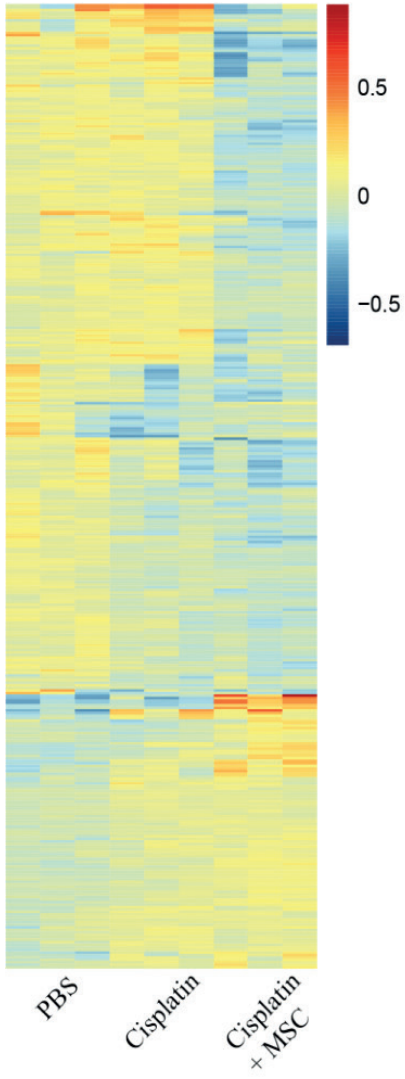


Supplementary Figure 2. Effect of nasally administered MSC on cisplatin-induced cognitive impairments in female mice

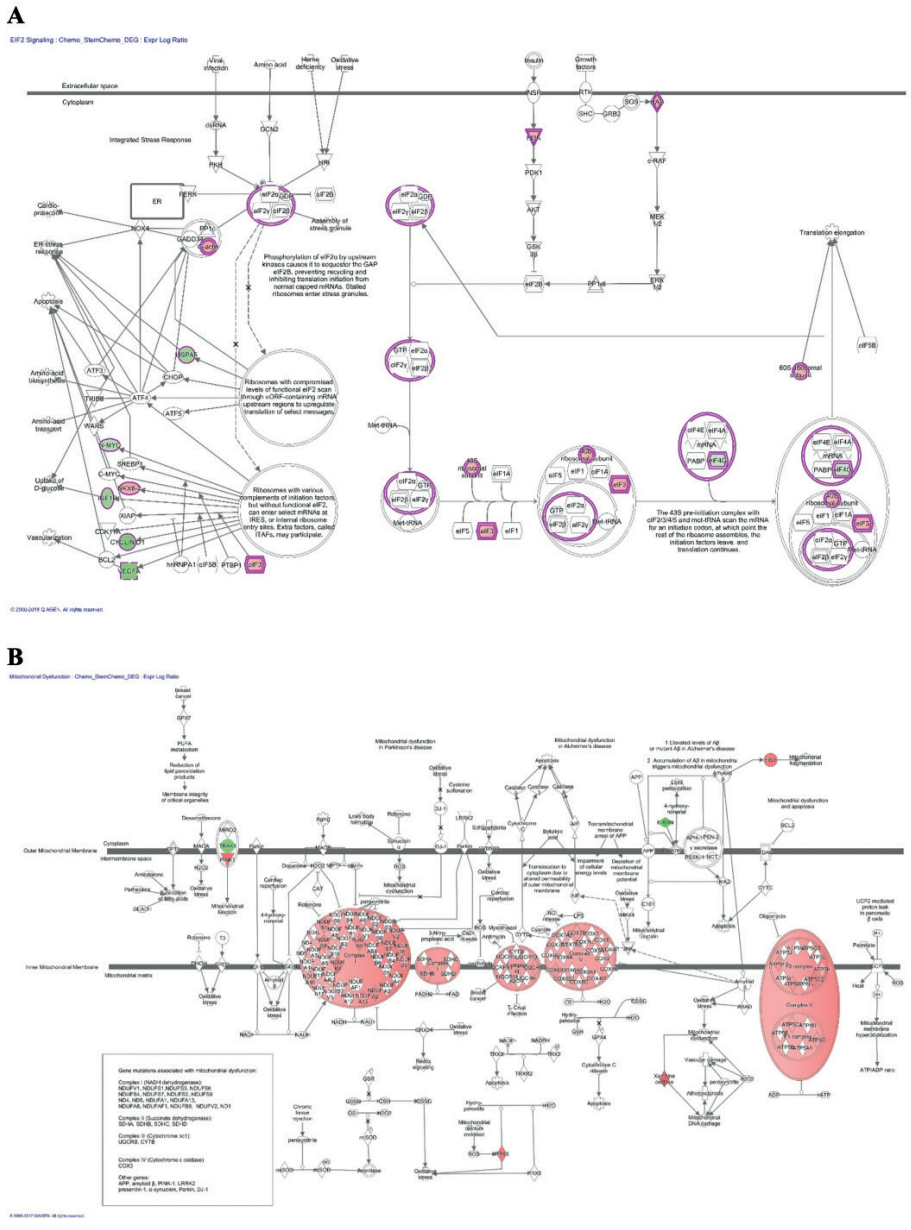
Supplementary Figure 2. Continued
(A) Female mice were treated with cisplatin and MSC, and the PBT was performed as with the male mice in Fig.1A. n = 4-7. *Tukey post hoc:* $*P < 0.05$ versus PBS controls. (B) Female mice were treated with cisplatin and MSC, and the NOPRT was performed. n = 4-7. *Tukey test:* $*P < 0.05$ versus PBS controls. (C) Total interaction times in the NOPR test were not affected by cisplatin and MSC treatment in female mice. n = 4-7. Two-way ANOVA Cisplatin x MSC interaction. (D) The percentage spontaneous alternation in a Y-maze was determined 1 day after the completion of the NOPRT in female mice. Dotted line indicates random chance. Results are expressed as means ± SEM; n = 4-7. *Tukey test:* $*P < 0.05$. (E) Total arm entries in the Y-maze were not affected by cisplatin and MSC treatment in female mice. n = 4-7.



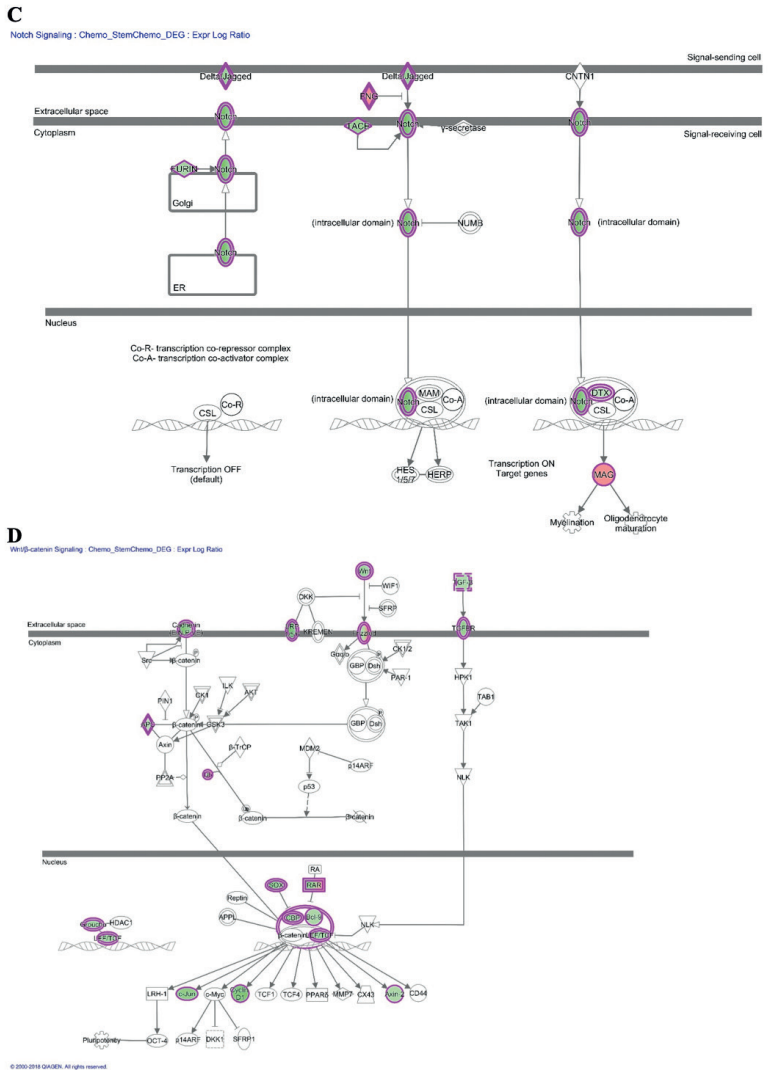
Supplementary Figure.3. Overview of location where MSC were detected.
Overview of brain (A) and olfactory bulb (B) depicting the location of the images depicted in Figure 2 in the hippocampus (A'), cortex (B'), thalamus (C'), and olfactory bulb (D').



Supplementary Figure 4: Effects of cisplatin and MSC on gene expression.
Heat map of differentially expressed genes between samples of PBS, cisplatin, and cisplatin + MSC treated animals (adjusted p<0.05).



Supplementary Figure 5: Details on differentially expressed genes in the top five canonical pathways.



Supplementary Figure 5: Continued
Genes that were upregulated (red) or downregulated (green) in response to nasal MSC administration to cisplatin-treated mice for EIF2 signaling (A), mitochondrial dysfunction and oxidative phosphorylation (B), Notch signaling (C), and Wnt/β-catenin signaling (D).

Supplementary Table 1: Genes upregulated by cisplatin and normalized by nasal administration of MSCs

Symbol	Entrez Gene Name
ACKR1	atypical chemokine receptor 1 (Duffy blood group)
ARC	activity regulated cytoskeleton associated protein
AXIN2	axin 2
Brd4	bromodomain containing 4
BTG2	BTG anti-proliferation factor 2
CALR	calreticulin
CEACAM1	carcinoembryonic antigen related cell adhesion molecule 1
CMIP	c-Maf inducing protein
COL1A1	collagen type I alpha 1 chain
CTSC	cathepsin C
CYR61	cysteine rich angiogenic inducer 61
DUSP1	dual specificity phosphatase 1
DUSP18	dual specificity phosphatase 18
FGF10	fibroblast growth factor 10
FLT1	fms related tyrosine kinase 1
FOS	Fos proto-oncogene, AP-1 transcription factor subunit
GPR151	G protein-coupled receptor 151
HEXIM1	hexamethylene bisacetamide inducible 1
HSP90B1	heat shock protein 90 beta family member 1
HSPA5	heat shock protein family A (Hsp70) member 5
HSPB1	heat shock protein family B (small) member 1
HSPG2	heparan sulfate proteoglycan 2
IER2	immediate early response 2
IGF1R	insulin like growth factor 1 receptor
INHBB	inhibin beta B subunit
JUN	Jun proto-oncogene, AP-1 transcription factor subunit
JUNB	JunB proto-oncogene, AP-1 transcription factor subunit
LRP1	LDL receptor related protein 1
NECTIN1	nectin cell adhesion molecule 1
NECTIN3	nectin cell adhesion molecule 3
NID2	nidogen 2
NPTX1	neuronal pentraxin 1

Supplementary Table 1: Continued

Symbol	Entrez Gene Name
NPTX2	neuronal pentraxin 2
NWD2	NACHT and WD repeat domain containing 2
PDIA3	protein disulfide isomerase family A member 3
PRDM16	PR/SET domain 16
RTN4RL1	reticulon 4 receptor like 1
SCUBE1	signal peptide, CUB domain and EGF like domain containing 1
SLC12A7	solute carrier family 12 member 7
SOX4	SRY-box 4
TGFBR2	transforming growth factor beta receptor 2
THBS1	thrombospondin 1
UBC	ubiquitin C
VWF	von Willebrand factor
WFIKKN2	WAP, follistatin/kazal, immunoglobulin, kunitz and netrin domain containing 2

Supplementary Table.2 Genes downregulated by cisplatin and normalized by nasal administration of MSCs

Symbol	Entrez Gene Name
1700048O20Rik	RIKEN cDNA 1700048O20 gene
2810468N07Rik	RIKEN cDNA 2810468N07 gene
ACTN2	actinin alpha 2
ALDH1A1	aldehyde dehydrogenase 1 family member A1
ANLN	anillin actin binding protein
Bhlhe41	basic helix-loop-helix family, member e41
CIRBP	cold inducible RNA binding protein
CPM	carboxypeptidase M
DBP	D-box binding PAR bZIP transcription factor
DLX6	distal-less homeobox 6
EPHA8	EPH receptor A8
EVI2A	ecotropic viral integration site 2A
Fus	fused in sarcoma
GATM	glycine amidinotransferase
GPR83	G protein-coupled receptor 83
HCN2	hyperpolarization activated cyclic nucleotide gated potassium channel 2
IL33	interleukin 33
INSIG1	insulin induced gene 1
KCNAB1	potassium voltage-gated channel subfamily A member regulatory beta subunit 1
LMO7	LIM domain 7
MAG	myelin associated glycoprotein
NKX6-2	NK6 homeobox 2
PLEKHB1	pleckstrin homology domain containing B1
PPP1R14A	protein phosphatase 1 regulatory inhibitor subunit 14A
PRKCH	protein kinase C eta
RAP1GAP	RAP1 GTPase activating protein
RND2	Rho family GTPase 2
S100B	S100 calcium binding protein B
SLC22A3	solute carrier family 22 member 3
TBC1D8	TBC1 domain family member 8

3

Mitochondrial transfer from mesenchymal stem cells to neural stem cells protects against the neurotoxic effects of cisplatin

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ABSTRACT

Mesenchymal stem cells (MSCs) transfer healthy mitochondria to damaged acceptor cells via actin-based intercellular structures. In this study, we tested the hypothesis that MSCs transfer mitochondria to neural stem cells (NSCs) to protect NSCs against the neurotoxic effects of cisplatin treatment. Our results show that MSCs donate mitochondria to NSCs damaged in vitro by cisplatin. Transfer of healthy MSC-derived mitochondria decreases cisplatin-induced NSC death. Moreover, mitochondrial transfer from MSCs to NSCs reverses the cisplatin-induced decrease in mitochondrial membrane potential. Blocking the formation of actin-based intercellular structures inhibited the transfer of mitochondria to NSCs and abrogated the positive effects of MSCs on NSC survival. Conversely, overexpression of the mitochondrial motor protein Rho-GTPase 1 (Miro1) in MSCs increased mitochondrial transfer and further improved survival of cisplatin-treated NSCs.

In vivo, MSC administration prevented the loss of DCX+ neural progenitor cells in the subventricular zone and hippocampal dentate gyrus which occurs as a result of cisplatin treatment. We propose mitochondrial transfer as one of the mechanisms via which MSCs exert their therapeutic regenerative effects after cisplatin treatment.

Keywords: mesenchymal stem cells, neuronal stem cells, mitochondrial transfer, cisplatin.

INTRODUCTION

Mesenchymal stem cells (MSCs) have been shown to stimulate tissue repair in various disease models, including cardiomyopathy, pulmonary damage, cerebral ischemic insults, and neurodegenerative disorders like Alzheimer and Parkinson disease [8]. Multiple mechanisms have been proposed to mediate these beneficial effects of MSCs, including suppression of inflammation and release of growth factors. Recently, it has become apparent that transfer of healthy mitochondria to damaged cells represents an important mechanism of endogenous regeneration. For example, astrocytes have been shown to transfer mitochondria to neurons after ischemic stroke in mice [15]. MSCs also transfer mitochondria to cardiomyocytes in a model of anthracycline-induced cardiomyopathy [46], to murine alveoli in acute lung injury [17], to murine lung epithelial cells in rotenone-induced airway injury [2], to cortical neurons in a model of cerebral stroke [5] and to human monocyte-derived macrophages as well as murine alveolar macrophages in models of acute respiratory distress syndrome [18].

Platinum-based chemotherapeutic agents, such as cisplatin, are widely used to treat solid tumors [19]. Increasing evidence indicates that cognitive deficits develop in cancer patients treated with chemotherapy. Cognitive deficits induced by cancer treatment are characterized by confusion, memory loss, reduced attention and processing speed, and decreased executive functioning [20-22, 39, 43]. Longitudinal neuropsychological studies report that up to 75 % of cancer patients experience cognitive problems during treatment and likely 35% of affected cancer patients have long-term cognitive effects that seriously impair their quality of life [1].

We recently reported that cisplatin induces cognitive impairment, synaptosomal mitochondrial dysfunction, and changes in neuronal mitochondrial morphology in mice [7]. Nasal administration of MSCs to cisplatin-treated mice restored cognitive function and normalized mitochondrial function [6]. Lomeli et al. have demonstrated that MSCs reduce cranial irradiation-induced brain damage as well [28].

Neurogenic precursor proliferation and differentiation, particularly in the dentate gyrus (DG) of the hippocampus, is crucial to formation of new

neurons, thereby enhancing neural circuity and improving learning and memory [37, 41, 47]. Disruption of adult neurogenesis contributes to the pathogenesis of many neurodegenerative diseases associated with cognitive impairment. *In vivo* and *in vitro*, cisplatin induces loss of neuronal precursors [3, 7, 28]. Gong el al. have shown that neuronal precursors are very sensitive to low cisplatin concentrations [13].

The aim of the present study was to test the hypothesis that MSCs can protect neuronal stem cells (NSCs) in culture, *in vitro* against the neurotoxic effects of cisplatin through mitochondrial donation. Moreover, we investigated whether nasal administration of MSCs *in vivo* protects the brain neurogenic pools against the damaging effects of cisplatin.

MATERIALS AND METHODS

Animals

9 weeks old C57BL/6J male mice (Jackson Laboratory) were used. Mice were housed on a 12/12 h reverse dark–light cycle. Animals had access to water and food ad libitum. All experiments were conducted at The University of Texas MD Anderson Cancer Center in Houston, Texas. Animals were used in accordance with Institutional Animal Care and Use Committee-approved protocols.

Cell Culture and Transfection

Mouse cortical NSCs (R&D Systems, Minneapolis, MN, USA) were cultured in monolayers in low-glucose Dulbecco's Modified Eagle's Medium (DMEM)/F12, supplemented with 100 U/mL penicillin and N-2 plus supplement (R&D Systems). Fibroblast growth factor basic and epidermal growth factor (both 20 ng/mL; R&D Systems) were added to the cultures daily. NSCs were cultured on surfaces coated with poly-L-ornithine (Sigma-Aldrich, St. Louis, MO, USA) and bovine fibronectin (R&D Systems) and detached using Accutase (Innovative Cell Technologies, San Diego, CA, USA).

C57BL/6 mouse MSCs (Invitrogen) were grown in 5% CO_2 at 37°C in DMEM/F12 medium with GlutaMax-I, supplemented with 10% MSC-qualified fetal bovine serum and 5 µg/mL gentamycin (all from GIBCO, Carlsbad, CA, USA). Before transfection, MSCs were seeded on plates coated with poly-L-lysine (Sigma-Aldrich). Cells were harvested using TrypLE-express

(GIBCO). For labeling mitochondria we used pLYS1-FLAG-MitoGFP-HA (Addgene plasmid # 50057; gift from Vamsi Mootha [35]) which contains the pore-forming subunit of the mitochondrial calcium uniporter to target the label to the mitochondria. The mito-mcherry plasmid was generated by cloning the mito-GFP insert of the pLYS1-FLAG-MitoGFP-HA plasmid into the mcherry2-N1 vector (Addgene plasmid # 54517, gift from Michael Davidson). The mitochondrial rho GTPase 1 (Miro1)-GFP overexpression plasmid (RhoT1, MG224107) was obtained from Origene (Rockville, MD, USA). Transfection of mito-GFP, mito-mcherry and Miro1-GFP plasmids were performed using either Lipofectamine LTX (Invitrogen) with Plus reagent for mito-GFP or mito-mcherry expression in MSCs or Lipofectamine 2000 (Invitrogen) and JetPrime (Polyplus Transfection, New York, NY, USA) for Miro1 overexpression, according to the manufacturer's instructions.

Chemotherapy and MSC treatment

Cisplatin (2.3 mg/kg/day; Teva, Petah Tiva, Israel) or phosphate-buffered saline (PBS) was administered daily to mice intraperitoneally, following a 5 days injections and 5 days rest scheme. Prior to MSC application, mice received 3 µl of hyaluronidase in PBS in each nostril (100 U per mouse, Sigma-Aldrich) to increase the permeability of the nasal mucosa [6, 10, 11, 16]. 30 minutes after, 3 µl of MSC cell suspension ($1x10^6$ cells per mouse per day) or PBS were administered to mice, twice in each nostril, for a total of 12 µl. MSCs were applied 48 and 96 hours after the last cisplatin injection.

Co-Culture of MSCs and NSCs

NSCs (35×10^4 cells) were plated on cell culture imaging dishes (ibidi, Fitchburg, WI, USA). Two days after plating, NSCs were treated with cisplatin (Teva, Petah Tikva, Israel) for 8 h, stained with 20 µM CellTracker Blue fluorescent probe (CTB; Invitrogen, Carlsbad, CA) for 45 min at 37°C, and washed in serum-free media. MSCs (15×10^4 cells) were added to the culture for 17 h. Co-cultures were stained with wheat germ agglutinin (WGA) conjugates (WGA 488 or WGA 594, 1/300 dilution; Invitrogen) for 10 min at 37°C, followed by 2 washes in Hank's Balanced Salt Solution (GIBCO) prior to imaging in Live Cell Imaging Solution (Invitrogen).

To inhibit actin polymerization, MSCs were treated with 2 µM Latrunculin B (Sigma-Aldrich) for 24 h prior to co-culture with NSCs. To assess NSC survival, CTB-positive NSCs were counted using the countess II FL automated cell

counter (Invitrogen). Mitochondrial transfer was quantified in representative confocal images of every condition used, and the percentage of either mito-GFP-positive or mito-mcherry-positive NSCs was determined.

Analysis of Mitochondrial Membrane Potential

After exposure to cisplatin and co-culture with MSCs, cells were stained with 250 nM of the fluorescent mitochondrial membrane potential-sensitive dye tetramethylrhodamine methyl ester (TMRM, Invitrogen) for 45 min at 37ºC. As a positive control, NSCs were treated with 10 µM carbonilcyanide p-triflouromethoxyphenylhydrazone (FCCP), a mitochondrial uncoupler, for 15 min. Cells were imaged by confocal microscopy or washed with serum-free media and collected in a single cell suspension for flow cytometry analysis. The fluorescence signal was detected with a BD accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA USA) at FL2 emission of 585/40 nm. We used the TMRM in sub-quench mode, as described previously [30, 33].

Analysis of Mitochondrial Bioenergetics

To assess mitochondrial bioenergetics, NSCs were grown in a Seahorse XFe 24 microplate (Seahorse Biosciences/Agilent Technologies, Santa Clara, CA, USA) coated with Poly-L-Ornithine and fibronectin to 80% confluency. NSCs were treated with 0.5-1 µM cisplatin or vehicle for 12 h, washed with serum-free media, and incubated for 1 h at 37ºC in XF base media (Seahorse Biosciences) supplemented with 11 mM glucose, 2 mM glutamine, and 1 mM pyruvate. Oligomycin (2 mM), FCCP (4 mM), and rotenone/antimycin A (2 mM each) were used with a 3-time repeat of a 2-minute mix, 3-minute wait, and 2-minute measure assay cycle. Oxygen consumption rates were normalized to the total protein content of each well. Basal respiration, adenosine triphosphate (ATP)-linked respiration, proton leak, and maximal respiratory capacity were determined as described previously [7, 23].

Immunohistochemistry

Immunostaining of neural precursors in the dentate gyrus (DG) and subventricular zone (SVZ) was performed as previously reported [7]. Briefly, mice were euthanized and perfused with PBS followed by 4% paraformaldehyde (PFA). Brains were removed and fixed in 4% PFA for 48h, paraffin embedded and sectioned at 8 and 10 µm for the SVZ and DG, respectively. Brain slices were stained for doublecortin (DCX, 1:50; Abcam, Cambridge, UK) followed by AlexaFluor 488 Donkey secondary

antibody (Invitrogen) and DAPI. Sections were imaged and positive staining was quantified using ImageJ. The number of DCX+ cells was quantified in representative images by researchers blinded to treatment.

To characterize NSCs, immunostaining was done as previously described [4]. Briefly, cells were fixed with 4% paraformaldehyde in PBS, treated with 0.25% Triton X-100, blocked in 2% BSA in PBS and stained with either anti-DCX antibody (1:50; Abcam, Cambridge, UK), anti Sox2 (1/200; Millipore, Burlington, MA), anti βIII-Tubulin (1/200; R&D Systems) or anti Nestin (1/1000, Abcam) and anti-GFAP (1/200, Acris, Rockville, MD) in blocking buffer followed by secondary antibody and DAPI. NSCs used for experiments were 52% Nestin+, 17% GFAP+, 16% Nestin/GFAP double-positive, 92% DCX+, 94% Sox2+ and 5% βIII-Tubulin+.

Confocal Microscopy

Live cell images of MSC and NSC co-cultures, fixed NSC staining as well as DCX expression in the DG and SVZ, were acquired using a SPE Leica Confocal Microscope (Leica Microsystems, Buffalo Grove, IL, USA) with a 63 X or 40 X objective, and analyzed with LAS X software. Image J was used to quantify TMRM intensity in individual cells. Data are expressed as the corrected total cell fluorescence = integrated density (area of selected cell × mean fluorescence of background readings) [31].

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM) of at least 3 independent experiments. Data were analyzed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). One-way or two-way analysis of variance (ANOVA) was used with or without repeated measure followed by either Bonferroni's or Tukey's correction for multiple comparisons, Dunn's multiple comparison or using Student's t-test, as appropriate and indicated in the legends.

RESULTS

MSCs rescue NSC from Cisplatin-Induced Cell Death in vitro and in vivo

NSCs were treated with 0, 0.5 or 1 μM cisplatin for 8 h and stained with Cell Tracker Blue fluorescent probe. Subsequently, the Cell Tracker Blue positive neurons were co-cultured with MSCs for 17 h and recovery of the NSCs was quantified. Figure 1A shows that cisplatin dose-dependently reduced NSC survival. Addition of MSCs significantly increased survival of NSCs.

Next, we tested whether MSCs also rescue NSCs from cisplatin-induced cell loss in the DG of the hippocampus and the SVZ. Mice were injected with cisplatin during two cycles of 5 days (2.3 mg/kg) with 5 days of rest in between [7, 23]. One month after completion of cisplatin treatment, we observed a 40 % decrease in the DCX+ neural progenitors in the DG of the hippocampus (Figures 1F) and a 50 % decrease in the SVZ (Figure 1K). Nasal application of MSCs at 48 and 96 hours after the last cisplatin dose reduced the cisplatin-induced loss of DCX+ neuronal progenitors in both the DG (Figures 1F) and SVZ (Figures 1K).

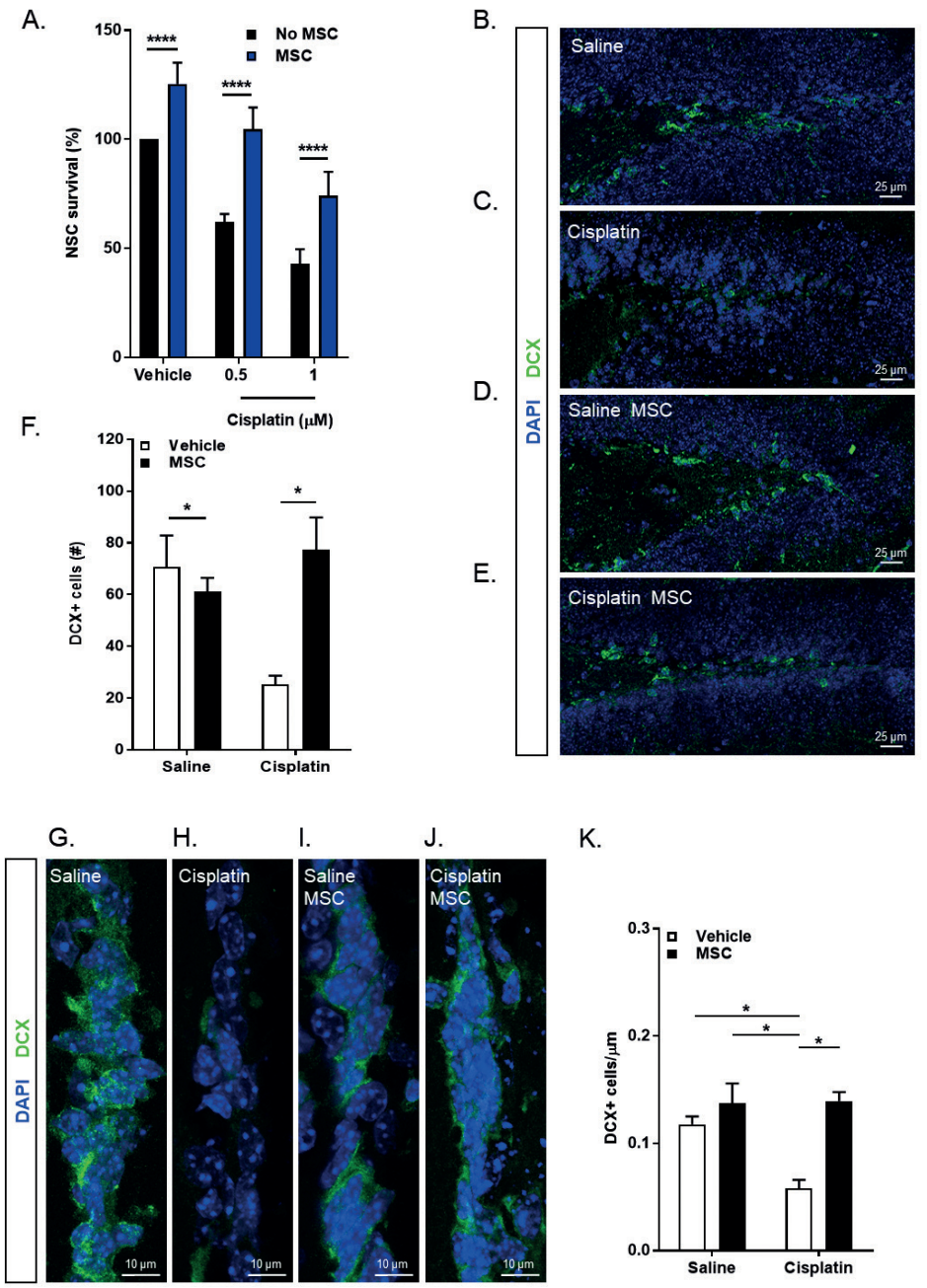

Figure 1. MSCs rescue damaged NSCs after cisplatin treatment and reverse the loss of neuroblasts in the brain

Figure 1. Continued
(A) Neuronal stem cells (NSCs) were treated with cisplatin or vehicle for 8 h, stained with cell tracker blue (CTB), and subsequently co-cultured for 17 h with or without mesenchymal stem cells (MSCs). Survival of NSCs was assessed by counting the number of CTB-positive cells. The graph shows the rate of NSC survival after 17 h co-culture with MSCs (blue bars) or without MSCs (black bars). Data are normalized to survival in the absence of MSCs and cisplatin in each experiment and represent the mean ± SEM of 6 independent experiments. Data were analyzed using two-way ANOVA, repeated measures (cisplatin × MSC interaction: $P<0.01$), followed by Bonferroni's post-hoc test. **** $P<0.0001$).
(B-K) Animals were treated with cisplatin for 2 cycles of 5 days. DCX+ neuronal progenitors were observed in the DG of the hippocampus **(B-E)** as well as the SVZ **(G-J)**. The number of cells were counted in the DG tip **(F)**. For the SVZ, the number of cells was normalized to the length of the SVZ **(K)**. Data were analyzed by two-way ANOVA followed by Tukey's post-hoc test. *$P<0.05$.

Cisplatin Induces Mitochondrial Damage in NSCs

To assess whether cisplatin induced mitochondrial damage in NSCs, we measured oxygen consumption rates of NSC using the Seahorse XF24 extracellular flux analyzer (Figure 2A). NSCs treated with cisplatin showed a marked decrease in basal respiration as well as in oxygen consumption related to ATP production in comparison to control conditions. Furthermore, cisplatin significantly reduced maximal respiration as measured in the presence of FCCP (Figure 2B).

As a second measure of cisplatin-induced loss of mitochondrial integrity, we assessed mitochondrial membrane potential. NSCs were treated with cisplatin and labeled with the mitochondrial membrane potential-sensitive dye tetramethylrhodamine methyl ester (TMRM). Using live-cell imaging, we observed that cisplatin decreased the TMRM signal, indicating a reduction in mitochondrial membrane potential (Figure 2C). Quantification of the change in TMRM staining by flow cytometry showed that exposure of NSCs to cisplatin decreased mean TMRM fluorescence intensity by approximately 40%. This decrease in mitochondrial membrane potential was similar to what we observed in response to the mitochondrial uncoupler FCCP, which was used as a positive control (Figure 2D).

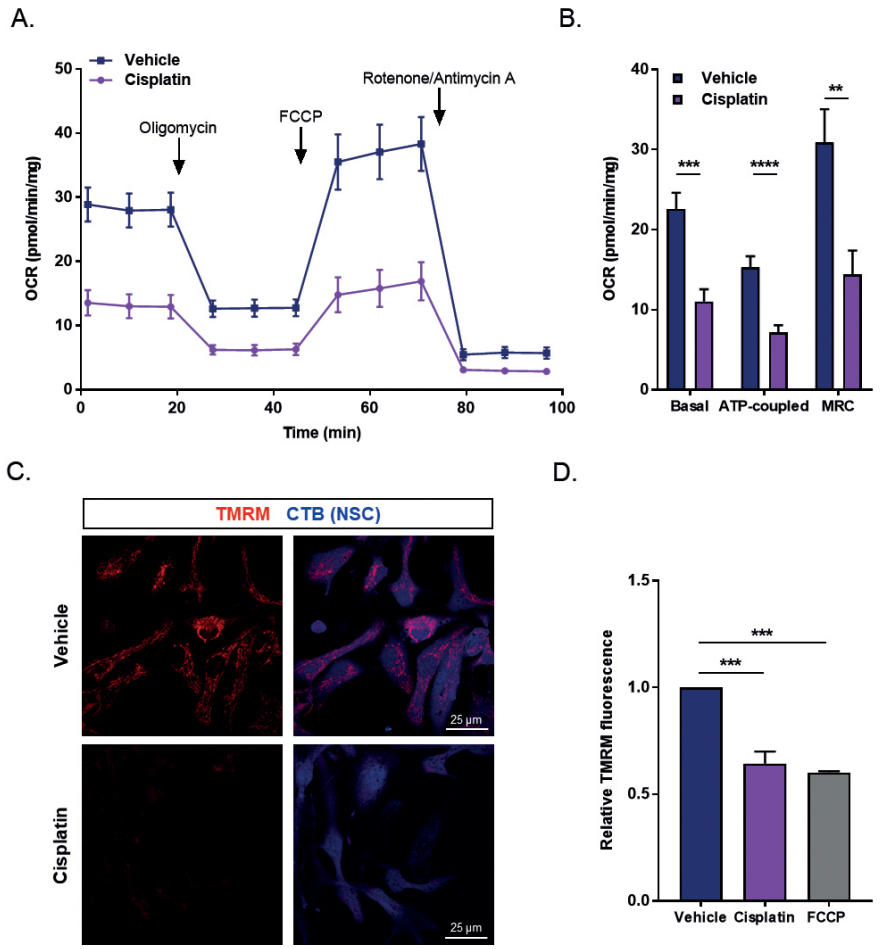

Figure 2. Cisplatin induces NSC mitochondrial dysfunction
Neuronal stem cells (NSCs) were treated with 1 µM cisplatin for 12 h. Oxygen consumption rates (OCR) were analyzed using a Seahorse XFe 24 Analyzer and normalized to protein content **(A).** Mean basal, ATP production-related, and maximum respiratory capacity (MRC) normalized to protein content were calculated **(B)**. Results are expressed as means ± SEM of 3 independent experiments. Data were analyzed using Student's t-test: **$P<0.01$; ***$P<0.001$, ****$P<0.0001$. NSCs were treated with 1 µM cisplatin for 8 h followed by 17 h in normal medium. Cells were stained with tetramethylrhodamine methyl ester (TMRM) and monitored immediately by live-cell imaging **(C)** or flow cytometry **(D)**. NSCs treated with carbonilcyanide p-triflouromethoxyphenylhydrazone (FCCP, 10 µM for 15 min) were used as a positive control. Bar graphs represent mean ± SEM of 3 independent experiments. Data are normalized to mean fluorescence intensity of vehicle-treated cells in each experiment. Data were analyzed using One-way ANOVA followed by Bonferroni's post-hoc test. *** $P\leq0.001$.

Mitochondrial Transfer from MSC to Cisplatin-treated NSC

Having shown that MSCs can protect NSCs against cisplatin-induced cell death, we hypothesized that the mechanism of the restorative effect of MSCs may involve donation of healthy mitochondria from MSCs to cisplatin-damaged NSCs. To test this possibility, we labeled the mitochondria in the MSCs using mito-GFP (Figures 3) or mito-mcherry (Figure 4) plasmids [35]. NSCs were treated with cisplatin or control medium for 8 h, followed by co-culture with MSCs containing the fluorescently tagged mitochondria. Confocal imaging revealed GFP+ or mcherry+ mitochondria in cisplatin-treated NSCs, suggesting mitochondrial transfer from MSCs to NSCs (Figures 3 and 4). Quantitative assessment of mitochondrial transfer showed that cisplatin dose-dependently increased the percentage of NSC that had received mitochondria from MSCs (Figure 3C). The orthogonal slice view in figure 4C demonstrates that the MSC-derived mitochondria are indeed localized inside the cisplatin-treated NSC.

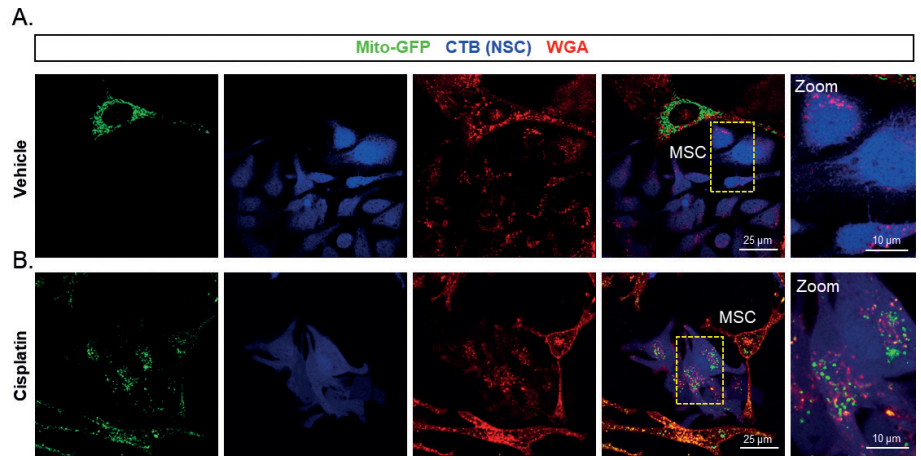

Figure 3. MSCs donate mitochondria to NSCs damaged by cisplatin
Representative confocal images of Neuronal stem cells (NSCs) treated with 1 µM cisplatin or vehicle for 8 h, stained with cell tracker blue (CTB) and subsequently co-cultured for 17 h with mesenchymal stem cells (MSC) transfected with mito-GFP (A and B) to label the MSC-derived mitochondria. Prior to confocal imaging, co-cultures were stained with wheat germ agglutinin (WGA) AF 594 (A and B) to reveal cell membranes. GFP-positive mitochondria were detected in NSCs treated with cisplatin (B, right panel), indicating mitochondrial transfer. The extent of mitochondrial transfer was quantified by counting the number of NSCs positive for the GFP signal in the co-cultures (C). Data are represented as the mean ± SEM of 3 independent experiments and were analyzed using One-way ANOVA followed by Bonferroni's post hoc-test. **** P<0.001.

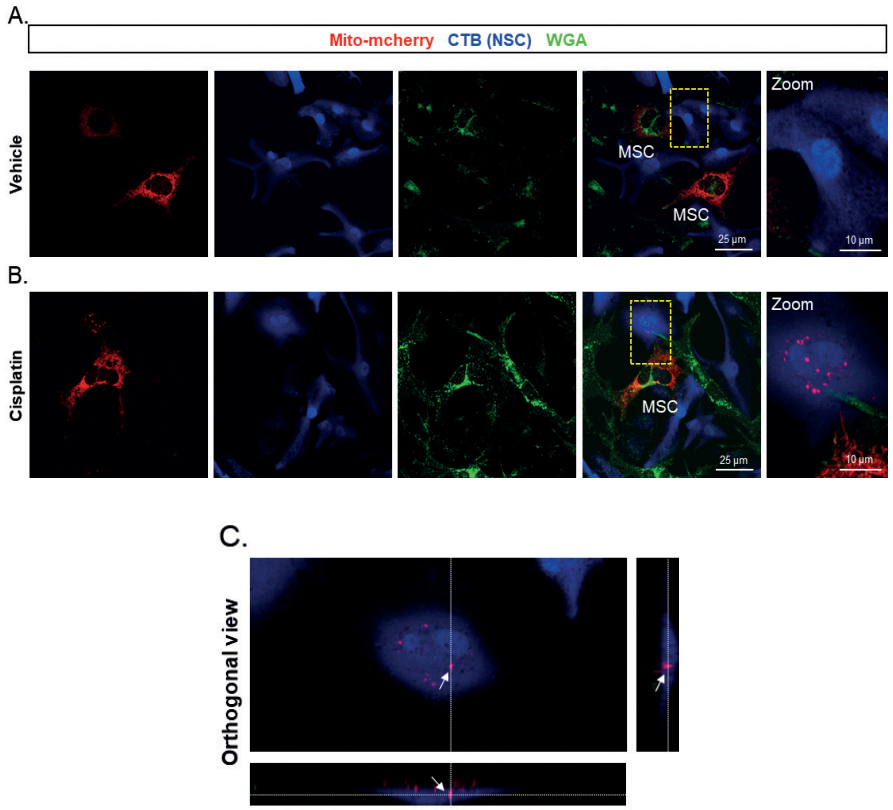

Figure 4: Representative confocal images of Neuronal stem cells (NSCs) and mesenchymal stem cells (MSCs) co-cultures.
NSCs were treated with either 1 µM cisplatin or vehicle for 8 h, stained with cell tracker blue (CTB) and co-cultured with MSCs transfected with mito-mcherry plasmid **(A** and **B)** to label the mitochondria. Co-cultures were stained with wheat germ agglutinin (WGA) AF 488 **(A** and **B)** to reveal cell membranes before confocal imaging. mcherry-positive mitochondria were observed in NSCs treated with cisplatin (**B,** right panel**)** showing mitochondrial transfer. **(C)** Orthogonal slice view of NSC containing mcherry-positive mitochondria derived from MSC.

MSC Normalize Mitochondrial Membrane Potential in Cisplatin-Treated NSCs

Cisplatin treatment decreased TMRM staining in NSCs, indicating a reduction in mitochondrial membrane potential (Figures 2 and 5). We next analyzed whether mitochondrial transfer of MSCs to cisplatin-treated NSCs restored their mitochondrial membrane potential. To that end, we compared TMRM staining in cisplatin-treated NSCs co-cultured with or without mito-GFP-transfected MSCs (Figure 5). TMRM staining was markedly increased in those NSCs that received mitochondria from the MSCs (as shown by the presence of mito-GFP-labeled mitochondria) in comparison with the NSCs that were negative for mito-GFP (Figure 5). The latter results indicate that MSC-derived mitochondrial donation restores mitochondrial integrity of NSCs treated with cisplatin.

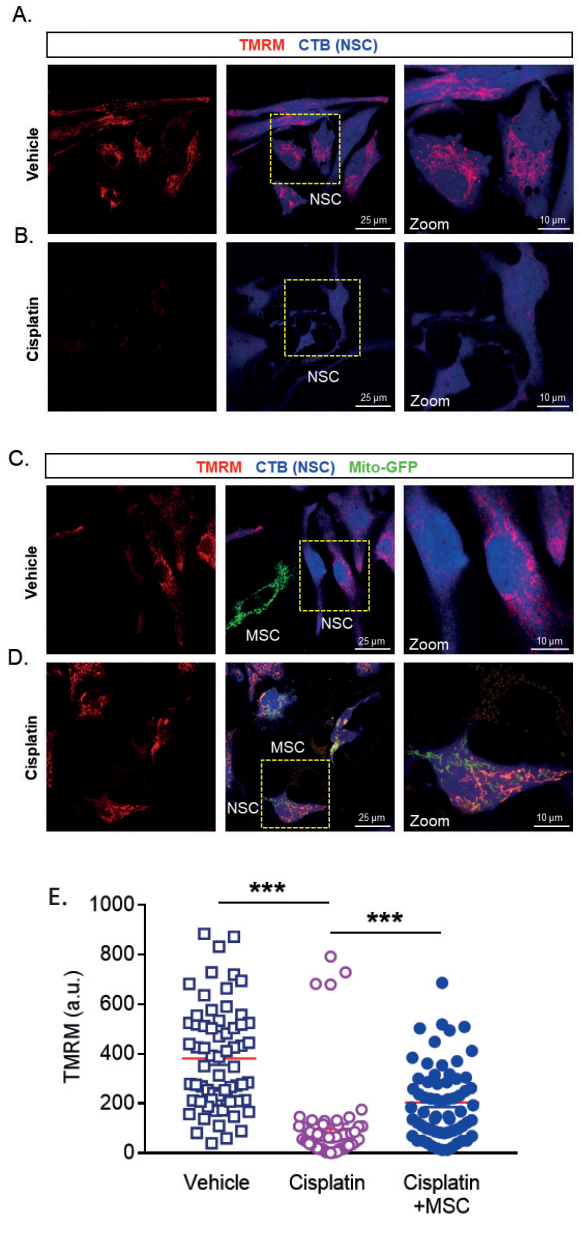

Figure 5. Mitochondrial transfer from MSCs to NSCs damaged by cisplatin restores mitochondrial membrane potential
Neuronal stem cells (NSCs) were treated with vehicle or 1 µM cisplatin for 8 h, stained with cell tracker blue (CTB), and subsequently co-cultured for 17 h with or without mesenchymal stem cells (MSCs) transfected with mito-GFP to label the mitochondria. Co-cultures were stained with tetramethylrhodamine methyl ester (TMRM) and imaged.

Figure 5: Continued
Confocal images of co-cultures show that NSCs exhibited a bright TMRM signal in control conditions **(A** and **C)** that was markedly reduced after cisplatin treatment **(B)**. The mitochondrial membrane potential in cisplatin-treated NSCs was restored after receiving MSC-derived mitochondria labeled in green **(D)**. MSCs do not uptake TMRM as well as NSCs (Supplementary Figure S1). TMRM fluorescence was quantified in individual NSCs **(E)**. N = 65 cells were quantified in each group. Data are represented as means ± SEM and were analyzed using One-way ANOVA followed by Dunn's multiple comparisons test. *** $P \leq 0.001$.

Effect of Latrunculin B on Transfer of Mitochondria and NSC Survival

Mesenchymal stem cells (MSCs) can transfer mitochondria to other cells via formation of tunneling nanotubes (TNTs) and protrusions [18, 40]. To determine whether mitochondrial donation is crucial for NSC survival, we pre-incubated MSCs with latrunculin B (LatB, an inhibitor of f-actin polymerization) which has been shown to inhibit TNT formation [34].We assessed the effect of LatB on mitochondrial transfer and NSC survival in the co-cultures with MSCs labeled with mito-mcherry. LatB reduced the transfer of mito-mcherry-labeled mitochondria to cisplatin-treated NSCs (Figure 6D and E). Interestingly, LatB did not affect transfer of mitochondria to untreated NSCs (Figures 6B and E). Moreover, LatB-treated MSCs were no longer able to promote survival of cisplatin-treated NSCs (Figure 6F) indicating that mitochondrial transfer by MSC contributes to NSC survival.

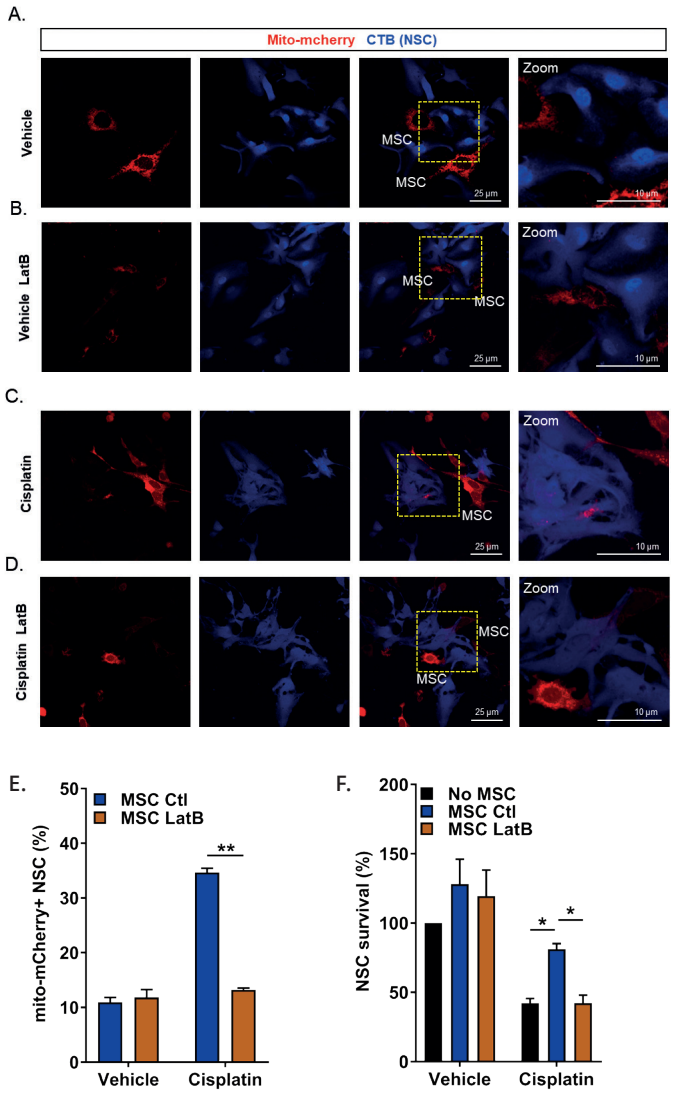

Figure 6. Effect of Latrunculin B on transfer of mitochondria and NSC survival
Representative confocal images of Neuronal stem cells (NSCs) stained with cell tracker blue (CTB) and subsequently co-cultured for 17 h with mesenchymal stem cells (MSC) transfected with mito-mcherry **(A-D)** to label the MSC-derived mitochondria.

Figure 6: Continued
Neuronal stem cells (NSCs) were treated with 1 µM cisplatin or medium for 8 h followed by co-culture with mesenchymal stem cells (MSCs) treated with 2 µm Latrunculin B (MSC LatB, **B** and **D**), control MSC (MSC Ctl, **A** and **C**), or without MSC for 17 h. Mitochondrial transfer **(E)** and NSC survival **(F)** were quantified as in Figures 1 and 3. Data are represented as means ± SEM of 4 independent experiments and were analyzed using two-way ANOVA followed by Bonferroni's post-hoc test. ** $P<0.01$; * $P<0.05$.

Overexpression of Miro1 in MSC Increases Mitochondrial Transfer and Promotes NSC Survival

Miro-1 is an essential mediator of microtubule-based mitochondrial motility and contributes to mitochondrial transfer between cells [2]. In our search to improve mitochondrial donation by MSCs, we overexpressed Miro1 in MSCs using a mitochondrial Rho GTPase 1 (Miro1)-GFP plasmid. NSCs were treated either with 1 µM cisplatin or vehicle for 8 h, and subsequently co-cultured with or without MSCs overexpressing Miro1 and mito-mcherry or MSCs transfected with control vector and mito-mcherry for 17 h. Overexpression of Miro1 in MSCs increased mitochondrial transfer to NSCs (Figures 7B, D and E). Moreover, overexpression of Miro1 in MSCs increased their positive effect on survival of cisplatin-treated NSCs (Figure 7F).

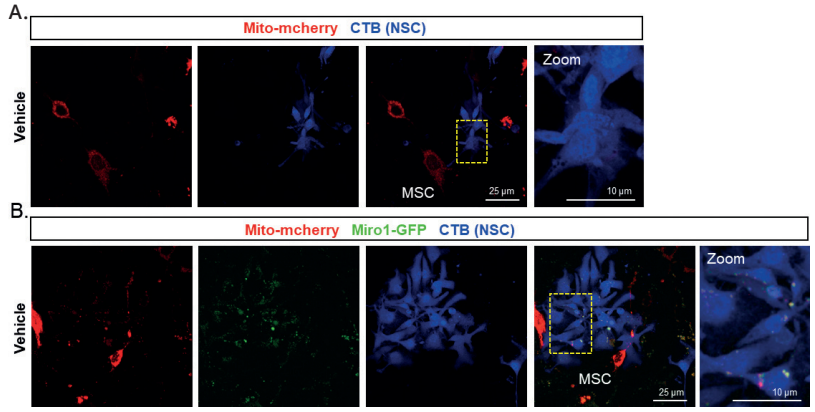

Figure 7. Overexpression of Miro1 in MSCs boosts NSC survival and enhances mitochondrial transfer to injured NSCs

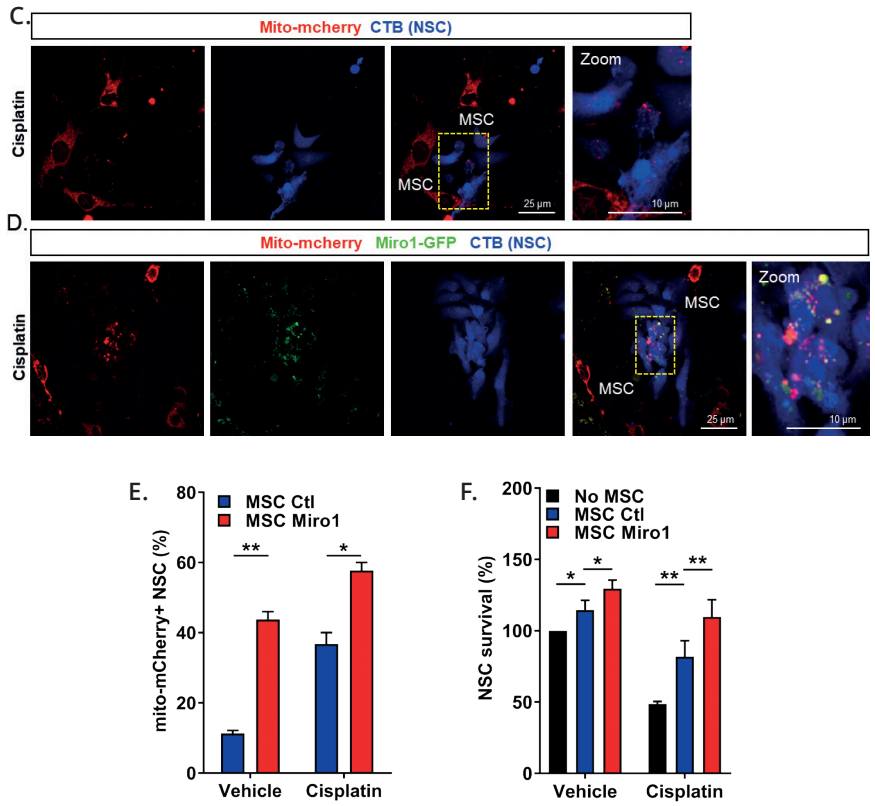

Figure 7: Continued
Representative confocal images of Neuronal stem cells (NSCs) stained with cell tracker blue (CTB) and subsequently co-cultured for 17 h with mesenchymal stem cells (MSC) transfected with mito-mcherry **(A-D)** and miro1-GFP (**B, D**) to label the MSC-derived mitochondria. Neuronal stem cells (NSCs) were treated with 1 µM cisplatin for 8 hours and then co-cultured for 17 h with mesenchymal stem cells (MSCs) overexpressing Miro1 GTPase (MSC Miro1), MSCs transfected with empty vector (MSC Ctl), or without MSCs. Mitochondrial transfer **(E)** and survival **(F)** were assessed as in Figure 6. Data were analyzed by two-way ANOVA followed by Bonferroni's post-hoc test. ** $P<0.01$; * $P<0.05$.

DISCUSSION

Here we show for the first time that MSCs donate mitochondria to NSCs when damaged by cisplatin *in vitro*. We also show for the first time that the loss of DCX+ neuronal precursors caused by administration of 2 cycles of cisplatin can be rescued by intranasal administration of MSCs. Transfer of mitochondria from MSCs to NSCs reversed the decrease in mitochondrial membrane potential of the cisplatin-treated NSCs and favored their survival after cisplatin treatment. Blocking MSC-derived mitochondrial transfer by inhibiting actin polymerization eliminated the beneficial effect of MSCs on survival of cisplatin-treated NSCs. Conversely, when mitochondrial transfer was enhanced by overexpression of the Rho-GTPase 1 (Miro1), a mitochondrial motor protein, NSC survival after cisplatin treatment further increased. Collectively, our data support the model that MSCs can transfer mitochondria to damaged NSCs via formation of protrusions and tubular structures to rescue the latter ones after cisplatin treatment.

MSCs have the potential to repair injuries by a variety of mechanisms that range from secretion of paracrine factors and transfer of proteins and RNA to the transfer of organelles such as mitochondria. It is now well recognized that MSCs can transfer mitochondria to various cell types, including epithelial cells, macrophages, cardiomyocytes, neural cells and endothelial cells, in highly toxic or cell-damaging conditions [2, 5, 18, 26, 27, 44, 46]. As mentioned in the introduction, the transfer of mitochondria has also been shown to occur *in vivo* and may assist in rescuing functional and bioenergetic properties of recipient cells in metabolic need [2, 5, 18, 26, 27, 44, 46].

In our *in vitro* model of cisplatin-induced neurotoxicity, we observed that cisplatin treatment markedly increased the number of NSCs that received mitochondria from MSCs. This finding implies that cisplatin-treated NSCs express a "danger signal" that prompts MSCs to initiate mitochondrial transfer and therefore promote reparative functions. Several danger signals have been hypothesized to function as inducers of mitochondrial transfer. One possibility is that cells in need release damaged mitochondria and mtDNA that can then be recognized by MSCs via receptors for damage-associated molecular patterns, including toll-like receptors [12]. The actual uptake of damaged mitochondria by MSCs was shown to be crucial for

activating MSCs to rescue damaged cardiomyocytes or human umbilical-vein endothelial cells, both *in vitro* and *in vivo* [29].

Intercellular communication is crucial for the development and maintenance of tissue growth, differentiation, and regeneration. Cells are capable of establishing direct contact through various types of cell connections, such as formation of cytoplasmic TNTs that enable the transfer of organelles such as mitochondria from one cell to another. Studies have reported that one of the means by which MSCs make contact with injured cells and transfer their mitochondria is via formation of these TNTs [2, 18, 26, 27]. Our finding that LatB reduces mitochondrial transfer indicates that MSCs use TNTs for delivering their mitochondria. The molecular signal inducing the formation of TNTs is still unclear and seems to differ between cell types [40]. Studies conducted in immune cells and HEK293T cells highlight the involvement of the M-Sec pathway, a 73-kDa cytosolic protein also known as tumor necrosis factor α-induced protein 2 or B94, in inducing the membrane protrusion that is one of the first steps in the formation of TNTs [14, 32]. In addition, the tumor suppressor molecule p53 and the Akt/PI3K/mTOR signaling pathway have been shown to play a role in TNT formation in astrocytes [42].

Wang et al. [42] also found that p53 activation is crucial for TNT formation since genetic ablation of p53 prevented formation of TNTs in rat hippocampal co-cultures of astrocytes and neurons. In line with these findings, we have preliminary data indicating that prevention of mitochondrial accumulation of p53 by the mitochondrial protectant pifithrin-μ, decreased the transfer of mitochondria to damaged NSCs *in vitro* (data not shown). Furthermore, we recently showed that *in vivo*, cisplatin treatment rapidly induced translocation of p53 to mitochondria in the brain. Inhibition of p53 translocation to mitochondria prevented cisplatin-induced mitochondrial dysfunction of neuronal synaptosomes [7]. Therefore, we propose that translocation of p53 to the mitochondria of the damaged acceptor cell is part of the complex that confers a danger signal to the donor cell and prompt MSCs to transfer healthy mitochondria to NSCs.

Mitochondrial transfer between donor and acceptor cells requires mitochondrial movement along the actin cytoskeleton of the cells. This movement of mitochondria is regulated by the mitochondrial membrane Rho-GTPase 1 (Miro1), which binds the cytoplasmic adaptor protein

milton and kinesin heavy chain through its cytoplasmic domains, thereby connecting mitochondria to the actin cytoskeleton. We show that Miro1 overexpression in MSCs increased the transfer of mitochondria and enhanced the rescue potential of MSCs when co-cultured with cisplatin-damaged NSCs. Ahmad et al. [2] demonstrated in an *in vitro* model using lung epithelial cells that genetic manipulation of MSCs to overexpress Miro1 resulted in higher efficiency of transfer from MSCs to epithelial cells. Interestingly, intravenous administration of MSCs overexpressing Miro1 improved the efficacy of MSCs to reduce rotenone-induced lung injury [2]. The mechanism of action of Miro1 is complex but Schuler et al. recently showed using mouse embryonic fibroblasts that increasing Miro1 in MSCs may lead to better positioning of mitochondria at the leading edge of the cytoplasm instead of in the perinuclear area, allowing higher cytoplasmic energy redistribution and thereby favoring protrusion formation and MSC migration [36].

We do not yet know how mitochondria of donor origin rescue mitochondrial health and promote survival of the damaged acceptor NSCs. One possibility is that the donor mitochondria fuse with mitochondria in the acceptor cell, thereby restoring bioenergetic efficiency. Another potential mechanism is that the acceptor cell discards the damaged mitochondria and instead hijacks the donated mitochondria for energy production. Future research is needed to investigate how the MSC-derived mitochondria communicate with the acceptor cell cellular machinery to foster cellular health.

MSCs are becoming excellent candidates for regenerative strategies to restore brain damage after traumatic brain injury, stroke, hypoxia-ischemia, and neurodegenerative disorders like Alzheimer disease [9-11, 24, 25, 45]. We recently observed that nasal administration of MSCs can restore the cognitive deficits that arise after cisplatin treatment [6, 16]. In relation to this finding, it is of interest that we observed that cisplatin-induced loss of DCX+ neuroblasts was reversed by intranasal administration of MSCs as well. We propose that MSC-mediated transfer of mitochondria to NSCs reverses the neurotoxic effects of cisplatin, thereby facilitating cognitive processes.

The improvement in mitochondrial integrity and survival of NSCs in the presence of MSCs is likely not limited to the transfer of mitochondria alone. MSCs are known to favorably influence the growth factor milieu,

which could also aid in rescuing cisplatin-damaged NSCs [38]. However, when we blocked the transfer of mitochondria to NSCs using LatB, which depolymerizes F-actin and thereby inhibits TNT formation, we no longer observed the positive effect of MSCs on the NSC survival in vitro. These findings indicate that mitochondrial transfer plays a major role in the rescue of NSCs damaged by cisplatin.

In conclusion, we propose that the transfer of healthy mitochondria is an important mechanism underlying the regenerative effects of MSCs in the brain.

List of abbreviations:

ATP	Adenosine triphosphate
CTB	Cell Tracker Blue fluorescent probe
DCX	Doublecortin
DG	Dentate Gyrus
FCCP	Carbonilcyanide p-triflouromethoxyphenylhydrazone
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
LatB	Latrunculin B
MSC	Mesenchymal stem cell
Miro1	Mitochondrial Rho GTPase 1
NSC	Neuronal stem cell
SVZ	Subventricular Zone
TMRM	Tetramethylrhodamine methyl ester
TNT	Tunneling nanotube
WGA	Wheat germ agglutinin

DECLARATIONS

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Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Authors' contributions

N.B., A.K. and C.J.H designed the study. N.B., G.C. performed the experiments. N.B., G.C., A.K. and C.J.H interpreted the data. N.B., A.K. and C.J.H wrote the manuscript. All authors read and approved the final version.

Ethics approval and consent to participate

All animal procedures were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of M.D. Anderson Cancer Center.

Consent for publication

Not applicable.

Competing interests

None.

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SUPPLEMENTARY FIGURES

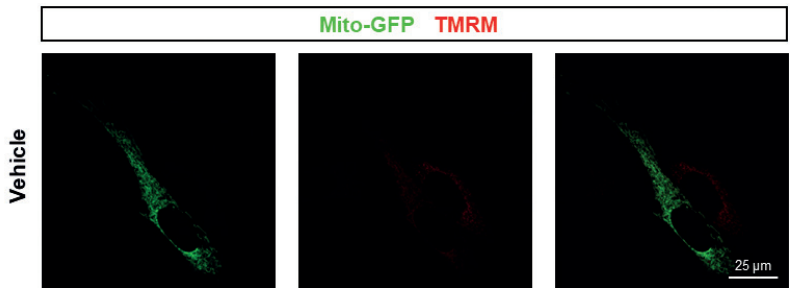

Figure S1. Representative images of MSC harboring GFP labeled mitochondria and TMRM staining.
MSCs were transfected with mito-GFP, stained with TMRM and imaged. MSCs do not exhibit a strong TMRM signal as compared to NSCs.

4

Nasal administration of mesenchymal stem cells reverses chemotherapy-induced peripheral neuropathy

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ABSTRACT

Chemotherapy-induced peripheral neuropathy (CIPN) is one of the most frequently reported adverse effect of cancer treatment. CIPN affects 30-80% of patients receiving chemotherapy, often persists after treatment completion, and has detrimental effects on patient's quality of life. Currently, there are no effective FDA-approved drugs to prevent or reverse CIPN. Mesenchymal stem cells (MSC) have been demonstrated to stimulate tissue repair and ameliorate the outcome of preclinical models of cerebral insults, neurodegenerative disorders and of some models of peripheral nerve damage. We have shown recently that nasal administration of MSC resolves cisplatin-induced cognitive impairment in mice. In this study, we show that nasal MSC administration also resolves cisplatin-induced neuropathy in male and female mice; nasal administration of two doses of $1x10^6$ MSC was sufficient to completely resolve established mechanical allodynia, spontaneous pain, and the loss of intraepidermal nerve fibers (IENFs) in mice treated with cisplatin. Growing evidence implicates mitochondrial dysfunction in the peripheral nervous system in the etiology of CIPN. Consistently, resolution of CIPN after MSC administration was associated with normalization of the cisplatin-induced decrease in mitochondrial bioenergetics in DRG neurons and in tibial nerve. In search for the underlying mechanism, we show that MSC act through an IL10-mediated pathway because MSC obtained from *Il10*$^{-/-}$ mice fail to promote resolution of CIPN. Moreover, we show that MSC cannot promote resolution of CIPN or restoration of mitochondrial function in mice deficient for IL10 receptors on peripheral sensory neurons (*Adv-Il10ra*$^{-/-}$ mice). In conclusion, we show that nasal administration of MSC is sufficient to reverse signs of cisplatin-induced peripheral neuropathy, including mechanical allodynia and spontaneous pain. Mechanistically, we show that MSC act through an IL10-dependent pathway and that resolution of CIPN is associated with normalization of mitochondrial bioenergetics in the peripheral nervous system. It remains to be determined whether the propagation of the IL-10 signal initiated by nasally administered MSC is mediated via migration of MSC or transferred through other pathways. Because nasal MSC administration does not only resolve cognitive deficits but also peripheral neuropathy induced by chemotherapy, nasal MSC treatment may become a powerful treatment for the large group of patients suffering from these neurotoxic side effects of cancer treatment.

Key words. Mesenchymal Stem Cells (MSC), cisplatin, peripheral neuropathy, Interleukin-10 (IL-10), sensory neurons, mitochondria.

INTRODUCTION

Chemotherapy induced peripheral neuropathy (CIPN) is a frequent side effect of many chemotherapy regimens, including platinum-based agents such as cisplatin. Approximately 68% of patients getting chemotherapy develop CIPN within the first weeks of treatment. At least 30 % of the affected patients still suffer from CIPN months to years after treatment completion and thus experience a long lasting negative impact on their quality of life [43, 46].

CIPN symptoms include pain, paresthesia and temperature sensitivity. CIPN develops in a symmetric, distal, "stocking and glove" type distribution [27]. To date, there are no effective FDA-approved treatments for CIPN. In view of the increasing number of cancer survivors, an efficacious therapeutic strategy is highly needed to treat CIPN.

The mechanisms associated with the pathogenesis of CIPN are not completely understood. It is well accepted that mitochondrial impairment in peripheral sensory neurons contributes to the etiology of CIPN [2, 15-17, 25, 29, 30, 37]. Indeed, our pre-clinical studies demonstrated that preventing mitochondrial damage protects against CIPN [30, 40]. We recently showed that preventing the early p53 accumulation at the mitochondria that occurs as a result of cisplatin using co-administration of the small molecule pifithrin-μ (PFT- μ), could completely prevent CIPN [30, 40].

Mesenchymal stem cells (MSC) when administered via the systemic route have the capacity to stimulate tissue repair and to ameliorate motor function and alleviate pain in models of neuropathic pain that are not associated with chemotherapy, including diabetic neuropathy as well as peripheral nerve injury [1, 6-8, 14, 20, 21, 26, 41]. However, systemic administration has the disadvantage that many MSCs will be sequestered in the lung and liver and only few of the transplanted MSCs will arrive at the site of action [18]. Moreover, local application including intrathecal administration of MSC, is highly invasive.

We have shown before that the nasal route is an effective and safe route of administration of MSC to reverse hypoxic-ischemic and subarachnoid hemorrhage-induced brain damage in rodents [9-12, 49-51]. Nasal

MSC administration also reverses cognitive deficiencies as a result of cisplatin treatment in association with restoration of brain synaptosomal mitochondrial function [5].

Several mechanisms have been suggested to mediate MSC's beneficial effects *e.g.* transfer of healthy mitochondria from MSCs to damaged neurons, secretion of neurotrophic and angiogenic factors, and increased production of immunosuppressive factors including IL10 [19, 32, 39].

We and others have shown that IL-10 signaling is a crucial pathway in the spontaneous resolution of pain in multiple models, including, CIPN, nerve injury and also critically contributes to exercise-induced analgesia [24, 35, 36]. Furthermore, recent literature suggests that IL10 is involved in the repair of mitochondria [22]. In this respect it is of interest that intrathecal administration of anti-IL10 prevents the spontaneous resolution of the CIPN whereas treatment with IL10 induces pain relief [13, 28, 31, 35, 55, 56].

In this study, we explored whether nasal administration of MSC reverses cisplatin-induced peripheral neuropathy including allodynia, spontaneous pain and regrowth of intraepidermal nerve fibers. In addition, we examined whether the resolution of CIPN after nasal MSC administration was associated with a repair of mitochondrial dysfunction in dorsal root ganglia and peripheral nerve. As a possible mechanism for nasal MSC action, we explored whether the mechanism of action of MSC involves their capacity to produce IL10 and whether IL10 needs to signal through IL-10 receptors expressed on peripheral sensory neurons for resolution of CIPN.

RESULTS

Nasal MSC administration reverses cisplatin-induced mechanical allodynia and spontaneous pain in both male and female mice

Male and female mice were treated with two cycles of cisplatin for 5 days (2.3 mg/kg) with 5 days of rest in between [5, 29, 40]. MSC (1 x 10^6 per dose in 2 x 3 ml per nostril) were administered nasally at 48h and 96h after the last dose of cisplatin. Mechanical allodynia was measured over time using Von Frey hairs. The results in figures 1A and B demonstrate that cisplatin treatment induces mechanical allodynia in both male and female mice. Nasal MSC administration completely reversed the already existing mechanical allodynia. The reversal of cisplatin-induced mechanical allodynia in response to nasal MSC developed slowly over time; the first beneficial effects were detected on day 8 after MSC administration and MSC-treated mice had completely recovered from cisplatin-induced mechanical allodynia by day 21 after the last dose of MSC. Mechanical allodynia persisted in mice that had received cisplatin only. This result is in accordance with what we have shown earlier in this model, as there is no evidence for spontaneous recovery for at least 70 days after the first cisplatin injection [29]. Nasal administration of bone marrow-derived MSC (hMSC) of human origin reduced mechanical allodynia in cisplatin-treated mice as well (Fig.1C).

Clinically, spontaneous pain is an important complaint by patients with CIPN. To assess the effect of MSC on spontaneous pain induced by cisplatin, we used a conditioned place preference test with the nerve blocker retigabine as the conditioning stimulus. During the four days of the conditioning phase, saline injections were paired with exposure to the dark chamber and retigabine injections paired with exposure to the bright chamber. On day 4, mice were allowed to freely explore both chambers and an increase in the time spent in the bright, retigabine-paired chamber was interpreted as evidence for ongoing pain. The CPP paradigm started 3 weeks after the last dose of MSC. Cisplatin-treated mice showed an increase in time spent in the retigabine-paired bright chamber indicating ongoing pain. In contrast, mice treated with cisplatin and MSC did not show an increase in time in the retigabine paired chamber, suggesting that these mice are no longer in pain (Fig. 1D).

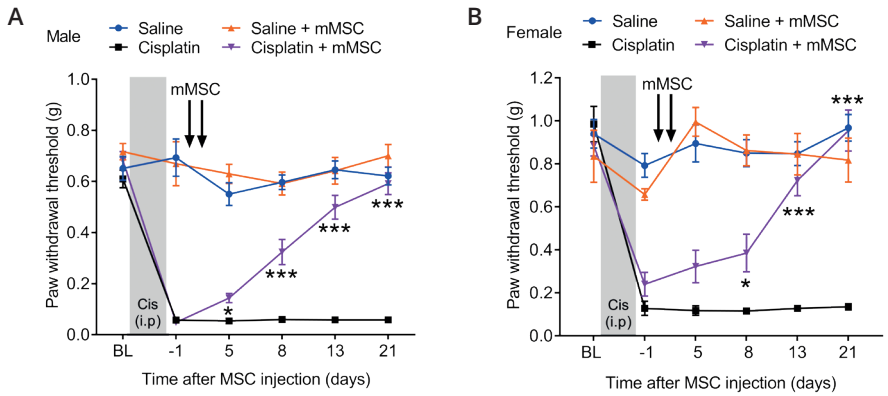

Figure 1. Nasal MSC application reverses cisplatin-induced allodynia.
Male **(A)** or female **(B)** mice were treated i.p. with 2 cycles of 5 days of cisplatin or saline; 48h and 96h after the last cisplatin dose, 1×10^6 mMSC **(A** and **B)** or hMSC **(D)** were administered via the nasal route. Mechanical allodynia was measured using Von Frey hairs. We calculated the 50% withdrawal threshold using the up-down method. Data are shown as mean ± SEM and were analyzed using multiple t tests **(A)** n=12/group**,** or Two-way repeated measure ANOVA followed by Bonferroni's post-hoc test **(B** and **C)**. **P*<0.05, *** *P*<0.001. n=4/group **(B)** and n= 8/group **(C). (D)** Spontaneous pain was measured by the conditioned place preference test, 3 weeks after mMSC treatment. The Y-axis indicates the change in time spent in light chamber (analgesic-paired chamber) between baseline and testing. Data are shown as mean ± SEM and were analyzed using Ordinary one-way ANOVA. **P*<0.05, ** *P*<0.01. n= 7-12/group.

Intraepidermal nerve density

Next, we determined whether nasal MSC administration affects the reduction in IENF density in the plantar surface of the hind paw after cisplatin treatment. Consistent with our previous report, we see a persistent

reduction of IENF density in cisplatin-treated animals. Interestingly, 3 weeks after nasal administration of MSC, the cisplatin-induced loss of IENF density was completely reversed (Fig.2 A and B).

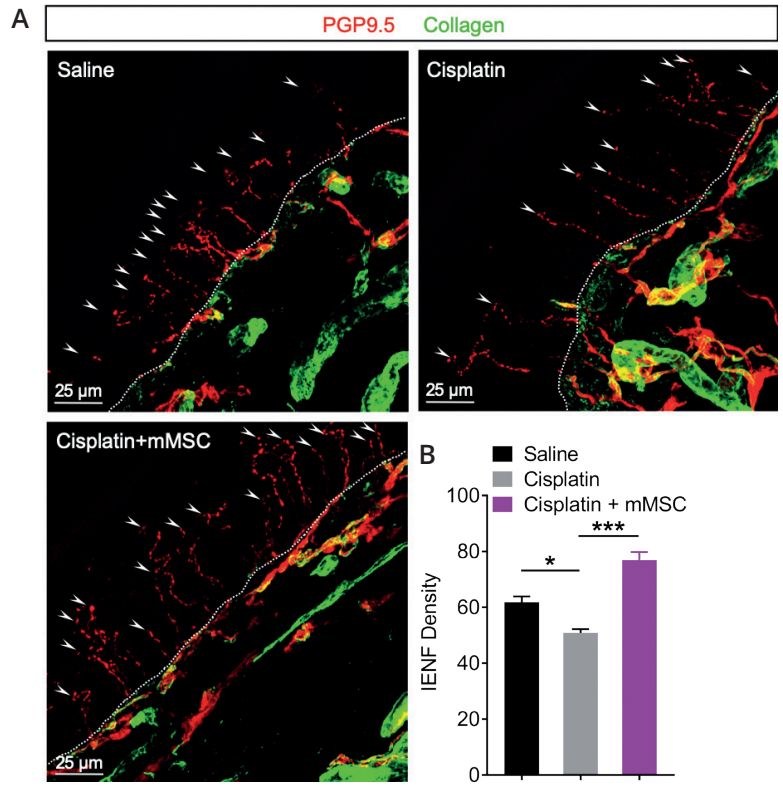

Figure 2. Nasal administration of mMSC reverses the loss of intra-epidermal nerve fibers in cisplatin-treated mice.
Mice were treated with cisplatin and MSC as in Figure 1. Paw biopsies were collected 24 days after the last mMSC administration and IENFs density was quantified using the pan-neuronal marker protein gene product PGP9.5 (red) and collagen (green). Representative confocal images from saline, cisplatin and cisplatin + mMSC-treated mice **(A).** The basement membrane is indicated by the dashed lines, the nerve fibers crossing the basement membrane are indicated by arrows. **(B)** Quantification of intra-epidermal nerve fiber (IENF) density is expressed as the number of nerve fibers (shown in red) crossing the basement membrane (shown in white)/length of the basement membrane (mm). 5-8 sections of each paw were quantified. n = 4 mice/group. Scale bars represent 25 µm. magnification 40 x. Data were analyzed using Ordinary one-way ANOVA followed by Bonferroni's post-hoc analysis. **P*<0.05, *** *P*<0.001. n=4 mice/group.

Effect of cisplatin and MSC on mitochondrial function in DRG neurons

Cisplatin-induced peripheral neuropathy is associated with impaired mitochondrial bioenergetics in dorsal root ganglion neurons and distal nerve endings [2, 15, 16, 29, 37, 38, 40]. Therefore, we assessed the effect of nasal MSC administration on mitochondrial bioenergetics in DRG neurons and tibial nerves 3 weeks after completion of MSC treatment. At this time point, cisplatin-treated mice still displayed mechanical allodynia, while mice treated with nasal MSC had fully recovered. Consistent with our earlier reports, cisplatin treatment decreased both the basal and maximal oxygen consumption rate of DRG neurons and peripheral nerve (Fig.3A and C). Notably, nasal MSC treatment normalized the cisplatin-induced decrease in basal and maximal mitochondrial respiration of DRG neurons and tibial nerve (Fig.3B). MSC treatment by itself did not have any effect on mitochondrial bioenergetics.

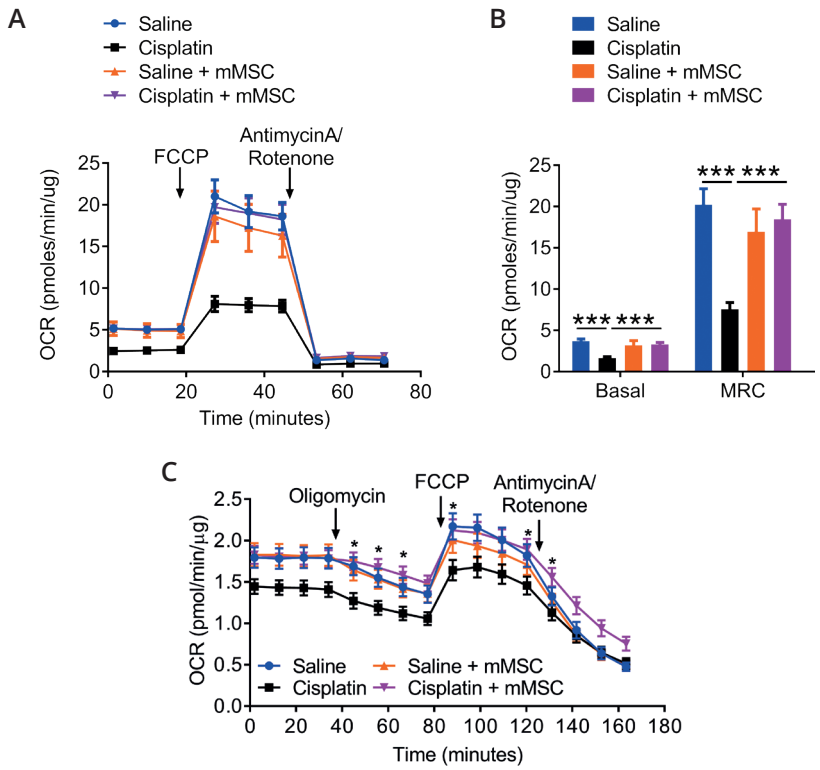

Figure 3. Nasally administered mMSC restore mitochondrial bioenergetics in DRG neurons and tibial nerve of cisplatin-treated mice.

Figure 3: Continued
Mitochondrial bioenergetics in DRG neurons **(A)** and tibial nerves **(C)** from mice that had received 2 rounds of cisplatin treatment and 2 doses of mMSC. Tissues were collected 24 days after the last mMSC administration. Oxygen consumption rates (OCR) were measured using a Seahorse XFe 24 Analyzer and normalized to protein content **(A).** Mean basal and maximum respiratory capacity (MRC) normalized to protein content were calculated **(B)**. Results are expressed as means ± SEM of 3 independent experiments. Data were analyzed using Two-way ANOVA followed by Tukey's post-hoc test. *** P<0.001. n= 11 mice/group.

MSC-derived IL10 is necessary for resolution of cisplatin-induced peripheral neuropathy

We have shown before that resolution of CIPN in cisplatin-treated mice depends on IL-10 signaling; blocking IL-10 action by intrathecal injection of anti-IL-10 prevented resolution of CIPN (Laumet et al, in preparation) [28]. To explore the mechanism of action via which nasally administered MSC reverse CIPN, we evaluated a possible contribution of MSC-derived IL-10. We compared the effect of nasal administration of WT MSC and MSC from *il10*$^{-/-}$ mice (*il10*$^{-/-}$ MSC) to cisplatin-treated mice on mechanical allodynia. The results in Figure 4A show that *il10*$^{-/-}$ MSC did not have any positive effect on cisplatin-induced mechanical allodynia, while mechanical allodynia resolved in response to the control WT MSC. These findings indicate that MSC act as provider of IL10 to stimulate the recovery of CIPN.

To test whether IL10 production by MSC is also needed for the resolution of the cisplatin-induced mitochondrial dysfunction in the peripheral nervous system, we assessed mitochondrial bioenergetics in DRG neurons isolated from mice treated with either WT or *Il10*$^{-/-}$ MSC. Interestingly, nasal administration of *Il10*$^{-/-}$ MSC failed to normalize the deficits in mitochondrial bioenergetics that were induced by cisplatin. In contrast, nasal administration of control WT MSC normalized mitochondrial bioenergetics in the DRG (Fig.4B and C).

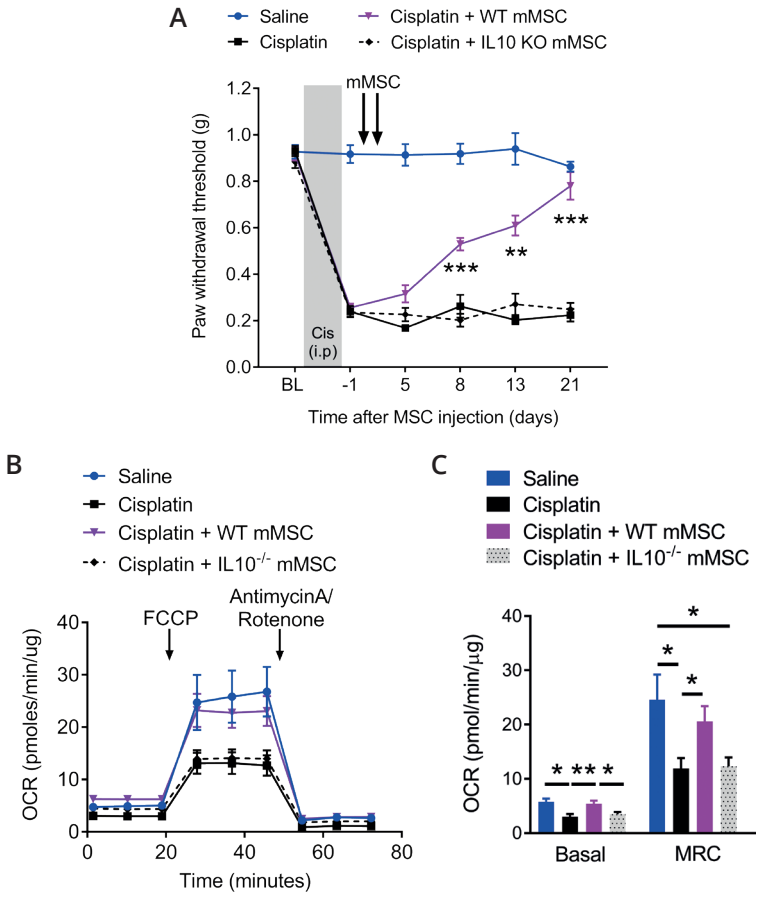

Figure 4. Nasal administration of *Il10*$^{-/-}$ mMSC fail to reverse cisplatin-induced mechanical allodynia and to restore mitochondrial bioenergetics in DRG neurons.

Mice received two rounds of cisplatin followed by two doses of either WT or IL10$^{-/-}$ MSC, 48 and 96 hours after the last cisplatin dose. Mechanical allodynia was measured as in figure 1. **(A)** Data were analyzed using Ordinary one-way ANOVA followed by Dunn's multiple comparisons. ** P<0.01, *** P<0.001. n= 12 mice/group. Mitochondrial bioenergetics in DRG neurons **(B)** and tibial nerves **(D)** was measured 24 days after the last MSC dose corresponding to the end of behavioral testing. Oxygen consumption rates (OCR) normalized to protein content are represented in **(C)**. Data were analyzed using Two-way ANOVA followed by Bonferroni's post-hoc test. *P<0.05, ** P<0.01. n= 8 mice/group.

Presence of IL-10R1 in sensory neurons is essential for resolution of cisplatin-induced mechanical allodynia

In a recent study, we showed that resolution of CIPN was associated with an increase in the expression of IL-10R1 in dorsal root ganglia [28]. In addition, we demonstrated that expression of IL-10R1 on advillin-positive sensory neurons (Advil10r1-/- mice) is required for the spontaneous resolution of CIPN (Laumet et al, in preparation). Here, we used Advil10r1-/- mice to determine the contribution of IL10 receptors on sensory neurons to the resolution of cisplatin-induced mechanical allodynia by nasal administration of MSC. *Advil10*ra$^{-/-}$ mice showed similar onset and intensity of allodynia as WT mice. More importantly, nasal MSC treatment did not normalize cisplatin-induced mechanical allodynia in mice lacking IL-10R1 in sensory neurons, whereas mechanical allodynia was completely resolved in control mice (Fig.5A).

Next, we probed whether the presence of IL-10R on sensory neurons is required for MSC to resolve cisplatin-induced mitochondrial dysfunction in response to nasal MSC administration. Interestingly, while MSC induced full recovery of mitochondrial bioenergetics in the DRG of control mice, we did not detect any improvement in mitochondrial function in *Advil10*ra$^{-/-}$ mice (Fig.5 B and C). These results clearly show that the IL10 receptor on sensory neurons is necessary for the resolution of both mechanical allodynia and mitochondrial dysfunction in peripheral neurons by nasal MSC administration.

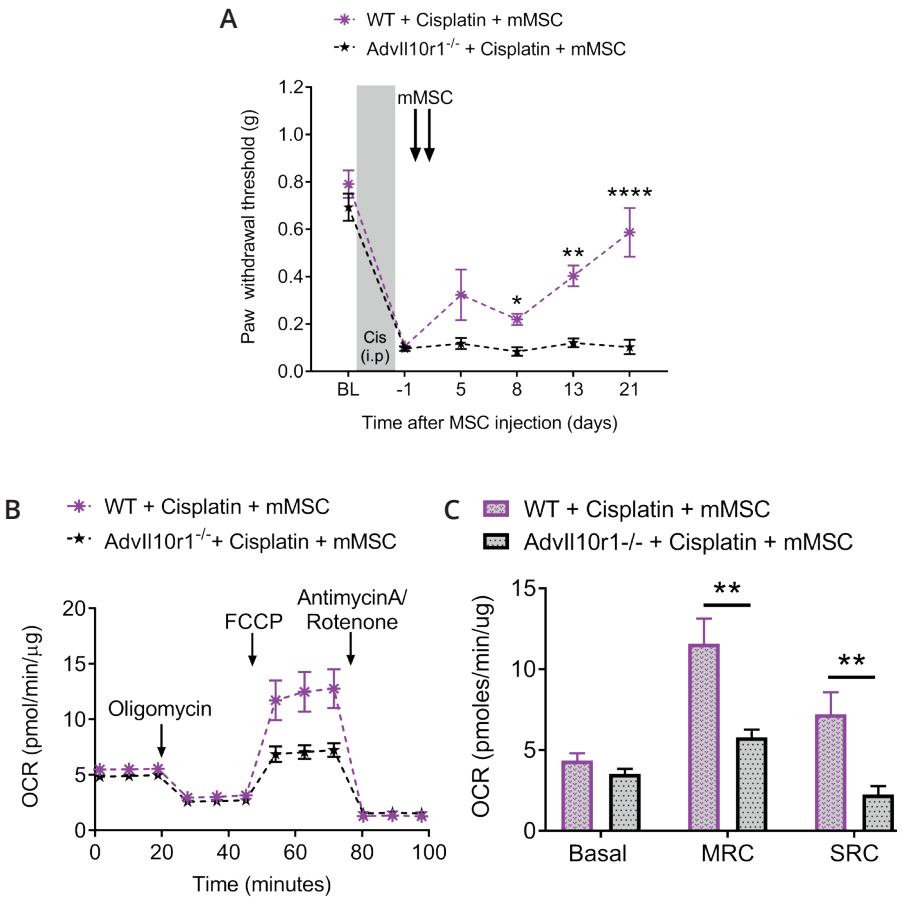

Figure 5. IL-10R1 expression in sensory neurons is necessary for resolution of cisplatin-induced mechanical allodynia.
Wild type (WT) and *AdvIl10r1*$^{-/-}$ mice were administered with two rounds of cisplatin and MSC as in Figure 1. Mechanical allodynia was measured as described in Figure 1. **(A)** Data were analyzed using Two-way ANOVA followed by Bonferroni's post-hoc test. **P*<0.05, ** *P*<0.01, **** *P*<0.0001. n= 6 mice/group. Mitochondrial bioenergetics was measured in DRG neurons **(B)** 24 days after the second MSC dose, corresponding to the end of behavioral testing. Oxygen consumption rates (OCR) normalized to protein content are represented in **(B)**. Mean basal, maximum (MRC) and spare respiratory (SRC) capacities normalized to protein are represented in **(C)**. Data were analyzed using Two-way ANOVA followed by Bonferroni's post-hoc test. ***P*<0.01. n= 6 mice/group.

DISCUSSION

CIPN is a frequent complication of cancer therapy and has a significant negative impact on long term quality of life of cancer patients. During chemotherapy CIPN often results in limited dosing or even termination of chemotherapy [42, 44]. Currently, no FDA-approved pharmacological therapies to mitigate CIPN are available. In view of the increasing number of cancer survivors, novel treatments for CIPN are urgently needed. In this study we used MSC as a therapeutic approach to treat cisplatin-induced peripheral neuropathy. We demonstrated for the first time that nasal administration of only a small number of MSC (2 doses of 1x10^6 MSC) is a disease-modifying intervention since it completely resolves multiple signs of CIPN, including mechanical allodynia, spontaneous pain, and the loss of IENF in the plantar surface of the paw. The MSC-mediated reversal of CIPN is associated with normalization of cisplatin-induced mitochondrial impairments in the dorsal root ganglia and tibial nerve. Mechanistically, we show that IL10 production by MSC is key to reversal of CIPN and the mitochondrial dysfunction in DRG and nerve. Moreover, we demonstrate that IL10 signaling through IL10 receptors expressed on peripheral sensory neurons mediates the beneficial effects of MSC on CIPN and normalization of mitochondrial function.

Using our mouse model of cisplatin-induced cognitive deficiencies, we recently showed that nasal MSC treatment reverses cognitive impairments after cisplatin treatment [5]. Now, we show that the same MSC treatment also reverses cisplatin-induced mechanical allodynia and alleviates ongoing pain as well. MSCs are known to be candidates for the treatment of peripheral nervous system injury including diabetic neuropathy, spinal cord injury and sciatic nerve constriction [1, 6-8, 14, 20, 21, 26]. In these studies MSC have been given either systemically or locally at the site of injury. We now show that nasal administration of MSC is a potent non-invasive route of MSC administration for at least two major signs of cancer treatment-related neurotoxicities, i.e. peripheral neuropathy and cognitive deficits.

The symptoms of cisplatin-induced peripheral neuropathy include sensory symptoms in a "stocking and glove" distribution, mainly affecting longest axons and most distal nerves first [27]. Several studies support the hypothesis that CIPN pathogenesis is related to bioenergetics deficits as

represented by mitochondrial dysfunction in peripheral neurons [2, 16, 29]. We indeed showed previously decreased mitochondrial bioenergetics in DRG neurons and tibial nerve of cisplatin-treated mice [29, 38]. Interestingly, we show now that nasal MSC administration improves mitochondrial bioenergetics in DRG neurons and tibial nerves of cisplatin-treated mice and restores IENF density. The unexpected finding that nasal MSC have such a long ranging effect is important from a clinical as well as mechanistic perspective.

MSC when administered nasally enter the meningeal compartment shortly after administration (See chapter 5) and can be found in the brain after 12-24 hours [5]. However, MSC do not survive a long time in the brain. MSC expressing the GFP transgene could only be traced in the brain for a maximum of 48-72 hours [5]. Interestingly, we could trace the MSC up to 7 days after administration in the meninges of the brain (see chapter 5). However, we did not trace any sign of MSC in spinal cord or DRG either by immunofluorescence or by genomic DNA GFP assessment.

Another possibility is that MSCs act in the brain and influence descending pain pathways originating e.g. in the periaqueductal grey-rostral ventromedial medulla (PAG-RVM) and the ventrolateral medulla [53]. However, the fact that MSC restore not only pain, but also peripheral mitochondrial function would argue for a (direct or indirect) peripheral action of MSC.

Interestingly, while WT MSC normalized all signs of CIPN in a slow and sustained manner and restored mitochondrial function in DRG neurons (as assessed on day 24 after first MSC dose), CIPN and DRG mitochondrial function of mice who had received IL-10 KO MSC remained impaired. This indicates that IL10 signaling is a central mechanism of action of MSCs to repair CIPN. We and others have shown that IL10 is an important cytokine in the spontaneous resolution of inflammatory and chemotherapy-induced pain [28, 54]. Specifically, we showed that intrathecal administration of anti-IL10 delayed the resolution of inflammatory pain and CIPN. Therefore, we propose that IL-10 production by MSC could therefore be viewed upon as an accelerator of spontaneous resolution of CIPN.

On the basis of our data, we propose that IL-10 secreted by MSC probably ‘educates’ cells in the brain meningeal compartment. Macrophages have

been shown to be capable of shifting from a pro-inflammatory state into an anti-inflammatory state or pro-repair state as a result of interaction with MSC overexpressing IL10 [45]. These anti-inflammatory macrophages could therefore migrate to the peripheral (damaged) nerves to resolve the pain and to repair the mitochondrial dysfunction though the production of anti-inflammatory cytokines including IL10. In this respect it is of interest that MSC overexpressing IL10 have been shown very efficient in the repair of brain damage as a result of traumatic brain injury as well as in reversing lung injury such as in E. coli pneumosepsis, where education of macrophages into the 2 type has also been shown [23, 45]. Moreover, we showed earlier that depletion of mice from macrophages delays resolution of inflammatory pain [54]. Therefore, we propose a two-step IL10 model’ since we also show in this report that IL10 signaling is needed at the site of the sensory nerve. The genetic deletion of the IL-10 receptors on sensory nerves led to a complete failure of MSC to alleviate CIPN. Another argument in favor of a two-step model is that the action of MSC is slow since it takes 8 days before we see the beginning of a positive effect of MSC administration. In case the action of MSC would be mediated by migration of IL-10 producing MSC to the periphery as a one-step model, we would expect a more acute effect of MSC especially because MSC are only short living in the periphery and the brain. Moreover, we have shown that intrathecal administration of IL-10 has an acute effect (within 30 minutes) on the resolution of mechanical allodynia, underlining the involvement of a two-step model in the resolution of CIPN via sequential IL10 secretion.

IENF loss has been reported both in patients and in rodent models with peripheral neuropathy as formation of IENFs is a bioenergetically active process, these regions are highly affected by chemotherapy-induced mitotoxicity. It is hypothesized that an insufficient energy supply results in a failure of the IENF to branch within the epidermis resulting in a loss of IENFs [2]. Consistent with our previous reports, we demonstrate here that cisplatin treatment decreased IENF density in the hind paws [29]. Importantly, we demonstrate that 2 doses of nasal MSC treatment fully reverses cisplatin-induced IENF loss. The restoration of mitochondrial bioenergetics in the distal nerve along with the re-innervation of the nerves is likely due to the normalization of mitochondrial function.

We have shown earlier that nasal MSC treatment is safe and has a lifelong efficacy in mice after hypoxic ischemic damage [11]. At 14 months after the hypoxic-ischemic event, we did not detect any neoplasia in the nasal turbinates, brain, or any other organ of hypoxic-ischemic mice treated with MSCs. Moreover, Chiu et al. demonstrated that MSC do not interfere with solid tumor formation (either in the presence or absence of cisplatin) using a heterotopic syngeneic murine model of human papilloma virus (HPV)-related head and neck cancer [5, 48, 52].

Taken together, our results identify nasal MSC administration as a novel therapeutic strategy for cisplatin-induced peripheral neuropathy. This is an important finding as to date, there are no available FDA-approved therapeutics for the treatment of established CIPN in cancer patients.

MATERIALS AND METHODS

Animals

Adult male and female C57BL/6J and female *Il10*-/- mice of 8 – 10 weeks were obtained from Jackson Laboratories. To to obtain adv-Il10ra-/- mice strain, we bred Avil-cre+/- mice with *Il10ra*floxflox mice [34]. Animals were housed at The University of Texas MD Anderson Cancer Center animal facility in Houston, TX on a reversed 12-hour dark/light cycle and had free access to water and food. Animals were randomly assigned to treatment groups and experiments were performed by investigators blinded to group. All procedures were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and the Ethical Issues of the International Association for the Study of Pain and were approved by the local Institution for Animal Care and Use Committee (IACUC).

Chemotherapy and MSC Treatment

Cisplatin (2.3 mg/kg/day; TEVA, Petah Tiva, Israel) or phosphate-buffered saline was injected i.p. for 2 cycles of 5 daily injections followed by 5 days of rest as described previously [29, 38]. C57BL/6 murine bone marrow MSC were either purchased from Invitrogen (Carlsbad, CA, USA) or isolated from tibia and femur of 8 weeks old female WT or IL-10 KO mice. Mouse MSC were cultured in 5% CO_2 at 37 °C in DMEM/F12 medium with GlutaMax-I, containing 10% MSC-qualified fetal bovine serum and 5 µg/mL gentamycin (all from GIBCO, Carlsbad, CA, USA). Cells were harvested using TrypLE-express (GIBCO). MSC were positive for MSC-associated antigens, CD29, CD44, CD73, CD105, CD106, Sca-1 and negative for hematopoietic markers, CD11b and CD45.

Human MSC (hMSC, Lonza, Basel, Switzerland) from female donors were a gift of the MD Anderson Cancer Center Cell Therapy laboratory. Human MSC were grown in 5% CO_2 at 37 °C in MEM-alpha medium (Corning, Corning, NY, USA) supplemented with 5% platelet lysate (PLTMax, Mill Creek Life Sciences, Rochester, MN, USA), 2 mM GlutaMax (GIBCO, Carlsbad, CA, USA), 100 units/ml of Penicillin/Streptomycin (Corning) and 2 units/ml of Heparin (Hospira, Lake Forest, IL, USA). Cells were harvested using 0.05% Trypsin (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Before MSC administration, mice received 3 µl of hyaluronidase in PBS in each nostril (100 U per mouse, Sigma-Aldrich) to temporarily increase the permeability of the mucosa lining the nasal cavity. MSC cell suspension (3 µl containing 1×10^6 cells per mouse per day) or PBS were given to mice 30 min later, twice in each nostril, for a total of 12 µl per mouse. MSCs were administered 48 and 96 h after the last dose of cisplatin as previously described [3, 5].

Behavioral tests

Mechanical allodynia. Mechanical allodynia was measured in mice using von Frey method, as previously described [29, 33, 38, 40, 47]. Briefly, during habituation, mice were placed in plastic cages on a mesh stand (IITC Life Science, Woodland Hills, CA) for 45 minutes. A series of filaments (0.02, 0.07, 0.16, 0.4, 0.6, 1.0, and 1.4 g; Stoelting, Wood Dale, Illinois, USA) were applied for up to 5 seconds to the plantar surface of the hind paw, starting with the application of the 0.16 g hair [29]. The “up and down” method was used to measure the force needed for 50% likelihood of paw withdrawal [4]. Data represent group means of the average of both hind paws for each mouse.

Spontaneous pain. Spontaneous pain was tested using a conditioned place preference (CPP) paradigm with retigabine (#R-100, Alomone Laboratory, Jerusalem, Israel) used as the conditioned stimulus to briefly relieve pain as described before [29]. During the first day, mice were first allowed to freely explore the CPP apparatus, consisting of 2 chambers (18 × 20 cm, 1 dark, 1 bright light) connected by a 15 cm hallway (Stoelting, Wood Dale, IL), for 15 minutes. The time spent in the bright light chamber was recorded. During the four days of the conditioning phase, intraperitoneal saline injections

were paired with exposure to the dark chamber and retigabine injections (10 mg/kg in PBS; #R-100; Alomone Labs, Jerusalem, Israel) paired with exposure to the bright chamber. On day 4, mice were allowed to freely explore the two chambers for 15 minutes without any injections. An increase in the time spent in the bright, retigabine-paired chamber as compared to the pre-conditioning session was interpreted as evidence for ongoing pain. Preconditioning and postconditioning test results were recorded and then analyzed using EthoVision XT video tracking software (Noldus Information Technology Inc, Leesburg, VA).

IENF density

Biopsies from the plantar surface of the hind paws were collected and processed as previously described [29, 33, 38, 47]. Briefly, frozen sections (25 µm thick) were incubated with primary antibodies for the pan-neuronal marker PGP9.5 (rabbit; Bio-Rad AbD Serotec, Oxford, United Kingdom) and collagen IV (goat; Southern Biotech, Birmingham, AL) followed by Alexa-594 donkey anti-rabbit and Alexa-488 donkey anti-goat secondary antibodies (Life Technologies, Carlsbad, CA). Images of IENF density were captured using an SPE Leica Confocal Microscope (Leica Microsystems, Buffalo Grove, IL, USA) with a 40 X objective, and analyzed using LAS X software. IENF density was expressed as the total number of nerve fibers crossing the basement membrane/length of the basement membrane (mm). 5 random pictures/mouse for a total of 4 mice/group were quantified by researchers blinded to treatment.

Assessment of mitochondrial bioenergetics

Tibial nerves were placed into islet capture XF24 microplate (Seahorse Bioscience, North Billerica, MA) containing XF media supplemented with 5.5 mM glucose, 0.5 mM sodium pyruvate, and 1 mM glutamine. Oxygen consumption rates (OCR) were measured with an XF24 Flux Analyzer (Seahorse Bioscience) as described before [29]. Oligomycin (12 µM), FCCP (20 µM), and rotenone/antimycin A (20 µM each) (Sigma-Aldrich) and an assay cycle of 3-minute mix, 3-minute wait, and 4-minute measure combination was repeated 4 times were used for measuring baseline rates as well as after each port injection.

Lumbar DRGs were collected and digested with 1.25% collagenase (Thermo Fisher Scientific, Waltham, MA) and 2% trypsin (Sigma-Aldrich). DRGs were

dissociated by triturating in Ham's F10 medium (Corning Inc., Corning, NY) using a fire-polished glass pipette as previously described. Dissociated neurons were layered on a 10 ml gradient of sterile 26% Percoll (GE Healthcare Life Sciences, Little Chalfont, United Kingdom) in PBS, and centrifuged at 800 × g for 20 minutes at room temperature. Cell pellet was resuspended in Ham's F10 medium supplemented with N2 supplement (Thermo Fisher Scientific). Cells were cultured in an XF24 microplate overnight. 1 hour before starting the assay, the cell maintenance medium was changed to XF base media (Seahorse Biosciences) containing 11 mM glucose, 2 mM glutamine, and 1 mM pyruvate. Oligomycin (2 µM), FCCP (4 µM) and rotenone/antimycin A (2 µM each) were used with a 3-time repeat of a 2-minute mix, 3-minute wait, and 2-minute measure assay cycle. OCR values were normalized to the total protein content of each well. Basal respiration, ATP-linked respiration, proton leak, and maximal respiratory capacity (MRC) were determined as described previously [29].

Statistical analysis

Data are shown as mean ± standard error of the mean (SEM) of at least 3 independent experiments. One-way or two-way analysis of variance (ANOVA) were used with or without repeated measure followed by either Bonferroni's or Tukey's correction for multiple comparisons, or using Student's t-test, as appropriate and indicated in the legends.

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5

Fate of nasally applied mesenchymal stem cells after cisplatin treatment

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ABSTRACT

Chemotherapy-induced peripheral neuropathy (CIPN) and chemotherapy-induced cognitive impairment are side effects of neurotoxic cancer treatment that represent a major challenge impacting both cancer patients and survivors. Treatments for these neurotoxicities are limited and new strategies need to be developed. Mesenchymal stem cells (MSC) therapy holds a great promise for the treatment of neurological disorders. Experimental evidence indicates that MSC migrate to sites of damage to contribute to tissue repair. Here we demonstrate that nasal MSC migrate to the meninges of the brain and spinal cord after cisplatin treatment. To fulfill their regenerative actions, we show that MSC connect with meningeal macrophages, as we found MSC-derived mitochondria within these macrophages. Moreover, we show that MSC modulate production of the anti-inflammatory cytokine IL-10 by meningeal macrophages. Finally, we identify that MSC cannot resolve CIPN in the absence of T cells.

Key words: MSC, meninges, macrophages, T cells, IL-10, CIPN, cisplatin, mitochondria.

INTRODUCTION

Neurotoxicity of cancer therapy is a common side effect of chemotherapy that is often dose-limiting and therefore negatively affecting the patient's prognosis. Chemotherapy damages both the central and peripheral nervous system. The adverse effects include cognitive deficits, fatigue, depression, and peripheral neuropathy. Cancer treatment-induced peripheral neuropathy (CIPN) includes pain, tingling in hand and feet, numbness and temperature sensitivity [8, 42, 52, 58]. These symptoms are present in a symmetric, distal, "stocking and glove" distribution [30, 66].

Even though the central nervous system is protected by the blood brain barrier as well as the cerebrospinal fluid, it is still subject to neurotoxic side effects of chemotherapy. 75% of patients develop cognitive impairments while undergoing chemotherapy and 35% of cancer survivors continue to experience cognitive deficiencies months to years following treatment termination [26, 65]. Depending on the type and dose of chemotherapy up to 90 % of patients receiving chemotherapy develop CIPN and a minimum of 30 % of the affected patients still suffer from CIPN months to years after treatment cessation [6, 8, 46, 47, 51-53, 58]. These side effects negatively affect patient's daily tasks and quality of life. Given the limited treatment strategies for chemotherapy-induced cognitive impairments (CICI) and CIPN, novel therapeutic strategies need to be developed.

Mesenchymal stem cells (MSC) are actively investigated as a novel therapy for the treatment of brain damage induced by insults such as traumatic brain injury and stroke as well as neurodegenerative diseases including Alzheimer and Parkinson disease [14, 16, 18, 36, 59, 63]. We recently reported that MSC administered via the nasal route reverse cognitive dysfunction in a model of chemotherapy-induced cognitive impairment [12]. Surprisingly, our recent findings show that nasal MSC treatment also resolved peripheral neuropathy resulting from cisplatin treatment (see Chapter 4). Furthermore, we have shown previously that nasally applied MSC improve both functional outcome as well as lesion size in experimental models of neonatal hypoxic-ischemic brain injury and stroke [16, 18, 63].

Intravenous administration of MSC leads to migration of MSC to damaged tissue sites as guided by the microenvironment [7, 50, 60]. However, before

reaching the site of damage, many intravenously administered MSC are sequestered in the lung and liver [19]. We propose that intranasal delivery of MSC provides an efficacious alternative strategy for delivery of MSC to the central nervous system that avoids entrapment of MSC in lungs and liver and has the advantage of being non-invasive, fast and targeted.

Our group demonstrated that nasally administered MSC migrate specifically to damaged brain areas in models of ischemic brain damage and cisplatin-induced cognitive dysfunction [12, 16, 17] and (Chapter 2). However, the number of MSC detected in the brain after nasal administration is relatively small and the cells do not survive longer than 48-72 hours. However, the restorative effect of MSC after stroke or chemotherapy is long-lasting. Notably, we do not have evidence of transdifferentiation of MSC into neurons or glia, consistent with the lack of long term engraftment. In contrast, MSC are thought to resolve damage by promoting endogenous repair mechanisms via local secretion of neurotrophic factors and suppressing neuroinflammation in brain and nerve injury models [9, 12, 16, 61, 62].

It is also possible that nasally administered MSC contribute to resolution of damage without actually entering the nervous system. One likely compartment to which nasally administered MSC may migrate to exert their pro-resolution effects are the meninges of the brain.

Here we investigated the route via which MSC may influence brain and peripheral nervous function and focused especially on the meningeal compartment of the brain and the spinal cord. Interestingly, a large body of recent evidence identified a key role of immune cells in the meninges surrounding the brain and spinal cord in controlling brain health. Originally, the meninges were thought to function as just a protective membrane [55]. However, it is now well documented that the meningeal compartment, and in particular the meningeal immune system, plays a prominent role in CNS physiology and response to injury [13, 15, 24, 41]. Moreover, our recent studies demonstrate that resolution of chemotherapy-induced peripheral neuropathy depends heavily on regulatory effects by the immune system including the meningeal compartment as well (Singhmar et al. bioRxiv, 2019) [34, 35]. Specifically, we identified a key role of T lymphocytes and IL-10 signaling in the resolution of CIPN [31, 34]. MSC have potent immunomodulatory effects including regulation of T cell function and

promotion of the production of anti-inflammatory cytokines including IL-10 by T cells and macrophages [11, 21, 37, 39, 44, 45, 48, 56].

In this study, we aimed to investigate first whether nasally administered MSC migrate from the nasal cavity to the meninges of the brain and spinal cord. We also tested the hypothesis that nasally administered MSC directly interact with macrophages and/or T cells in the meninges to promote IL-10 production which is necessary for the resolution of CIPN.

MATERIALS AND METHODS

Animals

7- 8 weeks adult male WT, female GFP, male Rag2$^{-/-}$ (no mature T and B cells) mice in a C57BL/6J background were obtained from Jackson Laboratories (Bar Harbor, Maine). Animals were housed at The University of Texas MD Anderson Cancer Center animal facility in Houston, TX and had free access to food and water. All procedures were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the local Institution for Animal Care and Use Committee (IACUC).

Chemotherapy and MSC Treatment

Cisplatin (2.3 mg/kg/day; TEVA, Petah Tiva, Israel) or phosphate-buffered saline were administered i.p. for 2 cycles of 5 daily injections followed by 5 days of rest as previously described [4, 12]. C57BL/6 murine bone marrow MSC were either purchased from Invitrogen (Carlsbad, CA, USA) or isolated from tibia and femur of 8 weeks old female GFP mice. Mouse MSC were grown in 5% CO_2 at 37 °C in DMEM/F12 medium with GlutaMax-I, supplemented with 10% MSC-qualified fetal bovine serum and 5 µg/mL gentamycin (all from GIBCO, Carlsbad, CA, USA). Cells were harvested using TrypLE-express (GIBCO).

Human MSC from female donors with DsRed labeled mitochondria (Mito-DsRed hMSC) were a gift of Dr Rodrigo Jacamo and Dr. Michael Andreeff, Department of Leukemia, Section of Molecular Hematology and Therapy, The University of Texas MD Anderson Cancer Center. For labeling mitochondria, a construct was generated by cloning the PDHA1 (pyruvate dehydrogenase) containing the mitochondria leader sequence with the N-terminal of

DsRed into a lentiviral vector. Human MSC were grown in 5% CO_2 at 37 °C in MEM-alpha medium (Corning, Corning, NY, USA) supplemented with 5% platelet lysate (PLTMax, Mill Creek Life Sciences, Rochester, MN, USA), 2 mM GlutaMax (GIBCO, Carlsbad, CA, USA), 100 units/ml of Penicillin/Streptomycin (Corning) and 2 units/ml of Heparin (Hospira, Lake Forest, IL, USA). Cells were harvested using 0.05% Trypsin (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Before MSC administration, mice were administered with 3 µl of hyaluronidase in PBS in each nostril (100 U per mouse, Sigma-Aldrich) to temporarily increase the permeability of the nasal mucosa. 3 µl of MSC cell suspension (1×10^6 or 2×10^6 cells per mouse per day) or PBS were administered to mice 30 min after, twice to each nostril, for a total volume of 12 µl per mouse. MSCs were given 48 and 96 h after the last cisplatin injection as described previously [4, 12] .

Mechanical allodynia

Mechanical allodynia was measured in mice using von Frey test, as previously described [32, 34]. Briefly, mice were placed in plastic cages on a mesh stand (IITC Life Science, Woodland Hills, CA) for 45 minutes for habituation. A series of filaments (0.02, 0.07, 0.16, 0.4, 0.6, 1.0, and 1.4 g; Stoelting, Wood Dale, Illinois, USA) were applied for up to 5 seconds to the plantar surface of the hind paw, starting with the application of the 0.16 g hair as previously described. We used the "up and down" method to calculate the force needed for 50% likelihood of withdrawal. Data represent means of the average of both hind paws for each mouse. Testing was performed by an experimenter blinded to treatment groups.

Confocal microscopy

Representative images of meninges were acquired using an SPE Leica Confocal Microscope (Leica Microsystems, Buffalo Grove, IL, USA) with either a 40 X or 60 X objective, and analyzed using the LAS X software.

Assessment of mitochondrial bioenergetics

Lumbar DRGs were collected and digested with 1.25% collagenase (Thermo Fisher Scientific, Waltham, MA) and 2% trypsin (Sigma-Aldrich), and then dissociated by triturating in Ham's F10 medium (Corning Inc., Corning, NY) with a fire-polished glass pipette as previously described [32]. Dissociated neurons were layered on a 10 ml gradient of sterile 26% Percoll (GE Healthcare Life Sciences, Little Chalfont, United Kingdom) in PBS, and

centrifuged at 800 × g for 20 minutes at room temperature to pellet cells. Cells were resuspended in Ham's F10 medium containing N2 supplement (Thermo Fisher Scientific) and cultured in an XF24 microplate overnight. 1 hour prior to starting the assay, the cell maintenance medium was changed to XF base media (Seahorse Biosciences) supplemented with 11 mM glucose, 2 mM glutamine, and 1 mM pyruvate. Oligomycin (2 µM), FCCP (4 µM) and rotenone/antimycin A (2 µM each) were used with a 3-time repeat of a 2-minute mix, 3-minute wait, and 2-minute measure assay cycle. OCR values were normalized to the total protein content of each well. Basal respiration, ATP-linked respiration, proton leak, and maximal respiratory capacity (MRC) were calculated as previously described [32].

Isolation and immunofluorescence analysis of brain and spinal cord meninges

Mice were euthanized by CO_2 exposure and transcardially perfused with 10 ml ice-cold PBS containing 5 units/ml of heparin. Meninges were collected as described by Louveau et al. [33, 40, 41]. Briefly, the skull was isolated and the inferior jaw, lower orbits, and nasal bone were removed. The top of the skull was removed by cutting at the post-tympanic hook and placed onto petri dish with ice-cold PBS. Whole-mount meninges were fixed attached to skullcap in PBS with 4% PFA for 24h at 4°C, then meninges were harvested from skullcap. Spinal columns were fixed in PBS with 4% PFA for 2 days at 4°C. The bone was cut to expose the spinal cord and meninges peeled off the spinal cord as a whole. Brain and spinal cord meninges were incubated in PBS containing 1% BSA, 2% of normal serum (either donkey or goat), 0.1% Triton-X-100 and 0.05% of Tween 20 for 1 h at room temperature, followed by incubation with appropriate primary antibodies: anti-GFP (Invitrogen , A-21311); anti-F4/80 (Cell Signaling, clone D2S9R, 70076), anti-CD206 (Bio-Rad, clone MR5D3, MCA2235), anti-mitochondria (Abcam, clone 113-1, ab92824) and anti-CD3 (Invitrogen, clone 17A2, 14-0032-82) overnight at 4°C in PBS with 1% BSA and 0.5% Triton-X-100. Meninges were washed 3 times for 5 minutes at room temperature in PBS and then incubated with Alexa-fluor 488 donkey anti-rabbit, Alexa-fluor 647 donkey anti-rat or Qdot 655 goat anti-mouse secondary antibodies (Invitrogen) for 1 hour at room temperature in PBS containing 1% BSA and 0.5% Triton-X-100. Meninges were then stained with DAPI and imaged.

Flow cytometry

Meninges were collected in PBS with 2% BSA and 1mg/ml collagenase IV and digested for 40 minutes at 37 °C. Meninges were then minced through a 70 µm cell strainer and homogenized in FACS buffer containing 2% FBS, 0.5 mM EDTA and 0.1 % sodium azide in PBS. Homogenized samples were centrifuged at 300 × g for 5 minutes to pellet cells and stored on ice. For intracellular IL-10 staining, cells were resuspended in MSC media and stimulated with protein transport inhibitor containing Brefeldin A (BD GolgiPlug, 555029) for 3 hours. Cells were collected and washed with FACS buffer. Cells were stained with the following extracellular antibodies: Brilliant Violet 570 anti-CD45 (Biolegend, clone 30-F11, 103136), Pacific Blue anti F4/80 (Biolegend, clone BM8, 123124), PE/Cy7 anti- CD206 (Biolegend, clone C068C2, 141720) and Alexa Fluor 700 anti-CCR2 (R&D systems, FAB5538N) for 30 minutes at 4 °C. Cells were washed with FACS buffer, fixed for 30 minutes at 4°C, and then permeabelized for 30 minutes at room temperature using mouse Foxp3 buffer set (BD Pharmingen, 560409) containing mouse Foxp3 fixation and permeabilization buffers. Cells were washed with FACS buffer and stained intracellularly with Alexa Fluor 647 anti-IL10 antibody (clone JES5-16E3, 505014) for 30 minutes at 4°C. Cells were resuspended in FACS buffer and acquired on a Gallios flow cytometer (Beckman Coulter). Data for all flow cytometric evaluations was analyzed using Kaluza 2.1.1 software (Beckman Coulter).

Statistical analysis

Data were analyzed using One-way or two-way analysis of variance (ANOVA) followed by either Bonferroni's or Tukey's correction for multiple comparisons, or using Student's t-test, as appropriate and indicated in the legends. Significant difference are indicated in graphs as ****$P < 0.001$, ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$.

RESULTS

Nasally delivered MSC migrate to the meninges of the brain

To determine whether nasally administered MSC migrate to the meninges, we treated mice with two cycles of cisplatin for 5 days (2.3 mg/kg) with 5 days of rest in between. MSC from GFP-transgenic mice (GFP⁺-MSC, 2 x 10^6 per dose in 4 x 3 ml per nostrils) were administered nasally at 48h after the last dose of cisplatin. We collected the meninges of the brain at 2, 24, 72 hours and 7 days after nasal administration and traced the presence of MSC by confocal microscopy (Figure 1). We detected GFP⁺-MSC within the superior sagittal sinus (SSS) (Figure 1A/B) in the meninges of the brain at all time points tested (Figure 1 C-D). The highest number of MSC in the meninges was detected at 2 and 24 hours after nasal administration. The number of MSC started to decrease at 72 hours. Notably, we still detected up to 50% of the number of MSC present at 2-24 hours in the meninges at least for 7 days after intranasal administration, which is in sharp contrast to what we detected in the brain, where the GFP signal had completely disappeared within 72 hours (Figure 1C and chapter 2).

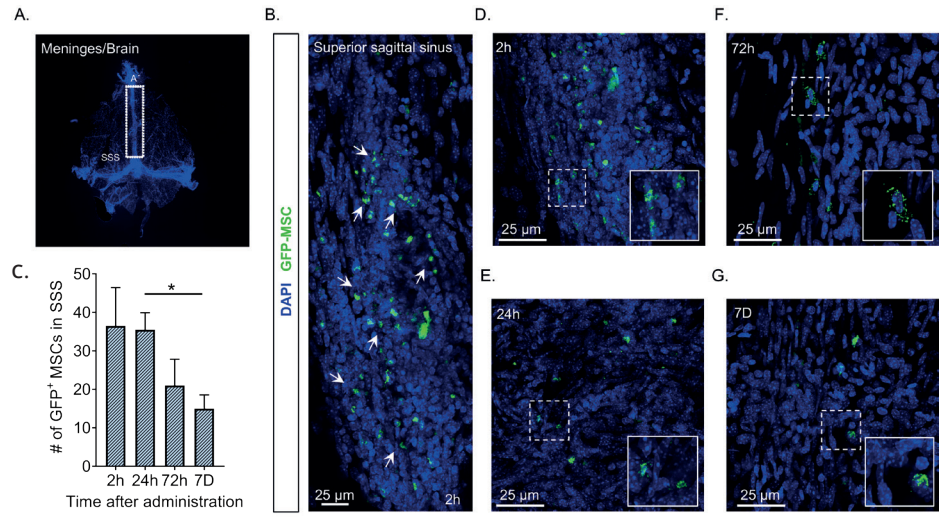

Figure 1. Nasal MSC migrate to the meninges of the brain.
Mice were treated i.p. with 2 cycles of 5 days of cisplatin; 48 hours after the last cisplatin dose, 2×10^6 GFP⁺-MSC were administered nasally. Meninges were harvested 2h, 24h, 72h and 7 days after administration to trace MSC migration. **(A)** Overview image of meninges of the brain stained with Dapi. The white line shows the superior sagittal sinus (SSS) **(A´)**. Representative images of nasally administered GFP-MSC in SSS, 2h **(B** and **D)**, 24h **(E)**, 72h **(F)** and 7 days **(G)** after nasal administration. **(C)** Quantification of the number of GFP⁺-MSC in the SSS. Data are represented as means ± SEM of 4 mice/group. Data were analyzed using multiple t test. *P<0.05. Regions denoted by a white line indicate magnified images (right panel). Arrows indicate GFP⁺-MSC.

The meninges of the brain extend towards the spinal cord forming the spinal cord meninges. In view of the positive effect of nasal administration of MSC

on neuropathic pain, we also examined the spinal cord meninges for GFP⁺-MSC. The data in Figure 2 demonstrates that nasally administered GFP⁺-MSC do reach the spinal cord meninges where we detected GFP⁺-MSC at 12 and 24 hours after nasal administration (Figure 2B and C).

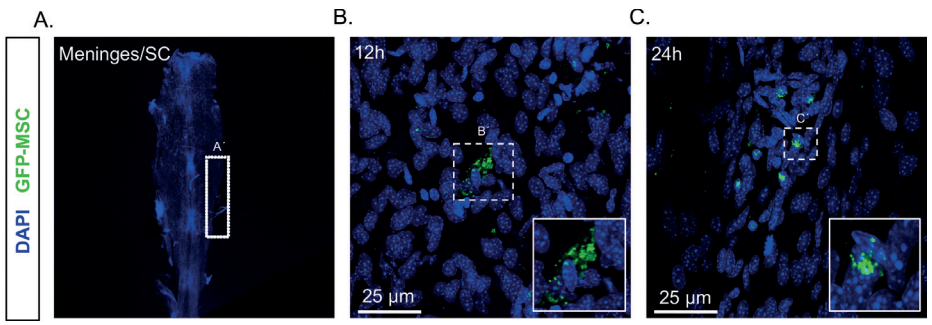

Figure 2. Distribution of GFP⁺-MSC in the spinal cord meninges.
Mice were treated with cisplatin and received GFP⁺-MSC as in Figure 1. Spinal cord meninges were dissected and GFP⁺-MSC were traced 2h, 24h, 72h and 7 days after nasal administration of cells. Regions denoted by a white line indicate magnified images (right panel). GFP⁺ cells were only detected at 12h and 24h after MSC administration.

MSC-derived mitochondria in the brain meninges

There is increasing evidence that at least part of the beneficial effects of MSC is mediated via transfer of mitochondria from MSC to damaged target cells [3, 4, 22, 28, 38, 54, 64, 68]. To determine whether MSC transfer mitochondria to target cells in the meninges of the brain of cisplatin-treated mice, we used mito-DsRed labeled MSC so that we could track cells positive for MSC-derived mitochondria. The majority of the DsRed positive signal was detected in the SSS and adjacent area (Figure 3A, B).

The highest number of DsRed⁺ cells was detected 30 min after administration (Figure 3C). The number of DsRed⁺ cells gradually decreased over time (Figure 3A, C). To confirm that the mito-DsRed signal we detect in the meninges is indeed identifying mitochondria derived from the nasally administered human MSC, we stained the meninges with an antibody specific for mitochondria of human origin. Figure 3D shows co-localization of mito-DsRed and the human mitochondrial labeling, confirming specificity.

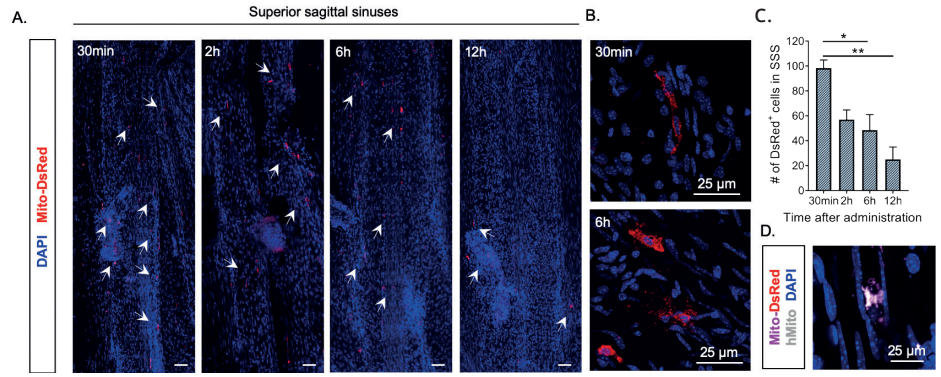

Figure 3. Distribution of MSC-derived mitochondria in the meninges of the brain.
Mice received cisplatin and nasal human MSC as in Figure 1. Representative confocal images showing distribution of the mito-DsRed signal in the superior sagittal sinus (SSS) at 30 min, 2h, 6h and 12h after nasal MSC administration **(A** and **B)**. **(B)** Zoomed-in images of mito-DsRed⁺ cells within the SSS. **(C)** Bar graph represents the number of mito-DsRed⁺ cells in the SSS. **(D)** Representative image of mito-DsRed⁺ cells co-localizing with human mitochondria. Data are represented as means ± SEM of 3 mice/group. Data were analyzed using One-way ANOVA followed by Bonferroni's post-hoc test. *P<0.05, ** P < 0.01.

It has been shown before that uptake of MSC-derived mitochondria by macrophages promotes phagocytosis and production of M2-type cytokines thereby contributing to tissue repair and regeneration [27, 28, 48]. We therefore hypothesized that at least part of the mito-DsRed MSC derived mitochondria in the meninges are present in macrophages. The results in Figure 4A demonstrate that we indeed detect DsRed⁺ human MSC-derived mitochondria in F4/80⁺ macrophages in the meninges. The mito-DsRed signal detected in the macrophages co-localizes with an antibody specific for human mitochondria, confirming that the mitochondria are from human MSC origin.

The mito-DsRed signal was not detected in meningeal T cells that were found in close proximity to the macrophages (Figure 4B).

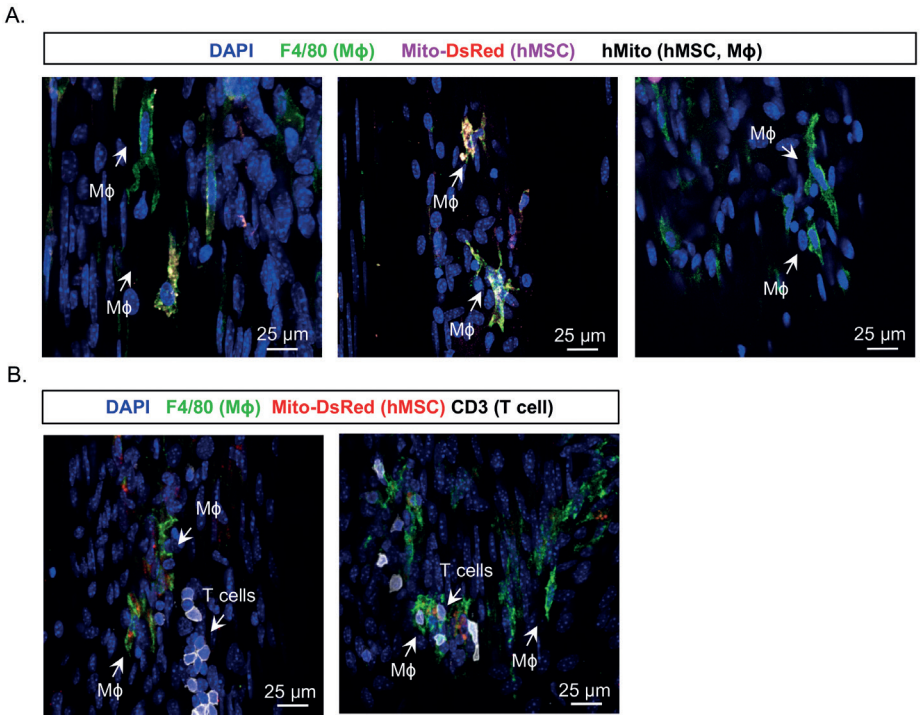

Figure 4. Meningeal macrophages harbor MSC mitochondria.
Meninges harvested 6h and 12h after cisplatin and nasal MSC administration were stained with anti-F4/80 (macrophages) and anti-CD3 (T cells) antibodies **(A** and **B)**. Representative images of meningeal areas showing the presence of MSC-derived mitochondria (mito-DsRed) in F4/80$^+$ macrophages **(A** and **B)**. Positive mito-DsRed mitochondria were not detected in CD3$^+$ T cells **(A)**. **(B)** mito-DsRed signal co-localized with human mitochondrial staining using anti-mitochondria of human origin antibody in F4/80$^+$ macrophages. Macrophages negative for mito-DsRed were also negative for human mitochondrial staining **(A,** right panel**)**.

We hypothesized that the presence of MSC and/or of MSC-derived mitochondria in meningeal macrophages would be associated with an M1 to an M2 phenotypic switch. Therefore, we compared the phenotype of meningeal macrophages from mice treated with cisplatin only or treated with cisplatin followed by MSC. Meningeal cells were stained for F4/80, CD11c and CD80 to identify M1-type macrophages and F4/80, CD206 markers to identify M2 type macrophages (Figure 5). The results in Figure 5 show that cisplatin does not affect the percentage of F4/80$^+$ macrophages staining for M1 markers (Figure 5A, B). However, cisplatin decreased the number of CD206$^+$ macrophages in the meninges (Figure 5C). Although there was a

tendency for MSC to reverse the decrease in CD206$^+$ macrophages, this did not reach statistical significance.

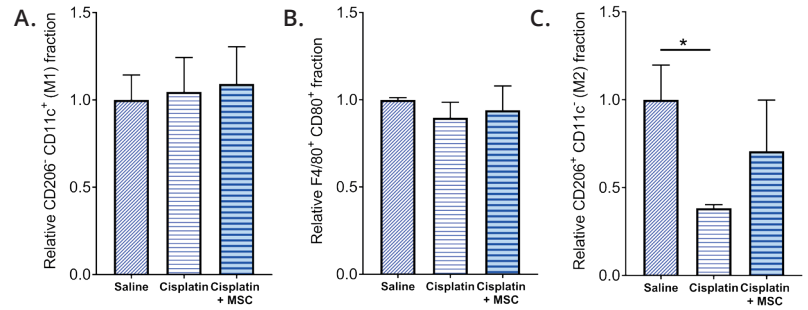

Figure 5. Effect of MSC on the phenotype of meningeal macrophages
Mice were treated i.p. with 2 cycles of 5 days of cisplatin or saline; 48h and 96h after the last cisplatin dose, 1×10^6 mMSC were administered nasally. 72h after the last MSC dose, meninges were harvested and cells were analyzed by flow cytometry. **(A-F)** Quantification of the percentage of CD206$^-$ CD11c$^+$(M1), F4/80$^+$ CD80$^+$, CD206$^+$ CD11c$^-$(M2) cells normalized to control. Data are expressed as means ± SEM of 3-5 mice/group. Data were analyzed using multiple t tests. *P<0.05.

Interestingly, however, nasal administration of MSC to cisplatin-treated mice significantly increased the percentage of IL-10 producing macrophages (Figure 6). Cisplatin treatment alone did not IL-10 production by meningeal macrophages (Figure 6D).

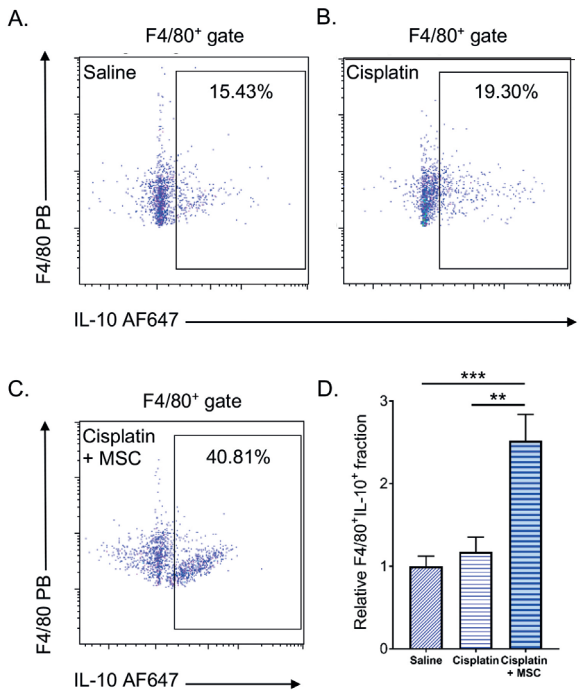

Figure 6. Nasal MSC treatment increases IL-10 production by meningeal macrophages.
Mice received either saline or cisplatin and MSC as in Figure 5. Meninges were harvested and cells stimulated with Brefeldin A for intracellular cytokine detection. Cells were then analyzed by flow cytometry. **(A-C)** Representative dot plots of macrophages ($F4/80^+$) in the meninges of mice treated with either saline **(A)**, cisplatin **(B)** or Cisplatin and MSC **(C)**. **(D)** Quantification of the percentage of $F4/80^+$ $IL\text{-}10^+$ macrophages normalized to control. Data are expressed as means ± SEM of 7 mice/group. Data were analyzed using One-way ANOVA followed by Bonferroni's post-hoc test. $^{**}P<0.01$, $^{***}P<0.001$.

Role of T cells and macrophages in the resolution of CIPN induced by nasal MSC treatment.

We showed that nasal administration of MSC promotes resolution of CIPN. Moreover, we showed previously that the spontaneous resolution of CIPN depends on T lymphocytes and endogenous IL-10 signaling [31, 34]. We therefore aimed at testing the contribution of macrophages to the resolution of cisplatin-induced mechanical allodynia. To that end we generated mice expressing the diphtheria toxin (DT) receptor in macrophages using transgenic mice that express a Cre recombinase under control of LysM promoter ($Lysm^{Cre}$) crossed with mice carrying the human diphtheria toxin receptor-mcherry fusion protein preceded by a loxP-flanked stop element under the control of the Csf1r promoter ($Csf1r^{LsL\text{-}DTR}$) [57]. These mice were treated with two rounds of cisplatin followed by nasal MSC. Unfortunately, however, treatment of these mice with DT after completion of cisplatin treatment and administration of MSC led to rapid weight loss and death of the mice. Control mice treated with cisplatin, MSC and DT or DTR-positive mice treated with cisplatin and MSC who did not receive the DT treatment did not show any problems. These findings indicate that macrophages are key to protect against cisplatin-induced mortality, but also precluded us from testing the hypothesis that macrophages mediate the beneficial effects of MSC.

Next, we addressed the potential role of T cells in the reversal of CIPN in response to nasal administration of MSC. We compared the effect of nasal MSC on cisplatin-induced mechanical allodynia and on DRG mitochondrial function in WT and $Rag2^{-/-}$ mice. Rag $2^{-/-}$ mice lack mature B and T cells and have similar onset and intensity of allodynia as WT mice (Figure 7A) [34].

The results in Figure 7 confirm our earlier findings that nasal administration of MSC reverses cisplatin-induced mechanical allodynia as well as the cisplatin-induced mitochondrial deficits in the dorsal root ganglia in WT mice. Cisplatin-induced mechanical allodynia and DRG mitochondrial abnormalities were similar in WT and $Rag2^{-/-}$ mice. However, MSC failed to exert their beneficial effects in $Rag2^{-/-}$ mice that do not have B and T cells, implying that MSC need T cells (and/ or B cells) to exert their positive effect on CIPN.

Figure 7. Nasal MSC administration does not reverse CIPN signs in Rag 2$^{-/-}$ mice. Male WT and Rag2$^{-/-}$ mice were treated i.p. with 2 cycles of 5 days of cisplatin or saline; 48h and 96h after the last cisplatin dose, 1×10^{6} mMSC were administered nasally. Mechanical allodynia was measured using Von Frey hairs. The 50% paw withdrawal threshold was calculated using the up-down method **(A)**. Data are shown as means ± SEM of 5 mice/group and were analyzed using Two-way ANOVA followed by Bonferroni's post-hoc test. **** *P*<0.0001. **(B)** Mitochondrial bioenergetics in DRG neurons was measured 24 days after the last MSC dose using Seahorse XFe 24 Analyzer as previously described [32].Oxygen consumption rates (OCR) were normalized to protein content**.** Mean basal and maximum respiratory capacity (MRC) were calculated **(C)**. Results are expressed as means ± SEM. Data were analyzed using One-way ANOVA followed by Tukey's post-hoc test. *** *P*<0.001. n= 5 mice/group.

DISCUSSION

Here we show that nasally administered MSC rapidly migrate to the meninges of the brain of cisplatin-treated mice where they are mainly

detected in the superior sagittal sinus. The MSC travel also to the spinal cord meninges where they can be found between 12-24 hours after nasal administration. Furthermore, we show that MSC-derived mitochondria end up in F4/80^{+} macrophages in the meninges of the brain of cisplatin-treated mice. Flow cytometric analysis showed that cisplatin decreases the number of CD206^{+} cells in the meninges of the brain, while MSC tend to restore this macrophage subset. Importantly, nasal MSC administration increased IL-10 production by meningeal macrophages, suggesting that MSC are programming macrophages toward a more anti-inflammatory regulatory phenotype. We showed earlier that nasal administration of MSC reverses cisplatin induced cognitive deficits as well as neuropathic pain (see chapter 2 and 4). Here, we show that MSC did not resolve cisplatin-induced mechanical allodynia in Rag2$^{-/-}$ mice, suggesting that the MSC-mediated resolution of pain is dependent on T and/or B cells.

We show that GFP^{+}-MSC reach the superior sagittal sinus (SSS) of the meninges as early as 2 hours after administration. Our previous study showed that nasally administered MSC also enter the parenchyma of the brain [12]. Galeano et al. [25] have reported that MSC, when given via the nasal route, pass the olfactory bulb epithelium, then cross the cribiform plate and arrive in the subarachnoid space via a channel which is an extension formed by the subarachnoid space. From the cerebral meningeal compartment, MSC can freely migrate to the meningeal compartment of the spinal cord by migrating along the meninges, the meningeal vasculature and/or via the cerebrospinal fluid. We indeed detected MSC^{+}-GFP in the meninges of the spinal cord at 12 and 24 hours after nasal administration.

From the meninges, MSC could enter the brain via the olfactory bulb and the rostral migratory stream, or they could penetrate the brain via passing through the vasculature, but this route has still to be investigated.

It is generally accepted that MSC do not survive longer than 24-48 hours after administration independently of the route of administration [19, 20, 67]. Consistently, we showed that MSC, either given intracranially or nasally, could only be detected in the brain of animals subjected to hypoxia-ischemia or cisplatin treatment until 24-48 hours after administration [12, 17]. In the meninges, however, MSC where detectable until at least 7 days after nasal

administration. This long lasting presence of MSC in the meninges likely contributes to their beneficial effects.

MSC have emerged as promising candidates for cell therapies based on their immunomodulatory and regenerative capacities [2, 11, 48, 69]. Accumulating evidence suggests that MSC promote the conversion of pro-inflammatory macrophage populations into anti-inflammatory cells producing IL-10 and expressing CD206 [1, 11, 23, 29, 49]. Our study demonstrates that administration of MSC to cisplatin treated mice promotes the production of IL-10 by meningeal macrophages as well.

The question that arises is how MSC signal to meningeal macrophages to increase their IL-10 production. Accumulating evidence indicates a role for direct cell-to-cell contact between MSC and macrophages. For example, Braza et al. reported that MSC applied to the lungs are phagocytosed by alveolar macrophages leading to an M2 phenotypic switch with increased IL-10 production and alleviation of inflammation in a murine asthma model [5]. Similarly, Jackson et al reported that MSC enhance the function of alveolar macrophages in a model of acute respiratory distress syndrome by transferring their healthy mitochondria to macrophages in the lung [28]. Both studies show that uptake of mitochondria is associated with enhanced macrophage bioenergetics and phagocytic capacities. Our data show that mitochondria from MSC origin are present in meningeal $CD206^{+}$ macrophages. We do not yet know if the presence of MSC-derived mitochondria in macrophages is due to a transfer of mitochondria via tunneling nanotubes, via paracrine action through vesicles, via membrane fusion or results from phagocytosis of MSC.

We reported before that nasal MSC administration reverses the mitochondrial dysfunction in the dorsal root ganglia of cisplatin-treated mice (chapter 4). The latter may imply that these MSC travel all the way to the DRG where they transfer healthy mitochondria to DRG neurons. However, we did not detect MSC in the DRG (data not shown) making this an unlikely explanation. Therefore, we propose that the beneficial effect of nasal administration of MSC on DRG neurons is mediated at least in part via indirect pathways.

We have shown before that the spontaneous resolution of CIPN is dependent on T lymphocytes and IL-10 production. Moreover, the resolution of CIPN and restoration of DRG mitochondrial function in response to pharmacological interventions, *e.g.* HDAC6 inhibition or sphingosine-1-phosphate receptor 1 inhibition has also been shown to be dependent on T cells and IL-10 [10, 43]. The results in Chapter 4 demonstrate that the beneficial effects of MSC administration on pain and mitochondrial function in the DRG is dependent on IL-10 signaling as well. Specifically, we showed that the MSC are not effective in mice lacking IL-10Receptors on sensory neurons. Our current data show that MSC also do not work in $Rag2^{-/-}$ mice that do not have T and B cells. Collectively these findings indicate that MSC act by promoting endogenous T cell and IL-10 dependent repair mechanisms. It remains to be determined how T cells and IL-10 exert their function, but in view of the above, IL-10 and T cells may probably be part of a final common pathway in the resolution of CIPN. We previously described that T cells do not function by producing IL-10 since $IL10^{-/-}$ T cells can still aid in reversing CIPN. Moreover, here we did not observe mitochondrial transfer from MSC to T cells in the meninges (Figure 4). It might be that T cells resolve CIPN by regulating the expression of the IL-10R1α on the sensory neuron. In another model of chemotherapy-induced CIPN using paclitaxel, we have observed that paclitaxel increases the IL-10R1α on DRG neurons, a phenomenon which does not occur in T cell-deficient mice [31].

In conclusion we hypothesize that the meningeal compartment represents the 'incubator' of macrophage-MSC interaction probably in part via transfer of MSC-derived mitochondria to macrophages turning these cells into migrating IL-10 producing macrophages. These 'educated' macrophages may then migrate to damaged areas in the brain, spinal cord and possibly the DRG to promote tissue repair. Moreover, T cells will be needed in the resolution of CIPN by nasal MSC treatment.

In summary, we propose here that after nasal administration of MSC, the cells pass the cribriform plate and end up in the meningeal compartment of the brain and spinal cord, where they 'educate' the meningeal macrophages and T cells to a more regulatory IL-10 producing phenotype. We suggest that both regulatory cell types are necessary to resolve CIPN. Future studies are needed to examine the precise cellular mechanisms involving

macrophages and T cells during the MSC-mediated resolution of cisplatin-induced neurotoxicities.

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6

Mitochondrial transfer from mesenchymal stem cells to damaged neurons to stimulate repair: Mechanisms and functions

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Review Article

Energy is synonymous with life. Animal cells generate over 90% of the energy within cellular organelles called mitochondria. Mitochondria are crucial cellular endosymbiotic organelles with their own genome, the mitochondrial DNA (mtDNA), consisting of the only circular double-stranded genome in the mammalian system. MtDNA encodes some of the proteins belonging to the electron transport chain, which ultimately defines the respiratory capacity of the cell [30]. The respiratory capacity of a cell can be measured by analyzing the oxygen consumption needed to maintain its basal state or in response to a stimulus. Mitochondrial respiration leads to a transfer of chemical energy derived from oxygen and food substrates into a transmembrane electrochemical potential. This energy will be used by mitochondria to produce adenosine triphosphate (ATP), to execute calcium buffering, and to achieve protein and molecular import and export, to produce signaling molecules, and to participate in apoptosis.

Mitochondria are dynamic organelles ranging between 0.75 and 3mm^2 in area. Mitochondria continuously fission and fusion to adapt to changes in the cellular and tissue micro environment [67]. A disruption of the balance between fission and fusion of mitochondria leads to dysfunction and ultimately to cell death. Mitophagy, the selective degradation of damaged mitochondria, is another crucial mechanism for keeping cells in a functional state. Damaged mitochondria can lead to depletion of the cell of ATP, increased production of ROS, occurrence of submitochondrial particles, and cytochrome-c from the inner membrane inducing release of pro-apoptotic proteins such as caspases. In order to preserve the integrity of the cell, proper elimination of damaged or even aged mitochondria is indispensable. This process occurs via sequestration and hydrolytic degradation by the lysosome. In addition to the critical elimination of damaged mitochondria, mitophagy also regulates mitochondrial numbers to allow cellular adaptation to changing metabolic needs and for mitochondrial turnover. Mitophagy and mitochondrial fission and fusion are regulated by multiple proteins, including PINK1 and Parkin proteins as carefully reviewed by Jin et al [28]. Dysfunction of mitochondria is often associated with morphological changes such as vacuolization of the organelle and disruption of the cristae. Cristae are invaginations of the inner mitochondrial membrane, making the inner membrane about 5 times as large as the outer membrane. Changes in cristae structure affect metabolic efficiency of the mitochondria and modulation of the cristae structure is necessary for adaptation to metabolic demands [10].

In case of injury to the mitochondria, increased ROS production, changes in Ca-buffering and energy production, and release of cytochrome c leading to apoptosis, represent various aspects of mitochondrial dysfunction and are often associated with a loss of mitochondrial cristae. As a result, cellular function will be severely hampered leading to maladaptation of the cellular system to the demands of the environment and eventually cell death.

Due to the fact that adult neurons are relatively high energy consumers and do not self-replicate, they are highly dependent on efficient mitochondrial functioning for long term maintenance of cellular health and to adapt to their constantly fluctuating energy supply. This review will focus on the maintenance and repair of neuronal mitochondrial integrity.

Role of neuronal mitochondrial health in brain function

Indeed, there are many neurological disease states where mitochondrial dysfunction is thought to be one of the key underlying mechanism of the pathology. Inborn errors consisting of mutations in mtDNA often lead to severe neuronal pathology. Moreover, brain injury as a result of internal or external trauma, and neurotoxicity caused by chemotherapy or other medications, are associated with neuronal mitochondrial dysfunction that is thought to play a major role in the cognitive, motor and sensory deficits that develop in these conditions [35, 40]. For example, accumulating evidence suggests that mitochondrial dysfunction in the cell bodies of substantia nigra neurons contributes to both sporadic and familial Parkinson disease [52]. Mitochondrial dysfunction has also been recognized as an important marker of Alzheimer disease [11]. Moreover, it is now recognized that impaired mitochondrial function is one of the hallmarks of natural aging rendering the brain more sensitive to traumatic stimuli [36, 39]. These diseases and the aging process go hand in hand with regulation of mitochondrial fission and fusion dynamics as essential mechanisms the cell employs to handle a decrease in mitochondrial activity leading to mitochondrial DNA damage, build-up of misfolded protein aggregates like Tau-1, and mitochondrial dysmorphology [58].

In line with the central role of mitochondrial health in controlling brain functioning, we showed that a selective mitochondrial protectant, the small molecule inhibitor pifithrin-μ (PFT-μ) that specifically prevents the accumulation of p53 at the mitochondrial membrane, completely protected

the brain from damage after chemotherapy. Co-administration of PFT-μ with the chemotherapeutic drug cisplatin prevented the increase in mitochondrial p53, leading to protection against the chemotherapy-induced loss of mitochondrial function in the brain and protected against cognitive deficits as well as structural changes that develop as a result of treatment with cisplatin alone [16]. Selective protection of the mitochondria by PFT-μ also prevented of structural and functional damage including hippocampal lesions, white matter loss, and cognitive deficits in a mouse model of cerebral neonatal ischemic damage [16, 43]. These data underline the key importance of maintaining mitochondrial integrity in brain trauma and neurotoxicities and suggest mitochondrial protectants as crucial therapeutic targets.

Intercellular transfer of mitochondria

Since the seminal paper of Lederberg and Tatum [32] where the transfer of genetic information via a donor and a recipient bacterium was described, the phenomenon of endogenous organelle transfer as a way to rejuvenate or rescue cells has been studied in several physiological and pathological models. Indeed, accumulating evidence indicates that the transfer of cellular organelles like mitochondria and endosomes/lysosomes and cytoplasmic content from one cell to the other is a common process that occurs in healthy as well as damaged tissue. Multiple structural adaptations contribute to the intercellular transfer of organelles, including vesicles, gap junctions and as shown most recently, tunneling nanotubes (TNTs) [41].

TNTs are thin non-adherent actin-rich membranous structures with diameters between 50-1500 nm that can span distances of several hundred nm up to 100 mm [62]. These TNT form direct connections between cells to allow transport of many cellular components including cytoplasm, ions, lipid droplets, viral and bacterial pathogens, genetic material and organelles like lysosomes and last but not least mitochondria [34]. In a study using cryo-electron microscopy, Sartori-Rupp et al. elegantly showed that the TNT we observe in neuronal cell cultures by confocal microscopy are in fact a bundle of individual open-ended TNTs, each surrounded by a plasma membrane and linked to each other by bridging filaments containing N-Cadherin [53]. Each individual TNT extended by the neuronal cell contains one parallel actin bundle along with vesicles and mitochondria that are being transferred by traveling along the actin filament.

In certain cell types like neurons, mitochondria must travel a long distance to reach the synapse or peripheral nerve endings. Therefore the transport machinery of a cell to transfer the mitochondria is extremely important to allow regulation of the metabolic demands at the required site of action. Essentially mitochondria move via the microtubules with the directives of two motor proteins, dynein and kinesin. To ensure a tight coupling between microtubules and motor proteins, the adaptor proteins Milton, as the motor adaptor, and Miro, taking care of mitochondrial coupling, are of great importance [38].

Ahmad et al. were the first to show that the adaptor protein and Rho-GTPase Miro1 is crucial for the mitochondrial transport from MSC to epithelial cells. MSCs overexpressing Miro1 exhibited an enhanced rescue of epithelial injury, while knockdown of Miro1 prevented rescue of the injury in conjunction with a reduction in mitochondrial transfer from MSC to the epithelial cells. In vivo, airway hyperresponsiveness and remodeling were reversed by treatment with MSC overexpressing Miro1 in three separate allergen-induced asthma models, while Miro1 knockdown led to a loss of the efficacy of MSC [1].

MSC to restore brain mitochondrial health and brain function

Mesenchymal stem cells, also known as mesenchymal stromal cells, are self-duplicating cells with strong regenerative and immunosuppressive capacities [64]. MSC have been described for the first time by Alexander Friedenstein in 1966 [22]. MSC have been applied for a long time as the most successful and safe therapeutic strategy for allogeneic cell therapy to e.g. accelerate the hematopoietic reconstitution after transplantation of hematopoietic stem cells or high dose chemotherapy in breast cancer [29, 31]. MSC are strongly immunosuppressive and have been proposed as a therapy for autoimmune diseases and other inflammatory conditions including Crohn's disease and ulcerative colitis [23, 44]. Because of their immunosuppressive actions, MSCs have also been regularly applied for the treatment of Graft versus host disease.

MSCs can differentiate into cells of mesodermic origin like chondroblasts, osteoblasts and adipocytes but it is now accepted that MSC can also differentiate into cells of ectoderm origin, including neuronal cells, especially under in vitro conditions. Because of their capacity to differentiate into cells of multiple lineages,

MSCs have been studied extensively for the use in regenerative medicine including for promoting brain health after insults like cerebral stroke, subarachnoid hemorrhage, hypoxic-ischemic events and traumatic brain injury [19, 20, 44, 59, 61]. Importantly from a therapeutic perspective, MSCs appear to have minimal to no immunoreactivity and lack MHC-Class II expression, which means that they can be used from a third party donor without fear of rejection.

On the basis of many preclinical studies focusing on the regenerative capacities of MSC transplantation for the resolution of brain damage, these cells are now emerging in the clinic as a potential candidate for ischemic stroke and even for perinatal arterial stroke. Preclinical studies have shown that the regenerative effects of e.g. intranasal administration of MSC on brain structure and function after neonatal hypoxia-ischemia are lifelong. Moreover, Donega et al. showed that this therapy is efficacious and safe [19]. Donega et al. demonstrated that nasal MSC administration could restructure a complete hippocampus that was no longer microscopically detectable after the hypoxic-ischemic event, showing the power of the nasal MSC therapy [18]. Other studies have shown that MSC administered intravenously, intracranially, or via the nasal route are also effective for the treatment of various forms of brain injury in adult rodents [17, 44, 45].

Moreover, in cisplatin-treated mice the synaptosomal mitochondrial dysfunction is completely restored when these mice receive MSC treatment via the nasal route after completion of the chemotherapy [15].

Interestingly, restoration of brain function by administration of MSC in models of ischemic or chemotherapy-induced brain damage is not associated with long term survival of cells of donor origin in the brain. In fact, the existing evidence indicates that MSCs or MSC-derived cells are no longer detectable in the host brain, 48-72 hours irrespective of the route of administration (intravenously, intracranially or intranasally) [18, 60]. Nevertheless, these administration of MSC is effective at normalizing brain function and structure. These findings indicate that the MSC promote endogenous repair mechanisms through cell-to-cell communication pathways.

Intercellular communication is essential for tissue and body homeostasis and coordinates responses to injury and internal/external cues. In order to maintain homeostasis, cellular communication pathways need to be highly specific, adaptive and efficient. This implies that there are many routes of communication including paracrine communication via diffusible factors, secretion of extracellular vesicles including microvesicles and exosomes, or via direct cell-to-cell contact allowing transfer of signals through membrane fusion or gap junctions.

Apart from the well-known paracrine action of MSC through the production of growth and differentiation factors such as VEGF and BDNF, it has been proposed that MSC may act by 'educating' other cell types to produce and release growth factors or cytokines. Macrophages are a good example of cells whose function can be redirected by MSC. Jin et al. showed that MSC promote type 2 macrophage polarization, meaning the macrophages switch from a damage promoting phenotype to a functional subtype that promotes repair rather than enhance damage. MSC can also communicate with neurons via secretion of extracellular vesicles or by direct cell-cell contact via gap junctions containing connexin 43 and/or by transferring part of their content, including mitochondria, to damaged target cells [26, 47, 49].

In this review, we will focus on intercellular communication via transfer of mitochondria as a way to maintain or restore neuronal health in response to MSC administration.

MSC as mitochondrial donors

In general it has been proposed that cells with a relatively high bioenergetic capacity are the best donors for delivering repair signals to cells in need and this includes the transfer of mitochondria via TNTs. MSCs have a relatively high bioenergetic activity and have been shown to be excellent donors of mitochondria to recipient cells whose mitochondria are damaged.

MSCs have been demonstrated to transfer mitochondria to many cell types including macrophages, neurons, stem cells, tumor cells and endothelial cells. For example, it has been suggested that the beneficial effects of MSC on the endothelial barrier after myelosuppressive and myeloablative treatments for leukemia and multiple myeloma is mediated by transfer of healthy mitochondria from the MSC to the damaged endothelial cells [21].

Other studies show that mitochondria transferred by MSCs ameliorate the energetic activity of the alveolar epithelium of mice treated with LPS, and of rodents with rotenone-induced airway injury [26, 50, 51, 56]. The transfer of mitochondria from MSC to macrophages via TNTs may explain also part of the mechanism of the beneficial effects of MSC on improving clearance of bacteria in models of Acute Respiratory Distress Syndrome (ARDS) and sepsis [27]. Jackson et al. elegantly showed that MSC enhance the phagocytic activity of macrophages after in vitro co-culture in association with transfer of mitochondria from MSC to the macrophages at least in part through TNTs. In this model, blocking of TNT formation prevented the change in phagocytic activity and lead to a failure to improve the bioenergetic activity of the macrophages [27]. Lung macrophages that had received mitochondria from MSC in vivo, had a higher phagocytic activity [27].

Mitochondrial transfer to maintain or restore brain health

Neurons are complex cells extending often very long dendrites and axons. It is therefore of great importance to maintain transportation and adequate long distance distribution of mitochondria to meet their high energy requirements especially during conditions of cellular stress [55]. It is therefore not surprising that mitochondrial transfer is part of the processes in place to maintain brain health under physiological conditions. Astrocytes are well known for their role in neuronal health and recent studies indicate that these cells exchange mitochondria with neurons. In the normal healthy brain, astrocytes can donate healthy mitochondria to neurons. Other studies have shown that neurons can export their damaged neurons to astrocytes. In this way the neurons can conserve energy by acquiring healthy neurons from astrocytes and by outsourcing mitophagy to astrocytes [25] . It has also been reported that astrocytes increase donation of mitochondria to neurons in conditions of damage, e.g. in a model of stroke [25]. However, when the negative pressure on a given cell or organ is too high, the endogenous repair processes including transfer of mitochondria are not sufficient to fully prevent or restore damage. We propose that in these situations therapeutic local or systemic administration of MSC which can donate mitochondria to rescue the bioenergetic status of neurons can help restore brain function.

Not a lot is known how MSC sense the damaged potential recipient cell and “know” to transfer healthy mitochondria or how TNT formation is regulated. Mahrouf-Yorgov et al. described that (damaged) mitochondria

from the recipient cell are ‘sensed’ by MSC in response to formation of contact between TNT extended by the damaged recipient cell to the MSC [37]. On the molecular level the accumulation of p53 at the damaged mitochondrial membrane has been suggested as the ‘danger signal’ for the mitochondrial donor cell to express TNT. When p53 function was deleted by a dominant negative construct or siRNA treatment, no TNT formation could be obtained in astrocytes under H_2O_2 or serum deprivation conditions [65]. The latter would imply that the damage-induced mitochondrial p53 accumulation is the signal for the recipient cell to extend a TNT structure which can be picked up as a ‘request for help’ to a cell such as MSC. How MSC sense the TNT structure and how they respond to it is unknown. When MSC are engineered to overexpress the Rho-GTPase Miro1, mitochondrial transfer is increased whereas genetic ablation of Miro1 in MSC donors blocks transfer of mitochondria to neurons [65]. It could be that the MSC-Miro1-mediated signaling pathways are actively promoting mitochondrial transport via the TNTs formation initiated by the recipient. There is also evidence that astrocytes release mitochondria via involvement of astrocytic CD38 to the recipient neuronal cells. CD38 is mainly expressed by glial cells and CD38-deficient mice show decreased recovery after brain injury. CD38 catalyzes the synthesis of a calcium messenger, cyclic ADP-ribose (cADPR) in mitochondrial membranes [2, 33]. Astrocytes increase the expression of CD38 in response to glutamate release from neurons allowing the astrocytic production of extracellular mitochondria [9]. Whether or not TNT formation is involved in CD38 mediated mitochondrial transfer is unclear but paracrine mitochondrial secretion might be a factor of importance as well in cellular communication between astrocytes and neurons.

Dividing neuronal stem cells and DCX^+ progenitor cells in neurogenic niches in the brain are extremely sensitive to the toxic effects of chemotherapy as well as to brain trauma [15, 18]. Especially DCX^+ progenitor cells lining the dorsal site of the subventricular zone as well as in the dentate gyrus, are important neurogenic niches in the brain and necessary for neurogenesis thereby supporting neural circuitry and maintaining optimal cognitive function [54, 63, 69]. Disruption of adult neurogenesis leads to the pathogenesis of several neurodegenerative diseases associated with cognitive dysfunction. Multiple studies have shown that chemotherapy strongly reduces the number of neuronal progenitors in the subventricular zone and in the dentate gyrus of the hippocampus. Our recent studies have shown that the beneficial

effects of MSC administration on cisplatin-induced cognitive impairments is associated with a restoration of the number of DCX^+ progenitor cells in these areas. In vitro, exposure of radial glial cells (Nestin/$GFAP^+$) and the neural progenitor (DCX^+) cells to cisplatin induces mitochondrial deficits characterized by rapid loss of mitochondrial membrane potential and reduced mitochondrial respiration leading to precursor cell death. Interestingly, these adverse effects of cisplatin where completely reversed when the damaged neuronal precursors were co-cultured with MSC. We demonstrated that the beneficial effects of the MSC were mediated at least in part by mitochondrial transfer via MSC; pharmacological disruption of the actin-based structures in MSC using latrunculin B led to an inability of mitochondrial transfer from MSC to the neuronal precursor [8]. Latrunculin B treatment of the MSC also prevented the MSC-induced increase in neuronal precursor survival. Moreover, mitochondrial transfer from MSCs to neuronal stem cells reversed the decrease in mitochondrial membrane potential induced by cisplatin. The contribution of mitochondrial transfer to the beneficial effects of MSC on survival of the DCX^+ precursors in cisplatin treated mice in vivo remains to be determined. However, we did show that overexpression of the Rho-GTPase Miro1 in MSC improved the regenerative effect of MSC in vitro, implying that stimulation of mitochondrial movement is involved [8, 42].

In this model of mitochondrial transfer by MSC to neural stem cells we proposed that it is the damaged neural stem cell that gives the initial signal for TNT formation and mitochondrial transfer. The first reason is that we detected only a very low level of mitochondrial transfer when co-culturing healthy neural stem cells with MSC. The number of neuronal stem cells that contain MSC-derived mitochondria increased dramatically when the neural stem cells had been damaged by previous exposure to the chemotherapeutic drug cisplatin. Second, preliminary data indicates that the small molecule inhibitor PFT-μ which specifically prevents accumulation of p53 at the mitochondrial neural stem cell membrane, prevented the transfer of mitochondria to the neural stem cell. Accumulation of PFT-μ after neuronal damage in vivo is a very early molecular event since it was only detectable till 4 hours after the cerebral insult [43]. Since mitochondrial p53 accumulation is a ubiquitous event after cellular damage in general, we would like to propose that the early mitochondrial event in the recipient cell is the danger signal for the MSC to donate mitochondria.

In this respect it is of interest that in vivo PFT-μ is capable of preventing synaptosomal mitochondrial dysfunction as a result of cisplatin administration or cerebral hypoxia-ischemia is associated with a rescue of neural progenitors and/or white and grey matter structure [8, 16, 43]. The neuroprotective effect of inhibition of p53 transcriptional activity by PFT-α was smaller and was independent of reduced oxidative stress, indicating that the p53 danger signal is given by the mitochondria that are at risk [43].

Most of the mitochondrial transfer studies focus on the effect of mitochondrial donation on the restoration of respiration and ATP production, but mitochondria are more than power plants. They regulate metabolic processes like long-chain fatty acid beta-oxidation, amino acid metabolism as well as urea, one-carbon, and TCA cycles. One way might be that mitochondrial transfer also leads to changes in the epigenome by changing histone acetylation and DNA methylation [14]. Mitochondria produce at least 346 distinct regulating enzymes that modify DNA and histones for modulation of gene expression of metabolites. One can expect that fusion of recipient mitochondria with donor mitochondria may lead to epigenetic changes that could also contribute to the observed restorative effects [3, 14, 48].

Optimizing mitochondrial transfer

Therapeutically it is important to explore how to select or engineer MSC to have an optimal mitochondrial donating capacity. Paliwal et al. described that MSC have a differential capacity to transfer mitochondria depending on the tissue they originate from. For example, adipose or bone marrow-derived MSC were less efficient mitochondrial donors as compared to dental pulp or Wharton jelly-derived MSC that have higher respiratory capacities [46]. MSC with high mitochondrial respiration have lower mitochondrial transfer capacities but higher suppressive capacities of mtROS. Although these data are interesting, they are contra intuitive since high transfer of healthy mitochondria may efficiently lead to suppression of mtROS. Probably other factors in addition to mitochondrial transfer could be responsible for the observed effects [46]. Another way of improving the transfer of mitochondria by MSC is by priming with damaged recipient cells in vitro before administration. MSC were shown to be much better donors when the recipient cells (*i.c.* astrocytes) were damaged by ischemic damage lading to elevated ROS levels. Babenko et al. and Ahmad et al. have elegantly shown

that MSC primed by co-culturing with cortical neurons, are better donors and have more therapeutic effect in a model of cerebral stroke than non-primed MSC [1, 4, 5]. Priming also increased the expression of the Rho-GTPase Miro-1. The latter is interesting since genetic overexpression of Miro-1 in MSCs facilitates mitochondrial transfer from MSC to neuronal stem cells (See above) [8] . Babenko et al. demonstrated the same effect of Miro-1 overexpression with respect to mitochondrial transfer from MSC to astrocytes leading to enhanced cell recovery [4, 37]. Interestingly, airway hyper responsiveness was mitigated by MSC overexpressing Miro-1 in three models of allergen-induced asthma, pointing towards a potential therapeutic effect whereas knockdown of Miro-1 had the opposite effect [1, 4].

Future directions

It is not surprising that donation of isolated mitochondria would be the next step to achieve regeneration of tissue including brain damage after ischemic stroke, traumatic brain injury and other organ injury like pulmonary and cardiac damage. Donation of isolated mitochondria could potentially be beneficial if the mitochondria are taken up adequately by the damaged recipient cell allowing restoration of mitochondrial respiration. There are already some reports about clinical trials with mitochondria. Although the effects were described to have some positive effect, the results have also been met with some reluctance [7].

Mitochondria once isolated, are sensitive organelles and easily damaged by ex vivo factors like temperature changes, calcium overload due to high calcium concentrations in the circulation, leading to a decrease in mitochondrial membrane pore formation, swelling of mitochondria and loss of cristae. However, it has been demonstrated that isolated purified mitochondria released by e.g. endothelial progenitor cells are still viable and capable of releasing ATP and have intact oxygen consumption. Hayakawa et al showed that these mitochondria were capable of restoring brain endothelial cell barrier function, increased levels of TOM40, mtDNA copy number and intracellular ATP after exposing brain endothelial cell cultures to oxygen glucose deprivation [24]. Interestingly, mitochondria can also be passively taken up by breast cancer cells which results in decreased proliferation and oxidative stress of the tumor cells and enhanced sensitivity to chemotherapeutics like doxorubicin and paclitaxel. The same phenomenon was observed when starved glioma cells were incubated

with mitochondria derived from human astrocytes by showing a rescue of aerobic respiration and a greater sensitivity to radiation [57]. Interestingly, when dysfunctional mitochondria were isolated from osteosarcoma cybrids containing mtDNA with the A8344G mutation, Sun et al. demonstrated that there was no recovery of cellular respiration and no growth inhibition of tumor cells [12].

There are not many data available if and how mitochondrial transplantation could support regeneration of the brain damage after cerebral insults or under neurodegenerative conditions. There are in vitro studies with cortical neurons (ischemia/reperfusion) or PC12 cells (UV light damage) showing incorporation of donated mitochondria leading to reduced apoptosis and transfer of mtDNA from the donor. In vivo data are still scarce. Hayakawa et al. reported an improvement in neuronal survival and plasticity after transient focal cerebral ischemia [25], although there was an immediate response to this report advising to approach the interpretation of these data with some caution [6]. Chang et al. showed that local injection of mitochondria at the median forebrain bundle could improve the locomotor activity in a rat model of Parkinson disease and an attenuation of the deterioration of dopaminergic neurons.

Wang et al. recently published that mitochondrial transplantation also attenuated LPS-induced depressive behavior in rats using outcome parameters like forced swim test, tail suspension test and sucrose preference test. Interestingly, mitochondrial donation significantly downregulated the neuroinflammatory response to LPS in association with the activation of glia, increased BDNF expression and neurogenesis and ATP production and oxygen consumption [66].

Before mitochondrial transplantation will become a realistic therapeutic strategy that promotes neuronal survival and regeneration for stroke, CNS injury and neurodegenerative diseases, several goals will have to be met. Importantly, functionalization of mitochondria to facilitate uptake and function should be established. Foremost the organelle delivery should be functionalized in a way that they can be used ready from the shelf. Wu et al. have published that cellular uptake of isolated mitochondria could be significantly improved by coating with a polymer conjugate of dextran with triphenylphosphonium [68]. Chang et al. have shown that coating

the mitochondria with the cell penetrating peptide, Pep-1, improved mitochondrial function and viability of human cybrid cells with the MELASA3243G mutation [13]. Moreover, more should be known about the mechanism of action and ultimately the safety of the procedure. However, the donation of healthy mitochondria to cells in need of energy, has become an exciting and interesting route to explore.

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7 Summary and Discussion

SUMMARY

In this thesis, we show for the first time that nasal administration of mesenchymal stem cells (MSC) promotes recovery from chemotherapy-induced cognitive impairment and chemotherapy-induced peripheral neuropathy, two major side effects of cancer treatment that frequently persist long into survivorship.

First we introduce the concept of neurotoxicities as a result of cancer treatment and possible underlying mechanisms in *chapter 1*.

In *chapter 2,* we demonstrate that nasal administration of MSC reverses cognitive impairments induced by cisplatin treatment, including deficits in working memory, executive functioning and spatial recognition in male and female mice. Using resting state functional magnetic resonance imaging study of network connectivity in the brain, we show that cisplatin treatment leads to a decrease in global neuronal connectivity. Moreover, connectome analysis shows a decrease in path length in cisplatin-treated mice, while MSC treatment reversed the abnormal path length. On the functional level nasally administered MSC reversed the cisplatin-induced synaptosomal mitochondrial dysfunction as well as abnormal mitochondrial morphology resulting from cisplatin treatment. Moreover, MSC led to a reversal of the cisplatin-induced morphological changes of cortical white matter structures. RNA sequencing analysis of the hippocampus of mice treated with cisplatin with and without nasally administered MSC revealed mitochondrial oxidative phosphorylation as a top pathway activated by MSC administration to mice treated with cisplatin, supporting the concept that MSC may act by resolving neuronal mitochondrial dysfunction leading to restoration of the cognitive deficits and associated brain damage as a result of cancer treatment.

In *chapter 3*, we show that cisplatin treatment leads to mitochondrial dysfunction and death of neural stem cells (NSC) *in vitro* as well as a decrease in the number of Doublecortin^{+} (DCX)$^{+}$ neural progenitor cells in the brain neurogenic niches. Furthermore, we show that nasal MSC treatment rescues damaged NSC from cell death *in vitro* and reverses the loss of DCX^{+} cells *in vivo*. As a potential mechanism of MSC action, we show for the first time that MSC donate mitochondria to NSC when damaged by cisplatin *in vitro,* contributing to the beneficial effects of MSC in rescuing damaged NSC as a result of cisplatin treatment. They transfer their healthy mitochondria to the NSC via tunneling nanotubes to the damaged NSC. Mitochondrial transfer is potentiated by overexpression of the Rho-GTPase Miro1. Donation of mitochondria by MSC leads to a restoration of the decrease in mitochondrial membrane potential induced by cisplatin. Moreover, mitochondrial transfer by MSC to NSC is associated with protection against cell death as a result of cisplatin.

In *chapter 4*, we demonstrate that nasal MSC administration not only has its effect on the brain but also resolves symptoms of CIPN, including mechanical allodynia and spontaneous pain. In addition, we show that nasal MSC treatment normalizes cisplatin-induced mitochondrial dysfunction in the DRG neurons and tibial nerves and reverses the retraction of peripheral nerves endings in the paw of the mice treated with cisplatin.
As a mechanism of action of the MSC, we show that IL-10 production by the nasally administered MSC is critical for reversal of CIPN. In addition, IL-10 signaling via IL-10 receptors expressed by peripheral sensory neurons appeared to be crucial for MSC-mediated recovery of CIPN, including the restoration of mitochondrial impairment in the DRG neurons.

In *chapter 5,* we describe the tracing of nasally administered MSC transgenically overexpressing GFP in the brain and meninges of the brain and spinal cord. We observed an abundant presence of MSC within 30 min-1 hour after administration in the meninges of the brain. MSC could still be detected in the meninges of the brain at 7 days after administration. MSC were also present in the parenchyma of the brain, in the deep cervical lymph nodes, and in the meninges of the spinal cord at 12-24 hours albeit in low numbers. Interestingly, mitochondria derived from MSC were taken up by macrophages in the meninges of the brain which induced the macrophages to increase production of IL-10. The latter suggest that the meningeal compartment may 'educate' immune cells to a more regenerative healing phenotype.

In *chapter 6*, we review the mechanisms and functions of mitochondrial transfer from MSC to damaged neuronal cells.

DISCUSSION

Cognitive impairment and associated brain damage after cisplatin treatment

Cisplatin treatment results in cognitive decline in humans and animals [1, 51]. We have developed a model where we used a dose of cisplatin that efficiently killed tumors in vivo (see chapter 2). Although cisplatin is known to only penetrate the blood-brain barrier in very low doses, adult neurons as well as neural stem cells have shown to be very sensitive to cisplatin. It has also been suggested that neural stem cells in the SVZ can actively sample the circulation (and the CSF) via their ciliae extending to the vessels [41]. In chapter 2, we show that cisplatin treatment clearly caused damage to the cortical white matter including loss of myelin density and reduction in myelin arborization. In chapter 3 we showed that cisplatin treatment led to a severe loss of DCX^+ neural progenitors as well. One of the hallmarks of our findings is that cisplatin treatment led to a mitochondrial dysfunction in synaptosomes. We did not test the mitochondrial function of the remaining DCX^+ cells specifically. However, the mitochondrial loss will not only be restricted to the synaptosomal fraction. We observed an abnormal morphology of mitochondria in myelinating cells in the sensory motor cortex as well (Chiang et al. man. in prep.). Interestingly, we observed here that cisplatin caused myelin protraction or unwrapping, a phenomenon also observed in the aging brain.

Advancements in neuroimaging suggest that patterns of functional connectivity bring a broadly applicable neuromarker of cognitive performance. The use of resting-state neuroimaging to characterize the neural basis of cognitive impairment has been well established [33-35]. Current studies suggest an association between chemotherapy and decreased functional and structural connectivity in the brain. Using rsfMRI, we show in chapter 2 that cisplatin treatment changes functional network connectivity in mice. Our data suggest that the brain network is overly integrated in mice treated with cisplatin and appears as a noisy network, consistent with the greater density of connectomes in the cisplatin-treated group.

Mitochondrial dysfunction in cisplatin-induced cognitive impairment and peripheral neuropathy

We and others have demonstrated that damage to neuronal mitochondria in brain and peripheral sensory nervous system is an underlying mechanism of the development of CICI and CIPN, respectively [8, 9, 18, 22, 58]. When we tested mitochondrial function as a result of cisplatin treatment, we observed a decrease in maximal (MRC) and spare respiratory capacity (SRC) after cisplatin. SRC reflects the ability of mitochondria to meet an increased energy demand with increased oxygen consumption and it is considered as a primary factor to define survival of neurons under stress [47]. A lower SRC has been linked to cognitive impairments in rodent models of Alzheimer's disease and aging [5, 26, 31, 42, 44, 49].

Morphologically, we showed that systemic cisplatin administration induces structural changes in mitochondria in brain synaptosomes including absence of cristae and a swollen vacuolized appearance [8]. A disassembly of cristae have been directly linked to a loss of membrane potential, as has been shown after cisplatin treatment (see chapter 3) leading to increased ROS production and cellular damage. This abnormal morphology is associated with impaired mitochondrial bioenergetic profile (Chapter 2, Figure 5A-5F).

Functional role of MSC treatment for CICI and CIPN via the nasal route

The nasal pathway represents a non-invasive route of administration for MSC which has been shown to be beneficial for restoring brain health after cerebral insults or neurotoxic events [3, 8, 11, 12, 17, 60, 62]. We clearly showed in Chapter 2 that nasally administered MSC can restore cognitive function on the level of executive functioning, spatial memory and novelty. RNA sequencing analysis of the hippocampus demonstrated that MSC treatment increased expression of nuclear and mitochondrial genes involved in mitochondrial respiration. Indeed, expression of nuclear encoded genes for components of the four complexes of the electron transport chain increased and mitochondrial transcriptome analysis revealed an upregulation of genes encoding components of complex I, III and IV of the electron transport chain (chapter 2). In line with these explorative data, we show for the first time that MSC treatment restores mitochondrial morphology in association with the reduction in synaptosomal mitochondrial function in the brain of cisplatin-treated mice. This underlines the central role of mitochondrial function in the neurotoxic effects of cisplatin.

Mitochondria are subjected to constant fission and fusion, which is a tightly regulated process by specialized proteins known as mitochondria-shaping proteins. Mitochondrial fusion is orchestrated by Mitofusin-1 and Mitofusin-2 (which coordinate the fusion of the outer mitochondrial membrane) and optic atrophy-1 (which regulates the inner mitochondrial membrane fusion) [55] . Another protein Dynamin-Related Protein 1 (Drp1) governs the fission of mitochondria and contributes to the shaping of mitochondria as well. Drp1 can also directly regulate the cristae of mitochondria [48, 55]. In preliminary studies, we have observed that cisplatin decreased the phosphorylation of Drp1 on serine 616, suggesting that inactive Drp1 might contribute to the change in morphology of mitochondria (Jamal et al. man. in prep). Newell et al. have shown using a contact co-culture system that MSC co-cultured with fibroblasts donate mitochondria and rescued fission morphology as the mitochondria in the recipient fibroblasts were shifted to an elongated fusion pattern. This indicates that MSC induce morphologic shifts and that MSC-derived mitochondrial transfer may play a role in the process of restoration of mitochondrial function [46]. Whether MSC can regulate the fusion and fission machinery in our cisplatin model is a subject of investigation in our group.

Analyzing the connectome of the brain of cisplatin-treated mice we observed that nasal MSC treatment increased the path length in cisplatin-treated mice, demonstrating normalization of the functional connectome and underlining the efficacy of nasal MSC administration. This rescue of neuronal efficiency by MSC could very well reflect a resolution of mitochondrial function since neurons are completely dependent of their energy production to ensure connectome efficiency.

Anyhow, it is important to mention that treatment with MSC appears to be disease-modifying as we showed in chapter 2, since it could resolve already existing synaptosomal mitochondrial dysfunction (see below).

Interestingly, nasally administered MSC can even have 'long ranging' actions as nasally administered MSC also restored deficits in the peripheral nervous system like CIPN. This data are very important from a therapeutic perspective since the physical and emotional burden of CIPN during and after cancer treatment is heavy and no adequate FDA-approved treatment is available.

Apart from the CNS, we have reported previously that cisplatin treatment impairs mitochondrial morphology and bioenergetics in the peripheral sensory system as well. Using whole genome RNA sequencing, we showed that cisplatin changes expression of genes involved in mitochondrial oxidative phosphorylation in DRG neurons of cisplatin-treated mice (Ma et al, 2019). In chapter 4, we show that cisplatin-treated mice have decreased basal and maximal respiratory capacity in the DRG neurons. Importantly, we are the first to demonstrate that nasal MSC treatment reverses long distance mitochondrial dysfunction in both DRG neurons and tibial nerves of cisplatin-treated animals. Scruteri et al. showed that MSC can rescue DRG from dying in vitro through direct cell to cell contact [53, 54]. This process could very well include transfer of mitochondria from MSC into DRG neurons via connexins or mitochondrial transfer via nanotubes (see Chapter 3 and 4). However, until now we have not observed MSC in the DRG after nasal administration, but only in the spinal cord meninges (see below). MSC may influence the sensory nerve projecting to the spinal cord allowing the signal to be propagated to the DRG soma. However, this mechanism has to be explored in depth in the future and will also be discussed below.

Intra-epidermal nerve fiber (IENF) loss has been proposed as the earliest sign of axonal pathology. IENFs represent bioenergetically active areas which are relatively far from the soma and are thus highly susceptible to mitotoxic insults induced by chemotherapy. In accordance with previous reports, we show in chapter 4 that cisplatin treatment decreased IENF density in the hind paws. Interestingly, we show that the loss of IENF was completely reversed, 24 days after MSC treatment. We do not know whether this represents actual regrowth of nerve endings after dying off or that the actual retraction process is mitigated and unwrapping the nerve endings occurs. However, both processes need energy and we therefore propose that MSC restore mitochondrial function in the nerve ending either via restoration of mitochondria in the DRG soma allowing more transport of proteins and organelles to the peripheral nerve ending or by a direct effect of MSC (or macrophages 'educated' by MSC, see below) on the nerve ending.

Consistent with previously reported data on CIPN in rodent models, we do not detect any sex differences in cisplatin-induced mechanical allodynia (see chapter 4). This observation is in line with the clinical data showing that gender does not predict susceptibility of cancer patients to develop CIPN

[32]. Furthermore, there is no difference in the response to MSC treatment, as MSC are equally efficient in reversing CIPN in both sexes.

Fate of nasally administered MSC

The meningeal compartment

The nasal route has the advantage that a lower number of MSC is needed for restoration of cognitive deficits compared to the intravenous injections (i.v.). It has been shown that most of the MSC when given i.v. end up in peripheral organs including the lung and liver [4, 38, 52, 57]. We propose that the nasal route can also be more effective. Preliminary data from our group shows that i.v. administration of MSC, even at higher doses, does not improve cognitive impairments in a mouse model of cranial irradiation and temozolamide used as a treatment for glioblastoma, while nasal administration of MSC is very effective (Chiu et al. man. In prep.). We have observed that the latter model of treatment for glioblastoma is aggressive in a sense that we observed a lot of structural damage to white and gray matter as well, underlining the superior route of MSC administration via the nasal route. Moreover, in our safety studies, it appeared that MSC when given nasally, do not migrate to the brain tumor as far as could be detected, whereas i.v. administration gave rise to extensive migration of MSC to the tumor (Chiu et al. man. In prep.).

We show that MSC, when given via the nasal route to cisplatin-treated animals, first end up in the meninges of the brain within 30 min to 1 hour, where they can interact with other cell types such as macrophages (see chapter 5). Interestingly, these exogenous MSC home for a long time in the meninges of the brain, at least for 7 days. This is in sharp contrast to all the published data on the survival of exogenous MSC in vivo [19, 20]. Our observation that most of the MSC were found in the superior sagittal sinus (SSS) and adjacent area of the meninges could mean that these cells travel through the lymphatic circulation and are drained into the DCLN by using a paravascular route and migrating between the brain interstitial fluid (ISF) and the CSF along the glymphatic routes [2]. In this respect it is of interest that we traced MSC back into the deep cervical lymph nodes (DCLN) see Figure 1.

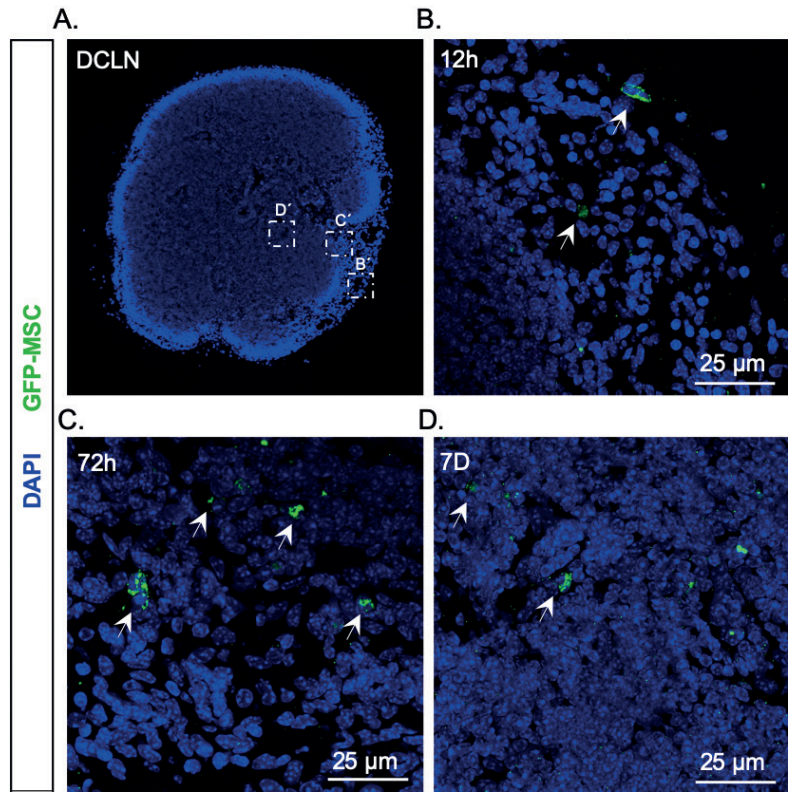

Figure 1. Nasally applied MSC migrate into the deep cervical lymph nodes of cisplatin-treated mice.
Mice received 2 cycles of 5 days of cisplatin i.p; 48h after the last cisplatin dose, 2×10^6 GFP$^+$-MSC were administered nasally and deep cervical lymph nodes (DCLN) collected 12h, 72h and 7 days after MSC administration. **(A)** Overview image of DCLN stained with Dapi. White line shows area positive for GFP$^+$-MSC **(B´-D´)**. **(B-D)** representative images of regions were GFP-MSC were traced, 12h **(A)**, 72h **(B)** and 7 days **(C)** after nasal administration. Arrows indicate GFP-MSCs.

Tracing of MSC in the brain

In chapter 2 of this thesis, we show that nasally administered MSC migrate also into the brain parenchyma of cisplatin-treated mice where they arrive between 12-24 hours after administration. This result is consistent with what was shown previously by Donega et al.[15, 16] and van Velthoven et al. [59]. These authors previously reported that MSC administered nasally migrated specifically to the lesioned hemispheres in a model of neonatal hypoxic-ischemic brain damage [16, 60]. In our cisplatin model, we were also able to detect MSC tagged with a fluorescent dye, in various brain

areas including the hippocampus, thalamus and cortex. However, we also observed that the number of MSC present in brain tissue remains relatively low. We did not detect any GFP$^+$ MSC in the brain 72h following nasal administration, showing that these MSC probably do not integrate in the brain and differentiate into neurons or glia. This observation is consistent with what others have shown previously in a model of myocardial infarction, where MSC injected through the i.v. route remain in the circulation no longer than 24 hours. Moreover, van Velthoven et al. [61] have shown that cranially administered MSC overexpressing GFP when administered after neonatal hypoxia-ischemia are no longer present after 72 h. Donega et al. showed that after nasal administration MSC had disappeared from the brain after 48-72 hours [16]. It should be mentioned that the meninges of the brain was the only site where we observed MSC for at least 7 days after administration, indicating the importance of this compartment (see below).

It still has to be unveiled whether the low number of MSCs is really enough to repair the brain. In our view this is questionable in view of the low number and the short time that MSC are present in the brain. Another possibility is that MSC 'educate' other cell types that actually take care of repairing the brain (see below). Macrophages can penetrate into the brain parenchyma when there is neuronal damage after e.g. cerebral insults [21, 23]. Therefore we suggest that MSC are not the main effectors in the brain parenchyma, but that they induce 'tissue healing macrophages' in the meningeal compartment which will migrate to the areas of damage to restore function and structure.

Regeneration of the neurogenic niche in the brain by nasal MSC administration

In chapter 3 we also show that MSC can regenerate the neurogenic niches in the brain such as the lateral side of the subventricular zone (SVZ) and the dentate gyrus of the hippocampus (DG). Cisplatin treatment has a strong effect on the proliferating DCX$^+$ neuronal progenitors as was shown in chapter 3. MSC treatment led to a complete restoration of this cell type. It has been shown that neural stem cells in the brain are very important for cognition and other types of behavior since the absence of DCX$^+$ cells leads to a strong cognitive deficit [27, 64]. There is a lot of controversy in the literature on whether they are involved in actual renewal of adult neurons [30, 45]. Whether or not MSC may directly interact with the cells in the neurogenic niches such as the SVZ is unknown but theoretically it could be the case, since neuronal stem cells in the SVZ can sample MSC-derived

proteins or organelles from the CFS via their ciliae [41]. Alternatively, MSC could reach the SVZ via migration from the OB. However, in our experiments we did not observe tagged MSC in the SVZ specifically, which could suggest that sampling by DCX$^+$ cells of MSC-derived signals via the CSF might be an option. It could also be that the neurogenic niches of SVZ and DG are restored by MSC-educated macrophages as discussed above.

In Chapter 3 we have clearly shown in an *in vitro* model that MSC can effectively transfer mitochondria to cisplatin-damaged NSC. Furthermore, we showed that transfer of mitochondria reverses the decrease in mitochondrial membrane potential in NSC and accounts for the increased NSC survival after cisplatin treatment. Interestingly, blocking mitochondrial transfer abrogated the beneficial effects of MSC. Moreover, enhancing mitochondrial transfer through overexpression of Miro1, a mitochondrial motor protein, facilitated mitochondrial transfer and boosted NSC survival. Several reports have shown that MSC have the potential to transfer mitochondria to damaged cells and thus replace the defective mitochondria or even compensate for their dysfunction and conferring metabolic benefits [6, 10, 46]. An important finding in our study is that nasal MSC treatment restores the loss of DCX$^+$ neural progenitors in the brain neurogenic niches. We have not explored yet the possibility of mitochondrial transfer in vivo in the DG and SVZ of cisplatin-treated animals but it is likely that MSC or MSC-educated macrophages contribute to the rescue of NSC through transfer of mitochondria, thereby ameliorating cognitive processes. We will investigate the role of mitochondrial transfer in vivo in the near future.

Tracing of MSC in the spinal cord meninges

We could trace low numbers of MSC back into the spinal cord meninges 12 and 24 hours after nasal administration. In view of the fact that the cerebral and spinal meninges are interconnected, we favor the explanation that MSC after passing the cribriform plate, end up in the subarachnoid space including the meningeal compartment, and will subsequently travel via the CFS to the damaged sites such as the DRG where they can contribute to resolution of the CIPN [24].

Mitochondrial transfer and education of macrophages by MSC

We have observed an interaction between MSC and macrophages in meninges of cisplatin-treated mice (chapter 5), detected by mito-DsRed

mitochondria derived from MSC in F4/80^{+}/CD206^{+} macrophages. Jackson et al. showed that MSC can transfer mitochondria to macrophages in a model of acute respiratory distress syndrome [29]. Braza et al. showed that alveolar macrophages phagocytose MSC, leading to an M2 phenotypic switch and alleviation of inflammation in a mouse model of asthma [7].

It is possible that our nasally applied MSC will be transferred to macrophages already in the meninges via tunneling nanotubes and/or phagocytosis of MSC.

After nasal MSC administration the percentage of CD206^{+} meningeal macrophages slightly increased in comparison to mice treated with cisplatin alone, whereas cisplatin decreased the number of CD206^{+} cells in comparison to saline control animals. No change was observed in M1 population. Although CD206 expression has been referred to as an M2 regenerative and healing phenotype, the microenvironment will determine the actual activation state of the macrophage with varying different phenotypic and functional characteristics [25]. However, it is of interest that the presence of MSC in the meninges induced meningeal macrophages to produce more IL-10, 72 hours after the last MSC dose, which represents a time point when MSC in most other compartments are gone. We have shown before that the cytokine Il-10 is a prerequisite for resolution of CIPN. We also show in Chapter 4 that selective ablation of the IL10R1a on the sensory neurons wipes out the positive effect of MSC [37] and (chapter 4).

The idea of MSC-educated macrophages has been described in several studies. Kim et al. have shown that macrophages adopt an M2 phenotype after 3 days of co-culture with MSC [36]. Maggini et al. showed that MSC educate macrophages which in turn show an increase of IL-10 when stimulated with LPS [43] Lonescu et al. reported that M2 macrophage activation is one of the mechanisms through which MSC alleviate lung injury [28]. Consistent with these reports, our findings suggest that MSC reprogram host macrophages to a 'healer' phenotype including increased IL-10 production. To further explore this theory it will be interesting to reconstitute mice with MSC-educated macrophages to determine whether they can resolve CICI and CIPN

Since the natural microenvironment for MSC is the bone marrow, we also investigated whether MSC could be traced back into the bone marrow of the tibia and femur (data not shown). Endogenous MSC are normally present in low numbers in the bone marrow (1:10.000 BM cells) [50]. We saw a sharp increase in GFP expressing MSC in the bone marrow, 72 hours after nasal transplantation. In the bone marrow, MSC could efficiently educate e.g. monocyte derived suppressor cells (MDSC) that could then migrate to the DRG to restore mitochondrial function of DRG. Future research will have to decide whether the bone marrow actually could contribute as a site for regenerating exogenous MSC.

Role of T cells

An interesting finding though is that nasal MSC treatment fails to resolve CIPN in T-cell-deficient (Rag2$^{-/-}$) mice, lacking T cells and B cells, while WT mice fully recovered. Our group have previously shown that T-cell-deficient mice when reconstituted with CD8^{+} T cells, recover from paclitaxel–induced pain. Moreover, we reported previously, a role of T cells (and not B cells) in recovery from CIPN [37]. These data indicate that T cells play an important role in MSC-mediated recovery from CIPN signs. Moreover, we have shown that CD8^{+} T cells and not CD4^{+} T cells are involved in the resolution of CIPN and inflammatory pain [37]. It will be very interesting to explore how MSC interact with CD8^{+} T cells in our model. We know that CD8^{+} T cells involved in the resolution of CIPN are acting in an antigen-nonspecific way [39]. It would be interesting to explore whether MSC can induce CD8^{+} T cells to become antigen-nonspecific suppressor cells that will in turn contribute to resolution of the peripheral neuropathy.

Role of IL-10

We and others have reported the role of IL-10 in the spontaneous resolution of CIPN including paclitaxel-, oxaliplatin- and cisplatin-induced neuropathy, while intrathecal administration of anti-IL10 prevents the resolution of CIPN [37, 40]. Several studies have shown that MSC produce IL-10 [20] . We demonstrated in chapter 4 that MSC lacking the capacity to produce IL-10, do not resolve signs of CIPN in cisplatin-treated mice. In addition, many publications have shown that IL10 itself can have a feed forward effect by inducing macrophages into a pro-repair phenotype [14, 56, 63]. In order to explore whether Il-10 production was necessary upstream of the peripheral nervous system in CIPN, we ablated the Il10R1a from the peripheral nerve.

To our surprise our data showed that we needed Il-10 signaling at the level of the DRG since MSC had no effect in a cell-specific IL10R1a knockout mouse (see chapter 4).

Concluding hypothesis

On the basis of our data we hypothesize that MSC act via a **two-step IL-10 dependent model**: *Step 1* involves IL-10 production by the MSC to contribute to the education of the recipient cell (the macrophage). The educated macrophage will be capable of increasing its IL-10 production and will migrate to the site of damage (Figure 2).

Step 2 involves IL10 production by the MSC-educated cell at the level of the 'effector' neuron to regulate cognition or the pain response.

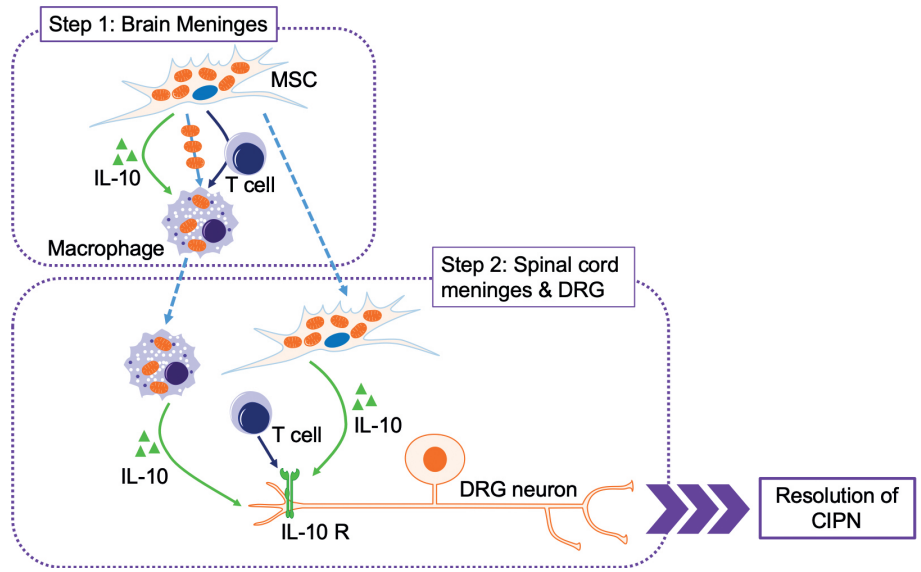

Figure 2. Potential mechanism of MSC-induced resolution of CIPN.
Resolution of CIPN may be achieved in 2 steps mediated by IL-10. MSC-derived IL-10 can educate macrophages in meninges and migrate to the spinal cord. IL-10 production by MSC and/or educated macrophages will act at the level of the nociceptive nerve to reverse CIPN. MSC: mesenchymal stem cell, IL-10: Interleukin 10, IL-10 R: IL-10 receptor, DRG: dorsal root ganglia.

In this respect it is of interest that IL10 deficiency has been shown to lead to defective mitochondrial respiration and changes in morphology as described by us before in brown adipose tissue [13]. Therefore we propose that the restorative effect of the MSC-educated macrophages will resolve the CIPN or cognition by restoring the mitochondrial respiration and morphology in the sensory neuron or the neural stem cells/adult neurons in the brain.

Taken together, the results presented in this thesis demonstrate that nasal administration of MSC is an effective treatment option for both CICI and CIPN. Nasal MSC administration offers a safe, non-invasive therapeutic strategy with a great potential in treating side effects of cancer therapy.

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8

Samenvatting in het Nederlands

SAMENVATTING IN HET NEDERLANDS

In dit proefschrift laten we voor het eerst zien dat behandeling met mesenchymale stamcellen (MSC) via de neus het herstel bevordert van cognitieve problemen en perifere neuropathie die optreden als gevolg van chemotherapie. Cognitieve problemen en perifere neuropathie representeren twee belangrijke klinische problemen veroorzaakt door de chemotherapie. Deze problemen blijven vaak lang bestaan na het beëindigen van de kankerbehandeling en hebben daardoor een negatieve invloed op de kwaiteit van leven van de patiënt na succesvolle behandeling van kanker.

In hoofdstuk 1 introduceren wij het begrip neurotoxiciteit als resultaat van de kankerbehandeling en bespreken mogelijke oorzaken.

In hoofdstuk 2 laten we zien dat nasale behandeling met MSC een succesvolle interventie is voor de cognitieve problemen als gevolg van chemotherapie, zoals defecten in werkgeheugen, executief functioneren en ruimtelijk geheugen, in mannelijke en vrouwelijke muizen. Met behulp van functionele magnetische resonantie imaging bestuderen wij netwerk connectiviteit van het brein en laten we zien dat cisplatin de connectiviteit verlaagt terwijl nasale behandeling met MSC deze verlaging in connectiviteit te niet doet. MSC behandeling herstelt ook de mitochondriële dysfunctie in de neuronale synaptosomen. Deze mitochondriële functie resultaten konden ook bevestigd worden met behulp van elektronen microscopische analyse van de mitochondrieën in de synaptosomen. MSC behandeling corrigeerde ook de defecten in witte stof structuur van het brein die ontstaan door behandeling met cisplatin. RNA sequencing van de hippocampus van muizen die wel of niet behandeld waren met cisplatin en/of met MSC liet zien dat de meest belangrijke cellulaire pathway die hersteld werd door MSC de mitochondriële oxidatieve phosphorylerings pathway is. Wanneer we deze data samenvoegen kunnen we concluderen dat MSC werken door de cellulaire mitochondriële dysfunctie in het brein te herstellen.

4In hoofdstuk 3 laten we zien dat cisplatin mitochondriële dysfunctie en celdood induceert in neuronale stamcellen in vitro en ook in vivo het aantal neuronale voorlopercellen vermindert. Nasale behandeling met MSC kan deze verschijnselen volledig corrigeren. We laten hier voor het eerst zien dat MSC hun effect hebben via donatie van hun mitochondrieën aan de

neuronal stamcellen via zogenoemde nanobuisjes. Interessant is dat deze mitochondriële donatie door MSC verhoogd wordt wanneer de MSC het Rho-GTPase Miro1 overexpresseren. Donatie van mitochondrieën door MSC aan de stamcellen leidt tot een herstel van de door cisplatin veroorzaakte verlaging van de mitochondriële membraan potentiaal van de neuronale stamcellen en de door cisplatin veroorzaakte celdood.

In hoofdstuk 4 laten we zien dat nasale behandeling niet alleen een herstellend effect heeft op het brein maar ook op de door cisplatin veroorzaakte perifere neuropathie. MSC herstellen mechanische allodynie en de spontane pijn. MSC gegeven via de neus corrigeren ook de mitochondriële dysfunctie in het dorsale wortel ganglion en het verlies van de periferen neuronale uiteinden in de poot van de muizen die met cisplatin behandeld waren. Als mechanisme stellen we voor dat de interleukine-10 (IL-10) productie door MSC van groot belang is voor het positieve effect. We laten zien dat IL-10 de IL-10 receptor op perifere sensorische neuronen activeert zodat de pijn verdwijnt en de mitochondriële dysfunctie van neuronen in het dorsale wortel ganglion normaliseert.

In hoofdstuk 5 beschrijven we de tracing van MSC wanneer we ze via de neus toedienen. MSC zijn binnen 30 minuten al in de hersenvliezen zichtbaar waar ze tot 7 dagen daarna nog steeds gedetecteerd kunnen worden. MSC konden ook aangetoond worden in het parenchym van het brein, in de diepliggende cervicale lymfklieren en in de meningen van het ruggemerg na 12-24 uur, hoewel in lage aantallen. In de meningen zagen we dat de macrofagen die mitochondrieën van MSC opgenomen hadden meer IL-10 gingen maken. Dit resultaat suggereert dat MSC de macrofagen instrueert tot cellen die gespecialiseerd zijn in de regeneratie van cellulaire schade.

In hoofdstuk 6 hebben wij de mechanismen en functies van mitochondriële donatie van MSC naar neuronale cellen beschreven die in de literatuur zijn vermeld.

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LIST OF PUBLICATIONS

Boukelmoune N, Chiu GS, Kavelaars A, Heijnen CJ. Mitochondrial transfer from mesenchymal stem cells to neural stem cells protects against the neurotoxic effects of cisplatin. Acta Neuropathol Commun. 2018 Dec 12;6(1):139. doi: 10.1186/s40478-018-0644-8.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BM	Bone marrow
CICI	Chemotherapy-induced cognitive impairments
CIPN	Chemotherapy-induced peripheral neuropathy
CNS	Central nervous system
CPP	Conditioned place preference test
CSF	Cerebrospinal fluid
CTGB	Cell tracker green BODIPY
DCLN	Deep cervical lymph node
DCX	Doublecortin
DG	Dentate gyrus
DRG	Dorsal root ganglia
FCCP	Carbonilcyanide p-triflouromethoxyphenylhydrazone
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
IENF	Intraepidermal nerve fibers
IL-10	Interleurkin-10
ISF	Interstitial fluid
KO	knockout
LN	Lymph nodes
MRC	Maximal respiratory capacity
MSC	Mesenchymal Stem Cells
NOPRT	Novel object/place recognition test
NSC	Neuronal stem cells
OB	Olfactory bulb
OCR	Oxygen consumption rate
PBS	Phosphate-buffered saline
PBT	puzzle box test
PFT- µ	pifithrin-µ
RMS	Rostral migratory stream
rsfMRI	Resting-state functional MRI
SEM	Standard error of the mean
SVZ	Subventricular zone
SSS	Superior sagittal sinus
Miro-1	Mitochondrial Rho GTPase 1
TMRM	Tetramethylrhodamine methyl ester
TNT	Tunneling nanotube
WGA	Wheat germ agglutinin
WT	Wild type

List of abbreviations

VITA

Nabila Boukelmoune was born in Oran, Algeria. She attended the University of Science and Technology of Oran, Algeria, where she obtained an engineer degree in Molecular Genetics. Upon graduation, Nabila worked as a medical representative for Julphar, one of the largest pharmaceutical companies in the Middle East and North Africa. In the fall of 2011, she entered the Graduate Program in Molecular Biology at The University of Oran and received a Master's of Science in Molecular Biology in 2012. Nabila moved to The United States of America and joined the Graduate School of Biomedical Sciences at The University of Texas Health Science Center in Houston in the fall of 2012. She obtained another Master's degree in the fall of 2014. Nabila then joined the laboratory of Neuroimmunology at The University of Texas M.D. Anderson Cancer Center in the summer of 2015. She began working on her PhD under the supervision of Prof. Dr. Cobi Heijnen (University of Texas M.D. Anderson Cancer Center), Prof. Dr. Manon Benders and Dr. Cora Nijboer (University Medical Center, Utrecht). Her research presented in this thesis focuses on mesenchymal stem cells therapy for the repair of chemotherapy-induced neurotoxicities.