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Abstracts for the 22nd Annual Meeting of the American Society for Neural Therapy and Repair

Presidential Symposium: Experimental Therapies for Behavioral Deficits in Rodent Models of Huntington's Disease

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During the past two decades, much of the research with my colleagues in the Field Neurosciences Laboratory for Restorative Neurology at Central Michigan University has focused on searching for treatments for Huntington's disease (HD). Our early work centered on adopting appropriate animal models of HD to test a wide array of potential therapeutics for cognitive and motor deficits that mimic what occurs in patients who suffer this dreaded disease. Prior to the advent and availability of transgenic rodent models of HD, our lab worked to develop ways to simulate the progression of HD-like behavioral pathologies via novel methods of administering quinolinic acid (QA) and 3-nitropropionic acid (3-NP). Using these models, we were able to show that treatments with GM_1 ganglioside and with the dietary supplement creatine could significantly reduce these neurotoxic-induced HD-like cognitive and motor deficits. Although pharmaceutical and nutraceutical interventions are still an important aspect of our research, we have concentrated our most recent efforts on the use of stem cell therapies in rodent models (both neurotoxic and transgenic) of HD. Like many other investigators, our goal was to find a means of replacing cells lost to HD, with the hope that these transplanted stem cells would be able to integrate into the host neurocircuitry in functionally significant ways. Our first efforts provided us with mixed results, as we were able to reduce QA-induced behavioral deficits but in the absence of any evidence that our transplanted, bone marrow-derived stem cells were differentiating into neuronal phenotypes in sufficient numbers to account for the functional improvements. As a result, we postulated that the ameliorative effects of the transplanted cells were due to the release of anti-inflammatory cytokines and/or neurotrophic factors. Our subsequent research confirmed this hypothesis, as we provided evidence indicating that bone marrow-derived mesenchymal stem cells (MSCs) produced a host of anti-inflammatory cytokines as well as several kinds of neurotrophic factors. As additional support for the hypothesis that neurotrophic factors were key to providing the MSC-induced benefits, we transplanted MSCs, which were genetically altered to overexpress brain-derived neurotrophic factor (BDNF) or nerve growth factor (NGF), and found that YAC 128 mice, which carry the full-length human HD transgene, displayed both behavioral sparing (as measured by the rotarod task) and neuronal sparing [of neuronal nuclei (NeuN)- and dopamine- and cAMP-regulated phosphoprotein 32 (DARPP32)-positive cells] in the striatum. In addition, we utilized the immunomodulatory capabilities of MSC transplants by cotransplanting these cells with neural stem cells (NSCs) and found evidence that the addition of the MSCs tended to increase the survivability and improve behavioral performance in 3-NP rats relative to transplants of MSCs or NSCs alone. Finally, our most recent work has indicated that induced pluripotent stem cells, implanted into the striatum, can differentiate into cells with region-specific phenotypes (DARPP32 positive) and promote functional recovery, albeit most likely via production of BDNF. Collectively, our work indicates that transplantation of stem cells provides neuroprotective properties and the potential for cell replacement therapy, and that, in combination with other therapies, offers significant hope for an effective treatment for HD.

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Presidential Symposium: Genetically Engineered Mesenchymal Stem Cells as a Proposed Therapeutic for Huntington's Disease

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We are developing a novel therapy for Huntington's disease (HD): implantation of human mesenchymal stem/stromal cells (MSCs) engineered to secrete brain-derived neurotrophic factor (MSC/BDNF). BDNF levels are reduced in the brains of HD patients. BDNF has been shown in numerous transgenic HD mouse studies to prevent cell death and to stimulate the growth and migration of new neurons in the brain and is thus a lead candidate for neuroprotection in HD. We are using MSCs as delivery vehicles to produce BDNF in the affected areas of the striatum. We are conducting detailed tests of MSC/BDNF in HD mouse models in preparation for a proposed Phase I clinical trial of MSC/BDNF implantation into the brain of HD patients (HD-CELL), with the goal of slowing disease progression. Our approach is based on the proof-of-concept data from the Dunbar Laboratory (Dey et al., 2010). We have manufactured and tested human MSC/BDNF using standard operating procedures (SOPs) from our UC Davis good manufacturing practices (GMP) facility. We have shown that MSC/BDNF produces high levels of BDNF and that a multiplicity of infection of 10 virus particles per cell generates a single intact integrant per cell, on average. This is data critical to the Recombinant DNA Advisory Committee (RAC), for whom we have prepared an Appendix M application. RAC approval is needed prior to FDA approval because it is a proposed stem cell gene therapy trial. We are currently refining our application to the FDA and will seek California Institute for Regenerative Medicine (CIRM) approval for submission. We are completing our double-blinded studies, examining the effects on disease progression of implantation of MSC/BDNF in two strains of HD transgenic mice: YAC 128 and R6/2 (CAG 120). The R6/2 (CAG 120) model has the early onset of neurologic dysfunction and dies much earlier than the wild type of YAC 128 models. For this reason, it is a more suitable model of juvenile HD. In the R6/2 model, we have successfully demonstrated that implantation of MSC/BDNF causes an improvement in deficits in open field exploration, a behavioral/anxiety assay. We have also shown that MSC/BDNF causes increased neurogenesis in the brains of treated mice, an important milestone. The YAC 128 model develops slowly progressive behavior symptoms in midlife and has loss of brain cells that mirrors changes seen in HD patients. In the YAC 128 model, we have shown that implantation of our MSC/BDNF product decreases striatal atrophy between 8 and 12 months of age. Wild-type mice have a typical lifespan of 2 years, so this age in the YAC 128 mouse roughly corresponds to the typical age at onset for early stage HD patients that we are proposing to treat in our future planned Phase 1 study, HD-CELL. In

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tandem with the ongoing preinvestigational new drug (preIND) studies in the lab, the clinical team is conducting an observational study, PRE-CELL. The goal of PRE-CELL is to establish baseline characteristics and track disease progression in a group of early stage HD patients. PRE-CELL subjects undergo detailed neurological, psychiatric, cognitive, imaging, and laboratory testing, including measurement of BDNF levels. PRE-CELL participants who have completed at least 1 year of follow-up and meet inclusion and exclusion criteria will be considered for the future planned cell therapy trial. PRE-CELL has been approved by the Institutional Review Board at UC Davis since July 2013 and is still enrolling (ClinicalTrials.gov Identifier: NCT01937923). Our progress to date supports the completion of our final preclinical studies and our plan to go forward toward regulatory approval. There are potential applications of our research beyond HD. Our biological delivery system for BDNF sets the precedent for adult stem cell therapy in the brain and could potentially be modified for other neurodegenerative disorders, such as amyotrophic lateral sclerosis (ALS), spinocerebellar ataxia (SCA), Alzheimer's disease, and some forms of Parkinson's disease. It also provides a platform for future gene-editing studies.

Presidential Symposium: Gene Network Analysis Reveals Modules Driving Neural Stem Cell Phenotypes in Huntington's Disease

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We have utilized induced pluripotent stem cells (iPSCs) derived from Huntington's disease patients (HD-iPSCs) as a human model of HD. We generated an isogenic HD-iPSC pair for HD modeling (CAG repeat of 21 and 72) using traditional homologous recombination and found that the HD phenotypes only manifested themselves in the differentiated neural stem cell (NSC) fate—not iPSCs. To understand the molecular basis for the CAG-dependent disease phenotype in NSCs, we have performed transcriptomic analysis of HD-iPSCs, corrected iPSCs, HD NSCs, and corrected NSCs by RNA-Seq. Differential gene expression analysis and standard pathway analysis have been performed. Top upstream regulators by ingenuity pathway analysis (IPA) are transforming growth factor-β1 (TGF-β1), β-estradiol, and tumor necrosis factor (TNF). We also performed unbiased bioinformatic analyses through weighted gene coexpression network analysis (WGCNA). Of the seven distinct modules defined, two modules are characterized by coexpression traits consistent with changes in gene expression in NSCs due to CAG expansion. Genemania and Enrichr tools were applied to these modules, returning enriched terms from a number of published gene set libraries. Coexpression network analysis of those genes by GeneMANIA allows a further determination of larger biological networks associated with these genes. We find protein phosphatase inhibitor dopamine- and cAMP-regulated neuronal phosphoprotein 32 (DARPP-32) to be associated with a number of these enriched networks. Further, both biological networks identified could be modulated to correct HD phenotypes. We believe the HD-NSC model derived from human HD patient(s) has revealed the dysregulation of genes involved in neuronal development and the formation of the dorsal striatum for HD.

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Delayed Intravenous Transplantation of Human Bone Marrow Stem Cells in a Chronic MCAO Model of Ischemic Stroke: A Cell Graft Biodistribution Study

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Currently in the US, there are more than 3 million chronic stroke survivors relegated to rehabilitation with limited treatment options months or years after stroke onset. To date, preclinical studies present adult stem cell therapy as a potential candidate to alleviate the toxic milieu of the stroke posttherapeutic window, yet our knowledge on the homing, biodistribution, and anti-inflammatory effectiveness of delayed intravenous injection of human bone marrow stromal cells (hBMSCs) at chronic stages is still limited. In the present in vivo study, we characterized the homing, biodistribution, and neuroprotective effects of delayed intravenous transplantation of hBMSCs in a chronic model of ischemic stroke. Animals were transplanted intravenously with 4×10^6 hBMSCs or stroke vehicle (saline) at 60 days postischemic stroke. An in vivo imaging system (IVIS) tracked near-infrared fluorescent cells in vivo and ex vivo and revealed that the biodistribution of hBMSCs in our chronic ischemic model is time and organ dependent, most notably the cells migrated and homed to peripheral organs during the first hours posttransplantation. Moreover, significant amelioration of the infarct and peri-infarct area was observed in the striatum of the transplanted group compared to the stroke vehicle group. There was also a significant downregulation of inflammatory cells in the gray and white matter areas. Altogether, hBMSCs present as a promising therapeutic intervention even at chronic time points whereby cells homed in peripheral organs after 60 days, decreased the progressive cell death of the ischemic penumbra, and downregulated inflammatory genes that are a hallmark of chronic stroke.

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Widespread Expression of GDNF Throughout Rat Brain After Intranasal Delivery of hGDNF Plasmid Nanoparticles

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Glial cell line-derived neurotrophic factor (GDNF) gene therapy is a promising therapeutic approach for central nervous system (CNS) disorders such as Parkinson's disease (PD), since it could provide a renewable source of GDNF within the brain, while avoiding the need for repeated dosing. Current approaches rely on viral vectors to carry the gene into cells and require direct intracranial injection. We are investigating the intranasal route of administration of polethylene glycolyated (PEGylated) lysine 30-mer DNA nanoparticles (NPs) encoding hGDNF. These NPs, developed by Copernicus Therapeutics, Inc., are compact single molecules of the expression plasmid and have minimal cross-sectional diameters of ~10 nm. We have previously shown that intranasal NPs incorporating hGDNF plasmid DNA (pGDNF), or a hGDNF-enhanced green fluorescent protein (eGFP) fusion plasmid (pUGG), transfect brain cells in vivo and that intranasal pGDNF NPs protect substantia nigra (SN) dopamine neurons in the rat 6-hydroxydopamine model of PD. The goal of this study was to assess the regional and cellular pattern of transfection in the brain 7 days after intranasal administration of these NPs to rats. GDNF enzyme-linked immunosorbent assay (ELISA) showed significant increases in GDNF levels throughout the brains of rats given intranasal pGDNF relative to controls. To examine cellular transfection patterns, eGFP immunohistochemistry (IHC) was performed on brain sections from rats that received intranasal doses of pUGG NPs, naked pUGG, or saline. The number of cells expressing eGFP was significantly higher across brain regions in rats given intranasal pUGG NPs compared to fluorescence background in saline controls, with the highest number of eGFP-positive cells in the midbrain. Double-label IHC was also carried out for eGFP and a cell-specific marker, that is, either rat endothelial cell antigen 1 (RECA-1), glial fibrillary acidic protein (GFAP), the neuronal nuclei marker NeuN, or the dopamine neuronal marker tyrosine hydroxylase (TH). Most of the eGFP-expressing cells found in the brain 7 days after intranasal delivery of pUGG NPs were abluminal and immediately adjacent to capillary endothelial cells staining for RECA-1. eGFP-positive cells were

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often found contiguous to GFAP-positive astrocytic endfeet enwrapping capillaries, recapitulating their perivascular localization. When eGFP-positive cells were observed adjacent to TH-positive neurons in the SN, or to NeuN-positive cells in any brain region, these neurons were also located within 15 μm of a capillary. This pattern suggests that the cells preferentially transfected after intranasal administration of these DNA NPs are most likely pericytes, consistent with distribution of the NPs by the perivascular transport system. Collectively, these results confirm successful delivery and transfection of cells in rat brain after intranasal administration of Copernicus' DNA NPs and support use of intranasal delivery of hGDNF NPs as a noninvasive means of gene therapy for PD and other CNS disorders.

Supported by a Northeastern University Tier 1 Interdisciplinary Grant and the Michael J. Fox Foundation for Parkinson's Research.

Anatomical, Cellular, and Functional Effects of Neural Stem Cell and/or Olfactory Ensheathing Cell Grafts in a Rat Model of Focal Cerebral Ischemia With Reperfusion

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Stroke is a leading cause of loss of disability-adjusted life years, and treatment options that significantly improve the functional outcome are few. Stem cell therapy in acute stroke has been shown to improve functional outcome and may be a method that provides a longer window of opportunity for treatment. Neural stem cells (NSCs) can differentiate into both neurons and glia, but graft survival is poor. Olfactory ensheathing cells (OECs) secrete a number of growth factors important for neuronal, glial, and endothelial cells and promote axonal sprouting and neovascularization in the damaged CNS. Cotransplantation of NSCs and OECs may thus improve NSC survival. The aim of this study was to investigate whether cotransplantation of NSCs with OECs improves NSC survival, differentiation, and function in an experimental stroke study. Transient middle cerebral artery occlusion (MCAO) with 60-min duration was induced in the right hemisphere of adult Sprague–Dawley rats ($n=21$). Twelve days after stroke, stereotactic injections of a 3-μl suspension of stem cells were performed into the ipsilesional globus pallidus. The stem cell suspensions were prepared immediately before transplantation and consisted of either 5×10^5 NSCs (NSC, $n=6$), or 5×10^5 NSCs and OECs at 50:50 (NSC+OEC, $n=7$); or saline ($n=4$). Rats were weighed on days 0, 10, 17, and 28 post-MCAO. The cylinder-rearing test was performed at days 10, 11, 17, and 28 post-MCAO. MRI was performed at 10 and 28 days post-MCAO: volumes of uninjured tissue in both cerebral hemispheres were estimated from T2w images and the ratio of ipsilesional/contralesional hemisphere volumes calculated. Diffusion tensor images (DTI) were used to calculate mean diffusivity (MD) and fractional anisotropy (FA) in regions of interest in the corpus callosum, hippocampal fimbria, external and internal capsules in both hemispheres. Immunohistochemistry was performed on 50-μm-thick cryostat sections obtained from postmortem brain tissue. There was a reduction in the hemispheric volume ratios, and body weight increased in all groups from day 10 (pretreatment) to day 28 (posttreatment) after MCAO. The NSC and NSC+OEC groups had higher body weights at day 28. There were no differences in hemispheric volume ratios, MD or FA values, or cylinder rearing test results between the groups. Preliminary tissue analyses show PKH26-labeled and nestin$^+$ cells at the graft site; however, relatively few OECs were found at the injection site of the cografted animals. Our findings suggest survival of the transplanted NSCs. Whether there is overall improved NSC survival or differentiation in the cografts remains to be determined. Transplantation of NSCs or cografts did not improve tissue loss or motor function compared to saline.

AAV Delivery of α Synuclein to the Enteric Nervous System Impairs Colonic Motility

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Gastrointestinal (GI) dysfunction is one of the most common nonmotor complaints associated with Parkinson's disease (PD). GI pathology affects virtually every level of the GI tract, from esophagus to anus, primarily presenting as gastroparesis and decreased colonic motility. Owing to the major role the enteric nervous system (ENS) plays in coordinating virtually every aspect of GI function, it is possible that ENS function is impaired in PD, leading to the functional GI disorders observed. In support of this notion, recent studies have demonstrated that the PD-associated protein, α-synuclein (α-syn) forms inclusions within neurons of the ENS. To determine if α-syn pathology within neurons of the ENS is sufficient to produce GI dysfunction, we aimed to overexpress human wild-type α-syn within the ENS using direct injections of adeno-associated virus (AAV) to the descending colon. However, as there have been no focused investigations into the targeted transduction of the ENS using AAV, the goal of this study was to 1) examine the efficiency, tropism, and vector spread using several AAV serotypes in the ENS and 2) determine the effects of α-syn overexpression on GI function. In order to optimize gene delivery to the ENS, rats received direct injections (6×5μl) of either AAV 1, 2, 5, 6, 8, or 9 expressing green fluorescent protein (GFP) into the descending colon. Transduction occurred in neurons and enteric glia within the myenteric and submucosal plexuses of the ENS. AAV6 and AAV9 showed the highest levels of transduction, with AAV9 primarily transducing neurons, and AAV6 transducing neurons as well as enteric glia. Transduction efficiency scaled with titer and time and produced no vector-related immune response. Following characterization of AAV transduction in the ENS, it was determined that AAV9 demonstrated the highest levels of neuronal transduction and was thus selected to deliver the α-syn transgene to the ENS. To evaluate the role of α-syn in GI dysfunction, adult male rats received direct injections of AAV expressing either human wild-type α-syn or a GFP control transgene into the descending colon. Four weeks postsurgery, colonic motility was assayed by quantifying the time necessary to transit and excrete a bead through 5 cm of the descending colon within ambulatory animals. Animals that received AAV–α-syn had significantly impaired colonic motility, with a mean colonic transit time of 171.5 ± 43.4 min compared to animals that received AAV–GFP with a mean colonic transit time of 15 ± 6.8 min. In summary, here we present a thorough characterization of the transduction profile of AAV in the ENS following direct injection to the descending colon. Further, α-syn overexpression in neurons of the ENS significantly decreased colonic motility. This work provides a platform upon which to model GI dysfunction associated with PD, with the aim of determining the role of α-syn in GI pathology and providing potential therapeutic targets.

Autologous Bone Marrow Mononuclear Cell Therapy of Ischemic Stroke in Sheep

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Autologous bone marrow mononuclear cell (BMMNC) therapy was proven to be effective in rodent stroke models, and the first phase I/II clinical investigations are under way to assess their therapeutic potential in patients. However, our knowledge on therapeutic mechanisms, proof-of-concept efficacy in gyrencephalic brains, as well as long-term impact on neurofunctional parameters and lesion size development is still fragmentary. Here 32 adult Merino rams were subjected to permanent middle cerebral artery occlusion after an initial health check and prestudy surveillance. Following baseline neurofunctional assessment, animals were subjected to control or autologous BMMNC IV administration ($\geq 4.0\times10^6$ BMMNCs per kg body weight) 24 h after stroke ($n=16$ each). Animals were monitored for 42 days by behavioral phenotyping, magnetic resonance imaging (MRI), [^{15}O]- H_2O-, and [^{18}F]-fluorodeoxyglucose positron emission tomography (PET) before postmortem histological assessment. All experiments were conducted randomized and blinded, and strict exclusion/inclusion criteria were applied. The therapeutic approach was safe, but eight animals (3× control; 5× BMMNC treated) had to be removed from the trial due to violation of preset inclusion criteria including insufficient BMMNC numbers per kg bodyweight. BMMNC therapy was associated with amelioration of functional deficits from day 7 ($p<0.01$) and reduction of MRI lesion size at day 42 ($p<0.01$) after stroke. Treatment also improved cerebral blood flow ($p<0.05$) and glucose metabolism ($p<0.01$) at day 42 as shown by PET. Moreover, there was an increase in cerebral capillary density and a reduction of glial reactivity in white matter areas ($p<0.05$), potentially indicating a more suitable environment for endogenous functional regeneration/brain plasticity. The therapeutic effect was preserved when controlling for animal age and weight at trial induction, initial lesion size, and poststroke functional deficits. Therapeutic effects were confirmed after controlling for confounding factors including animal age, weight, and initial functional impairment. The approach seems promising, but current findings of reduced efficacy in aged and hypertensive subjects may call for a more detailed investigation of optimal therapeutic circumstances before moving ahead to large-scale clinical trials.

Arteriogenesis as a Therapeutic Target for Traumatic Brain Injury

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Arteriogenesis is the most important adaptive process known to occur following vascular injury throughout the body. Unlike angiogenesis (the long-term sprouting of new capillary networks), arteriogenesis refers to the immediate growth/enlargement of preexisting arterioles, which are a principal delivery route for oxygen, nutrients, and potential therapeutic agents. Studies have shown that the speed of arteriogenesis is not limited to its natural time course and that enhancing this process may aid in long-term tissue preservation. Although improvements in arteriogenesis correlate with tissue protection in stroke, the endogenous repair response has not been investigated following traumatic brain injury (TBI) nor are the mechanism(s) regulating this event fully understood. The *objective* of our studies is to elucidate the cellular and molecular mechanism(s) limiting arteriogenesis following TBI. In support of our objectives, we have determined that ephrin type-A receptor 4 (EphA4) tyrosine kinase suppresses endothelial cell proliferation, migration, and tube formation in vitro. Using endothelial cell (EC)-specific EphA4 knockout (KO) mice, we further demonstrate that there is a significant increase in the size of the pial arterioles compared to wild-type (WT) mice in the cerebral cortex following TBI. Based on these findings, we *hypothesize* that EphA4 signaling on ECs negatively regulates arteriole remodeling, blood flow recovery, and neurorestoration following TBI. To test our hypothesis, we evaluated motor behavior and blood flow at 1, 3, 7, and 14 days post-TBI then euthanized the mice and analyzed brain tissue sections using immunohistochemistry. Our findings indicate that EC-specific ablation of EphA4 improves the restoration of blood flow in the cerebral cortex as soon as 48 h after TBI. This correlated with a significant increase in motor recovery, using rotorod assessment, and a reduced injury volume on tissue sections. Future studies will address the role of angiopoietin-1 (Ang-1) signaling in the enhancement of arteriogenesis following EphA4 ablation using soluble Ang-1 inhibitors after TBI. These data highlight a novel healing response that occurs following TBI that may be limited by the expression of EphA4.

Worsening of Dizziness Impairment Is Associated With BMX Level in Patients After Mild Traumatic Brain Injury

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Over 2 million people suffer from a mild traumatic brain injury (mTBI) each year. Predicting symptoms of mTBI and the characterization of those symptoms has been challenging. Biomarkers that correlate clinical symptoms to disease outcome are desired to improve understanding of the disease and optimize patient care. Bone marrow tyrosine kinase gene in chromosome X protein (BMX), a member of the TEC family of nonreceptor tyrosine kinases, is upregulated after traumatic neural injury in a rat model of mTBI. The objective of this investigation was to determine if BMX serum concentrations can effectively be used to predict outcomes after mTBI in a clinical setting. A total of 63 patients with mTBI [Glasgow Coma Score (GCS) between 13 and 15] were included. Blood samples taken at the time of hospital admission were analyzed for BMX. Data collected included demographic and clinical variables. Outcomes were assessed using the Dizziness Handicap Inventory (DHI) questionnaire at baseline and 6 weeks postinjury. A participant was assigned to the "case group" if the subject's complaints of dizziness became worse at the 6-week assessment; otherwise, the participant was assigned to the "control group." A receiver operating characteristic (ROC) curve was constructed to explore the BMX level. Significant associations were found between serum levels of BMX and dizziness. Areas under the curve (AUCs) for prediction of change in DHI postinjury were 0.76 for total score, 0.69 for physical score, 0.65 for emotional score, and 0.66 for functional score. Specificities were between 0.69 and 0.77 for total score and emotional score, respectively. Therefore, BMX demonstrates potential as a candidate serum biomarker for exacerbating dizziness after mTBI.

Designing $CGRP_{8\text{-}37}$ Recombinant Peptide Construct to Evaluate Model of Nerve Injury-Induced Pain in Rats

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Neuropathic pain induced by peripheral or spinal cord injury insufficiently responds to current pharmacological treatment. Therefore, it is necessary to identify new therapeutic targets and approaches. Calcitonin gene-related peptide (CGRP) is produced by neurons in the dorsal root ganglia and thought to play a key role in nociceptive neurotransmission in the spinal dorsal horn. Hypersensitivity to CGRP and/or sprouting in response to injury may contribute to allodynia and hyperalgesia in persistent neuropathic pain conditions. A truncated CGRP peptide, $CGRP_{8\text{-}37}$, acts as a CGRP antagonist and can reverse symptoms of neuropathic and inflammatory pain in animal models. This study aims to test the analgesic potential of the neuropathic pain gene therapy candidate cDNA-encoding $CGRP_{8\text{-}37}$. Human CGRP cDNA from Open Biosystems was used to amplify the analgesic $CGRP_{8\text{-}37}$ sequence. The $CGRP_{8\text{-}37}$ fragment was subcloned downstream of the peptidylglycine-amidating monooxygenase (ssPAM/pGEMT) signal sequence to allow $CGRP_{8\text{-}37}$ to be amidated and secreted, and subcloned into adeno-associated virus-enhanced green

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fluorescent protein-woodchuck hepatitis virus posttranscriptional regulatory element (AAV–EGFP–WPRE) viral vector. Immunocytochemical colocalization of anti-CGRP and Golgi marker anti-Giantin antibody confirmed the production of secretable $CGRP_{8-37}$ peptide. For initial screening, supernatant of $CGRP_{8-37}$-transfected human embryonic kidney (HEK) cells was intrathecally injected into animals with the chronic constriction injury (CCI) neuropathic pain model and formalin-evoked inflammatory pain to study its effect on tactile, cold allodynia, and inflammatory pain. Authentic $CGRP_{8-37}$ peptide (10 nM, Phoenix Pharmaceuticals, Inc.) was used as a positive control. Results in the CCI model showed that reduction of mechanical allodynia in rats treated with AAV–$CGRP_{8-37}$ supernatant was comparable with the effect of 10 nM $CGRP_{8-37}$ peptide. Paw withdrawal thresholds were significantly higher in treated rats at 60 and 90 min postinjection compared with control saline-injected rats. Similar effects were observed for cold allodynia in CCI rats, which was significantly reduced in supernatant- or peptide-injected animals, but not controls. Results using the formalin test showed reduction of formalin-evoked pain responses in rats treated with AAV–$CGRP_{8-37}$ supernatant, comparable with the effect of 10 nM $CGRP_{8-37}$ peptide. Both phase I (0–10 min) and phase II (10–30 min) paw flinches/min were significantly lower in treated rats postinjection compared to control rats. These findings suggest that engineered $CGRP_{8-37}$ recombinant peptide constructs have the potential to alleviate inflammatory and nerve injury-induced pain.

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Dysregulation After Blast-Induced TBI and Neuroprotective Transcription Factor Modulation

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Traumatic brain injury has a prevalence of approximately 0.5% per year worldwide, and there is a 30-fold higher rate among deployed service personnel. Mild injuries are the most common, yet they do result in significant deficits in brain function and cognitive performance. We have identified transcription factor pathways involved in inflammatory responses that impact on neuronal damage. Most of our previous work focused on a closed head impact injury model. With an explosive blast injury model system, mice exposed to a sharp pressure wave from a TNT detonation suffer memory deficits. The injury is accomplished by a calibrated explosion in a controlled field environment and the distance from the blast serves to titrate the degree of injury. The advantages of the explosion model are that it most accurately reproduces the battlefield environment and the microsecond pressure wave. Protein and mRNA levels for nuclear factor erythroid 2-related factor 2 (Nrf2) were measured after mild TBI exposures at different distances and times from the blast, and we found that the injury exposure itself induced an upregulation in Nrf2 levels. This indicated that Nrf2 may be able to partially respond to this form of TBI insult. Postinjury treatment tested mice receiving *tert*-butylhydroquinone, an activator of the transcription factor Nrf2. We have also surveyed several protein changes that could affect neuronal health, focusing on the hippocampus as a sensitive region in this brain injury model. In summary, the examination of altered expression levels in regulatory pathways should help advance the identification of therapeutic targets that will benefit Veterans and other individuals suffering TBI.

This study was supported by the Department of Veterans Affairs (Veterans Health Administration, Office of Research and Development, Rehabilitation Research and Development), The Bay Pines Foundation, and the Florida Department of Health James and Esther King Biomedical Research Program. Disclaimer: The contents do not represent the views of the Department of Veterans Affairs or the United States Government.

Intimacy Hormone, Oxytocin, Protects Against Neuronal Death Mediated by $GABA_AR$ Modification in an In Vitro Stroke Model

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Even with the advancements in stroke management, ischemic–reperfusion (IR) injury is still a major cause of morbidity and mortality in patients. Oxytocin (OXT), a neuromodulator of intimacy, is secreted by the hypothalamus and has demonstrated neuroprotective effects against inflammation and oxidative stress in association with γ-aminobutyric acid (GABA) signaling transduction in neurons. The particular mechanism by which OXT influences protection of neuronal cells in stroke remains unclear. Primary rat neuronal cells were exposed to OXT for 3 days before induction of an experimental stroke model via oxygen–glucose deprivation-ischemia–reperfusion (OGD-IR). Pretreatment of OXT increased cell viability, prevented high mobility group box 1 (HMGB1; a molecular marker of stroke) secretion from the cells, decreased cell damage against oxidative stress during OGD-IR progression, and amplified the phosphorylation ratio of the $GABA_AR$ $Ser^{408/409}$ β-3 subunit before OGD treatment. Atoshiban, an OXTR antagonist, abolished these beneficial effects. OXT has no significant influence on cell growth, glutathione synthetase (GSH)-, glucose-6-phosphate dehydrogenase (G6PD)-, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity. The study of the signal transduction pathway between OXTR and $GABA_AR$ offers a new venue for developing treatment strategies against the early stages of stroke and further reveals the pivotal role of OXT as a key regulatory hormone-linking social interaction and stroke.

Funding: USF Department of Neurosurgery and Brain Repair Funds.

Peripheral Infusions of Human Umbilical Cord Blood-Derived Monocytes Improve Cognitive Deficits and Reduce Amyloid-β-Associated Alzheimer's Disease-Like Pathology in PSAPP Transgenic Mice

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Alzheimer's disease (AD) is the fourth major cause of mortality in the elderly in the US and the leading cause of dementia worldwide. While pharmacological targets have been discovered, current strategies for the symptomatic or disease-modifying treatment of AD do not significantly slow or halt the underlying pathological progression of the disease. We have recently discovered that multiple low-dose infusions of human umbilical cord blood cells (HUCBCs) ameliorate cognitive impairments and reduce Aβ-associated neuropathology in presenilin–amyloid precursor protein (PSAPP) transgenic mice. During recent years, functional recovery has also been observed from the use of HUCBCs in preclinical animal models of brain and spinal cord injuries. Monocytes, as important components of HUCBCs, have essential functions. Consequently, in the present study, we examined whether

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monocytes purified from HUCBCs would have beneficial outcomes on the reduction of AD-like pathology and rescue of cognitive impairments in PSAPP transgenic AD model mice. PSAPP mice and their wild-type littermates were treated monthly with a peripheral HUCBC-derived monocyte infusion over a period of 2 and 4 months, followed by behavioral evaluations and by biochemical and histological analyses. The principal finding of the present study confirmed that monocytes derived from HUCBC (CB-M) play central roles in HUCBC-mediated cognition-enhancing and Aβ-pathology-ameliorating activities. Most importantly, we found that compared with CB-M, aged monocytes showed a poor ability to phagocytose Aβ, while exogenous soluble amyloid precursor protein-α (sAPPα) can reverse this deficiency. Our further studies suggested that sAPPα could form a heterodimer with Aβs, and the APP672-688 ($A\beta_{1-16}$) region is responsible for this heterodimerization, which consequently binds to scavenger receptors and the resultant enhancement of Aβ clearance. In summary, our findings suggest an interesting hypothesis that peripheral monocytes contribute to Aβ clearance through heterodimerization of sAPPα and Aβ. Declined or impaired sAPPα production or heterodimerization with Aβ will cause a decreased Aβ clearance and finally pathogenesis of AD.

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Mitochondrial Electron Transport Chain Alterations in the Spinal Cord of ALS Patients

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Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by progressive motor neuron loss in the brain and spinal cord, leading to muscle atrophy, paralysis, and death. Mitochondrial dysfunction has been shown to contribute to disease progression, but it is unclear which complexes of the mitochondrial electron transport chain (ETC) are affected. It has been shown that deficits in the ETC complexes I, IV, and V are mainly associated with neurodegeneration. Complex I, nicotinamide adenine dinucleotide (NADH) dehydrogenase complex, oxidizes high-energy carrier NADH, pumping the protons into the intermembrane space while electrons are sent down the electron transport chain. Complex IV reduces oxygen molecules using these electrons, further consuming hydrogen from the matrix. This movement of electrons and hydrogen out of the mitochondrial matrix creates a membrane potential, enabling complex V to generate adenosine triphosphate (ATP). Disruption of the ETC and oxidation of NADH can result in reactive oxygen species (ROS), decreased membrane potential, lower ATP yield, and decreased oxygen consumption. Although previous studies have reported decreases in all ETC complexes in the spinal cords of ALS patients, suggesting a total loss of mitochondria, the particular role of each ETC complex in ALS pathogenesis is still unclear. The aim of this study was to determine the enzymatic activity of mitochondrial ETC complexes I, IV, and V in the spinal cords from ALS patients. These ETC complexes were investigated in gray and white matter postmortem cervical and lumbar spinal cord tissue from ALS patients ($n=18$) and age-matched controls ($n=5$), obtained from human tissue banks (Human Brain and Spinal Fluid Resource Center, Los Angeles, CA, and NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD, USA). Activities of complexes I, IV, and V were determined by using standard assays and measured on a Biotek Synergy 2 microplate reader. Our results showed a significant decrease in complex IV activity in the gray matter of cervical and lumbar spinal cords from ALS patients versus controls. A slight decrease in this complex was also noted in white matter. No significant differences were noted in the activity of complexes I and V versus controls. Since we have shown that ALS mitochondrial dysfunction is mostly associated with complex IV, previously reported ETC complex deficits are more likely due to mitochondrial dysfunction than to a reduction in mitochondria. Currently, immunohistochemical analysis of mitochondria in motor neurons, lateral funiculus, and capillaries in the cervical and lumbar spinal cord, including quantitative analysis by ImageJ is in progress. In summary, our finding of substantially reduced complex IV activity, mainly in gray matter of both lumbar and cervical spinal cord, is novel confirmation of the important role of mitochondrial dysfunction in ALS pathogenesis.

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Endothelial Progenitor Cell-Derived Conditioned Medium Promotes Brain Microvascular Cell Viability After Ischemic Insult In Vitro

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Recent observations suggest that impairment of vascular tissue in the brain is involved in the pathogenesis of neurodegenerative disorders. It is known that endothelial progenitor cells (EPCs) play an important role in revascularization and regeneration of several tissues. We have previously demonstrated that EPC-derived conditioned medium (EPC-CM) supports brain microvascular endothelial cell functions including number of viable cells, migration, and tubule network formation. Importantly, these effects were found to be significantly reduced by the blockage of the phosphoinositide-3-kinase (PI3K)/AKT and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) signaling pathways (Di Santo et al., 2014). In the present study, we aimed at investigating the type of factors mediating the effects of EPC-CM. For that purpose, brain endothelial cell line cultures were incubated with EPC-CM under standard conditions or after oxygen and glucose deprivation challenge (OGD). Cell viability was assessed by means of the Presto Blue assay. Serial dilutions, chloroform extraction, and heat inactivation experiments were performed to identify key effectors of the EPC-CM-mediated effects. The involvement of vascular endothelial growth factor (VEGF) as a canonical survival factor for endothelial cells was analyzed by use of a neutralizing antibody. As expected, we found that the viability of microvascular cells was significantly increased following incubation with EPC-CM. This effect was observed with up to five times diluted EPC-CM administration. Importantly, EPC-CM supported cell viability after OGD. Both heat inactivation and lipid extraction significantly reduced the EPC-CM-induced increase in microvascular cell viability. In contrast, neutralization of VEGF did not affect the endothelial supportive capacity of EPC-CM. In sum, our findings demonstrate that EPC-derived paracrine factors substantially support viability of brain microvascular cells and that these effects require the combined actions of both proteinaceous factors as well as lipidic molecules.

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Allele-Specific Modification of the Mutant Huntingtin Gene With Transcription Activator-Like Effectors

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Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by an abnormal expansion of CAG repeats encoding a polyglutamine sequence in the N-terminal region of the HD gene. It has been suggested that postnatal reduction of mutant huntingtin through protein interference or conditional gene knockout could prove to be an effective therapy for patients suffering from HD. The current study explored novel methods to reduce or silence expression of the mutant Huntingtin allele using transcription activator-like effectors (TALEs) in primary human HD fibroblasts. For allele-specific targeting, three TALEs designed to target single-nucleotide polymorphisms (SNPs) in the mutant allele were packaged into a vector backbone containing a Krüppel-associated box (KRAB) domain to promote transcriptional repression of the disease-associated allele. Each SNP site was carefully selected based on proximity to the promoter region, global minor allele frequency score, and specificity of the TALE to its target region. Three additional TALEs used in unique combinations with each other and designed to cause a CAG collapse in the expanded, mutant allele were packaged into a vector backbone containing heterodimeric *Flavobacterium okeanokoites* restriction endonuclease (FokI). In this study, human HD fibroblasts were transfected with each TALE–SNP independently or in combination to test the synergistic effects of multiple KRAB domains to test allele repression. Human HD fibroblasts were also transfected with the paired heterodimeric TALE–FokI to examine CAG collapse. Allele expression was measured using a SNP-genotyping assay, and mutant protein aggregation was quantified with Western blots for anti-ubiquitin and anti-huntingtin antibodies. The TALE–SNP and TALE–FokI were not inherently toxic to the cells and reliably demonstrated a 20–40% reduction in aggregated proteins and mutant allele repression using Western blots and SNP genotyping, respectively. This study demonstrates the potential of gene modification using TALE and provides a foundation for personalized treatment for individuals suffering from Huntington's disease.

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Global Brain Expression of Fractalkine to Reduce Neurodegeneration

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Microglial activation has been suggested as one of the mechanisms of neurodegeneration in a number of diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). There are several signals produced by neurons that have an anti-inflammatory action on microglia, for example, cluster of differentiation 200 (CD200), CD22, CD47, and fractalkine [FKN, chemokine C-X3-C motif ligand 1 (CX3CL1)]. FKN is a unique chemokine in that it is initially expressed as a membrane-bound molecule that can be proteolytically cleaved to form soluble FKN (sFKN). The sFKN is composed of a mucin-like stalk and a chemokine domain. Activation of fractalkine receptors on brain microglia can suppress secretion of proinflammatory cytokines and result in neuroprotection. We have observed neuroprotection in PD animal models and an AD model with increased tau deposition and neurodegeneration. In order to develop a gene therapeutic approach in diseases such as AD, we would need a global increase in FKN levels to reduce inflammation throughout the brain. Since we are working with a secreted soluble protein, and it is known that ependymal cell expression of recombinant proteins can deliver therapeutic levels of proteins to the parenchyma, we are examining the overexpression of FKN in the ependymal cells of the lateral ventricle. To this end, we injected recombinant adeno-associated virus (rAAV) serotype 4 expressing sFKN into the lateral ventricles of mice. Immunohistochemical analysis of brain sections demonstrates that we can generate sFKN expression in the choroid plexus and ependymal cells lining the ventricular system. We observed expression in the ependymal cells throughout both lateral ventricles and the third and fourth ventricles. We observed FKN secretion into the cerebrospinal fluid (CSF) as well as the brain parenchyma by Western blot analysis. In order to enable greater secretion and spread of the chemokine, we proposed that removal of the mucin-like stalk from the chemokine domain may allow for greater diffusion throughout the parenchyma. However, it is unclear whether the mucin-like stalk is contributing to fractalkine's neuroprotective effect or if the chemokine domain alone could suffice. We generated conditioned media with either sFKN or scFKN (chemokine domain only). This conditioned media was used to treat lipopolysaccharide (LPS)-activated murine microglial BV2 cells. As expected the sFKN-conditioned media significantly reduced the amount of tumor necrosis factor-α (TNF-α) released by the activated BV2 cells. However, the scFKN-conditioned media failed to reduce the amount of TNF-α. This would suggest that the mucin-like stalk is potentially contributing to the neuroprotective effects of sFKN and that a gene therapeutic approach would have to include this domain.

Neuroprotective Potential of Subthalamic Nucleus Deep Brain Stimulation in a Viral Vector-Mediated Nigrostriatal α-Synuclein Overexpression Model

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Deep brain stimulation (DBS) is the most common neurosurgical treatment for the alleviation of Parkinson's disease (PD) motor symptoms. However, the current practice of employing subthalamic nucleus (STN) DBS as a treatment of late stage disease, after the majority of nigral dopamine (DA) neurons and DAergic innervation in the putamen has degenerated, has prevented our ability to evaluate its disease-modifying potential. Our laboratory and others have demonstrated that STN DBS provides neuroprotection for DA neurons of the substantia nigra (SN) in neurotoxin models of PD. One major limitation of these previous studies is that the predictive validity of the PD neurotoxin models is low. In the present study, we sought to determine whether STN DBS applied in a model of α-synuclein (α-syn) nigrostriatal toxicity is neuroprotective. A large body of evidence points to α-syn involvement in PD. Further, α-syn overexpression targeted to the nigrostriatal system via direct, intranigral injections of a viral vector overexpressing α-syn results in a neuropathological and behavioral phenotype that recapitulates key features of PD. Young-adult, male rats received two, 2.0 µl, unilateral, intranigral injections of recombinant adeno-associated virus pseudotype 2/5 (rAAV2/5) expressing human wild-type α-synuclein (α-syn, 1.2×10^{13} genome copies per ml). In our laboratory, these rAAV2/5-α-syn injection parameters result in ≈40% SN and ≈10% striatal terminal loss 4 weeks after vector administration that progresses to ≈60% SN and ≈50% striatal terminal degeneration by 8 weeks. Rats were implanted ipsilaterally with a DBS electrode in the STN 18 days following vector injections and assigned to either active stimulation ($n=9$) or no stimulation (inactive, $n=13$) treatment groups. An additional group of rats received α-syn vector injections with no electrode implantation ($n=8$). Active rats received continuous STN stimulation for 4 weeks starting week 5 after vector surgery (130 Hz, 60 µs, amperage adjusted below the level of dyskinesia). Rats in the inactive group received no stimulation during the same 4-week interval and served as a critical control for the effects of electrode implantation.

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Cylinder task was used to track functional effects over the lesion time course and verify electrode patency. Nigrostriatal α-syn transduction and STN electrode placement were verified using immunohistochemical and histochemical methods, respectively. Stereological quantification of tyrosine hydroxylase immunoreactive (TH-ir) SN neurons and TH-ir striatal terminal density is ongoing. These pending results will determine whether STN DBS can provide neuroprotection from α-syn-mediated nigrostriatal degeneration. The demonstration that STN DBS provides neuroprotection in a nontoxin preclinical model of PD would provide support for a large-scale clinical trial examining whether STN DBS applied in early stage PD is disease modifying.

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Proteomic Analysis of Aged Microglia: New Clues in the Cause of Priming

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Microglia play a pivotal role in the homeostasis of the brain, and their proper functioning requires dynamic control over migration, expansion/contraction, and proper response to environmental signals. The objective of this study was to identify age-dependent changes in microglial cellular components that could potentially contribute to priming, a state where microglia are overresponsive to proinflammatory and underresponsive to anti-inflammatory signals. To accomplish these objectives, we used stable isotope labeling by amino acids in cell culture (SILAC) mass spectrometry. We attained primary microglia from the brains of 5-month and 22-month C57BL/6 mice. Across two biological samples (young, old) Scaffold identified 2,625 proteins with a 1% false detection rate (FDR), 2,539 of which were detected in both groups. Twenty-five proteins were unique in old microglia, while 61 were unique to young. Of the 2,625 proteins detected, 245 contained only light amino acids preventing their quantification leaving 2,380 quantifiable protein groups. Of those, 250 were differentially expressed ($p<0.05$) between young and old. Ingenuity pathway analysis was performed on all differentially expressed proteins with at least a 1.5-fold change and a $p<0.05$ statistical cutoff. Top canonical pathways were eukaryotic translation initiation factor 2 (EIF2) signaling, isoleucine degradation, ketolysis, ketogenesis, and glutaryl-CoA degradation. Further analysis of biological functions revealed alterations in RNA posttranscriptional modification, protein synthesis, posttranslational modification, and cellular growth and proliferation. Analysis of possible upstream regulators predicts an increase in tumor necrosis factor (TNF), interferon α, and trichostatin A, a nonselective histone deacetylase (HDAC) inhibitor, has a predicted activation indicating a predicted decrease in HDAC activity, while rapamycin-insensitive companion of mechanistic target of rapamycin (RICTOR), presenilin 2 (PSEN2), PSEN1, and mitogen-activated protein kinase 1 (MAPK1) have a predicted decrease in activity. Alterations in Rictor signaling is one explanation for the observed changes in amino acid metabolism. Impairments in amino acid metabolism may represent a first hit to the downstream disruption in protein translation and cytoskeletal organization. Signaling cascades are highly dependent on subcellular localization; disruptions here could contribute to the blunted response to M2 activators and other modulators of inflammatory response that characterizes "primed" microglia. In addition several recent studies have shown that knockdown of RICTOR and a component of the mechanistic target of rapamycin complex 2 (MTORC2) polarizes peripheral macrophages to an M1 phenotype by impeding its ability to regulate forkhead box O (FOXO). Here we identified RICTOR as an upstream regulator of effected pathways. In addition, the disruption in amino acid metabolism itself may be sufficient to disrupt inflammatory signaling, as one function FOXO3, a protein regulated by mTOR, is to integrate signals from the mitochondria to regulate stress response pathways. Understanding the alterations in cellular machinery is key to developing therapies to address age-dependent neurodegenerative diseases.

The Role of Blood–Brain Barrier Alterations in Subacute and Chronic Ischemic Stroke Rat Models

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Stroke is the fourth leading cause of death in the US. Approximately 87% of strokes are ischemic. Following ischemic insult, cerebral vascular perturbations lead to blood–brain barrier (BBB) damage. Despite intensive research into BBB complications from ischemic stroke, the bulk of these studies have focused on the acute poststroke stage and the cerebral hemisphere of initial ischemic insult. In our recent studies, we investigated the BBB status in subacute (7 days) and chronic (30 days) stroke using a transient middle cerebral artery occlusion (tMCAO) rat model. Results demonstrated significant BBB alterations in the ipsilateral hemisphere in both subacute and chronic ischemic stroke. BBB breakdown was primarily characterized by damaged endothelial cells containing numerous autophagosomes, pericyte degeneration, and perivascular edema. Additionally, vascular leakage, increased reactive astrocytes, and activated microglia, demyelinization, and neuronal pyknosis were noted. Similar vascular damage was also identified in contralateral brain areas (striatum, motor, and somatosensory cortices) remote from initial ischemic lesion. These widespread microvascular alterations in ipsilateral and contralateral brain hemispheres suggest persistent and/or continued BBB damage in ischemic stroke. The pathological changes in remote brain areas likely indicate postacute ischemic diaschisis, which should be considered in the development of treatment strategies for stroke.

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Induction of Lysosomal Biogenesis by Activating Peroxisome Proliferator-Activated Receptor α

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Lysosomes are ubiquitous, membrane-enclosed organelles filled with an acidic interior and are central to the autophagic-, endocytic-, or phagocytic pathways. In contrast to its classical function as waste management machinery, nowadays lysosomes are considered to be an integral part of various cellular signaling processes. Lysosomes have implications in not only lysosomal storage disorders (LSDs) but also play an important role in other diseases. The diverse functionality of this single organelle requires a very complex and coordinated regulation of its activity with transcription factor EB (TFEB), a master regulator of lysosomal biogenesis, at its core. TFEB has evolved as a therapeutic target for many diseases. However, the mechanisms by which TFEB is regulated are poorly understood. This study demonstrates that gemfibrozil, an agonist of peroxisome proliferator-activated receptor α (PPARα), alone and in conjunction with all-*trans*-retinoic acid (ATRA), is capable of enhancing TFEB in brain cells. We also observed that retinoid X receptor, α (RXRα) and PPARα,

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but not PPARβ and PPARγ, are involved in the gemfibrozil-mediated upregulation of TFEB. Reporter assay and chromatin immunoprecipitation studies confirmed the recruitment of RXRα, PPARα, and PPARγ coactivator 1 α (PGC1α) at the PPAR binding site on the *Tfeb* promoter as well. Subsequently, the drug-mediated induction of TFEB caused an increase in lysosomal protein and the lysosomal abundance within a cell. Collectively, this study reinforces the link between lysosomal biogenesis and lipid metabolism with TFEB at the crossroads. Also, gemfibrozil may be of therapeutic value in the treatment of certain LSDs in which the autophagy–lysosome pathway plays an important role.

ECM Hydrogel Injection for the Treatment of Stroke: Characterization of Acute Host Cell Invasion

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Stroke is a severe cerebrovascular accident that results in localized brain tissue loss, affecting nearly 800,000 Americans each year. Extracellular matrix (ECM) derived from urinary bladder lamina propria and basement membrane has shown therapeutic potential in rats following intracerebral injection for experimentally produced traumatic brain injury in rats. ECM can be prepared in liquid form at room temperature and dependent on concentration creates a hydrogel under physiologic conditions, providing ideal material properties for intracerebral injection through a thin needle. To determine an appropriate concentration of ECM (0, 1, 2, 3, 4, 8 mg/ml) for injection and retention of material in the stroke cavity, ECM was injected in liquid form with injection parameters (coordinates and volume) determined by magnetic resonance imaging (MRI) 12 days post-middle cerebral artery occlusion, while simultaneously draining necrotic liquefied brain tissue. Retention of ECM, as well as host cell invasion and their phenotypes were assessed 24 h postinjection using immunohistochemistry. ECM concentrations <3 mg/ml did not gel and were not retained in the cavity. A significant host cell invasion was observed with 20–30% of these being putative microglia/macrophages [ionized calcium-binding adapter molecule 1 positive ($Iba1^+$)] across all concentration groups. The number of astrocytes [glial fibrillary acidic protein positive ($GFAP^+$)] cells invading the ECM acutely was negligible, and there was no evidence of neuronal progenitor invasion. This characterization demonstrates that an ECM hydrogel can be readily injected and retained within a lesion cavity, while attracting host cells into the damaged region. Further time points and behavioral studies will be required to further evaluate the therapeutic potential of this approach.

Long-Term White Matter Pathology and Cognitive Deficits Following a Novel Model of Repetitive Mild Traumatic Brain Injury

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It is estimated that 5.6 million Americans are currently living with a disability from a traumatic brain injury (TBI). In 2010, 2.5 million Americans had a severe enough TBI to seek medical attention at a hospital; however, 70–90% of all TBIs are actually considered to be of mild severity, and most of these patients do not seek medical attention. Of growing concern is the emerging evidence of the detrimental effects from suffering several mild TBIs (mTBIs). Repeated mTBI (rmTBI) can manifest its own unique set of behavioral consequences as well as neuropathological changes, defined postmortem as chronic traumatic encephalopathy (CTE). To date, very few reproducible models exist to mimic the long-term effects of rmTBI both behaviorally and neuropathologically. We have combined components of two classic rodent models of TBI: the controlled cortical impact (CCI) model and the weight drop model to address this shortcoming and to ascertain whether there are long-term deficits (in behavior and pathology). We take advantage of the precision and reproducibility from a commercially available CCI device to deliver impacts at specific speeds and depths, as well as the "Marmarou" foam pad from the weight drop model to allow rotational, acceleration–deceleration forces, which may lead to specific white matter tract damage, specifically from shearing forces. Our rmTBI model produces long-term deficits in several behavioral tasks for rodents receiving rmTBI, but no deficits after receiving a single mTBI. Rotarod performance was impaired at 1 and 4 days post-rmTBI, but reached control levels by 10, 21, and 35 days postinjury. Mice suffering rmTBI performed differently on the elevated plus maze at 1 month postinjury (mpi), spending more time in open arms, as well as exhibiting hyperlocomotion; these deficits persist when retested at 6 mpi. rmTBI mice performed significantly worse than single mTBI and control sham mice at 2 and 6 mpi in a classic learning task, the Morris water maze. In addition to behavioral changes following rmTBI, we observed significant white matter tract pathology similar to clinical presentations of rmTBI. Mice suffering rmTBI exhibited myelin changes in corpus collosum, as well as atrophy of the corpus collosum. Via stereological analysis, the corpus collosum of mice receiving rmTBI were significantly smaller than both single mTBI and sham controls. This model for rmTBI allows for further studies investigating the mechanisms underlying both the white matter damage as well as behavioral changes following injury. Moreover, we will use this model to investigate the endogenous stem cell populations' response to rmTBI, as well as test the efficacy of human fetal neural stem cells as a therapeutic intervention to alleviate both neuropathological and behavioral deficits.

Human Parthenogenetic Stem Cell-Derived Neural Stem Cells Are Safe and Well Tolerated by a Nonhuman Primate Model of Parkinson's Disease

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Clinical studies have shown that grafted fetal neural tissue can achieve considerable biochemical and clinical improvements in Parkinson's disease (PD). However, the source of fetal tissue grafts is limited and ethically controversial. Human parthenogenetic stem cells offer a good alternative because they are derived from unfertilized oocytes without destroying viable human embryos and can be used to generate an unlimited supply of neural stem cells (NSCs) for transplantation. We have previously reported that human parthenogenetic stem cell-derived NSCs (hpNSCs) successfully engraft and survive for at least 3 months following transplantation, increase nigrostriatal dopamine levels, and have no adverse effects in rodent and nonhuman primate models of PD. Here we report the results of two studies: a comprehensive long-term safety and efficacy study of hpNSCs in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned African green monkeys with moderate to severe clinical

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parkinsonian symptoms and a 9-month tumorigenicity and biodistribution study in the athymic nude rats. hpNSCs were manufactured under current good manufacturing practices (cGMP) conditions, and different doses were injected bilaterally into the striatum and substantia nigra of the monkeys and rats. In the monkeys, behavioral changes and motor movements were evaluated against vehicle control based on a parkinsonian summary score. Necropsy, histopathology, and biodistribution analyses were used to determine the safety profile of the implanted hpNSCs. Monkeys had stable body weights throughout the study, and there were no significant differences between hpNSCs and vehicle control animals. Clinical pathology did not show statistically significant differences between vehicle control and treated samples in any of the hematological parameters tested. No serious adverse events, such as dyskinesia, were detected. There were no obvious lesions, gross pathology, or tumors seen on necropsy, and histopathology analysis did not detect adverse morphological changes or evidence of tumor formation. The results of our study demonstrate that intracranial administration of hpNSCs into the striatum and substantia nigra was safe and well tolerated by the nonhuman primates and supports its potential clinical application in Parkinson's disease.

Vagus Nerve Stimulation for the Treatment of Parkinson's Disease

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Vagus nerve stimulation (VNS) is an FDA-approved therapy for treatment-resistant forms of epilepsy and depression. Several groups have shown that VNS causes neurochemical changes in brainstem nuclei and along the projections of these regions, including an increase in brain-derived neurotrophic factor (BDNF) in the locus coeruleus (LC) target regions. In both Parkinson's disease (PD) and Alzheimer's disease (AD), it has been shown that LC noradrenergic (NE) neurons are lost prior to dopaminergic (DAergic) and cholinergic neurons, respectively. Owing to the increase in firing rates of LC-NE neurons after VNS, this method could offer a novel treatment option for both of these diseases. Therefore, our hypothesis is that VNS will attenuate the DAergic/NAergic loss in PD by increasing BDNF expression. To evaluate this hypothesis, we utilized a double-lesion rat model that mimics the cognitive and motor deficits of the disease [N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) + 6-hydroxydopamine (6-OHDA)]. Adult male Long Evans rats were administered the noradrenergic neurotoxin DSP-4 (50 mg/kg, IP), and 7 days later, the rats were given bilateral intrastriatal injections of the dopaminergic neurotoxin 6-OHDA (6 µg). At the same time as the 6-OHDA lesions, animals in the VNS group were implanted with a VNS cuff and head cap, which could be connected to a stimulator that emits precise bursts of stimulation of a set amplitude, duration, and rate. Starting 2 weeks after 6-OHDA injections, VNS was applied twice daily (a.m. and p.m.) for 2 weeks with locomotor activity recordings taken during the afternoon session for all rats. Total distance (cm) was measured for each rat, and we found that after day 3 of VNS, those rats receiving VNS had greater locomotion than the nonstimulated double-lesioned rats and with locomotor activity similar to saline control rats. Following the last stimulation/locomotor activity session on day 14, the rats were euthanized, and tissue was dissected for enzyme-linked immunosorbent assay (ELISA) detection of growth factors or for processing via immunohistochemistry. At this time point, in addition to the nonstimulated double-lesioned rats having lower motor activity, they also had a significant reduction in BDNF tissue levels in LC and substantia nigra (SN) target regions, as well as tyrosine hydroxylase immunoreactivity (TH-ir) in the dorsal striatum compared to saline controls. Data indicate that the 2-week stimulation of VNS following the double lesion of the NAergic and DAergic systems increased BDNF tissue levels compared to the nonstimulated double lesion rats in the frontal cortex and dorsal striatum. In addition, the 2-week stimulation paradigm in the double-lesioned rats resulted in greater expression of TH-ir in the dorsal striatum compared to nonstimulated double-lesioned rats. Future studies will be conducted to determine the mechanism by which VNS works in PD models. Taken together, these data point to the potential use of VNS as a therapeutic for individuals with Parkinson's disease.

This work was supported by a pilot project from the Barmore Foundation at MUSC.

Chromatographically Isolated Extracellular Vesicles Improve Behavioral Defects After Acute Traumatic Brain Injury

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Traumatic brain injuries (TBIs) produce both direct tissue damage and a secondary inflammatory response that prolongs and increases the injury. A major consequence is cognitive and behavioral defects that can be long-lasting. However, developing effective drugs for the therapy of TBI has been notoriously difficult. A series of recent reports have indicated that excessive inflammation seen in a number of animal models can be reduced by administration of mesenchymal stem/stromal cells (MSCs) or by administration of the vesicles they secrete. We have recently developed a scalable chromatographic protocol for isolating cluster of differentiation 63 positive ($CD63^+$) and negatively charged extracellular vesicles (n-EVs) from human MSCs (Kim, Nishida et al., in preparation). Here we injected n-EVs intravenously into mice after TBI was produced by controlled cortical contusion. Administration of the n-EVs reduced neuroinflammation in a dose-dependent manner as reflected by reductions in interleukin (IL)-1β ($p < 0.001$) in the ipsilateral brain. Also administration of the n-EVs improved several measures of behavior in Morris water maze tests ($p < 0.05$) 28 days after the TBI and therapy. The results suggest that intravenous administration of n-EVs can provide a novel therapy for acute traumatic brain injuries.

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Cryopreserved IPSC-Derived Midbrain Dopamine Neurons Survive and Maintain Dopaminergic Phenotype in the Rodent Brain

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Recent studies have indicated that induced pluripotent stem cells (iPSCs) differentiated into midbrain dopamine (iPSC-mDA) neurons provide functional benefit in animal models of Parkinson's disease (PD) (Kriks et al., 2011). Cryopreservation of postmitotic iPSC-mDA neurons represents a significant vertical advancement for clinical translation of pluripotent stem cell technologies as they are reliably and reproducibly thawed, allowing for rapid access to large numbers of highly pure, patient-specific cells. In the present study, we examined the engraftment potential of iPSC-mDA neurons (cryopreserved at different time points) after transplantation into the rodent brain. iPSC-mDA neurons were derived from human blood samples and cryopreserved in large master cell banks. After thawing, iPSC-mDA neurons retained high viability and maintained gene and protein expression patterns consistent with the midbrain lineage phenotype >1 month in vitro. Patch clamp recordings demonstrated normal electrophysiological characteristics with firing of evoked and spontaneous action potentials, postsynaptic currents, as well as functional ion channels with characteristic inhibitor responses. In addition, biochemical analysis of iPSC-mDA neurons indicated production of dopamine. To determine in vivo survival, cryopreserved iPSC-mDA neurons were thawed and prepared for transplantation without manipulation or additional subculturing. Cyclosporine-A-immunosuppressed Sprague–Dawley rats received bilateral stereotactic injections of iPSC-mDA neurons (4.5×10^5 cells/hemisphere; 1 injection/hemisphere) into the striatum or substantia nigra. Rats were sacrificed at 2 or 6 weeks post-transplantation. Immunohistochemistry indicated robust graft survival

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and maintenance of the dopaminergic phenotype with extensive fiber innervation into the host. Importantly, there was little to no cell proliferation indicating safety in our initial studies. Long-term studies are currently underway to ascertain whether cryopreserved iPSC-mDA neurons will provide functional benefit in the 6-hydroxydopamine (6-OHDA)-lesioned rat and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned primate models of Parkinson's disease.

Newly Developed TDP43 Targeting ICW Drug Screening Revealed B10 as a Novel Potent Drug for Treatment of TDP43 Proteinopathies

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Tat-activating RNA regulatory element (TAR) DNA-binding protein 43 (TDP43) accumulation and loss of function is linked to a group of neurodegenerative diseases known as "TDP43 proteinopathies" that prominently includes amyotrophic lateral sclerosis (ALS), frontotemporal lobe dementia (FTLD), and Alzheimer's disease (AD). We optimized a 96-well plate-based In-Cell Western (ICW) technique for screening TDP-43-targeting drugs. A number of compounds from a drug library were tested and compared with vehicle-treated control. One drug molecule, referred to as B10, showed a potent reduction in TDP43 levels. The effect of B10 on TDP43 was validated using rat N9 microglial cells, which express endogenous TDP43, and human embryonic kidney 293 (HEK293) cells transiently transfected with TDP43 constructs. We also analyzed whether B10 has a similar effect on pathological forms of TDP43. Indeed, we found that B10 dramatically reduces the insoluble form of TDP43, high molecular weight TDP43, ALS-linked TDP43 mutants Q331K and M337V, and the FTLD-linked C25-cleaved fragment form that localizes to the cytosol. These potent and novel effects of B10 on pathological forms of TDP43 suggest that B10 may serve as a promising candidate for the treatment of ALS and other TDP43 proteinopathies.

Spontaneous White Matter Damage, Cognitive Decline, and Neuroinflammation in Middle-Aged Hypertensive Rats: An Animal Model of Early Stage Cerebral Small Vessel Disease

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In cerebral small vessel disease (cSVD), the progressive remodeling of microvessels due to arterial hypertension causes subtle, but constant, cognitive decline and substantially increases stroke risk. The immune system is believed to contribute to the disease. Since most cSVD animal models are biased toward microhemorrhages, a more detailed understanding is impaired by the unavailability of appropriate animal models. Here we investigated the spontaneously hypertensive rat (SHR) as a possible model for early onset cSVD. Male SHR and normotensive Wistar Kyoto rats (WKY, $n=16$ each) were assigned to four groups and observed for 24 weeks (starting at age of 11 weeks). In group 1 ($n=3/3$), blood–brain barrier (BBB) integrity was assessed by fluorescein isothiocyanate (FITC)-lectin and Evans Blue. A brain tissue leukocyte profile was obtained from group 2 ($n=3/3$) by fluorescence-activated cell sorting (FACS). In groups 3 and 4 ($n=5/5$ each), blood pressure (RR) was measured from weeks 12 to 22. Animals were subjected to the novel object recognition (week 30) and Morris water maze (week 34) tests. Total brain, ventricle, and corpus callosum (CC) volumes were determined by T2 magnetic resonance imaging (MRI) in week 35. Postmortem analyses included cerebrospinal fluid/peripheral blood analysis by FACS and detailed brain histology. RR in SHR was significantly higher and increased over time ($p<0.01$). SHR, but not WKY, exhibited nonspatial memory deficits ($p<0.01$). MRI showed brain atrophy (increased ventricle volumes and decreased CC, and brain volumes; $p<0.01$) as well as an increased myelin index, indicating myelin loss. Histological analyses confirmed white matter demyelination and unveiled a circumscribed BBB dysfunction in conjunction with micro- and macrogliosis in the deep cortical regions (DCR; $p<0.05$). Flow cytometry and histological analyses revealed substantial disparities in cerebral highly expressed cluster of differentiation 45 ($CD45^{high}$) leukocyte counts and distribution patterns between SHR and WKY. SHR showed lower T-cell counts in the choroid plexus and meningeal spaces as well as decreased interleukin (IL)-10 levels in the cerebrospinal fluid ($p<0.05$ or lower). Both T-cells and natural killer (NK) cells were significantly augmented in the SHR brain microvasculature. Our results indicate that SHR share behavioral and neuropathological characteristics with human patients and undergird the relevance of immune responses for the initiation and progression of cSVD.

Small Proline-Rich Repeat 1A Protein Protects Nigrostriatal Axons From Degeneration in the 6-Hydroxydopamine Lesion Rat Model of Parkinson's Disease

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Multiple lines of evidence suggest Parkinson's disease (PD) is a dying-back axonopathy, and axonal degeneration is quickly becoming recognized as a critical early event in PD pathogenesis. A recent study demonstrated that patients lose their striatal dopamine (DA) projections early in the disease, well before significant somatic loss occurs. This observation explains why DA somatic preservation strategies have not been successful in clinical trials: They do not prevent the dying back of axonal DA projections in PD. To address the problem of early axonal degeneration in PD, we examined the progressive gene expression changes in the rat ventral midbrain from an intrastriatal 6-hydroxydopamine (OHDA) lesion over 16 weeks. We hypothesized that patterns of gene expression within the substantia nigra (SN) characterized by early, highly upregulated expression would be indicative of potential protective responses to a striatal 6-OHDA insult. The largest group of genes fitting this expression pattern was the regeneration-associated gene (RAG) family. The most highly upregulated RAG was small proline-rich repeat 1a (Sprr1a), which has been shown to facilitate postinjury axonal regeneration in peripheral nerves likely through stabilizing the cytoskeleton at the growth cone. We then confirmed that Sprr1a is specifically upregulated (using RNAscope in situ hybridization) in degenerating SN DA (tyrosine hydroxylase, TH; immunohistochemistry) neurons. We then overexpressed Sprr1a or green fluorescent protein (GFP) with recombinant adeno-associated virus (rAAV) constructs in the SN followed 1 month later with a striatal 6-OHDA lesion. Animals receiving rAAV-Sprr1a exhibited significant protection of nigrostriatal axons in the striatum compared to rAAV-GFP as measured by TH densitometry. The protective effect of Sprr1a was further confirmed by both stereological measurements of TH fiber density and volumetric assessments of striatal zones exhibiting severe, intermediate, or no lesion. Animals treated with rAAV-Sprr1a had significantly higher TH^{+} axon density in the striatum and significantly smaller zones of severe denervation compared to GFP controls. These data show that Sprr1a is part of the DA neuron's natural armamentarium to resist axonal degeneration and that it can be manipulated to mitigate the loss of striatal DAergic fibers in a rat 6-OHDA model.

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Locus Coeruleus Projection System Impairment in Mild Cognitive Impairment

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A major feature of Alzheimer's disease (AD) is the loss of noradrenergic locus coeruleus (LC) projection neurons that mediate attention, memory, and arousal. However, the extent to which the LC projection system degenerates during the initial stages of AD remains unclear. To address this question, we performed tyrosine hydroxylase (TH) immunohistochemistry and unbiased stereology of LC neurons in tissue harvested postmortem from subjects who died with a clinical diagnosis of no cognitive impairment (NCI), amnestic mild cognitive impairment (aMCI, a prodromal AD stage), or mild AD ($n=5$–6/group). Stereologic estimates of total LC neuron number revealed a 30–35% decrease in aMCI versus NCI ($p<0.01$) and a 45% loss of cells in mild AD compared to NCI ($p<0.01$). Furthermore, LC fiber density was selectively reduced in the hippocampus compared to the neocortex of aMCI subjects, suggesting that coeruleohippocampal pathway degeneration marks the transition from normal cognition to prodromal disease. To examine the molecular pathogenic processes underlying LC neurodegeneration in aMCI, we combined laser capture microdissection with custom microarray technology to quantify gene expression patterns in individual TH-immunopositive neurons accessed from LC tissue samples. These studies revealed significant reductions in select functional classes of mRNAs regulating mitochondrial metabolism (e.g., cytochrome c1, cytochrome oxidase subunit 5a, $p<0.01$), redox homeostasis (e.g., superoxide dismutase 2, glutathione peroxidase 1, $p<0.01$), and cytoskeletal plasticity (e.g., microtubule-associated binding protein 1a, utrophin, $p<0.01$) in both aMCI and AD subjects compared to NCI. Taken together, these observations show that LC projection system degeneration is a prominent feature during the transition from NCI to aMCI. In this regard, we are currently examining the extent of LC neuropathology in tissue from "preclinical AD" subjects who died with a clinical diagnosis of NCI but who displayed high postmortem Braak pathology. Targeting the noradrenergic LC system may present a novel disease-modifying strategy for cognitive protection in the elderly.

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Chromatographically Isolated Extracellular Vesicles Reduce Inflammation in Acute Traumatic Brain Injury

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Human mesenchymal stem/stromal cells (hMSCs) have been reported to produce beneficial effects in numerous disease models through their paracrine actions. The paracrine factors released from the hMSCs are, at least in part, in the form of extracellular vesicles (EVs) that contain a number of components such as cytokines, proteins, lipids, saccharides, and nucleotides (mRNAs and/or microRNAs). Here we report that hMSCs can be activated by incubation for 48 h in a chemically defined and protein-free medium (CDPF) to produce negatively charged EVs (n-EVs). The n-EVs can then be purified by anion exchange chromatography. Importantly, intravenous injection of 30 μg of n-EVs significantly reduced inflammation as reflected in reduced brain levels of interleukin (IL)-1β levels ($p<0.001$) in a mouse model of traumatic brain injury (controlled cortical contusion). Our results present a scalable chromatographic process for purifying n-EVs from hMSCs that have the potential to be effective therapies for a large number of human diseases that are characterized by excessive inflammation.

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Protective Effect of N-Acetylcysteine (NAC) and Methylsulfonylmethane (MSM) Against Acetaminophen (APAP)-Induced Oxidative Stress In Vitro

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Acetaminophen (APAP), a nonsteroidal anti-inflammatory drug (NSAID), is one of the most popular over-the-counter drugs that possesses both antipyretic and analgesic effects. Acetaminophen is metabolized by cytochrome P450 family 2, subfamily E, polypeptide 1 (CYP 2E1) to its toxic metabolite n-acetyl-para-benzoquinonimine (NAPQI). This toxic metabolite is highly electrophilic and covalently binds with biomolecules, thereby depleting the endogenous antioxidant glutathione reduced form GSH, oxidized form GSSG. There has been emerging evidence that acetaminophen may also have adverse effects not only in the liver but also in the brain. In addition, p-aminophenol (4AP) is another metabolite produced from APAP by deacetylation and is found within the brain. Despite the fact that APAP can cross the blood–brain barrier (BBB), and CYP 2E1 is expressed in the brain, the available research is inadequate to explain the neurotoxic effect of APAP and its metabolites. The brain is more prone to oxidative stress as it has a higher oxygen consumption rate leading to production of a high amount of free radicals, and being composed with a high concentration of polyunsaturated fatty acids makes it a leading target for lipid peroxidation. In this nature, the availability of the major endogenous antioxidant GSH is important to balance redox homeostasis. As NAPQI and 4AP are known to deplete GSH in the liver and kidney, we hypothesized that APAP disrupts GSH homeostasis in the neuronal cellular system by generating its toxic metabolites thereby causing apoptosis. Furthermore, we will explore the neuroprotective effects of N-acetylcysteine (NAC) and methylsulfonylmethane (MSM), a bioavailable form of L-cysteine or organic sulfur-containing dietary supplement, respectively. GSH is a tripeptide consisting of L-cysteine, glycine, and glutamic acid with L-cysteine being the rate-limiting step of GSH de novo synthesis. Therefore, we propose NAC and MSM may protect neurons by increasing cysteine availability. To test our hypothesis, APAP, NAPQI, and 4AP were administered to N2a neuronal cells and cell viability, membrane integrity (lactate dehydrogenase activity, LDH), reactive oxygen species (ROS), and GSH/GSSG were measured to assess the neurotoxic effect of APAP and its metabolites. Furthermore, NAC and MSM were added prior to drug administration and improvements in cell viability, LDH, ROS, and GSH/GSSG were assessed to determine the neuroprotective effects of NAC and MSM on disrupted redox homeostasis.

Targeting Cell Cycle-Associated Phosphorylation of APP and tau as a New Therapeutic Intervention for Alzheimer's Disease

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Mature neurons are thought to be postmitotic and terminally differentiated; however, recent studies in Alzheimer's disease (AD) reveal a paradigm shift in this classic notion. Compromised neurons in the AD brain show evidence of cell cycle deregulation, and neither the cause nor consequence of the phenomenon is clear. This ectopic expression of cell cycle regulators has been correlated with AD pathology development, and it is hypothesized that it is an event preceding neuronal death. The amyloid precursor protein (APP) is a central protein in AD, and our lab studies both its physiological and pathological functions. APP is a phosphoprotein, and we and others find that its specific phosphorylation (P-) at residue threonine 668 (Thr668) enhances pathogenic proteolysis and generation of the plaque-associated Aβ peptide. Our lab has recently demonstrated that this modification is largely cell cycle dependent. Additionally, we find that P-Thr668 APP associates with the centrosomes, suggesting a role for APP in the cell cycle deregulation observed in AD. Here we use human H4 neuroglioma cells overexpressing APP to demonstrate that hyperphosphorylation of APP at Thr668 occurs in mitotic cells and is minimal to absent in growth-arrested cells. Treatment of cells with early cell cycle inhibitors such as Aphidicolin or Roscovitine attenuates this modification. APP and tau have been shown to be phosphorylated by kinases such as cyclin-dependent kinases

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(cdks) and glycogen synthase kinase 3 (GSK-3). We find that GSK-3 activation is maximal in mitosis-arrested cells, and it coincides with hyperphosphorylation of APP. These cells also show very high levels of tau hyperphosphorylation, as seen with APP, implying that pathogenic modifications in both tau and APP occur under similar conditions. These data imply that inhibitors of cell cycle will not only prevent activation of cdks, but also GSK-3, thus interfering with unwarranted phosphorylation of APP and tau; this in turn will reduce both plaque and tangle pathology development in AD. We believe that targeting these specific kinases with cell cycle inhibitors is a potential therapeutic strategy for treatment of AD.

Running Exercise Does Not Promote Forgetting in Adult Rats

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Multiple studies have demonstrated that regular physical exercise bestows a variety of health benefits including improved cognitive and mood function. Physical exercise, such as running, may also prevent and treat a variety of neuropsychiatric disorders. While mechanisms underlying the various beneficial effects of running exercise are likely multifactorial in nature, enhanced neurogenesis in the hippocampus has been suggested as one of the major structural changes in the brain promoting improved cognitive and mood function in runners. Nonetheless, a recent study has shown that running induced increased neurogenesis-promoted degradation of hippocampus-dependent spatial memories in mice. This is supportive of the notion that increased neurogenesis reconfigures the circuitry between the dentate gyrus (DG) and the hippocampal CA3 region and hence would reduce the ability of a given set of cues to reinvoke the same pattern of activity that occurred at the time of memory formation (Akers et al., 2014). However, it was unclear whether such running-induced forgetting is a phenomenon restricted to mice or all mammalian species. Therefore, we examined whether running exercise would promote forgetting in adult male Sprague–Dawley rats. A larger cohort ($n = 29$) of young adult (3-week-old) rats were first subjected to a water maze test (WMT) for analysis of spatial learning and memory function. The test comprised eight blocks of learning sessions (four trials/day) over 8 days and a memory retrieval (or probe) test at 24 h after the last learning session. Following this, rats exhibiting similar memory retrieval abilities were randomly classified into two groups, a running exercise group ($n = 14$) and a sedentary group ($n = 15$). Rats belonging to the exercise group were housed continuously for 28 days in larger cages with easy access to running wheels, whereas rats in the sedentary group were housed in standard cages. Rats housed in cages fitted with running wheels ran an average of 0.3 kilometers per day (mean ± SEM = 0.3 ± 0.05) and 8.4 ± 1.4 kilometers during the entire 28-day period. Subsequently, on the 29th day, the rats in both groups were subjected to two consecutive probe trials, in order to examine their ability to retrieve memory at an extended timepoint after memory formation. The first probe trial was used to reorient the animal to the maze, and the second probe trial was used to examine the ability of the rats to retrieve long-term memory (Clark et al., 2007). Interestingly, animals in both exercise and sedentary groups displayed capability for memory retrieval, which was evidenced through increased dwell time in the platform quadrant in comparison to all other quadrants ($p < 0.01$–0.0001, one-way ANOVA). Comparison of additional parameters of memory retrieval (dwell times in platform quadrant, platform area, and target area; latencies to target area) across groups also revealed no differences between groups. Analyses of hippocampus neurogenesis are currently in progress, to measure the extent of exercise increased neurogenesis and its relationship to memory recall. The results clearly underscore that running exercise does not interfere with the recall of long-term memories that were formed prior to exercise in a rat model, in sharp contrast to the running-induced forgetting reported in a mouse model earlier (Akers et al., 2014). Since rats are considered evolutionarily more advanced than mice, it may be that running-induced forgetting is a phenomenon restricted to lower-order mammals.

Differential Effects of Isoxazole-9 on Neural Stem/Progenitor Cells, Oligodendrocyte Precursor Cells, and Endothelial Progenitor Cells in Primary Culture

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The adult mammalian brain can be plastic after injury and disease. Therefore, boosting endogenous repair mechanisms may be a relevant therapeutic approach for neurological disorders. Recently, isoxazole-9 (Isx-9) has been reported to enhance neurogenesis from neural stem/progenitor cells (NSPCs). However, the effects of Isx-9 on other types of progenitor/precursor cells remain mostly unknown. In this study, we investigated the effects of Isx-9 on the three major populations of progenitor/precursor cells in the brain: NSPCs, oligodendrocyte precursor cells (OPCs), and endothelial progenitor cells (EPCs). Cultured primary NSPCs were prepared from rat embryo, cultured primary OPCs were from rat neonatal cortex, and cultured EPCs were from mouse spleen. Cultured primary NSPCs, OPCs, or EPCs were treated with various concentrations of Isx-9 (6.25, 12.5, 25, 50 μM), and their cell number was counted in a blinded manner. To assess effects of Isx-9 on neurogenesis, samples from NSPCs after Isx-9 treatment were subjected to Western blots using anti-neuronal nuclei (NeuN) and anti-neurofilament antibodies (neuron markers). To assess the effects of Isx-9 on angiogenesis, outgrowth EPCs and rat brain endothelial RBE.4 cells were used for the tube formation Matrigel assay (in vitro angiogenesis assay). Isx-9 slightly increased the number of NSPCs but significantly decreased OPC number in a concentration-dependent manner. Isx-9 did not affect cell numbers in both early and outgrowth EPCs. As expected, Isx-9 effectively induced neuronal differentiation of NSPCs. But in a Matrigel assay of angiogenesis, Isx-9 significantly inhibited tube formation in outgrowth EPCs. This potential antitube formation effect of Isx-9 was confirmed in brain endothelial RBE.4 cells. Taken together, the proneurogenic compound Isx-9 exhibited different effects on the three major populations of progenitor/precursor cells in brain. Our data suggest that mechanisms and targets for promoting stem/progenitor cells in the central nervous system may significantly differ between cell types.

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Long-Term Pathological Deficits After Traumatic Brain Injury: Characterization of the Progressive Secondary Cell Death in Adult Rats Exposed to Cortical Impact Injury

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Long-term consequences of traumatic brain injury (TBI) have long been recognized as a risk factor for the development of dementia and neurodegenerative disease. The persistent and the progressive damage of chronic secondary injuries specifically at the molecular level to cortical and subcortical regions are not very well understood. In the present in vivo study, we examine the histopathological outcomes of experimental chronic TBI in proximal and distal areas after 6 months

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from the initial injury with special emphasis on inflammation and chronic axonal injury, which potentially contribute to the significant neurological impairments seen in TBI survivors. Six months after moderate controlled cortical impact injury, adult Sprague–Dawley male rats were euthanized and brain tissues harvested. Antibodies against activated antigen-presenting cells (OX6), cell cycle-regulating proteins (Ki-67), immature neurons, doublecortin (DCX), and axonal damage, β-amyloid precursor protein, (β-APP), were used to estimate activated inflammatory cells, cell proliferation, neuronal differentiation, and progressive chronic axonal damage, respectively, in cortex, subventricular zone (SVZ), hippocampus, striatum, thalamus, and cerebral peduncle. Stereology-based analyses revealed significant exacerbation of OX6-positive activated cells in both the ipsilateral and contralateral side of subcortical regions including the gray and white matter regions. In parallel, significant increments of β-APP expression throughout the brain were detected relative to sham control. Results indicate a persistent progressive deterioration of the TBI brain especially to the white matter over time characterized by elevated inflammation and chronic axonal injury. Therapeutic intervention at the chronic stage of TBI may confer abrogation of these deleterious cell death processes.

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Aging Disturbs Regenerative Responses in Neural Stem/Progenitor Cells and Oligodendrocyte Precursor Cells in Spontaneous Hypertensive Rats

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Aging and high-blood pressure are both major risk factors for stroke and other cerebrovascular diseases. However, the effects of these two comorbidity factors on the mechanisms of brain repair after injury remain to be fully elucidated. Here we ask if, compared to young brains in spontaneous hypertensive rats (SHRs), older brains may exhibit decreased rates of compensatory proliferation in neural stem/progenitor cells (NSPCs) and oligodendrocyte precursor cells (OPCs) after injury. Three-month- and 12-month-old SHRs were subjected to transient (70 min) middle cerebral artery occlusion by filament insertion. Regional blood flow was monitored by laser Doppler flowmetry. At day 3 or 14, rats were sacrificed, and brains were analyzed for (i) infarct volume by hematoxylin and eosin staining, (ii) NSPC proliferation by immunohistochemistry using anti-nestin antibody (NSPC marker), and (iii) OPC proliferation by double staining with anti-platelet-derived growth factor receptor α (PDGF R-α; OPC marker) and anti-Ki-67 (proliferative cell marker) antibodies. There were no significant differences in the regional blood flow ratio after filament insertion/removal and the mortality ratio between young and aged SHR. Hematoxylin/eosin staining showed that both young and aged SHR exhibited similar infarction volumes at days 3 and 14. Immunostaining showed that the number of nestin-positive cells increased in the peri-infarct region and subventricular zone of the ipsilateral side in both young and aged SHR, but younger SHR had a larger number of nestin-positive cells in both areas, especially at day 3. Similarly, compared to aged SHR, OPC proliferative responses in the corpus callosum region (i.e., white matter region) were also larger in young SHR. Taken together, our data suggest that although infarction volume by ischemic stress was similar in young and aged SHR, regenerative responses in NSPC and OPC proliferation were larger in young SHR. To find an effective therapeutic strategy, future studies are warranted to examine the underlying mechanisms for how the combination of aging and high-blood pressure disturbs regenerative responses after stroke in aged SHR.

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Effects of Tongue Force Training on Bulbar Motor Function in Female SOD1-G93A Rats

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The use of exercise to maintain muscle function in amyotrophic lateral sclerosis (ALS) is controversial. Recent preclinical studies suggest that moderate exercise may be beneficial in ALS, while intensive exercise may be detrimental. The effects of exercise on bulbar motor function have not been studied, however. In this study, we compared the effects of tongue force training on bulbar motor function in female SOD1-G93A rats and healthy controls. Half of each group underwent afternoon tongue force training sessions, while all rats were tested under minimal force conditions in the mornings. Tongue force did not differ between the SOD1-G93A rats and healthy controls during the testing sessions, nor was it affected by training. Surprisingly, tongue motility deficits emerged sooner and were greater in the tongue force-trained SOD1-G93A rats. This effect extended to the number of licks per session in affected rats. Decreased forelimb grip force and body weight with disease progression did not differ between the trained and untrained SOD1-G93A rats. Nor did tongue force affect survival or denervation of the genioglossus (tongue protruder muscle) in SOD1-G93A rats. These results indicate a deleterious effect of tongue force training on bulbar motor function in female SOD1-G93A rats. The facts that tongue force and neuromuscular junction innervation of the tongue were not affected suggest that factors other than lower motor neuron integrity likely accounted for this effect.

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Knockdown of α-Synuclein in the Substantia Nigra of the Nonhuman Primate Results in Dopaminergic Neurodegeneration

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Parkinson's disease (PD) is characterized by the loss of midbrain nigral dopamine (DA) neurons and the presence of Lewy bodies; proteinacious inclusions that are enriched in the protein α-synuclein (α-syn). Alterations of the α-syn gene are also implicated in some familial forms of PD. Thus, α-syn has been proposed to directly contribute to DA neuron loss in both idiopathic and familial forms of PD. The predominant hypothesis contends that α-syn-mediated pathology arises due to a toxic gain of function. Accordingly, some therapeutic strategies are aimed at removing α-syn from DA neurons. However, we have shown that knockdown of α-syn in midbrain DA neurons of adult rodents causes DA neurodegeneration. This suggests that the reduction of α-syn protein can also be toxic and that maintaining α-syn levels is crucial to DAergic neuron survival. As a result of this finding, we propose that α-syn is not a toxic species in PD per se, but rather that α-syn aggregation decreases the pool of soluble α-syn within a neuron to sufficiently impair cellular function and produce pathology. In other words, α-syn-mediated toxicity is due to a de facto toxic loss of function. In the current study, we tested this hypothesis in the nonhuman primate. African Green monkeys ($n=1$ for each treatment) were injected unilaterally in the substantia nigra (SN) with low [5×10^{12} vector genomes (vg)/ml] or high (2×10^{13} vg/ml) titer adeno-associated virus serotype 2/5 (AAV2/5) expressing a short hairpin RNA (shRNA) targeting α-syn or scrambled shRNA as control (total of four subjects). Animals were monitored for behavioral impairments indicative of nigral degeneration

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for 3 months until sacrifice. Brains were thereafter analyzed for striatal catecholamine content and neuropathology of the SN. High-dose α-syn shRNA treatment resulted in a progressive deficit in a summary score of healthy behaviors, but no increase in Parkinsonian signs. Analysis of tissue indicated that all α-syn shRNA-treated animals exhibited reduced striatal DA levels compared to the intact hemisphere. Stereological quantification of midbrain tyrosine hydroxylase-positive (TH^+) neurons suggests that neurodegeneration was preferentially present in the ventral tier of α-syn shRNA-treated SNs, whereas the dorsal tier only displayed modest neurodegeneration. The ventral tegmental area remained intact. Scrambled shRNA-treated subjects showed no behavioral or neural pathology. Transduction, as measured by the marker green fluorescent protein (GFP), was observed throughout the SN in control subjects. In contrast, GFP was only seen in dorsomedial neurons of α-syn shRNA-treated animals, presumably due to the loss of DA neurons in the ventral tier. Additionally, several TH^-/neuromelanin$^+$ neurons were observed, suggesting that α-syn shRNA treatment caused a loss of the TH phenotype in some surviving neurons. Our results indicate that nonhuman primate nigral DA (particularly ventral tier) neurons require α-syn for survival and that the experimentally induced neuron loss is not a product of nonspecific shRNA toxicity.

Transplantation of Midbrain Dopamine Neurons Derived From Human Embryonic Stem Cells in Rodent and Primate Models of Parkinson's Disease

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The degeneration of dopamine (DA) neurons in the substantia nigra and subsequent loss of dopaminergic transmission in the striatum largely accounts for the cardinal motor symptoms associated with Parkinson's disease (PD). The gold standard treatment for PD, levodopa, helps to ameliorate these symptoms, but can lead to "on–off" affects and levodopa-induced dyskinesias. Transplantation of authentic midbrain DA neurons from human embryonic stem cells could allow for a more physiological release of DA into the host to relieve motor symptoms and avoid the adverse effects of levodopa. Human embryonic stem cells were differentiated into authentic midbrain dopamine neurons (hESC-mDA) by mimicking normal human floorplate development (Kriks et al., 2011). hESC-mDA neurons were bilaterally transplanted into the striatum of cyclosporine-A immunosuppressed Sprague–Dawley rats, athymic NUDE rats, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned monkeys. Grafted cells were analyzed at multiple time points using multiple human-specific antigens to assess survival and maintenance of midbrain identity [forkhead box A2/tyrosine hydroxylase-positive ($FoxA2^+/TH^+$)]. The potential for rejection of grafted cells by the host immune system and posttransplant proliferation were also examined. We found that grafted hESC-mDA neurons matured over the course of 6 months, with large, $FoxA2^+/TH^+$ angular soma characteristic of midbrain lineage DA neurons. Fibers from the grafted hESC-mDA neurons were observed coursing through white matter tracks caudally, reaching the midbrain as early as 2 weeks, and extending through the brainstem by 6 months. Furthermore, when grafted into the striatum of four MPTP-lesioned monkeys, hESC-mDA neurons matured over time (between 1 month and 3 months posttransplantation) and maintained their $FoxA2^+/TH^+$ midbrain lineage. Grafted neurons innervated the striatum with long distance fiber outgrowth. Long-term functional studies in MPTP-lesioned monkeys are currently underway to determine safety and therapeutic value of pluripotent stem cell therapy for PD.

Optimizing Transplantation of Bone Marrow-Derived MSCs in a Rat Model of Spinal Cord Injury: Facilitating Functional Recovery Through Improvement in Microenvironment

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Spinal cord injury (SCI) is widely considered to be a permanently disabling condition, constraining those affected by it to wheelchairs and requiring intense daily care and assistance. However, it is now apparent that the loss of function due to SCI may not be permanent, as people are able to regain movement and sensation years after sustaining an injury. However, spontaneous recovery is limited, and therapy alone may not be enough to fully restore function. Therefore, strategies targeting regeneration of cells in the injured cord are currently gaining momentum in the field of SCI research, particularly those of cell replacement therapies. Human trials involving the transplantation of autologous stem cells have been conducted in several countries. However, determining the types of cells and the concentrations that are most efficacious is still widely debated. In addition, neuropathic pain and spasticity is a potential, undesirable consequence of these transplants. Therefore, it is important to determine what cell types, concentrations, and locations would provide the maximum benefit in functional recovery while also producing the fewest adverse reactions, before conducting extensive clinical trials. The current study explored the optimization of bone marrow-derived mesenchymal stem cells (MSCs) by transplanting various concentrations of them into the injured rat spinal cord and testing various locations of transplantation. Results indicate that MSCs are capable of facilitating functional recovery after moderately severe, incomplete SCI, without producing signs of neuropathic pain, and that these cells are able to do so at reasonably low doses. Animals receiving a moderate dose of 200,000 cells suspended in 4 µl demonstrated greater functional recovery than other cell concentrations, as measured by foot slips in the horizontal ladder and distance traveled across a rotating balance beam, and those whose cells were transplanted bilaterally rostral to the lesion showed the most rapid rate of recovery, as measured by the days postinjury to capable of traversing the horizontal ladder. No significant increase in visible muscular spasms of the hindlimbs was observed post-transplant when compared to postinjury. These findings indicate that a moderate concentration of cells is able to effectively improve functional recovery in rats following an incomplete spinal cord injury, without evidence of adverse side effects.

Preliminary Study of Autologous Mesenchymal Stem Cell Transplantations in Children With Drug-Resistant Epilepsy Derived From Hypoxic–Ischemic Encephalopathy

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Cell-based therapy is an evolving modality shown in preclinical studies to bring long-term improvement in hypoxic–ischemic encephalopathy (HIE). Initially cell therapy for epilepsy has focused on temporal lobe epilepsy (TLE). The purpose of this study was to assess the safety and potential benefit of cell therapy in drug-resistant epilepsy derived from HIE. A group of six children (three boys and three girls) ages 15 months to 6 years with hypoxic–ischemic encephalopathy was enrolled to receive cell therapy of autologous bone marrow-nucleated cell (BMNC) and/or mesenchymal stem cell (MSC) transplantation with intense neuro and neuropsychological rehabilitation. Four children

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experienced hypoxia and ischemia of the central nervous system during labor, while the other two (boys) experienced sudden hypoxia at the age of 7 months for an unexplained reason, followed by sepsis on the second day. Neuropsychological examination at admission revealed retardation of their mental and physical state. All children experienced regular drug-resistant epileptic seizures and were treated with multidrug therapy without any reduction of epilepsy episodes within 6 months. The combined treatment protocol—BMNCs injected intravenously (1×10^9) and via lumbar puncture (LP) (0.5×10^9) followed by four repeated rounds of MSC implantation via LP (2.5×10^7)—was performed in four cases. Two children enrolled were treated only with four repeated rounds of MSC implantations via LP. Efficacy was assessed based on the reduction of epileptic seizures, changes of their character, and electroencephalography examination. Neurodevelopmental improvement was evaluated using Polish children DSR scale. There were neither serious complications from the transplantation procedures nor any side effects of the cell therapy during an 18-month to 2-year follow-up period. One child experienced an increased temperature 2 days after MSC infusion. Reduction of the number of seizures has been seen in all of the epileptic children. In two (of the four) children treated with the combined protocol, 2 weeks after the second MSC transplantation, significant reduction of epileptic seizures (intensity and number) appeared (from 40 to 14 per week). In the two other cases from this group, after the second MSC implantation, only a slight reduction of epileptic episodes was reported. The number of seizures considerably decreased after a third MSC implantation (from 30 to 3 per week). In the group of children treated only with repeated rounds of MSCs, the significant reduction of intensity and number of epileptic seizures appeared after the second MSC implantation (from 40 to 10 and from 100 to 30 episodes per week). Four to 5 weeks after BMNC infusion, the first sensomotor improvement was noticed in the whole group of children treated with combined protocol. Two weeks after MSC infusion, the first cognitive improvement appeared in all children. The second MSC infusion caused further sensomotor improvement in both groups.

Endogenous Brain-Derived Neurotrophic Factor Promotes Oligodendrogenesis by Mediating Signals From Astrocytes to Oligodendrocyte Precursor Cells

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Oligodendrocyte precursor cells (OPCs) in the adult brain contribute to white matter homeostasis. After white matter damage, OPCs tend to compensate for oligodendrocyte loss by differentiating into mature oligodendrocytes. However, the underlying mechanisms remain to be fully defined. Here we test the hypothesis that astrocytes support the maturation of OPCs by secreting brain-derived neurotrophic factor (BDNF) under pathological conditions. A mouse model of prolonged cerebral hypoperfusion was prepared by bilateral common carotid artery stenosis in mice. Matching in vitro studies were performed by subjecting OPCs to sublethal 7-day $CoCl_2$ treatment to induce chemical hypoxic stress. In mouse cerebral white matter, astrocytes were one of the major sources for BDNF, especially under pathological conditions. Notably, BDNF-positive astrocytes were closely located to OPCs. We then examined whether astrocyte-derived BDNF would increase OPC maturation under pathological conditions both in vitro and in vivo. For in vitro experiments, cultured primary OPCs and astrocytes were prepared from postnatal day 2 rat cortex. When OPCs were exposed to chemical hypoxic stress by sublethal $CoCl_2$ for 7 days, in vitro OPC differentiation to oligodendrocytes was significantly suppressed. Conditioned medium from astrocytes (astrocyte-media) promoted the OPC maturation even under the stressed conditions via the phosphoinositide-3-kinase (PI3K)/Akt pathway. When astrocyte media were filtered with the tropomyosin-related kinase B-fragment crystallizable domain of human IgG (TrkB-Fc) to remove BDNF, the BDNF-deficient astrocyte media did not support the OPC maturation. For in vivo experiments, we generated a novel transgenic mouse line (*gfap*^{cre}*/bdnf*$^{wt/fl}$ mice), in which BDNF expression is downregulated specifically in glial fibrillary acidic protein (GFAP)-positive astrocytes. Both wild-type and transgenic mice were subjected to prolonged cerebral hypoperfusion by bilateral common carotid artery stenosis. As expected, compared to wild-type mice, the transgenic mice exhibited a lower number of newly generated oligodendrocytes and larger white matter damage at day 28. Taken together, these findings demonstrate that endogenous brain-derived neurotrophic factor promotes oligodendrogenesis by mediating signals from astrocytes to oligodendrocyte precursor cells.

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Traumatic Brain Injury Alters Chemokine Signaling in Aged Mice Permitting Exacerbated Infiltration of CCR2$^+$ Macrophages

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Epidemiologically, traumatic brain injury (TBI) is the greatest risk factor for the onset of multiple neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, as well as early onset dementia. Recent data suggest that there are approximately six million hospitalized incidents of TBI each year. Problematically, the incidence rates of hospitalized TBI rise dramatically in the aging population. Despite the prevalence of TBI and its associated neurodegenerative comorbidities, no viable therapeutic strategies exist. Following the initiating physical trauma, multiple cellular signaling cascades are activated involving resident macro- and microglia of the brain, often impacting primarily unaffected brain regions. Neuroinflammation has consistently been shown to propagate these injury-induced sequelae. However, the effect of aging on the brain's innate response to injury is poorly understood in the context of systemic mediators of the inflammatory response, notably circulating monocytes/macrophages. Herein, we examined the effect of aging on TBI-induced recruitment of circulating chemokine C-C motif receptor 2-positive (CCR2$^+$) macrophages into the injured parenchyma. Using both young (3 m) and aged (23 m) chemokine C-X3-C motif receptor 1$^{\text{green fluorescent protein/+}}$ CCR2$^{\text{red fluorescent protein/+}}$ ($CX3CR1^{GFP/+}CCR2^{RFP/+}$) reporter mice, we were able to reliably delineate the TBI-induced accumulation of CCR2$^+$ peripheral macrophages into the brain. Notably, aged animals exhibited an exacerbated response to TBI in the context of CCR2 macrophage recruitment. The recruitment of CCR2 macrophages to injured tissue is predicated on the expression of soluble chemotactic ligands. Our data show that aging significantly alters the expression of these ligands following injury, compared to their young counterparts. Moreover, we also observed significant alterations in the inflammatory response as a consequence of age and injury. Chronically, the effect of aging in combination with injury significantly impacted hippocampal-dependent cognitive function as assessed by the radial arm water maze. Cumulatively, our current data suggest that the increased recruitment of blood-borne CCR2$^+$ macrophages significantly augments TBI-induced neuroinflammatory sequelae, which may potentiate cognitive dysfunction.

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Fractalkine Overexpression Suppresses α-Synuclein-Mediated Neurodegeneration

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In Parkinson's disease, α-synuclein is known to activate microglia, and this activation has been proposed as one of the mechanisms of neurodegeneration. There are several signals produced by neurons that have an anti-inflammatory action on microglia, including chemokine C-X3-C motif ligand 1 (CX3CL1; fractalkine). We have shown that a soluble form of CX3CL1 is required to reduce neuron loss in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice and that fractalkine agonism can reduce neuron loss in a 6-hydroxydopamine lesion model. Here we show that fractalkine can reduce α-synuclein-mediated neurodegeneration in rats. Rats that received fractalkine showed abrogated loss of tyrosine hydroxylase and neuronal nuclei (NeuN) staining. This was replicated in animals where we expressed fractalkine in astrocytes with the glial fibrillary acidic protein (GFAP) promoter. Interestingly, we did not observe a reduction in major histocompatibility complex II (MHCII) expression suggesting that soluble fractalkine is likely altering the microglial state to a more neuroprotective one rather than reducing antigen presentation.

Experimental Study of Olfactory Mucosa Transplantation for Chronic Spinal Cord Injury

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Spinal cord injury is a serious disorder that leads to major neurological deficits. Some types of cell transplantation have been performed with some degree of success for its early stage, but we still do not have an effective treatment for its chronic stage. In our institution, olfactory mucosa transplantations have been performed since 2002 for chronic complete spinal cord injury patients. Nine patients have so far been operated on. In six cases, new voluntary activities in response to voluntary effort were documented by electromyography (EMG). In particular, two patients showed dramatic change. One began to walk with short leg braces and canes 3 years after surgery, and the other could stretch his leg just after 6 months postoperatively. Though efficacy of this treatment might depend on the level or length of the lesion, several experiments held by us support its appropriateness as a spinal cord injury therapy. In this presentation, we highlight four key factors within olfactory mucosa that contribute to neurological recovery. Those are 1) multipotent stem cells, 2) neurotrophic factors, 3) relay neurons, and 4) scaffold. 1) Olfactory mucosa consists of an olfactory epithelial layer and lamina propria. The olfactory epithelial layer contains horizontal basal cells, which differentiate into olfactory sensory neurons. Also olfactory sphere cells generated from adult rat olfactory mucosa differentiate into oligodendrocytes within injured spinal cord and can also differentiate into Schwann cells within transected saphenous nerve ends. These facts suggest that olfactory mucosa may include multipotent stem cells. 2) We confirmed that axonal outgrowth from cortical slices was significantly enhanced in primary olfactory mucosal cells compared with controls, and also it was severely reduced after treatment with antineurotrophin cocktails. Olfactory mucosa cells produce neurotrophin such as nerve growth factor (NGF) and neurotrophin 3 (NT-3). The latter was significantly rich compared with controls, and it might be the key neurotrophin associated with axonal outgrowth. 3) The existence of trans-synaptic neurons or relay neurons at 10 weeks after tissue transplantation for chronic spinal cord contusion model rats was confirmed by using neural tracer wheat germ agglutinin (WGA) and biotinylated dextran amine (BDA). 4) All the results mentioned above support the fact that olfactory mucosa is effective as a scaffold for axonal regrowth. In the rat spinal cord gap model, the corticospinal tract has been seen to extend over transplanted olfactory mucosa. Therefore, it can be said that olfactory mucosa is a suitable resource for covering the gap. In addition, clinically, intensive rehabilitation might be integral to activate those factors mentioned above and to reconstruct new neural pathways. Summarizing the above, olfactory mucosa transplantation could be a promising treatment for complete spinal cord injury.

Investigation of Hemicraniectomies in Patients With Malignant Stroke and Traumatic Brain Injuries—A Retrospective Study

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Occlusion of the middle cerebral artery (MCA) will cause a brain infarction. A malignant middle cerebral artery infarct (mMCAI) develops secondary to cytotoxic and vasogenic brain edema. Traumatic brain injury (TBI) can be subdivided into primary and secondary injury. The primary injury may result from mechanical stress such as fractures of the cranium, stretch, or tear of brain tissue and vessels and diffuse axonal damage. Secondary injury occurs because of damage to the blood–brain barrier, release of inflammation mediator factors, cerebral hypoxia, cerebral edema, and increase in the intracranial pressure (ICP). Decompressive hemicraniectomy (DHC) is the surgical procedure when a part of the cranium is removed and the dura is opened to provide extra space. DHC aims to decrease ICP and may therefore reduce morbidity and mortality. This study was designed as a retrospective cohort study of patients with either stroke or TBI submitted to the ICU, Department of Neurosurgery, Umeå University Hospital, Sweden, during the period of 2002–2012 and which underwent a DHC in order to relieve intracranial pressure. Statistical analysis was performed using Excel. There was no significant reduction in Reactive Life Scale (RLS) on arrival at the ICU compared to discharge from the hospital for either the stroke or the TBI group of patients. Of the patients, 83.9% diagnosed with mMCAI and 73.3% of the TBI group of patients were operated within 48 h of the onset of their symptoms. In the mMCAI group of patients, only one patient had been given an ICP monitoring device. In this patient, the ICP measurements decreased from a peak of 30 mmHg, prior to DHC, down to 6 mmHg afterward. In the TBI group, 23 patients out of 36 received ICP monitoring during their hospital stay. In these patients, the mean ICP value dropped from 43.2 mmHg before DHC to 12.1 mmHg after, an average reduction in ICP of 31.1 mmHg ($p<0.001$). Among the MCAI cohort of patients, the mortality was 5%, while in the TBI group it was 19%. The study has documented that performing DHC on TBI patients with elevated ICP refractory to conventional noninvasive medical treatment significantly decreases ICP. The equivalent data for the mMCAI group are insufficient in order to draw any conclusions, but a similar trend was observed. The study documented a positive outcome correlation for both cohorts of patients in terms of improvement in RLS score at discharge from our institution after DHC.

EphA4 Negatively Regulates Vascular Remodeling After Ischemic Stroke in Mice

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Stroke is the leading cause of morbidity and the fourth leading cause of death in US adults, for which there undoubtedly remain few available treatment options. A subtype called ischemic stroke is a result of vascular occlusion and occurs in 80% of stroke cases. Vascular occlusion in the brain has been shown to induce the active remodeling (arteriogenesis) of preexisting collateral arterioles or "bypass vessels" into functional conduits for blood that can reduce neural damage. However, the mechanisms regulating this remarkable adaptive response remain unclear. The objectives of this project focus on the role of ephrin type A receptor 4 (EphA4), an implicated axon guidance molecule, in limiting arteriogenesis after permanent middle cerebral artery occlusion (pMCAO). In support of our objectives, we have determined that (1) there is an extensive arteriole collateral network and reduced infarct volume in global EphA4$^{-/-}$ mice after

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pMCAO, (2) in vitro studies reveal an increase in the proliferation and migration rates of endothelial cells (ECs) derived from EphA4$^{-/-}$ mice, and (3) angiopoietin-1 (Ang-1) and multiple cytokines and chemokines known to regulate arteriogenesis are also increased in EphA4$^{-/-}$ ECs. Based on the extensive preliminary data, we hypothesize that EphA4 suppresses EC arteriole remodeling after pMCAO. To test this hypothesis, we generated EC-specific EphA4 knockout (KO) mice then induced stroke by cauterizing the main branch of the MCA. Blood flow (BF) was examined pre-, immediately after, and 4 days postinjury using a laser Doppler. We found an increase in BF in KO compared to wild-type (WT) mice. To assess arteriole remodeling, as a route for the observed BF increase, mice were euthanized 4 days after injury, stained for arteriole identification by vessel painting, and the brain sectioned by cryostat. Interestingly, KO mice had almost a twofold increase in arteriole diameter, a denser arterial network, and reduced injury volume compared to WT. These initial findings suggest that EC-specific ablation of EphA4 improves arteriogenesis in the brain after stroke. Further studies will focus on the mechanism(s), involving Ang-1 signaling, that regulate this effect and whether behavioral improvements are observed using this model system. Understanding the molecular mechanisms underlying the role of EphA4 in arteriogenesis is essential as potential therapies for ischemic stroke may depend on vascular remodeling and blood circulation. These studies will provide a platform for translational stroke research by promoting therapeutic arteriogenesis as a novel strategy for improving patient care.

Differential Effects of Two MRI Contrast Agents on the Integrity and Distribution of rAAV2 and rAAV5 in the Rat Striatum

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Intraoperative magnetic resonance imaging (MRI) is being used to optimize intracerebral targeting and for tracking infusate distribution in gene therapy trials for nervous system disorders. We have investigated possible effects of two MRI contrast agents, Gadoteridol (Gd) and Galbumin (Gab), on the distribution and levels of transgene expression in the rat striatum, and their effect on integrity and stability of recombinant adeno-associated virus (rAAV) particles. MRI studies showed that contrast agent distribution did not predict rAAV distribution. However, green fluorescent protein (GFP) immunoreactivity revealed an increase in distribution of rAAV5-GFP, but not rAAV2-GFP, in the presence of Gd when compared to viral vector injected alone. In contrast, Gab increased the distribution of rAAV2-GFP but not rAAV5-GFP. These observations pointed to a direct effect of infused contrast agent on the rAAV particles. Negative-stain electron microscopy (EM), DNAase treatment, and differential scanning calorimetry were used to monitor rAAV2 and rAAV5 particle integrity and stability following contrast agent incubation. EMs of rAAV2-GFP and rAAV5-GFP particles pretreated with Gd appear morphologically similar to the untreated sample; however, Gab treatment resulted in surface morphology changes and aggregation. Consistently, a compromise of particle integrity was suggested by sensitivity of the packaged genome to DNAse treatment following Gab incubation but not Gd for both vectors. However, neither agent significantly affected particle stability, although an increase in T_m was observed for AAV2 in lactated Ringer buffer. These results thus highlight previously overlooked interactions between MRI contrast agents and AAV that might affect vector distribution and stability, as well as the stabilizing effect of lactated Ringer on AAV2.

Tricyclic-Mediated Attenuation of α-Synuclein Aggregation in Models of Parkinson's Disease

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We previously demonstrated that tricyclic antidepressants (TCAs), including amitriptyline (AMI), delay the need for dopaminergic therapy in an early cohort of patients with Parkinson's disease (PD), suggesting they may have disease-modifying properties (Paumier et al., 2012). TCAs have been shown to be more efficacious for treating depression associated with PD, suggesting they may provide dual benefit to patients (Menza et al., 2009). Preliminary studies demonstrate select TCAs [i.e., nortriptyline (NOR), AMI] reduce α-synuclein (α-syn) aggregation in several in vitro aggregation assays (unpublished data). These findings prompted us to examine the effects of TCAs on α-syn propagation and accumulation in our recently established progressive, neurodegenerative synucleinopathy rat model that more closely resembles the spread and deposition of α-syn found in idiopathic PD. All rats received unilateral, intrastriatal injections (8 μg/4 μl) of preformed fibrillar (PFF) α-syn. Daily injections (IP) began 2 weeks prior to and 8 weeks following PFF injections. One group received saline, and the remaining groups received either AMI (5 or 15 mg/kg) or NOR (5 or 20 mg/kg). Two months post-PFF injection, phosphorylated α-syn (pSyn)-positive inclusions were readily apparent in the substantia nigra (SN) of saline-treated rats; however, α-syn pathology was substantially reduced in rats that received either AMI or NOR. Results indicate AMI and NOR significantly reduce the spread and accumulation of α-syn in a dose-dependent manner. NOR was more potent than AMI and completely prevented α-syn accumulation at the high dose, highlighting the potential for NOR pretreatment to slow disease progression. Next, we examined whether NOR impacted existing α-syn aggregates by beginning treatment after α-syn pathology was evident (2 weeks after PFF injection). Over the course of 2 months, α-syn continued to accumulate in the SN of saline-treated animals; however, there was a slight reduction in α-syn aggregates with NOR treatment. Although not significant, there was a trend for fewer α-syn aggregates with low (−30%) and high (−29%) NOR compared to saline ($p = 0.07$), suggesting NOR may halt ongoing disease progression. To establish the mechanism, we examined the interaction between NOR and α-syn in an in vitro kinetics assay that models the earliest stages of α-syn aggregation. According to this model, NOR interacts with the monomer and slows dimer formation by approximately fivefold, which is better than curcumin, a confirmed inhibitor of α-syn aggregation. Follow-up studies using tryptophan fluorescence indicate NOR binds to α-syn at physiologically relevant concentrations and may have multiple binding sites. Taken together, these preliminary findings suggest NOR directly interacts with monomeric α-syn and prevents aggregation in vitro. As this occurs at physiologically relevant concentrations (nM), it also suggests a potential mechanism for how NOR may attenuate α-syn aggregation in vivo.

Novel Neurotherapeutic Synergism and Biochemical Insights Into the GSK3β Pathway via Human iPSC-Derived Neurons

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Mechanistic understanding and development of novel drug treatments for neuropsychiatric disorders has been restricted due to difficulties in acquiring live diseased human neural cells for experimentation. With advancements in stem cell technologies and neuronal differentiation, it is now possible to perform drug screens on human neurons derived from embryonic and induced pluripotent stem cells (hESCs and hiPSCs), including those from disease-affected patients. The glycogen synthase kinase-3β (GSK3β) signaling pathway has long been associated with a myriad of neurodevelopmental and neuropsychiatric disorders, and modulation of GSK3β and its substrates represents intriguing targets for novel neurotherapeutics. We have utilized hiPSC-derived neurons to screen for compounds that modulate GSK3β. Unexpectedly, we found

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synergistic drug combinations that significantly impacted GSK3β and known direct GSK3β substrates. Fascinatingly, the efficacious drug synergisms we found are known to operate through divergent mechanisms of action, which appear to be converging on our proteins of interest. The effect of the compounds we found were compared between hiPSC-derived neurons from individuals affected with a neuropsychiatric disorder and hiPSC-derived neurons from unaffected individuals. Surprisingly, the affected hiPSC-derived neurons had a different biochemical response to the screened compounds than the unaffected neurons, possibly highlighting key GSK3β pathway abnormalities in individuals with neuropsychiatric disorders. Our results identified a possible novel synergistic therapeutic approach for treating neuropsychiatric disorders caused by GSK3β pathway dysfunction and identified compounds that may have the potential to be developed as neurotherapeutics.

Functional Improvement by Hematopoietic Growth Factor Treatment in Chronic Stroke in Aged Animals: A Treatment Frequency Study

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Stroke is a cerebral vascular disease characterized by obstruction of blood flow to a brain region. This disease has a high incidence in the elderly and is a worldwide leading cause of adult long-term disabilities. Stroke has three phases following its onset: the acute phase, the subacute phase, and the chronic phase. Generally, the chronic phase starts 3–6 months after stroke. In the chronic phase, a patient's pathological status is relatively stable, and patients are recommended to physical therapy, which is the only therapy available for stroke recovery. In our earlier studies, we have demonstrated the restorative role of two hematopoietic growth factors, stem cell factor (SCF) and granulocyte-colony-stimulating factor (G-CSF), in chronic stroke using rodent stroke models. However, it remains unclear which treatment paradigm would be the optimal intervention for enhancing stroke recovery. The aim of this study was to determine whether a multiple treatment paradigm is better than single treatment. Mice (male C57BL/6) at the age of 20 months were subjected to cerebral cortical ischemia by permanent occlusion of both the right middle cerebral artery and common carotid artery. Three months after brain ischemia, mice were randomized to receive either a 7-day treatment or a 2-week treatment of SCF + G-CSF. Blood samples were collected after the final injection of SCF + G-CSF as well as by the end of the experiment. Plasma levels for fibrinogen, an essential protein for blood clot formation, were determined by enzyme-linked immunosorbent assay (ELISA). Motor function was assessed at 2 and 6 weeks after final treatment using rotarod testing. Plasma fibrinogen levels showed a significant decrease in the 2-week treatment group right after the treatment, while this change did not show a significant difference 6 weeks after the treatment. At week 2 of rotarod testing, there was no significant difference between the two treatment groups. However, at the week 6 testing for motor function, a stronger recovery was observed in the 7-day treatment group compared to the 2-week treatment group. These results suggest that in the acute response to treatment, the 2-week treatment may show beneficial effects on blood flow, whereas in long-term evaluation of motor function the 7-day treatment appears to be more effective. How the treatment paradigm differently affects blood parameters and functional recovery remains to be determined in future studies.

Bone Marrow Cell Transplantation Time-Dependently Reverses G-CSF Effects After Stroke in Hypertensive Rats

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Granulocyte colony-stimulating factor (G-CSF) is a hematopoietic cytokine and preclinically proven neuroprotectant. A potential reason for the clinical failure of G-CSF may be that relevant G-CSF effects such as the mobilization of mononuclear hematopoietic stem/progenitor cells from the bone marrow may take too long in humans (up to 9 days) to counter initial stroke consequences. Systemic transplantation of bone marrow mononuclear cells (BMMNCs) is feasible within a relatively short time after stroke onset and may provide an external resource of aforementioned stem/progenitor cells, thereby "bridging the gap" until G-CSF comes to full effect. Male spontaneously hypertensive rats (SHR) were randomly assigned into four groups after permanent middle cerebral artery occlusion (MCAO). Groups 1–3 received IP G-CSF treatment (50 μg/kg) for 5 days starting 1 h after stroke onset. Groups 2 and 3 also received 1.5×10^7/kg BMMNCs IV at 6 or 48 h following stroke, respectively. Group 4 received placebo treatment. Functional deficits (adhesive removal test), infarct volume, and edema (T2 TSE MRI) were repeatedly assessed for 1 month. Peripheral leukocyte counts and BMMNC biodistribution were analyzed by flow cytometry during the first week after stroke. G-CSF monotreatment reduced functional deficits ($p < 0.05$) and partially reversed poststroke immune depression [overall leuko-/monocyte as well as B-, natural killer (NK), and T-cell counts; $p < 0.01$] and as expected increased peripheral leukocyte counts massively ($p < 0.01$). G-CSF did not affect infarct volume or edema. BMMNC cotransplantation at 6 h did not further improve functional deficits ($p > 0.05$ each). Surprisingly, BMMNC transplantation at 48 h abolished G-CSF effects. Early biodistribution studies (at 52 h after stroke onset) revealed splenic accumulation of granulocytes and BMMNCs as well as a granulocyte overload in the peripheral circulation and the brain ($p < 0.05$). Splenic accumulation of transplanted BMMNCs may have impaired peripheral granulocyte clearance. Subsequently, increased granulocyte numbers in the circulation and the poststroke brain prompted a proinflammatory bias of the innate immune system's response to stroke, ultimately abolishing G-CSF effects. These surprising findings indicate that systemic effects of experimental stroke therapies need to be carefully considered when assessing the therapeutic potential of such novel approaches.

Combining Deep Brain Stimulation With Neuroregenerative Therapy to Alter the Progression of Parkinson's Disease: Is It Possible?

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For over 10 years, deep brain stimulation (DBS) therapy has been used to ameliorate the symptoms of Parkinson's disease (PD). However, many PD-related symptoms, especially nonmotor symptoms, are not relieved through the use of DBS. In addition, DBS therapy has not been shown to halt or reverse the progression of the disease; therefore, new therapies need to be developed to enhance the therapeutic benefits of DBS. Based on the wealth of results of previous nonclinical, preclinical, and early clinical trials showing that neurotrophic factors help restore dopaminergic cell function, we think neurotrophic factors may serve the role of a therapy that complements DBS. While direct delivery of neurotrophic factor(s) is fraught with complications, a potential alternative source of neurotrophic factors is the Schwann cell from the peripheral nervous system. After injury, Schwann cells release a host of growth factors including glial cell line-derived neurotrophic factor (GDNF), nerve growth factor (NGF), brain-derived growth factor (BDNF), and neurotrophin 3 (NT-3). The added benefit of using Schwann cells is that patients can supply their own tissue, thereby minimizing the risk for immune rejection. We are conducting a pilot study to examine the safety and feasibility of implanting an autologous

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peripheral nerve graft into the substantia nigra of PD patients undergoing DBS surgery. Multistage, DBS surgery targeting the subthalamic nucleus was performed using standard procedures. After the DBS leads were implanted, a section of sural nerve (approximately 5 mm in length) containing Schwann cells was excised and unilaterally delivered, using a custom-designed cannula, into the area of the substantia nigra. Adverse events were continuously monitored. Quality of life and Unified Parkinson's Disease Rating Scale (UPDRS) evaluations were monitored preoperatively and at 1, 3, 6, 9, and 12 months after surgery. We have implanted eight participants (average age: 62.9 ± 9.2 years; duration with the disease: 9.8 ± 9.2 years; mean ± SD) with no significant adverse events. Immediate, postoperative magnetic resonance scans did not indicate evidence of abnormal tissue disruption. For the five participants who have completed the 12-month study, UPDRS Part III (motor) scores off medication/off stimulation were 24.6 ± 13.5 points while at baseline they were 34.2 ± 8.0 points (moderate clinically important differences are defined as >5 points; Shulman et al. 2010). On medication/on stimulation scores were 14.8 ± 9.7 points at baseline and 9.6 ± 7.2 at 12 months. All the while, medication levels decreased from 775 ± 618 daily levodopa equivalents, preoperatively, to zero after 12 months. Based on our initial safety outcomes and early efficacy results, combining Schwann cell delivery with DBS therapy may provide a means of offering neuroregenerative therapy that augments the benefits of DBS in patients with PD.

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Developments in Intracerebral Stem Cell Grafts

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The field of stem cell therapy has emerged as a promising research area for brain repair. Optimizing the safety and efficacy of the therapy for clinical trials will require revisiting transplantation protocols. The cell delivery route stands as a key translational item that warrants careful consideration in facilitating the success of stem cell therapy in the clinic. Intracerebral administration, compared to the peripheral route, requires an invasive procedure to directly implant stem cells into the injured brain. Although invasive, intracerebral transplantation circumvents the prohibitive blood–brain barrier in allowing grafted cells when delivered peripherally to penetrate the brain and reach the discreet damaged brain tissues. In this presentation, we will highlight milestone discoveries in cell therapy for neurological disorders, with emphasis on intracerebral transplantation in relevant animal models and provide insights necessary to optimize the safety and efficacy of cell therapy for the treatment of Parkinson's disease, Huntington's disease, stroke, and traumatic brain injury. Despite scientific advances that led to limited clinical applications of cell therapy, the challenges of implementing large-scale stem cell therapy in the clinic remain, requiring transplant regimen optimization and investigations into mechanisms of action underlying this treatment. The cellular and molecular changes in the brain microenvironment that accompany the progression of the disease warrant a careful consideration of the cell delivery route that will facilitate enhanced regeneration of the injured brain. A review of experimental and clinical data is deemed critical in guiding scientists in finding the most effective and safe transplant methodologies in an effort to improve outcomes in both animal models and patients with neurological disorders.

Immune System Changes After Adult Brain Injury Define Scar Formation

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Upon traumatic brain injury, the restoration of tissue architecture and function begin with the replacement of the lost neurons. This is not an easy task, not only due to the restricted neurogenesis in the adult brain but also to the exacerbated reaction of the glial cells during the regenerative process. Microglia, astrocytes, and oligodendrocyte progenitors (OPCs) are able to react upon injury orchestrating the wound healing process; however, the long-term effects (glial scar) of the glial reactivity might be very detrimental. The glial scar is the hallmark of the reaction to injury in the mammalian brain, and it is well known to create a very "hostile" environment that reduces tremendously neural integration and survival. Therefore, understanding glial scar formation is the first step to modulate the scarring process in order to promote functional recovery. To tackle these important questions, we use an animal model with great regenerative potential even in adulthood: the zebrafish. We have established two types of stab wound injuries in the zebrafish telencephalon with different scarring processes, a "scarless" injury (nostril injury) and the skull injury with oligodendrocyte lineage transcription factor 2-green fluorescent protein-positive (Olig2-GFP$^+$) cells and microglia reacting to the injury similar to the mammalian system. Importantly, the scar formed by the Olig2$^+$ cells remains in the regenerating brain for at least 4 weeks. The characterization of these two types of injuries shows major differences in the microglia and OPC activation. Microglia react strongly after both injuries, but the morphology, distribution, and kinetics of activation of 4C4$^+$ microglia differs greatly between the two paradigms, suggesting a prime role for 4C4$^+$ microglia in inducing glial reactivity. To identify the molecular mechanisms underlying the different scarring processes, we performed a transcriptome analysis after both types of injury at 1, 2, 3, and 7 days postinjury (dpi). We observed several signaling pathways exclusively regulated in each injury paradigm. Strikingly, the molecular signature during the scar formation (skull 3 dpi) is radically different compared to the scarless injury. The majority of these pathways are related to the immune response signaling. We hypothesize that differences in the control of the immune response activation are responsible for the resolution of glial scar and therefore successful regeneration. Our data suggest that the modulation of these pathways might be the key to achieving the scarless regeneration and therefore, potentially better survival of newly generated neurons.

Targeted Gene Knockout in Midbrain Nigral Neurons of Adult Rats Using the CRISPR/Cas9 System Technology

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Targeted gene editing using customizable nucleases enables the introduction of changes in the DNA of cells at a precise genomic location. This technique can be used to elucidate gene function and possibly as a therapy for many genetic diseases. Recently, a new class of nucleases was developed from the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated genes (CRISPR/Cas) microbial adaptive immune system. In this approach, the protein component, Cas9, is targeted to the desired DNA sequence by a guide RNA (gRNA), which is easily designed by following basic Watson–Crick base pair complementarity rules. To date, the CRISPR/Cas9 system has largely been used to generate transgenic animals, and its use to modulate endogenous expression in the adult animal has been limited. Here we utilized a gene therapy approach to evaluate whether we could achieve efficient CRISPR/Cas9-mediated gene inactivation in vivo, specifically targeting the dopaminergic neurons of the midbrain of adult rats. We selected to target the gene that encodes the protein tyrosine hydroxylase (TH), the rate-limiting enzyme in the production of the neurotransmitter dopamine (DA). Inhibition or removal of this protein results in an easily measurable reduction in DA and subsequent effects on motor behavior. To this end, we designed two gRNAs complementary to Exon 1 and Exon 2 of

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the TH gene and tested these in vitro. Initial assessment of the gRNA in cultured cells of the rat pheochromocytoma cell line, PC12, demonstrated robust DNA cleavage efficiency at the desired genomic location. To further test the capabilities of the selected CRISPR/Cas9 pair in vivo, we designed a dual recombinant adeno-associated virus (rAAV) vector system: one rAAV vector carries the protein Cas9 under the control of the neuron-specific synapsin promoter, and a second rAAV vector carries an expression cassette for the gRNA under the H1 Pol III promoter as well as green fluorescent protein (GFP) as a marker for transduction efficiency. A cohort of 2-month-old Sprague–Dawley rats received a single dose of a rAAV-Cas9/rAAV-TH gRNA mix or a rAAV-Cas9/rAAV-GFP mix as an "unguided" control, stereotaxically delivered to the substantia nigra. Experiments are ongoing using genetic, biochemical, and behavioral methods to determine the efficiency of TH-specific gene knockout. Results from this study will validate the use of CRISPR technology in the nigrostriatal dopaminergic system in mature brains and offer a very powerful tool to elucidate gene function in specific neuronal cell types while still in their native context.

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Effect of Neurite Aggregations and Abnormal Calcium Levels on Mitochondria Dynamics in Parkinson's Disease iPSC-Derived Sensory Neurons

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Less prominent features of Parkinson's disease (PD) are nonmotor symptoms, which are generally underappreciated and can severely impact quality of life. The majority of PD patients suffer from nonmotor symptoms including pain, depression, constipation, cognitive deficits, and hyposmia, which can occur before motor symptoms appear and may be independent of dopamine neuron loss. We have previously found that peripheral sensory neurons generated from homozygous leucine-rich repeat kinase 2 (LRRK2) G2019S induced pluripotent stem cells (iPSCs) exhibit neurite aggregates and altered calcium dynamics. Interestingly, kinase inhibition using three distinct LRRK2 kinase inhibitors partially rescued the neurite aggregation phenotype but fully rescued the abnormal calcium phenotype. Mitochondria require proper calcium signaling to maintain health and function, so we hypothesize that LRRK2 G2019S iPSC-derived peripheral sensory neurons will display altered mitochondrial respiration compared to control sensory neurons. Moreover, as mitochondria traffic along microtubules, we hypothesize that cytoskeletal aggregates will impede mitochondrial movement to the distal neurites. To test these questions, we are evaluating mitochondrial respiration in the Seahorse Bioanalyzer to measure oxygen consumption rates, live cell mitochondrial movement with Mitotracker, and mitochondrial localization with immunocytochemistry. Staining for the mitochondrial import receptor translocase of outer mitochondrial membrane 20 homolog type II (TOM20) indicates that mitochondria are highly associated with the cell body and proximal neurites in LRRK2 G2019S iPSC-derived sensory neurons compared to control sensory neurons, which display uniform TOM20 localization throughout the entire neurite projection. This raises the possibility that hyperactivity of LRRK2 G2019S may directly affect mitochondrial function and trafficking in peripheral sensory neurons. A better understanding of mitochondrial dynamics in LRRK2 G2019S peripheral sensory neurons may provide insight into the underlying mechanisms of nonmotor symptoms in PD thereby aiding therapeutic development.

Modulation of Nurr1 in the Striatum of the Parkinsonian Rat Alters the Severity and Onset of Levodopa-Induced Dyskinesia

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Levodopa (L-DOPA) is the current gold standard treatment for alleviating motor symptoms that arise in Parkinson's disease (PD) due to loss of striatal dopaminergic innervation. Chronic treatment with L-DOPA leads to the development of L-DOPA-induced dyskinesias (LIDs) in a majority of patients. These debilitating motor symptoms can become more severe and devastating than the Parkinsonian motor symptoms that L-DOPA was being used to treat. It is unknown why certain patients do not develop LIDs, while others with similar disease duration on comparable doses of L-DOPA inevitably do. It is imperative to understand the molecular differences between these types of patients in order to better understand the mechanism of LID development. In order to identify transcriptional differences between animals that develop LIDs (LID^+) and those that do not (LID^-), we performed a full genome array on striatal tissue from the rat Parkinsonian 6-hydroxydopamine (6-OHDA) model to identify differential expression between these groups. Nuclear receptor related 1 (Nurr1), an orphan nuclear receptor required for dopaminergic neuron development and health, was found to be significantly upregulated (greater than 30-fold transcript expression) in the striatum of LID^+ animals compared to LID^- animals. In the present study, we assessed Nurr1's role in the development and severity of LIDs. Adult male Sprague–Dawley rats were unilaterally injected with 6-OHDA to the striatum. Animals that did not show a significant lesion—indicated by forelimb akinesia postlesion—were excluded from the study. Remaining animals received an intrastriatal injection of recombinant adeno-associated virus (rAAV) type 2/5 expressing either 1) Nurr1 [or green fluorescent protein (GFP) as a control, 5×10^{13} vector genomes/ml] to investigate whether ectopic striatal Nurr1 overexpression exacerbates LIDs or 2) Nurr1 short hairpin RNA (shRNA) (or a titer-matched scrambled shRNA control, 2×10^{13} vg/ml) to examine whether striatal Nurr1 is required for LIDs. Four weeks after vector delivery, animals received escalating doses (0–24 mg/kg) of L-DOPA/benserazide every other day and were evaluated for LID severity using the abnormal involuntary movement (AIM) rating scale. L-DOPA doses of 12 mg/kg and higher resulted in significantly higher AIM scores and longer-lasting LIDs in animals overexpressing Nurr1 in the striatum when compared to rAAV-GFP-treated controls. Animals receiving Nurr1-shRNA displayed attenuation of AIMs with doses of L-DOPA as high as 18 mg/kg. Postmortem analysis confirmed the successful inhibition of Nurr1 upregulation in Nurr1-shRNA-treated animals. Our results show that Nurr1 maladaptive upregulation in the striatum of Parkinsonian rats is not a compensatory event of LIDs but rather a driver of dyskinesia development. Blockade of Nurr1 upregulation effectively prevents the development of LIDs in this model. These data suggest that Nurr1 plays a critical role in the molecular mechanism behind LIDs and may be a possible therapeutic target for these debilitating symptoms.

Generation of Dopaminergic Neurons From Rat Bone Marrow-Derived Mesenchymal Stem Cells

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Parkinson's disease (PD) is a neurodegenerative disorder characterized by the loss of dopaminergic neurons from the substantia nigra that innervate the striatum through the nigrostrial pathway. Loss of this pathway leads to significant motor impairments and affective symptoms. Although an effective long-term therapy has not been developed, research focusing on the transplantation of human fetal neuroblasts into the neostriatum of PD patients has provided evidence that long-lasting therapeutic benefits can be achieved through this method. Nevertheless, human fetal neuroblasts are limited in availability, raise ethical concerns, and have been shown to result in tumor formation in vivo. Owing to these limitations, other avenues of cell-based therapies aimed at alleviating the symptoms associated

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with PD are being investigated, including the use of mesenchymal stem cells (MSCs). MSCs can be easily isolated and expanded from different adult tissues, including bone marrow, peripheral blood, vasculature, adipose tissue, and umbilical cord blood. Further, studies have shown that they also have the ability to differentiate in vivo and in vitro into neural-like stem cells and, more specifically, into dopamine-producing cells using growth factors and morphogens. However, there are discrepancies among studies regarding the optimal time and method for dopaminergic induction in vitro. In the current study, we compared the ability of early (P4) and later (P40) passaged rat bone marrow-derived MSCs to differentiate into dopaminergic neurons when using two growth factor-based approaches; a direct dopaminergic induction using sonic hedgehog (SHH) and fibroblast growth factor-8 (FGF-8), and an indirect dopaminergic induction using basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), SHH, and FGF-8. Results from flow cytometry and immunocytochemistry (ICC) analyses indicated that treatment of both early and later passaged MSCs with bFGF and EGF induced neurosphere-like formation and expression of the neural progenitor markers doublecortin (DCX) and nestin. Also, early and later passaged MSCs, that underwent both the direct and indirect dopaminergic inductions, exhibited a change in morphology toward a neuronal phenotype and expressed neuronal [i.e., neuron specific nuclear protein (NeuN) and class III β-tubulin], and dopaminergic markers [i.e., tyrosine hydroxylase (TH), dopamine transporter (DAT), and LIM homeobox transcription factor 1 (LMX1a)] as determined by flow cytometry, ICC, and reverse transcription polymerase chain reaction (RT-PCR). Overall, findings from this study indicate that early and later passaged rat MSCs, which have undergone either the direct or indirect induction protocols, have the potential to differentiate into dopaminergic neurons and ultimately be used in cell-replacement therapies for the treatment of PD.

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Loss of Catecholaminergic Innervation in the Colonic Myenteric Plexus of Systemic 6-OHDA-Treated Rhesus Monkeys

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Gastrointestinal (GI) dysfunction, such as constipation, is a common nonmotor symptom of Parkinson's disease (PD) and can manifest decades before PD cardinal motor symptoms. Lewy bodies, the pathological hallmark of PD, which are composed largely of aggregated α-synuclein, are found in both dopaminergic and vasoactive intestinal peptide (VIP)-ergic nerves of the enteric nervous system (ENS) in the earliest stages of PD, prior to central nervous system pathology. Additionally, dopaminergic immunoreactivity is decreased in the ENS of PD patients with severe GI symptoms. Systemic dosing of the catecholaminergic neurotoxin 6-hydroxydopamine (6-OHDA) to rhesus monkeys induces cardiac sympathetic nerve loss and GI dysfunction (i.e., diarrhea), which suggests that the neurotoxin may also affect ENS catecholaminergic neurons. To assess this possibility, the colonic myenteric plexus of six control (9.09 ± 2.01 years old) and five 6-OHDA-treated rhesus monkeys (50 mg/kg IV; 7.0 ± 1.57 years old) were evaluated in this study. Three months postneurotoxin, the animals were euthanized by cardiovascular perfusion of phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Proximal colon samples were postfixed in 4% PFA for 24–48 h, followed by 70% ethanol, and then blocked in paraffin. The tissue was sectioned on a standard rotary microtome at a thickness of 5 μm and processed for immunohistochemistry to visualize tyrosine hydroxylase (TH; catecholaminergic marker), protein gene protein 9.5 (PGP9.5; panneuronal marker), VIP, and α-synuclein, and counterstained with hematoxylin. Data collection and analysis were performed by an investigator blind to the treatment groups. The NIH ImageJ program was used to define regions of interest (ROIs) around each ganglion (identified at 40×) and quantify immunoreactivity (-ir) as area above threshold (AAT). A significant reduction in myenteric ganglia TH-ir ($p < 0.001$) was found in 6-OHDA-treated animals compared to controls with an average 89.4% loss. In contrast, no differences in expression between treatment groups were found for PGP9.5-ir ($p = 0.1432$), VIP-ir ($p = 0.7796$), or α-synuclein-ir ($p = 0.6916$). Immunofluorescence colocalization of PGP9.5-ir with TH-ir, VIP-ir, or α-synuclein-ir confirmed myenteric intraneuronal expression of these markers. Analysis of colocalization of α-synuclein-ir with TH-ir or VIP-ir revealed similar expression and distribution in both neuronal populations of both treatment groups. In conclusion, systemic administration of 6-OHDA to rhesus monkeys significantly reduces TH-ir in the colonic myenteric plexus without affecting α-synuclein expression and distribution, as observed 3 months postintoxication. This reduction in TH-ir resembles ENS catecholaminergic loss found in PD patients with severe GI symptoms and suggests that systemically 6-OHDA-treated monkeys can be used to model this pathology.

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Supportive Effect of Human WJ-MSCs on Vascular Network of Postischemic Hippocampal Slice Culture

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Ischemic stroke results in violent impairment of tissue homeostasis, leading to perturbation of the proper interactions within the neurovascular unit (NVU). Some researchers emphasize the notion that brain ischemia is a vascular disorder, suggesting a significant role of endothelial progenitor cells (EPCs) in the recovery of the injured brain. Our studies of this issue consisted of several stages. At the beginning, the aim was to assess the potential of mesenchymal stem cells originating from Wharton jelly (WJ-MSCs) to differentiate into cells of endothelial lineage able to support vasculature. The next step was to investigate and characterize the effect of these cells on the vascular network in the model of hippocampal organotypic culture (OHC) injured by oxygen–glucose deprivation (OGD). The final step of the work was to evaluate the neuroprotective impact of MSCs primed by the stimulation of specific toll-like receptors (TLRs) on the coculture with postischemic OHC slices. WJ-MSCs were cultured in expansion medium (MSCGM) or in endothelial differentiating medium (EGM-2). Phenotypic characterization was performed by flow cytometry, immunocytochemistry, and RT-PCR based on expression of typical markers for mesenchymal and endothelial lineage [cluster of differentiation 90 (CD90), CD73, CD105, von Willebrand Factor (vWF), CD31, vascular endothelial growth factor (VEGF), and VEGFR2]. Expected angiogenic activity of WJ-EPCs was estimated in vitro by DiI-acetylated low-density lipoprotein (DiI-Ac-LDL) uptake assay and capillary-like structure formation test. Priming of WJ-MSCs/WJ-EPCs was achieved by stimulation of specific toll-like receptors with the TLR4 agonist lipopolysaccharide (LPS) and/or TLR3 agonist polyinosinic-polycytidylic acid [poly (I:C)]. Hippocampal slices after 5 days of incubation were exposed to OGD. Afterward, primed or nonprimed WJ-MSCs/WJ-EPCs were cocultured in the typical Transwell system with the intact or postischemic slices for the next 2 days. The supportive effect of cocultured MSCs on the vascular network was evaluated immunohistochemically with rat endothelial cell antibody 1 (RECA-1) and the apoptosis marker caspase 3 (CASP3) or propidium iodide (PI). The putative mechanism of this protection was studied by estimation of the MSC-derived human interleukin (IL)-2 and VEGF released into the culture medium, using the bead-based cytometric immunoassays (BD system). Our findings showed that WJ-MSCs after 7 days of endothelial differentiation acquired cobblestone endothelial-like morphology, were able to form capillary-like structures, and take up DiI-Ac-LDL. Both cell types were positive for CD73, CD90, CD105, VEGFR2, and VEGF, but only WJ-EPCs expressed vWF and CD31. Moreover, in MSC-treated OHC cocultures, a significant decrease in cell death with parallel attenuated atrophy of blood vessels has been observed in the hypoxia-sensitive CA1 region. These results may suggest a paracrine, cytokine-related

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mechanism of MSC neuroprotection in this model of postischemic hippocampal slice injury. Thus, finally we stimulated (primed) MSC cultures by levels of TLR3/TLR4 agonists prior to seeding them into coculture with rat OHC slices. These treatments transformed MSCs into proinflammatory or immunosuppressive phenotypes, confirmed by cell phenotypic changes and cytokine release, which influenced the effectiveness of stem cell-based neuroprotection.

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Promotion of Brain Self-Repair Mechanisms by Stereotaxic Microneedle Lesions

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Long-term implantation of a fine metal electrode, even without chronic electrical stimulation may produce unwanted effects. Neuropathological examination of brain tissue from patients with deep brain stimulation (DBS) revealed activated astrocytes and microglia regardless of the underlying disease. Electrical stimulation is not required to see signs of neuroinflammation; inflammatory changes have been observed around recording electrodes used for characterizing epileptogenic tissue and around cerebral spinal fluid (CSF) shunt catheters. To understand the earliest reactions to microinjury, we studied the cellular and cytokine responses over time to transient insertion of a fine needle (maximum diameter of 200 μm) into the dorsal hippocampus of the mouse. We tested the hypothesis that creation of a focal microlesion in the hippocampus elicits self-repair mechanisms mediated by cytokines, which activate microglia, promote astrocytosis, and stimulate stem/progenitor cells to proliferate and generate new neurons. Brief stereotaxic insertion and removal of a microneedle into the right hippocampus resulted in a) significantly increased expression of granulocyte-colony-stimulating factor (G-CSF), the chemokine macrophage inflammatory protein 1-α (MIP-1a), and the proinflammatory cytokine interleukin 12 subunit p40 (IL12p40), b) pronounced activation of microglia and astrocytes, and c) increase in hippocampal neurogenesis. This study describes immediate and early humoral and cellular mechanisms of the brain's response to microinjury that will be useful for investigation of potential neuroprotective and deleterious effects of microinjury by needle in various neuropsychiatric disorders, and eventually replace the DBS.

Neural Progenitor Transplantation Promotes Recovery of Breathing Following Cervical Spinal Cord Injury

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Cervical spinal cord injury (SCI) results in a host of devastating functional consequences, including impaired breathing. Not only can respiratory deficits require costly, lifelong care and significantly diminish quality of life, but they also remain a leading cause of morbidity and mortality among SCI patients. Breathing dysfunction largely results from disruption of the phrenic circuit, which controls diaphragm function—the primary muscle of inspiration. Our research team has identified populations of prephrenic interneurons that likely contribute to the spontaneous respiratory plasticity that occurs following cervical SCI. The central hypothesis of our ongoing research is that enhancing this endogenous spinal plasticity will likely yield the greatest improvements in long-term respiratory recovery. Furthermore, this interneuronal cell population may represent an ideal therapeutic target to optimize respiratory recovery. We hypothesize that the transplantation of embryonic spinal cord tissue, inherently rich in interneuronal progenitors, can provide an additional substrate to facilitate the formation of novel relay pathways to restore input to the diaphragm. Adult, female Sprague–Dawley rats received lateralized C3/4 contusions using the Infinite Horizons Impactor Device (preset force = 200 kilodynes). Following a 1-week recovery period, mechanically dissociated spinal cord tissue obtained from E13.5 day rats was transplanted into the injury cavity (~1–2 million cells per transplant). Four weeks later, a transynaptic, retrograde tracer—pseudorabies virus—was applied to the ipsilateral hemidiaphragm or injected directly into the mature transplant. Animals were sacrificed 72 h later. Respiratory function was assessed using terminal diaphragm electromyography (EMG) recordings or phrenic nerve recordings, conducted under normal conditions as well as during exposure to a respiratory challenge [hypoxia (10% oxygen) or hypercapnia (7% CO_2)]. In a subset of animals, electrophysiological activity of transplanted tissue was recorded simultaneously. Immunohistochemical results from tracing experiments revealed both graft-to-host and host-to-graft connectivity, respectively. Electrophysiology demonstrated improved phrenic function, during both eupneic breathing and respiratory challenge. In addition, intratransplant recordings detected both respiratory- as well as nonrespiratory-related firing patterns of donor neurons. Ongoing experiments will analyze the direct contribution of transplanted neurons to functional improvements and examine the neuronal phenotype of transplanted neurons. The survival and subsequent proliferation of transplanted tissues will be assessed during the acute period (1–7 days) following transplantation to establish whether there is selectivity for specific interneuronal progenitors. Results from these experiments can provide insight into optimizing transplantation strategies to promote integration and enhance functional recovery.

Kainic Acid-Induced Golgi Complex Fragmentation/Dispersal Shifts the Proteolysis of Reelin in Primary Rat Neuronal Cells: An In Vitro Model of Early Stage Epilepsy

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The endoplasmic reticulum–lysosome–Golgi network plays an important role in Reelin glycosylation, and its proteolytic processing. Separation of Reelin from this network is associated with Golgi complex fragmentation. Kainic acid (KA) is an excitotoxic agent commonly used to induce epilepsy in rodents. The relationship between KA-induced neuronal damage and Golgi complex fragmentation has not been investigated, leaving a major gap in our understanding of the molecular mechanism underlying the development of pathophysiology in epilepsy. We cultured primary rat cortical neurons in either the ambient condition (control) or treated them with a dose range of KA to reveal whether Golgi complex fragmentation impaired neuronal function. The half-life maximal inhibitory concentration (IC_{50}) value of KA was measured to be approximately 5 μM, whereby at these concentrations KA impaired neuronal viability, which was closely associated with Golgi complex fragmentation and reduction in both the expression and glycosylation patterns of Reelin. These findings implicate Golgi complex fragmentation and Reelin dysfunction as key contributors to neuronal cell death in the early stage of epilepsy pathophysiology, thereby they are representing novel disease biomarkers, as well as potent therapeutic targets for epilepsy.

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Neural Circuitry for Locomotion Recovery After Thoracolumbar Contusion Injury and Peripheral Neurotization

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The Teng Laboratories earlier reported that peripheral nerve anastomosis post-thoracolumbar hemisection spinal cord injury (SCI) could reactivate central pattern generation (CPG) in rats (Konya et al., 2008). We have now tested our hypothesis that peripheral nerve rerouting may enable reactivation of CPG after thoracolumbar contusion. Female Sprague–Dawley (SD) rats (220–235 g) received T13–L1 contusion (mild: 10 g×12.5 mm; moderate: 10 g×25 mm). T12 intercostal nerve was neurotized to L2 or L3 nerve root (total: $n=22$) or sham operated ($n=8$/group) either 1 week or 13 weeks after either mild or moderate SCI. For chronic SCI, neurotization was done 10 days following human mesenchymal stromal stem cell (hMSC) injection (4×50k cells/µl; 1 µl injection at 1 mm rostral and caudal to the epicenter, respectively, and 2 µl into the injury site; $n=8$ for each SCI group). Behavior tests were performed for 8–24 weeks following neurotization. Also used were multimodal analyses of neural tracing and 5-ethynyl-2′-deoxyuridine (EdU)-labeled endogenous neural stem cell proliferation (50 mg/kg, QD×5 days, IP), plus electrophysiological and neuropharmacological assessments. Our data demonstrates that subacute T12–L2 or L3 neurotization markedly improves hindlimb locomotion. Moreover, there are discernible propriospinal and somatic sensory improvements. Neurotization after hMSC transplantation also restores locomotion in rats with chronic contusion. Our investigation reveals that neurotization triggered reorganization of neural circuits, increased propriospinal projection connectivity across the injury site, increased serotonin innervation, and maintained neuromuscular junctions. Therefore, peripheral nerve neurotization builds unique neural circuits for Recovery Neurobiology after thoracolumbar SCI.

Downregulation of P2X7 Receptor Activity Is Required for Neuronal Differentiation of Embryonic Stem Cell Differentiation and Regeneration of Dopaminergic Neurons in an Animal Model of Parkinson´s Disease

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Purinergic receptors participate in developmental functions, stem cell biology, and tissue regeneration. Recent data suggest that the purinergic receptor P2X ligand-gated ion channel 7 (P2X7R) promotes proliferation and regulates neural differentiation. Here we used mouse embryonic stem cells (ESCs) for further studying P2X7R functions in proliferation and neural differentiation. P2X7R expression together with the pluripotency marker octamer-binding transcription factor-4 (Oct-4) was highest in undifferentiated ESCs. In undifferentiated cells, the P2X7R agonist 2′(3′)-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate (Bz-ATP) accelerated cell cycle entry, which was blocked by the P2X7R blocker KN-62. ESCs, induced to neural differentiation by retinoic acid, reduced Oct-4 together with P2X7R expression. In agreement with differential P2X7R expression patterns, receptor-promoted intracellular calcium fluxes were obtained at lower Bz-ATP ligand concentrations in undifferentiated than in differentiated cells. The presence of KN-62 led to increased numbers of cells expressing stage-specific embryonic antigen 1(SSEA-1), doublecortin (Dcx), and β3-tubulin and the cells positive for both SSEA-1 and β3-tubulin immunostaining confirm that onset of neuroectodermal differentiation and neuronal fate determination depends on suppression of P2X7R activity. Moreover, an increase in the number of Ki-67-positive cells under conditions of P2X7R inhibition indicates rescue of progenitors into the cell cycle, increasing the neuroblast population and consequently promoting neurogenesis. In agreement with the proliferation-stimulating effect mediated in ESCs, downregulation of P2X7R expression resulted in diminished proliferation of murine P19 embryonal carcinoma cells and reduction of gliogenesis. In summary, P2X7R expression and activity is upregulated in embryonic cells for maintenance of pluripotency and proliferation. Downregulation of P2XR expression and activity favors neurogenesis, while gliogenesis rates are decreased under these conditions. P2X7R inhibition has been suggested as a strategy for prevention of neuronal cell death. For this purpose, unilateral hemisphere lesions of the nigrostriatal pathway of adult male Sprague–Dawley rats were induced by stereotactic injection of 6-hydroxydopamine (6-OHDA). One week after lesion, the animals presented rotational behavior when challenged with apomorphine. Treatment with brilliant blue G (BBG) had a beneficial functional effect. Animals that were treated with 6-OHDA and received BBG ($n=6$) during the following 7 days at a 50 mg/kg dose showed a statistically significant decrease in the number of rotations per minute (13 to 4, $p<0.05$) whereas animals receiving only saline did not reveal any significant improvement in rotational tests (11 to 8, $p>0.05$). In agreement, levels of regeneration of dopaminergic neurons in BBG-treated animals, as revealed by antityrosine hydroxylase staining, were twice as high as those observed for the saline-treated control group. The results shown here demonstrate the importance of P2X7 for neurogenesis and regeneration in brain diseases.

Mitochondrial Transplantation to Restore Cellular Bioenergetics After Spinal Cord Injury

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Contusion spinal cord injury (SCI) is a devastating traumatic event in which a mechanical insult to the spinal cord results in excitotoxicity, excessive reactive oxygen and nitrogen species (ROS/RNS) production, and necrotic cell death. Each of these factors then contributes to the development of secondary pathophysiological cascades resulting in oxidative stress, lipid peroxidation, calcium dysregulation, decreased cellular bioenergetics, and delayed apoptotic cell death, which are responsible for increasing the spread and severity of injured tissue. Notably, the loss of mitochondrial function is implicated in many of these pathways, thus a single mitochondrial therapeutic can address many of these issues and could be far reaching in its potential benefits after SCI. Pharmacological treatments that improve mitochondrial bioenergetics after experimental SCI, including providing antioxidant compounds and alternative energy sources, have been shown to foster both neuroprotection and long-term functional recovery. Mitochondrial transplantation has been investigated in various models of tissue injury both in vitro and in vivo and was found to decrease injury size, increase adenosine triphosphate (ATP) production, and normalize cellular respiration. While these transplantation approaches show promise in alleviating tissue damage in other models of injury, no studies have examined the effects of transplanting healthy mitochondria into spinal cord tissue after traumatic injury in an attempt to normalize overall cellular bioenergetics. Such maintenance of cellular homeostasis acutely is expected to be correlated with increased neuroprotection and improved long-term recovery of hindlimb function. With this in mind, experiments were designed to assess whether supplementing the injured spinal cord with healthy mitochondria can potentially provide a multimechanistic neuroprotective approach by targeting critical upstream effectors of the secondary injury cascade, possibly salvaging at-risk tissue that would have otherwise been compromised. Therefore, using a rat model of severe contusion SCI we are testing the hypothesis that intraspinal transplantation of exogenous mitochondria after acute injury increases overall cellular bioenergetics to preserve damaged tissue and provide neuroprotection. Preliminary data indicate that mitochondrial transplantation increases the overall mitochondrial bioenergetics of the injured cord, acutely, and ongoing studies are using transgenically labeled mitochondria to evaluate host cell phenotypes, which incorporate these exogenous organelles. This comprehensive novel approach to target mitochondrial dysfunction may be applied to other central nervous system traumas and diseases that involve mitochondrial pathophysiology.

Common Marmoset Monkey iPSC-Derived Neurons

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The differentiation of neurons for applications in both in vitro disease modeling and translational regenerative medicine is facilitated by somatic cell-derived induced pluripotent stem cells (iPSCs). In that regard, the common marmoset monkey (*Callithrix jacchus*) has been identified as an ideal species for modeling age-related disorders, such as Parkinson's disease (PD), due to their shorter lifespan compared to larger nonhuman primates. While neural differentiation has been achieved from marmoset embryonic stem cells (Cj-ESCs) and iPSCs derived from fetal tissues, production of mature neurons from adult marmoset fibroblast-derived iPSCs has not been reported. The aim of this study was to fill this gap by producing a Cj-iPSC line from adult marmoset skin fibroblasts, generate a protocol for neuronal differentiation of Cj-iPSCs, and characterize the expression of pluripotent, neural ectoderm, neural progenitor, and mature neuronal subtype genes throughout the differentiation process of both marmoset Cj-ESCs (Cj367) and Cj-iPSCs (M8). To reprogram the marmoset skin fibroblasts, skin punch biopsy tissue obtained from a single adult marmoset was immediately cut to smaller samples and explanted to a six-well plate. Once the fibroblasts emerged from the biopsy and expanded to an appropriate number of cells, nonintegrating pluripotency-inducing plasmids were electroporated into the fibroblasts. To verify pluripotency of selected colonies, RT-PCR was performed using primers to the marmoset octamer-binding transcription factor 4 (OCT4), sex-determining region Y box 2 (SOX2), NANOG, Kruppel-like factor 4 (KLF4), LIN28, and C-MYC genes. Neural differentiation was initiated by applying neural induction media at day 0 (1 day after passaging). By day 8 colonies were released from the culture surface and grown in suspension until day 28. While in suspension, polarized neural ectoderm rosettes could be seen developing within the neurospheres. The cells were then dissociated and replated on coverslips for immunocytochemistry (ICC) and treated with neural differentiation medium. The rosette formations, as well as stereotypical neuronal morphology, were observed in as little as 4 h after replating. ICC revealed that nearly all plated cells expressed either nestin, a marker for neural progenitor cells, BIII-tubulin, a marker for neurons, or both. Neurons that were not immediately allocated for ICC displayed dense fiber network outgrowth in subsequent weeks. Our results demonstrate that the M8 line reprogrammed from adult marmoset fibroblasts is morphologically identical to the Cj367 ESC line, and pluripotency mRNA expression was similar between the two cell lines, each expressing all six genes mentioned above. We also observed that both M8 and Cj367 cell lines differentiated efficiently to neural progenitors under basic differentiation parameters, and both produced BIII-tubulin-positive neurons with very sparse glial fibrillary acidic protein (GFAP)-positive glial differentiation. We conclude that adult marmoset fibroblast-derived iPSCs can be differentiated into mature, postmitotic neurons.

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Transdifferentiation of Mesenchymal Stem Cells Into Dopaminergic Neurons for Cell Transplantation

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Parkinson's disease (PD) is a progressive and continuous neurodegenerative disorder. Transplantation of human embryonic dopaminergic (DAergic) progenitors within the striata of PD patients has given the field encouraging results, but ethical concerns and tissue availability limit this approach. The use of mesenchymal stem cells (MSCs) and induced pluripotent stem cells as an alternative cell source for transplantation circumvents the ethical issues and provides a readily available source of cells, as they are derived from adult tissue. This two-part study (in vitro and in vivo) explored the use of MSCs as a cell source for DA neuronal induction prior to transplantation as a means to increase integration within the striatum. To this end, our lab developed a novel adenovirus for the polycistronic expression of multiple genes [achaete-scute complex-like 1 (*Ascl1*), LIM homeobox transcription factor 1, α (*Lmx1a*), and nuclear receptor-related 1 (*Nurr1*)] that are involved in DA neuron differentiation and used green fluorescent protein (GFP) to track transfection. MSCs were cultured with the adenovirus, which resulted in morphological changes as well as expression of GFP as evidenced by fluorescence microscopy. The presence of the viral DNA within the transfected cells was confirmed with PCR. Immunocytochemistry and RT-PCR analyses revealed that cells expressing GFP have nuclear colabeling of ASCL1, LMX1a, and, NURR1, as well as an upregulation of these genes, along with an upregulation of downstream gene targets, such as tyrosine hydroxylase, and the dopamine transporter. These results are indicative of active ASCL1, LMX1a, and NURR1 promoting dopaminergic differentiation. Furthermore these induced DA neuronal-like cells produced dopamine, which has been quantified utilizing high-performance liquid chromatography. Our in vitro results suggest that the approach used in this study may provide a new means of facilitating cell replacement therapy. Following the in vitro study, the in vivo study consisted of transplanting induced DA neuronal-like cells into the unilateral 6-hydroxydopamine (6-OHDA) lesion rat model of PD. The unilateral 6-OHDA lesion was assessed utilizing the cylinder test and amphetamine rotation. Induced DA neuron-like cells were transplanted into the dorsal striatum of rats at 8 and 4 weeks, following verification of the 6-OHDA lesion. The behavioral test exhibited a modest improvement, while histological findings exhibited cell survival and modest integration.

HLA Interactions With Human Cord Blood Cells in a Humanized Mouse Model of Stroke

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The current emphasis in the cell therapy field for stroke is on stimulation of repair processes that improve functional outcome. As a result, less traditional cell therapy approaches have emerged using non-neural cells, unorthodox delivery routes, and targeting nonintuitive physiologic processes. Our approach has been to use cells from human umbilical cord blood (HUCB) to treat stroke. These cells reduce infarct size and improve functional outcome when they are injected intravenously (IV) at delayed time points after stroke onset in rodent models. However, with IV administration the HUCB cells are subject to the full surveillance of the host/recipient immune system. The immune interaction between donor and host in a human-to-human allograft situation is not the same as the interaction in a human-to-rat xenograft paradigm. Therefore, clinically relevant issues such as whether the cells must be human leukocyte antigen (HLA) matched and if immune suppression is required cannot be addressed in the xenograft model. The overall objective of this research project was to establish a mouse model with a functioning human immune system and use it to characterize the cellular interactions between the host/recipient immune cells and the HUCB cells in a model more akin to the clinical situation. Neonatal nonobese diabetic severe combined

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immunodeficient γ chain of the interleukin 2 receptor null (NOD SCID γ; NSG) mice were sublethally irradiated and transplanted with 1×10^5 cluster of differentiation 34-positive ($CD34^+$) HUCB cells intrahepatically. Nonirradiated NSG mice and CD1 mice served as controls. At 8 weeks of age, blood was analyzed to determine the degree of human cell engraftment. The mice were then stroked and transplanted with HLA-matched or mismatched $CD14^+$ HUCB cells. Stroke outcome was measured with the cylinder test and measurement of infarct size at 1 month poststroke. Survival was similar in the "humanized" and nonhumanized NSG mice, although the humanized NSG mice gained weight at a slower rate than did NSG controls. Engraftment of the human cells was 25.3 ± 3.1% of the total number of $CD45^+$ cells. The percent of lymphocytes in normal CD1 mice was 64.8 ± 1.7% but only 24.7 ± 3.3% in NSG controls. In the humanized NSG mice, there were 44.7 ± 1.5% lymphocytes. Liver enzymes indicated the transplants were well tolerated. Analysis of behavioral data and infarct size is underway. Initial results suggest that the humanized NSG mouse may be a useful model for testing interactions between HUCB cells transplanted IV and host immune cells.

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Derivation of Dopaminergic Neurons by Inducing Pluripotency in Malignant Glioma Cells

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Malignant glioma is a well-known primary brain tumor with an unmet medical need in curative therapy. Previously, we have successfully isolated cluster of differentiation 133 (CD133)-positive glioma stem cells (GSCs) from brain tumors and examined the percentage of CD133 expression ratio in different types of brain tumor cells by using flow cytometry. Using lentivirus carrying short hairpin (sh)*CD133*, sh sex-determining region Y box 2 (*Sox2*), or sh*Nanog*, about 40–50% of the cell numbers of glioma cells were reduced within 3 days compared to control group. In addition, we found that manipulation of *CD133*, *Sox2*, or octamer-binding transcription factor 4 (*Oct4*) stemness genes inhibits cell growth in glioma cancer. These data implied that stemness genes influence cell fate. Although cancer is a disease with genetic and epigenetic origins, the possible effects of reprogramming by defined factors remain to be fully understood. We studied the effects of stemness genes on malignant glioma cells. Retroviral-mediated introduction of four inducible pluripotent stem cell (iPSC) genes *c-Myc/Kruppel-like factor 4(Klf4)/Oct4/Sox2* was able to induce the expression of immature status-related proteins, including Tra-1-60, and Tra-1-81 in glioma cancer cells. For in vitro analysis, induced pluripotent stem glioma cells (iPSGCs) showed slow proliferation and were sensitized to differentiation-inducing treatment, and an in vivo teratoma test demonstrated pluripotency of iPSGCs in severe combined immunodeficient (SCID) mice. We further differentiated the iPSGCs into dopaminergic neurons. After differentiation of iPSGCs, the cells expressed neurofilament (NF), microtubule-associated protein 2 (MAP2), and neuronal β III tubulin (Tuj1 antibody clone). Additionally, dopamine transporter (DAT) and tyrosine hydroxylase (TH) were expressed in the differentiated iPSGCs. This study demonstrated not only that pluripotency was induced in the malignant glioma cells but also that the iPSGCs could be differentiated to dopaminergic neurons.

Granulocyte Colony-Stimulating Factor Diminishes Delayed tPA-Induced Hemorrhagic Transformation in Ischemic Stroke Rats via Angiogenesis and Vasculogenesis

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Although treatment with tissue plasminogen activator (tPA) is currently the only drug approved by the FDA for the treatment of stroke, there are two risks; short therapeutic time window (>4.5 h poststroke) and the possibility of hemorrhagic transformation (HT). Thus, extending the narrow time window of tPA is of necessity for future stroke patients. Male Sprague–Dawley rats (9 weeks, 250 ± 30) underwent middle cerebral artery occlusion (MCAo) surgery, and all drugs were administered intravenously at 6 h after MCAo. Experimental groups consisted of vehicle (5% dextrose), tPA (10 mg/kg), and tPA with granulocyte colony-stimulating factor (G-CSF, 300 μg/kg). Infarct size was determined by triphenyltetrazolium chloride (TTC) staining, and modified neurological deficit tests were used to reveal the functional differences for treatment groups. Western blot and immunohistochemistry staining were performed to confirm angiogenesis and vasculogenesis markers such as vascular endothelial growth factor receptor 2 (VEGFR2) and angiopoietin 1 and 2 (Ang-1, -2). Twenty-four hours posttreatment, G-CSF + tPA-treated stroke rats displayed 25% progression in neurological functions and 38.9% reduction of hemorrhage, with Western blots showing 1.9- and 1.2-fold increments in Ang-2 expression in the ischemic cortex and striatum, respectively, and a threefold increase in phosphorylated endothelial nitric oxide synthase expression in the ipsilateral cortex relative to tPA-treated rats. Immunohistochemistry also showed 2- and 2.8-fold increase in von Willebrand factor expression, 3.2- and 2.2-fold increased cluster of differentiation 34-positive ($CD34^+$) expression, and 4- and 13-fold upregulation of VEGFR-2 expression in the ischemic cortex and striatum, respectively, in G-CSF + tPA-treated stroke rats relative to tPA-treated subjects. Taken together, these findings indicate that G-CSF diminished delayed tPA-induced HT likely through the improvement of angiogenesis and vasculogenesis. The ability of G-CSF to protect the vasculature may not only overcome the adverse outcome of tPA but also extend the narrow therapeutic window for stroke patients.

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In Vitro Preconditioning of Human Mesenchymal Stem Cells for Stroke Treatment

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Human mesenchymal stem cells (hMSCs) have been shown to enhance stroke lesion recovery through secretion of trophic factors that mediate inflammation and tissue repair. However, low cell survival and reduced secretory functions posttransplantation of culture-expanded hMSCs are the major barriers limiting hMSC therapeutic efficacy in stroke treatment. In this study, we report the impact of in vitro preconditioning via (a) hypoxia preconditioning and (b) three-dimensional (3D) aggregation on hMSC secretory properties, resistance to ischemic stress, in vivo resistance at stroke lesion site, and stroke lesion recovery. For hypoxia treatment, hMSCs were expanded in 2% O_2. For 3D aggregation, hMSCs spontaneously formed 3D aggregates on ultralow adherent (ULA) culture plates. Prior to hypoxia expansion or 3D aggregation, hMSCs were labeled with superparamagnetic iron oxide (SPIO) nanoparticles for in vivo magnetic resonance imaging (MRI) analysis and then injected intra-arterially in a stroke rat model induced via a middle cerebral artery occlusion. The influence of hypoxia and 3D aggregation on the secretion of anti-inflammatory, proangiogenic, and antiapoptotic cytokines was analyzed. To assess the influence of preconditioning on hMSC in vivo lifespan and stroke lesion recovery, serial MRI at 21.1 T was performed to acquire ^1H and ^{23}Na images of lesion progression and cell migration. The results show that hypoxia preconditioning increases hMSC resistance to in vitro ischemia. On the other hand, aggregate-derived hMSCs are smaller in size and exhibit enhanced stromal cell-derived factor 1 (SDF-1)α-induced migration with increased chemokine C-X-C motif receptor 4 (CXCR4) expression through a caspase-mediated mechanism compared to monolayer cultured cells. Aggregate-derived hMSCs showed enhanced resistance to

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in vitro ischemic stress and a dramatic increase in secretion of multiple growth and anti-inflammatory factors, including interleukin (IL)-10, hepatocyte growth factor (HGF), Stanniocalcin 1 (STC-1), prostaglandin E2 (PGE2), and IL-6. In vivo MRI images of the ischemic stroke lesion showed increased ^{1}H and ^{23}Na signal as evidence of the influx of extracellular water and disruption of ionic homeostasis. Lesion volume analysis show increased recovery for hypoxic hMSCs with statistical significance for ^{23}Na MRI, demonstrating the increased sensitivity with high field ^{23}Na MRI and its importance in stroke and cell therapies. SPIO-labeled 3D cultured hMSCs are localized only within the stroked hemisphere, and after 1 week of initial MRI a decreases in SPIO contrast is evident on T_2*-weighted images. Together, the results demonstrated that hMSC preconditioning via hypoxia or 3D aggregates is an effective strategy that enhances their trophic effects and functional improvement in stroke lesion recovery compared to adherent cells.

Engineered hNSCs for Treating Experimental Spinal Cord Gliomas

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Currently there is no efficacious treatment for clinical spinal cord gliomas. Genetically engineered neural stem cells (NSCs) have been used to convert precursor compounds into oncolytic drugs to kill cancer cells locally due to NSC tumor tropic capability. For the goal of developing a NSC-based therapy for spinal cord gliomas, we first investigated cell interactive outcomes between human NSCs (hNSCs) carrying either cytosine deaminase (F3.CD) or CD-thymidine kinase double genes (F3.CD-TK) and G55 human glioblastoma cells in vitro. F3.CD and F3.CD-TK eliminate tumor cells by metabolizing nontoxic 5-fluorocytosine (5-FC) and 5-FC + ganciclovir (GCV) into 5-fluorouracil (5-FU) and 5-FU + GCV-triphosphate, respectively, to trigger premature DNA chain termination and apoptosis of tumor cells. We determined that prototype hNSCs did not stimulate tumor cell growth, and F3.CD-TK regimen had a significantly stronger tumor suppression effect than F3.CD treatment in vitro. We next developed a rat spinal cord tumor model that emulates representative clinical pathophysiology. G55 cells were implanted into C6 spinal cord. Seven days later, DiI-labeled F3.CD, F3.CD-TK 7, or control cell debris was implanted at 1 mm rostral and caudal to the tumor site, followed with repeated 5-FC and GCV administrations. Data collection included systematical evaluation of autonomic parameters of respiratory function, blood pressure, and body temperature in addition to locomotion performance. Postglioma survival was defined by capability to perform consistent weight-bearing hindlimb locomotion. All rats developed spinal cord pathologic signs resulting from cervical glioma growth. Rats treated with F3.CD-TK plus 5-FC and GCV showed significantly increased survival relative to controls receiving either F3.CD or cell debris, as well as significantly less respiratory deficits and better maintained mean artery blood pressure. Moreover, less severe body temperature decrease in F3.CD-TK and F3.CD-treated groups was observed relative to the cell debris-treated controls. Pathological outcome shows that F3-hNSCs migrated into the tumor mass and were in close contact with glioma cells. There was significantly increased G55 apoptosis around the infiltrated F3.CD-TK and F3.CD, relative to cell debris-treated group. The data suggest that F3.CD-TK cell therapy significantly improved autonomic function in rats with cervical glioma and markedly increased their survival posttumor implantation.

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