

## Abstracts for the 11th International Neural Transplantation and Repair Meeting Held in Conjunction With the 18th Annual Meeting of the American Society for Neural Therapy and Repair

### Presidential Symposium: What Are the Important Challenges for Neural Therapy in the 21st Century?

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In June of 1984, a seminal scientific meeting entitled “Transplantation in the Mammalian CNS” was held in Sweden. This meeting brought together the world leaders in the field of neurological repair to address the state of knowledge regarding the biology underlying the response of the CNS to neural transplantation. This meeting also marked the first forum assessing the therapeutic prospects for grafted cells to restore neurological function. This meeting marked the advent of a new era for research into CNS repair and, following subsequent meetings in Rochester (USA) and Cambridge (UK), these triannual scientific congresses were established as the International Neural Transplantation and Repair (INTR) Meetings. At the time of the 1984 meeting, transplanted cells were somewhat mysterious “spare parts” that integrated into the circuitry with various degrees of success. The CNS was seen as an immunoprivileged recipient tissue and grafted cells, though stimulating regeneration in certain conditions, did so by unknown mechanisms. Manipulating gene expression in cells through *ex vivo* or *in vivo* genetic therapy approaches had not yet been envisioned. The existence of endogenous neural stem cells in the mature CNS was a discovery (or rediscovery) that was still in the future. Now 27 years later, we are again on the brink of a new era in research with progress in many areas including stem cells, epigenetic regulation, gene and cell therapy, and medicinal chemistry of small molecules. We have experimental and analytical tools that far surpass the resources available to investigators in 1984. Likewise, our understanding of neurodegenerative pathophysiology and the biology of CNS regeneration has substantially evolved. In the 11th INTR meeting, we will hear of the latest advances in neurological restoration. But what key areas still represent *terra incognita*? What fundamental understanding of cell biology do we lack or technological barriers still exist that, once overcome, will usher in new advances? What research focus will turn out to be truly transformative in regard to how we understand disease, injury, and regeneration in the CNS? To help us envision the answers to these questions, the Presidential Symposium of the 11th INTR meeting features perspectives from three leading scientists, Anders Björklund (Lund University, Sweden; one of the organizers of the 1984 meeting), Ron McKay (NIH, USA), and Hideyuki Okano (Keio University, Japan). Each speaker will present a talk describing their perspective of our state of knowledge and the challenges that need to be overcome to advance neurological repair. This will be followed by a forum discussion with the three speakers. We anticipate the stimulating discussion that will arise from this symposium will provide a conceptual roadmap that will help guide the research efforts of the next generation of investigators.

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### Human Neural Stem Cell Transplantation Restores Cognitive Function Following Cranial Irradiation

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Radiotherapy of brain tumors is beneficial, but can cause debilitating side effects on cognitive function. Nearly 200,000 patients/year receiving cranial radiotherapy are at risk for developing cognitive dysfunction, especially pediatric patients can lose up to 3 IQ/year. Currently, there are no satisfactory, long-term solutions for this serious side effect. Radiation-induced stem cell depletion and the resultant inhibition of neurogenesis is one of the pathogenic explanations for cognitive decline. Previously, we successfully showed that transplanted human embryonic stem cells (hESCs) could reverse radiation-induced cognitive impairment, thereby providing the first evidence that such a strategy might hold promise to improve quality of life (Acharya et al., PNAS, 2009). While encouraging, use of hESCs is not without risk because of the possibility of teratoma formation. Thus, to circumvent these concerns, we explored the capability of using human neural stem cells (hNSCs) as alternative therapeutic agents for transplantation after cranial irradiation. Prior to grafting, hNSCs were expanded and labeled with bromodeoxyuridine (BrdU). Athymic nude rats subjected to 10 Gy head-only irradiation were transplanted 2 days afterward with hNSCs at four distinct hippocampal sites (100,000 cells/site; 400,000 cells/hemisphere). Control (nonirradiated) and irradiated animals receiving vehicle served as sham surgery groups. At 1 or 4 months post-grafting, rats were tested on hippocampal-dependent novel place recognition task. Irradiated animals exhibited significant impairment on short- and long-term memory function compared to nonirradiated controls. In contrast, irradiated animals transplanted with hNSCs showed significant improvements in spatial memory and cognitive performance that was comparable to controls. These results strongly suggest that hNSC grafting ameliorated radiation-induced cognitive impairment by preserving short- and long-term hippocampal-dependent memory. Immunocytochemical analyses of brain sections from 1- and 4-month postgrafting animals revealed extensive migration of grafted cells throughout the host hippocampus. Grafted cell survival, quantified by unbiased stereology, revealed that 23% and 12% of the grafted cells survived at 1 and 4 months, respectively. These numbers indicate that as few as 100,000 surviving cells in the irradiated brain were sufficient to ameliorate radiation-induced cognitive deficits. Dual immunofluorescence analyses and confocal microscopy revealed that grafted hNSCs differentiate into neuronal/glial lineages. Notably, 11% of the grafted hNSCs expressed behaviorally induced activity-regulated cytoskeleton-associated protein (Arc) and displayed neuronal morphology, suggesting their capability to functionally integrate into the host hippocampus. These findings provide evidence that grafted hNSCs can survive, differentiate along neural lineages, and functionally integrate into the irradiated hippocampus. Importantly, engrafted cells restored cognitive impairments following cranial irradiation. Therefore, stem cell-based therapy may provide a long sought after clinical intervention for reversing the adverse effects of radiotherapy on cognition that may benefit millions of cancer survivors worldwide.

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### One-Week Treatment With VEGF Decreases Cell Proliferation in the Subgranular Zone of the Dentate Gyrus

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Vascular endothelial growth factor (VEGF) is critical for blood vessel growth in the developing and adult nervous system of vertebrates. Several recent studies demonstrate that VEGF also promotes neurogenesis, neuronal patterning, neuroprotection, and glial growth. For example, the circulating levels of VEGF increase after exercise, and blocking the action of peripheral VEGF abrogated the exercise-induced production of new neurons. Furthermore, short-term treatment of VEGF (3 days) stimulated an increase in proliferating 5-bromo-2-deoxyuridine (BrdU<sup>+</sup>) labeled cells and neurogenesis in the hippocampus *in vitro* and *in vivo*. This study suggested a mechanism of action through the VEGF receptor FLK1 present on neural progenitor cells (NPC). However, relatively little evidence exists on longer term treatment effect of *in vivo* use of VEGF. Therefore, with this *in vivo* study, we aim to investigate the effect of VEGF treatment for 7 days on cell proliferation and neurogenesis. Three groups of male Fischer 344 rats, 3, 12, and 22 months old, were used. VEGF or control was infused in the left lateral ventricle, using an osmotic minipump (Alzet, Model 2004 pumping rate, 0.25  $\mu$ l/h; total volume 200  $\mu$ l). Before implantation, the pumps were incubated in sterile saline for at least 48 h at 37°C to prime the pumps. For implantation, the rats were anesthetized with isoflurane and placed in a stereotaxic frame. A guide cannula was stereotaxically implanted in the left lateral ventricle and connected to the osmotic minipump, which was inserted subcutaneously. Pumps were weighed before implantation and at the end of the experiment to ensure complete delivery of their content. The infusion started on the day of the surgery and continued for 14 days. During the first 7 days all animals were infused with saline. On day 8 the minipump was replaced with a new pump filled with VEGF (10  $\mu$ g/ml). On day 15, all animals received intraperitoneal injection of BrdU (50 mg/kg, IP). Twenty-four hours later animals were anesthetized and perfused with saline followed by 4% paraformaldehyde and brains were removed and cut at 40  $\mu$ m. Immunohistochemistry for BrdU was performed on every third section throughout the entire hippocampus. Unbiased stereological analyses with an optical fractionator revealed that a 7-day treatment of VEGF significantly decreased neural cell proliferation in the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus. These results were consistent in all age groups. The results of this study suggested that treatment with VEGF for 7 days negatively affects cell proliferation, in contrast to work demonstrating that 3 days of treatment with the same dose of VEGF increased proliferation of neural progenitors in aged rats. In order to further test this hypothesis we are planning on looking at doublecortin (DCX) neurogenesis marker, NeuN mature neuronal marker, and apoptotic marker caspase 3.

### Intracortical Delivery of an Adeno-Associated Viral Vector Expressing the Glutamate Transporter (GLT-1) Decreases Stroke-Induced Extracellular Glutamate Overflow and Cerebral Infarction After Transient Middle Cerebral Artery Occlusion in Rats

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Following the onset of an ischemic brain injury, the excitatory neurotransmitter glutamate is released. The excitotoxic effects of glutamate are a major contributor to the pathogenesis of a stroke. The aim of this study was to examine if overexpression of a glutamate transporter (GLT-1) reduces ischemic brain injury in a rat model of stroke. Clearance of glutamate was characterized by *in vivo* amperometry through glutamate-specific electrodes and microdialysis at 3 weeks after intracerebral injection of adeno-associated viral vector expressing the rat GLT-1 cDNA (AAV-GLT-1) or GFP (AAV-GFP) in nonstroke and stroke rats. Tissue damage was assessed at 1 and 2 days post-middle cerebral artery occlusion (MCAo) using TUNEL and TTC staining, respectively. Behavioral testing was performed at 2, 8, and 14 days poststroke. Expression of the rat GLT-1 protein was confirmed by Western blot analysis and immunostaining in HEK293 cells and primary cortical neurons after transfection. Pretreatment with AAV-GLT-1 increased glutamate clearance rate in nonstroke rat brain, measured by amperometry. Animals receiving AAV-GLT-1, compared to AAV-GFP, showed significant decreases in the duration and magnitude of extracellular glutamate, measured by microdialysis, during the 60-min MCAo. A significant reduction in brain infarction and DNA fragmentation was observed in the region of AAV-GLT-1 injection. Animals that received AAV-GLT-1 showed significant improvement in behavioral recovery following stroke compared to the AAV-GFP group. Overexpression of the glutamate transporter, GLT-1, significantly reduces ischemia-induced glutamate overflow, decreases cell death, and improves behavioral recovery.

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### CDNF Protects the Nigrostriatal Dopamine System and Promotes Recovery After MPTP Treatment in Mice

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Cerebral dopamine neurotrophic factor (CDNF) is a recently discovered protein, which belongs to the evolutionarily conserved CDNF/MANF (mesencephalic astrocyte-derived neurotrophic factor) family of neurotrophic factors. CDNF has been shown to contribute to the survival of midbrain dopamine neurons *in vivo*. The degeneration of dopamine neurons following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment is well characterized and efficacy in this model is considered a standard criterion for development of parkinsonian therapies. MPTP is a neurotoxin that produces parkinsonian symptoms in humans and in C57/Bl6 mice. There have been no reports to date about the effects of CDNF on dopamine neuron survival or

function in the MPTP rodent model, a critical gap. Therefore, we studied whether bilateral CDFN injections into striatum of C57/Bl6 mice have neuroprotective and neurorestorative properties for the nigrostriatal dopamine system after MPTP injections. We found that bilateral striatal CDFN injections, given 20 h before MPTP exposure, improved horizontal and vertical motor behavior when measured 2 weeks afterwards. In addition, CDFN pretreatment increased tyrosine hydroxylase (TH) immunoreactivity in the striatum and in the substantia nigra pars reticulata (SNpr), as well as number of TH-positive cells in substantia nigra pars compacta (SNpc). Posttreatment with CDFN, given 1 week after MPTP injections, increased horizontal and vertical behavior of mice. Furthermore, dopamine fiber densities in striatum and the number of TH-positive cells in SNpc were increased after CDFN injections. We conclude that intra-striatal CDFN administration is both neuroprotective and neurorestorative for the nigrostriatal dopamine system in the MPTP model, which supports the development of CDFN-based treatment strategies for Parkinson's disease.

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#### Developing MRI-Based Biomarkers for Early Diagnosis of Parkinson's Disease

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Parkinson's disease (PD) is currently considered a systemic disease with complex motor disorders and nonmotor deficits that appear before or in parallel with motor deficits and then worsen with disease progression. It has been well documented that PD is also characterized by a long preclinical phase (from the onset of dopamine neuron loss to the onset of motor symptoms), which could last for many years or even decades. Thus, there is an urgent need to develop imaging modality to screen individuals who may be in the preclinical phase of PD for earlier diagnosis and treatment to slow down or stop progression of the disease. Our group has been trying to develop magnetic resonance imaging (MRI)-based imaging biomarkers for early detection of PD because the imaging modality (MRI) is noninvasive, sensitive, specific, reproducible, and cost-effective. In addition, we also use MRI techniques to map a specific dopamine agonist such as apomorphine- or *d*-amphetamine-induced activation in a living brain. The imaging methods, called pharmacological MRI (phMRI), have demonstrated that phMRI can detect functional deficiency of the nigrostriatal system by showing stronger activation in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned striatum versus the unlesioned side of the same structures in hemiparkinsonian monkeys rendered by unilateral administration of MPTP (a neurotoxin for dopamine neurons) and correlate with severity of parkinsonian features (Zhang et al., 2006). We also demonstrated that phMRI can be used to monitor therapeutic effects in PD monkeys (Luan et al., 2008). Recently, we found that phMRI can also detect functional deficiency of the nigrostriatal system even in asymptomatic parkinsonian monkeys in which there were no obvious motor deficits but with dysfunctions of the dopamine system judged by potassium- or *d*-amphetamine-evoked dopamine release measured by *in vivo* microdialysis, number of dopamine neurons in the midbrain, and dopamine fiber density in the striatum. The asymptomatic PD model was developed by using later middle-aged rhesus monkeys and a relatively low dose of MPTP. Those animals were monitored up to 18 months by a computerized behavioral testing battery. The results of phMRI regarding dopamine deficiency were further validated by a positron emission tomography (PET) study with [<sup>11</sup>C]DTBZ BP as a vesicular monoamine transporter-2 (VMAT-2) tracer. In the PET study, asymptomatic PD monkeys showed about 49.5% dopamine deficiency in the MPTP-lesioned putamen (unpublished pilot data). Taken together, these novel findings support the notion that phMRI effect has the potential to be developed as a nonin-

vasive, sensitive, specific, reproducible, and cost-effective state marker for PD, especially for detection of earlier stages of the neurodegenerative disease.

#### Microinjection of the GABA-Enhancing Drug Vigabatrin in an Acute Seizure Model as a Strategy for Identifying Targets for Neural Transplantation With GABA-Producing Cells

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Resistance to antiepileptic drugs (AEDs) is a major problem in epilepsy treatment and affects 30–40% of epileptic patients and an even higher portion of patients suffering from temporal lobe epilepsy. One promising approach to overcome this problem is the neural transplantation of  $\gamma$ -aminobutyric acid (GABA)-producing cells into appropriate target regions within the brain. Inhibition of the substantia nigra pars reticulata (SNr), a main basal ganglia output structure, has repeatedly been shown in experimental epilepsy to be a highly interesting approach for modulation of different seizure types emanating from the limbic system. The AED vigabatrin irreversibly inhibits the degradation of GABA, resulting in an elevation of the GABA concentration in the synaptic cleft. In the present study, we used microinjection of vigabatrin to identify the most promising target region within the basal ganglia network for inducing robust anticonvulsant effects by subsequent grafting of GABA-producing cells. Because pentylenetetrazole (PTZ) acts as a GABA antagonist, the PTZ seizure threshold test is particularly sensitive to GABA-potentiating manipulations, thereby representing an efficient nonlaborious model for the screening of therapeutic approaches that increase GABAergic inhibition. By using the timed intravenous PTZ infusion seizure threshold test, we now examined (1) whether and with which time course systemic application of vigabatrin is effective in the PTZ seizure threshold test, (2) whether and with which time course local application of vigabatrin into functionally distinct subregions of the SNr is anticonvulsant, and (3) whether and with which time course anticonvulsant effects also occur after local application of vigabatrin into the subthalamic nucleus (STN), which in comparison to the SNr is poorly investigated as a target structure for focal epilepsy therapy. We evaluated the seizure susceptibility of adult female Wistar rats before and after systemically or locally applied vigabatrin, respectively. The PTZ seizure threshold was determined at least 48 h prior to vigabatrin injection and again either 6, 24, 48, or 96 h after vigabatrin treatment. We found a significant anticonvulsant effect after systemic treatment with vigabatrin (600 mg/kg, IP), with a maximum efficacy 6 h after application. For local microinjection of vigabatrin, rats were anesthetized with isoflurane (induction 3%, maintenance 1.5%), which did not influence PTZ seizure threshold 6 h after anesthesia. Vigabatrin (40  $\mu$ g/ $\mu$ l) was injected bilaterally in a volume of 250 nl into the SNr or STN, respectively, during surgery. Microinjection of vigabatrin into subregions of the SNr as well as into the STN clearly proved that local increase of inhibition within these target regions is as effective as systemic treatment with vigabatrin. The maximum efficacy here was 24 h after microinjection. Our studies demonstrated that, apart from the SNr, the STN is a promising target structure for neural transplantation of GABA-producing cells in epilepsy (see Abstract by Gernert et al., this Meeting).

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### Behavioral Improvement Without Dopamine After Hematopoietic Stem Cell Transplantation in MPTP-Treated Primates

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Previous findings from our laboratory indicate that human umbilical cord blood, particularly the fraction of CD133 stem cells, yields significant behavioral and histological improvements in a rodent model of Parkinson's disease. The present study examined the effects of striatal CD133 transplantation with and without pretreatment with glial cell line-derived neurotrophic factor (GDNF) striatal transfection. The specific aim of our study was to determine the viability and potential of CD133s in a GDNF-enriched environment to repair, replace, or regenerate the loss of dopamine in the striatum of in bilateral 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated parkinsonian-like primates. To test the hypothesis, cynomolgus monkeys ( $n = 10$ ) were lesioned with both a right intracarotid and systemic injections of MPTP. The monkeys were symptomatically stable for over 2 years before adeno-associated virus (AAV) transfection with GDNF ( $n = 5$ ) or needle sham lesion ( $n = 5$ ) 1 month prior to transplantation with CD133 stem cells ( $n = 10$ ) into the right putamen. Timed arm-reach tasks and clinical rating scores on/off L-dopa were performed weekly and before sacrifice. One monkey from each group was sacrificed at 1 month after transplantation and the remainder at 4 months. Tissue sections were stained for tyrosine hydroxylase (TH), dopamine transporter (DAT), CD133 human nuclei (CD133), or double labeled for DAT and CD133 for cell counts. All subjects demonstrated improvement in both behavioral assessments. Clinical rating scores were unchanged ( $p = 0.122$ ) in "off" 2 years after MPTP but significantly improved in only the GDNF group at 4 months postop ( $p = 0.018$ ). The GDNF group Gash tests (timed movement) also significantly improved ( $p = 0.04$ ). There was also improvement ipsilateral to the graft ( $p = 0.03$ ). When transplanted into a GDNF-enriched striatum more CD133 stem cells remain at the injection site. TH-positive cells were rare. CD133 cells were found in the substantia nigra. The potential benefit of the CD133 in the striatum and nigra remains to be explored.

### Dopaminergic Neurons Derived From Human Embryonic Stem Cells for the Treatment of Parkinson's Disease by Transplantation. A Characterization and Functional Analysis Study

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Human embryonic stem cells (hESC) from cell line H1 (hESC H1) were studied at two planned stages of differentiation: human neural stem cells and hESC-derived dopamine neurons in order to carry out in vivo studies in monkeys. To identify transplanted donor cells, hESC H1 were infected with a lentivirus carrying a GFP fluorescent marker protein. Characterization of the pattern of expression of genes involved in the dopaminergic differentiation process was performed on our GFP-positive cells at each step of the differentiation protocol by reverse transcription polymerase chain reaction (RT-PCR). Differentiation studies on these cells using in vitro methods such as immunofluorescence analysis revealed that 90% of the cells had a neuronal phenotype and 17% of the cells were tyrosine hydroxylase (TH)-posi-

tive cells. Functional analyses were also performed on TH-positive neurons measuring dopamine (DA) release in response to membrane depolarization. Preliminary studies suggest that the TH-positive neurons are able to secrete DA and the release of DA is activity dependent. Finally, we have performed implantations of these ESC-derived TH neurons into MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-exposed monkey brains. We have also implanted DA neurons from the H9 hESC line in collaboration with M. Daadi. Preliminary data allowed us to confirm the short-term cell survival and stability of the dopamine phenotype of differentiated cells implanted.

### A Model of Human Embryonic Stem Cell (hESC)-Based Neocortical Development

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Development of the cerebral cortex involves sequential specification of forebrain cortical progenitors to various neuronal subtypes that will subsequently form the different cortical layers. Modeling of this temporally sensitive process would enable mechanistic studies of neocortical development and consequently open new doors for drug discovery and treatment of neurological afflictions. Previous studies have described the generation of cortical tissue from human embryonic stem cells (hESCs); however, only the early events of cortical development were able to be reconstructed. Furthermore, the intrinsic differentiation mechanisms of hESCs to forebrain cortical progenitors remain poorly elucidated. Here we studied effects of Wnt and Nodal signaling on the differentiation of hESCs to forebrain progenitors. Previous findings in mouse ESCs suggest that Wnt signaling inhibition but not inhibition of Nodal signaling promotes a dorsal forebrain fate. To examine Wnt and Nodal signaling effects, undifferentiated hESCs were cultured in the presence of fibroblast growth factor-2 (FGF-2), differentiated to embryoid bodies on day 6, and to neural tube-like rosettes on day 16, with periodic treatment with a Wnt antagonist (Dkk-1) and/or Nodal antagonists (LeftyA and SB431542) once every 2 days. In contrast to mESCs, the inhibition of Nodal signaling in hESCs promoted specification to dorsal forebrain progenitors, as determined by the expression of forebrain markers BF1 and Otx2, and dorsal forebrain marker Pax6 and EMX1. Wnt antagonists had no effect on the dorsal forebrain differentiation, and the combination of Wnt and Nodal antagonists was similar to that of the Nodal antagonists alone. Notably, Nodal signaling inhibitors induced a dorsal forebrain fate through hindering hESC pluripotency and suppression of mesodermal and ectodermal fates. Rosettes that had formed by day 16 were manually isolated and grown in the presence of FGF-2, brain-derived neurotrophic factor, and neurotrophic factor-4. This yielded neurospheres consisting of homogeneously Pax6<sup>+</sup> rosettes at day 24, which were seeded on poly-ornithine/laminin-coated plates and differentiated for 3 weeks. As a result of neurons migrating from the colonies along radial glia scaffolding, deep-layer CTIP2<sup>+</sup> cortical neurons appeared after the first week of differentiation and upper-layer SATB2<sup>+</sup> cortical neurons had formed after 3 weeks. These findings illustrate that layer-specific neurons generated from Pax6<sup>+</sup> forebrain progenitors follow a temporal patterning similar to that observed in the developing human neocortex. After 3 weeks, more than 80% of neurons generated were glutamatergic, expressing glutamate transporters VGLUT1 and VGLUT2, while less than 20% of neurons were GABAergic ( $\gamma$ -aminobutyric acid-ergic) and expressed the GABA transporter, VGAT. This ratio is similar to that found in human embryonic cortex. This model recapitulates neocortical development and can be applied to the study of various neurological disorders, and thus will be an invaluable tool for understanding mechanisms involved in disorders of neocortical development and for the development of pharmaceutical agents for application to disorders involving development of the neocortex.

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### Zebrafish *Dbx1a*<sup>+</sup> Cells Are a Resident Neuronal Stem Cell Population Contributing to De Novo Neurogenesis After Spinal Cord Injury

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*Dbx1a* labels a multipotent progenitor population in the embryonic zebrafish spinal cord. These cells also exist in the embryonic mammalian spinal cord, and in both animals give rise to multiple spinal cord cell types: neurons, oligodendrocytes, astrocytes, and radial glia. Using a transgenic line expressing enhanced green fluorescent protein (eGFP) under predicted *dbx1a* enhancers, we show that labeled cells dramatically slow their proliferation and neurogenesis at larval stages. Interestingly, following spinal cord transection, zebrafish are able to regenerate neurons, allowing functional recovery, while mammals cannot. Following spinal cord transection in larvae, *dbx1a*<sup>+</sup> cells proliferate and undergo de novo neurogenesis. These findings now give us a tool to determine the regulatory pathways responsible for the ability to regenerate the spinal cord after injury.

### The Proliferative Effects of FGF-2 Depend on Activation of the VEGF Receptor, Flk-1, in the Adult Hippocampus

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Stem cells are readily expandable and can differentiate into the various neural cell phenotypes. The use of stem cells for CNS repair will require their directed recruitment into the needed phenotypes, regardless of whether the cells are endogenous to the CNS or exogenous cell transplants. Furthermore, as many diseases needing stem cell therapies have increased incidence with aging, it is important that preclinical studies evaluate therapeutic potential for directing stem cell differentiation in the aging CNS environment. At present, there is little information about how cell therapies will be affected by an aged environment. We investigated age-related environmental changes in neurogenic regions containing stem cells with the potential to contribute to endogenous repair, as well as nonneurogenic regions that lack neural stem cells but are likely targets for cell recruitment. We found age-related and heterogeneous changes in cell phenotype gene expression, and protein levels for two astrocytic growth factors, vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2). Both factors have neuroprotective and neurogenic properties following injury and disease, and we asked if their impaired signaling could alter the behavior of endogenous stem cells. First, we looked at the effects of blocking VEGF signaling via its receptor Flk1 on both the proliferation of endogenous neural stem cells under baseline conditions and following an insult to the CNS. We determined that VEGF is necessary for both cycling under normal conditions and during an endogenous neural stem cell-mediated regenerative response triggered by AraC administration. Next, we looked at how the lack of VEGF would affect the mitotic effects of FGF-2, as both work synergistically in other systems, and found that FGF-2 depends upon VEGF to promote its mitotic effects. These results indicate that growth factors not only directly influence cells necessary for endogenous repair, but they may also affect how other factors function. Understanding the potential interactions of growth factors in young and aged tissue improves our understanding of endogenous repair, and provides insights necessary to recruit stem cells to contribute to repair.

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### Altered Striatal Dopamine Release in DSP-4 Lesioned Rats

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In addition to loss of dopamine neurons in Parkinson's disease (PD), noradrenergic neurons in the locus coeruleus degenerate. Increased dopamine neuron vulnerability has been demonstrated in animals with noradrenergic depletion in the locus coeruleus, and a recent study demonstrated increased ventral mesencephalic graft survival when cografed with locus coeruleus. To elucidate the effects of the noradrenergic input to the dopamine system, Sprague-Dawley rats were systemically injected with DSP-4 [*N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine], causing selective degeneration of noradrenergic neurons. Because the cerebral cortex is extensively innervated by locus coeruleus, the noradrenergic nerve fiber content was evaluated by measuring the dopamine  $\beta$ -hydroxylase (DBH) immunoreactivity in the cortex. Significant decrease in DBH nerve fiber density was seen. Approximately 20% of the cortical DBH-positive nerve fibers were still present at 3 days and ~10% at 3 months after DSP-4 administration. At 6 months postinjection, some regeneration had occurred. In vivo chronoamperometry was utilized to measure dopamine release in the striatum at 3 days, 3 and 6 months after DSP-4 administration. The measurements revealed significantly increased peak amplitudes of potassium-evoked dopamine release over time, at 3 days and 3 months, in DSP-4-lesioned rats compared to normal control rats. However, at 6 months after DSP-4 administration, peak amplitudes were comparable to that found in controls. In conclusion, these findings shed light on the interaction between the dopaminergic and the noradrenergic system and its possible relevance in the etiology of PD.

### Hydrogel Characteristics That Are Beneficial to the Fate of Neural Cell Populations: An Eye on the Future of Cell Transplants

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The survival rate for neural stem cells or fetal brain tissue grafts after transplantation into the brain is typically very low, leaving some to wonder if more effective therapeutic results could be obtained if the numbers of surviving cells were increased. Most cell death occurs 7–14 days after transplantation when the neuroimmune response has been triggered by penetrating the brain in order to place the cells. One potential way to improve cell survival is to incorporate the transplanted cells into a protective carrier, such as a biodegradable hydrogel. Hydrogels have been used for decades in drug delivery and tissue reconstruction of the body, but they have only begun to be exploited for use in the brain recently. Before hydrogels can be used as cell carriers in the brain two issues must be addressed: 1) What physical characteristics of the hydrogel allow cells to survive and thrive on the inside? 2) How does the brain, as an organ, react to the presence of the hydrogel once implanted? We have tried to address both questions in a series of papers whose main conclusions will be presented. Using hydrogels as cell carriers, we found that primary neural cell populations, derived from the forebrain of an e14–15 rat embryo, were significantly affected by the physical stiffness (compressive modulus) of the hydrogel, affecting survival, proliferation, and the ratio of neurons to astrocytes to neural progenitor cells. Furthermore, at compressive modulus measures permissive for cell survival, the neural cells initially survived better when incorporated into a hydrogel carrier (3D) than

when seeded on the hydrogel surface (2D); however, cells growing on the surface showed greater proliferation. When hydrogel strands were implanted into normal rodent brain, the host brain had a significantly attenuated neuroimmune response compared to sham implants, which were given a needle penetration only. Astrocytes and microglia were found in fewer numbers around the hydrogel and typically had a less reactive morphology. While the initial compressive modulus for all hydrogel strands remained constant, we manipulated the rate of degradation of the hydrogels implanted into the brain, using a fast degrading, slow degrading, and a nondegrading hydrogel composition. While all hydrogels were less immunoreactive than the needle penetration, the neuroimmune response was significantly different between the fast degrading hydrogel and the slow or nondegrading hydrogel. In these studies, we explored how cell survival and the host brain response are functions of the compressive modulus and degradability of the hydrogel. These are only two physical variables in a sea of many physical and chemical variations that are possible. Optimizing hydrogels as cell carriers for transplantation into the brain may be an arduous task; however, with these studies we have begun to establish rules of thumb regarding at least the physical characteristics of hydrogels.

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### Neural Stem Cells Improve Cognition Via BDNF in a Transgenic Model of Alzheimer's Disease

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Neural stem cell transplantation represents an unexplored approach for treating neurodegenerative disorders associated with cognitive decline. In Alzheimer's disease, beta-amyloid (A $\beta$ ) plaques and tau-laden neurofibrillary tangles accumulate in several brain regions, leading to synaptic dysfunction and cognitive deficits. Here we show that neural stem cells (NSCs) transplanted into the hippocampus of Alzheimer's transgenic mice rescue spatial learning and memory deficits. Remarkably, cognitive function is improved without altering A $\beta$  or tau pathology. Instead, the mechanism underlying the improved cognition involves a robust enhancement of hippocampal synaptic plasticity, mediated by brain-derived neurotrophic factor (BDNF). Gain-of-function studies show that delivery of recombinant BDNF mimics the beneficial effects of NSC transplantation. Furthermore, loss-of-function studies show that depletion of NSC-derived BDNF fails to improve cognition or restore synaptic plasticity. Taken together, our findings demonstrate that neural stem cells can ameliorate complex behavioral deficits associated with widespread Alzheimer's disease pathology via BDNF.

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### Differential Effects of the Inflammogen, Lipopolysaccharide, on Animals With a Partial Deletion of the GDNF Receptor, GFR $\alpha$ 1, Gene

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Previous studies have demonstrated that in order for glial cell line-derived neurotrophic factor (GDNF) to exert its dopaminergic (DAergic) neuroprotective effects, the GDNF receptor, GFR $\alpha$ 1, is required in combination with rearranged during transfection (RET) receptor tyrosine kinase. Inflammation is thought to contribute to the supply of

neurotrophic factors, including GDNF, by increasing its expression/excretion from activated microglia/astrocytes. Studies using the endotoxin lipopolysaccharide (LPS), a potent inflammogen, show that systemic insults can trigger prolonged microglial activation and proinflammatory cytokine production and neuronal degeneration. LPS administration is known to lead to detrimental effects on hippocampal-dependent behaviors in rodents; however, the role of GDNF signaling for these effects has not been explored. To examine effects of the combined insult of LPS and reduced GDNF receptor levels, we administered LPS (1 mg/kg, IP) once a month for 3 months starting at 7 months of age to male wild-type (WT) and GDNF receptor heterozygous (*Gfra1*<sup>+/-</sup>) mice. One month following the last LPS injection, the mice underwent a battery of behavioral testing, including locomotor activity, accelerating rotarod, and water radial arm maze (WRAM), using a win-shift paradigm with four of the eight arms baited with escape platforms. At 10 months of age, *Gfra1*<sup>+/-</sup> mice treated with LPS demonstrated a greater reduction in motor activity compared to saline-treated (*Gfra1*<sup>+/-</sup> and LPS-treated WT mice. Furthermore, *Gfra1*<sup>+/-</sup> mice treated with LPS performed worse when tested in the WRAM than the other treatment groups, especially during trial 4, when memory load was highest. The impaired performance of *Gfra1*<sup>+/-</sup> mice treated with LPS at the highest memory load was present in both the reference and working memory component of the task. The behavior data will be combined with immunohistochemical analysis to correlate these behavioral effects with structural alterations in the limbic system. Thus, these data demonstrated that memory and motor impairment following repeated LPS exposure was significantly exacerbated by a partial genetic reduction of Gfr $\alpha$ 1.

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### Stem Cells and Gene Therapies for Adenosine Augmentation in Epilepsy

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Adenosine is an endogenous anticonvulsant of the brain and is implicated in seizure termination and postictal refractoriness. Research from our lab has identified dysregulation of adenosine-based neuromodulation in epilepsy as a major contributor to seizure generation. Thus, astrogliosis—a pathological hallmark of the epileptic brain—leads to overexpression of adenosine kinase (ADK), the major adenosine metabolizing enzyme. Overexpression of ADK results in focal adenosine deficiency, which per se and in the absence of any other epileptogenic event is sufficient to trigger seizures. Likewise, transgenic or virus-induced overexpression of ADK triggers seizures. Based on this conceptual framework, focal adenosine augmentation therapies (AATs) constitute a neurochemical rationale for therapeutic intervention. To meet the therapeutic goal of focal adenosine augmentation, mouse and human stem cells have been engineered to secrete adenosine, either based on a biallelic genetic disruption of the *Adk* gene, or based on the introduction of a lentiviral vector expressing an inhibitory microRNA directed against *Adk*. Intra-hippocampal implants of neural progenitor (NP) cells derived from either human or mouse embryonic stem cells (ESCs) form dense grafts within the infra-hippocampal fissure and differentiate into neurons within the CA1 region. Intra-hippocampal implants of ADK-deficient mouse ESC-derived NPs robustly suppressed kindling epileptogenesis in the rat. Most importantly, when transplanted into the infra-hippocampal fissure of mice 24 h after the intra-amygdaloid injection of kainic acid, the same cells significantly reduced astrogliosis, normalized the expression levels of endogenous ADK, and completely prevented the emergence of spontaneous recurrent seizures 3 weeks after kainic acid injection. This is a time point at which recipients of wild-type cells or of a sham injection displayed prominent astrogliosis, overexpression of ADK, and recurrent seizures at a rate of more than four seizures per hour. In a first step to develop personalized stem cell therapies for epilepsy, human mesenchymal stem cells with a knockdown of ADK were shown to suppress acute

hippocampal injury as well as the development of spontaneous seizures. In a complementary approach, adeno-associated virus (AAV)-based vectors have been engineered to modify ADK expression in a cell type-selective manner. Overexpression of ADK in astrocytes of the hippocampus of mice was shown to trigger spontaneous seizures, whereas the knockdown of ADK—mediated by antisense expression of an *Adk* cDNA—effectively suppressed seizures in adenosine-deficient mice. Our findings suggest that stem cell- or gene therapy-based focal adenosine augmentation is a rational and efficient approach to suppress seizures in epilepsy and possibly to prevent epileptogenesis.

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### **Intracranial Injection of Tau-5 Antibody Reduces Histological Tau Deposits in Middle-Aged Tg4510 Mice**

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In the last decade, immunotherapies targeting amyloid beta peptides have been widely tested in both animal models and clinical trials. A more recent immunotherapeutic approach is to target tau protein instead, another major feature of Alzheimer's disease (AD). We investigated the effects of intracranial injections of 2 µg of tau-5 antibody (against the mid-domain of the tau peptide) into one hippocampus and 2 µg of a green fluorescent protein (GFP) antibody (both murine IgG1) in the contralateral side in 13.5-month-old Tg4510 mice ( $n = 6$  per group). The GFP antibody does not stain normal mouse brain proteins when used in immunostaining reactions. Tissue was collected 4 days later and sections were stained for several markers of tau deposition [anti-phospho-Ser199/202; anti-phospho-Ser396; H150 against total tau (aa 1–150); all rabbit polyclonal antibodies; or Gallyas silver staining against deposited fibrillar tau]. In addition, we examined staining for microglia (Iba-1) and neurodegeneration (Nissl). Sections were analyzed for reaction product area using a digital scanning microscope (Mirax). The results indicated that staining for anti-phospho-Ser 199/202 and Gallyas was significantly reduced 50–65% by tau-5 injection, compared with GFP-injected hippocampi. A nonsignificant trend for reduction was found with immunostaining by antisera directed against phospho-Ser396. However, no differences between GFP-injected and tau-5-injected hemispheres were discerned for total tau staining or microglial activation, nor was there any indication of neurotoxicity (both by measurement of Nissl sections and visual evaluation of the tissue). These initial data suggest that the tau-5 antibody stimulated clearance of phosphorylated tau deposits, but not total tau staining, and that the mechanism of clearance did not require activation of microglia or death of neurons.

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### **Multipotent Adult Progenitor Cells Promote Neurite Outgrowth and Ameliorate Axonal Dieback After Spinal Cord Injury**

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The long-distance retraction of severed axons, a phenomenon known as axonal dieback, occurs after many types of CNS injury,

and infiltrating macrophages contribute directly to this process. Adult adherent stem cells are known to have immunomodulatory capabilities, but their potential to ameliorate this detrimental process has not been investigated. We have developed an in vitro model in which adult rat dorsal root ganglion (DRG) axons are confronted with a gradient of increasing inhibitory proteoglycan and decreasing laminin. Growth cones in this environment develop a characteristic dystrophic morphology and, when contacted by activated macrophages, undergo dramatic axonal dieback. In this study, we sought to determine if culturing DRG neurons with rat multipotent adult progenitor cells (MAPC) or MAPC-conditioned media (MAPC-CM) could prevent macrophage-mediated axonal dieback. In the presence of MAPC or MAPC-CM dystrophic axons became remarkably active. Macrophages contacted these axons extensively, but axonal retraction was prevented. We found that MAPC significantly decreased matrix metalloproteinase-9 release from macrophages, effectively preventing induction of axonal dieback. MAPC also induced a shift in macrophages from an M1, or “classically activated” proinflammatory state, to an M2, or “alternatively activated” anti-inflammatory state. We extended these findings to human MAPC, or MultiStem®, and determined that these cells were also able to prevent macrophage-mediated axonal dieback. To determine the growth-promoting capacity of MAPC-CM and MultiStem-CM in vitro, we compared dissociated DRG neurons on a laminin substrate treated with MAPC-CM or control media and measured the longest neurite of every neuron. Both MAPC-CM- and MultiStem-CM-treated neurons exhibited a significant increase in outgrowth over control media and conditions after 24 h. We sought to determine if MAPC could prevent axonal dieback or promote regrowth of injured axons in vivo in a dorsal column crush model of spinal cord injury. We transplanted MAPC into the spinal cord immediately following injury, and measured axonal position at 2, 4, and 7 days postinjury. The transplanted cells integrated into the lesioned tissue and associated with the endings of injured axons. Four days postlesion, MAPC-transplanted animals demonstrated a significant attenuation of axonal dieback normally observed at this time. Seven days postlesion, MAPC-transplanted animals showed a significant increase in the extent of axon extension into the lesion core compared to vehicle controls. Our results demonstrate that MAPC have therapeutic benefits after spinal cord injury and provide evidence that these cells exert positive immunomodulatory and neurotrophic influences. We are currently examining the effects of MultiStem in a contusive injury model, which will allow for long-term behavioral analysis and accelerate the translation of MultiStem into clinic for treatment of spinal cord injury.

### **The Blood-Brain Barrier (BBB) and Angiogenesis as Therapeutic Targets in the Treatment of Parkinson's Disease (PD)**

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The endothelium of the neurovasculature is a dynamic structure that separates the periphery from the CNS. An emerging literature suggests BBB dysfunction occurs in several models of Parkinson's disease (PD), although not all, as well as several other neurodegenerative conditions. Given that neuroinflammation is a component of all neurodegenerative diseases and is known to compromise BBB integrity, the resulting barrier dysfunction could facilitate entry of elements of the peripheral vasculature including inflammogens and cells of the adaptive immune system that could facilitate disease progression. This would necessitate rethinking the neurocentric views that are currently unable to explain neurodegenerative disease progression. We have shown that all dopamine (DA) neurotoxins studied to date are associated with barrier compromise. These toxins produce acute punctate areas of leakage to the nondiffusible marker fluorescein isothiocyanate (FITC)-labeled albumin. We also recently showed more widespread reductions in the tight junction protein ZO-1. We and others demonstrated that DA toxin exposure is associated with increased entry of levodopa, decarboxylase inhibitors, and other factors that normally do

not readily enter the brain, including T cells. We also recently demonstrated that systemic administration of 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>), which did not produce DA neuron loss in normal mice, produced further DA neuron losses when administered 4 days after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment and further increased numbers of activated microglia. This suggests that BBB function is compromised, allowing entry of water-soluble peripheral toxins. Because our earlier studies demonstrated that a marker for angiogenesis (increased expression of integrin  $\beta$ 3), colocalized with FITC-albumin leakage, we examined  $\alpha$ v $\beta$ 3 expression in PD patients. The PD patients exhibited significant increases in  $\alpha$ v $\beta$ 3 relative to aged-matched controls, indicating that angiogenesis is ongoing at the time of death in the substantia nigra pars compacta (SNpc) and putamen. Because newly created vessels are unlikely to have developed a BBB and chronic exposure to angiogenic factors can produce vessels that are outright leaky, these results are consistent with a dysfunctional BBB. To determine if angiogenesis is instrumental in BBB dysfunction, inflammation, and DA neuron loss, we posttreated MPTP-treated mice with a cyRGDfV peptide that binds to  $\alpha$ v $\beta$ 3 and prevents angiogenesis. cyRGDfV prevented the FITC-albumin leakage, changes in microglia, and the DA neuron loss normally produced by MPTP, suggesting that other antiangiogenic drugs currently used to treat tumors may slow PD progression. It is also known that tight junction integrity can be increased using a number of drugs (e.g., caffeine and other phosphodiesterase inhibitors) and may reverse barrier compromise. Taken together, these data argue that BBB dysfunction may contribute to PD progression, and therapies designed to prevent angiogenesis or enhance tight junctions may slow disease progression.

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#### **Distinguishing Contact-Mediated Effects From Soluble Factors of Human Endothelial Cells on the Differentiation of a Clinical-Grade Human Neural Stem Cell Line**

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The vascular compartment plays an intricate role within the neurovascular niche, where neurogenesis and angiogenesis are considered coupled processes. Endothelial cells, together with astrocytes and pericytes, form the blood-brain barrier, and they are certainly involved in cerebrovascular events, such as stroke, in which the neurovascular unit is disrupted. Transplantation of neural stem cells (NSCs) within this environment can affect angiogenesis, but conversely, angiogenesis can also affect the fate of NSCs. To develop a better therapeutic use of NSCs, we therefore investigated how human umbilical vein endothelial cells (HUVEC) influence the differentiation of a clinical-grade human NSC line (STROC05) in a transwell culture system. Soluble factors increased glial fibrillary acidic protein (GFAP) expression in differentiated NSCs, but also maintained the expression of nestin at the level of undifferentiated cells. These changes were the result of the emergence of a subpopulation of cells coexpressing nestin and GFAP. These nestin/GFAP double-labeled cells may represent reactive astrocytes present in the glial scar surrounding stroke damage. To determine the contact-mediated effects of endothelial cells, as would be the case within the intact neurovascular unit, human endothelial cells (HUVEC and brain) were cocultured with green fluorescent protein expression hNSCs. These studies will potentially provide evidence as to how the neurovascular microenvironment can influence the fate of NSCs and indicate particular sites of injection to target-specific mechanisms of repair.

#### **Differentiation and Transplantation of Human Pluripotent Stem Cell-Derived Ventral Midbrain Dopaminergic Neurons Into Animal Models of Parkinson's Disease**

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Human pluripotent stem cells are a potential source of cell therapy for Parkinson's disease (PD) patients or to examine neurodegenerative processes. However, therapeutically relevant ventral midbrain (VM) dopaminergic (DA) neurons are not generated by standard differentiation protocols. We present a new approach that generates a practical yield of human VM DA neurons from human embryonic stem (ES) cells or induced pluripotent stem (iPS) cells derived from PD patients. Importantly, these human VM DA neurons express the transcription factor forkhead box protein 2 (FOXA2), confirming the ventral midbrain phenotype. Next we show that human DA neurons integrate and function in a rodent model of PD. Finally, we show that flow cytometry can purify human ES/iPS cell-derived neurons that survive transplantation and function in the dopamine-denervated rat striatum without forming tumors. In summary, these data provide compelling evidence for translational studies in our robust primate model of idiopathic PD. Towards this goal, we are generating and differentiating primate iPS cells to test the function of isogenic and allogeneic neuronal transplantation.

#### **Optogenetic Stimulation of Locus Coeruleus Norepinephrine Neurons in Rats Performing Set-Shifting Tasks: Modulation of Cognitive Flexibility**

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Deficits in cognitive flexibility are early and debilitating symptoms of neurodegenerative diseases, including Parkinson's disease (PD). Modern theories posit a pivotal role for the locus coeruleus (LC) norepinephrine (NE) system in mediating attention and cognitive flexibility. The Adaptive Gain Theory (Aston-Jones and Cohen, 2005), derived from unit recordings in behaving animals and neural network modeling, proposes that low levels of tonic LC impulse activity with concurrent task-related phasic activation facilitates focused attention and execution of task decisions to maximize utility in the task at hand. However, when behavioral utility wanes (reward decreases) phasic LC activation declines and tonic (baseline) LC activity increases. The ensuing tonic mode of LC activity is proposed to disengage behavioral focus from the current strategy or goal and increase cognitive flexibility to permit identification of a new, more remunerative goal in the current context. The ability to focus attention on a goal, but to also perform flexibly and find a new goal when the context changes, is a key element in high-level cognitive function and adaptive behavior. LC NE neurons degenerate early in PD. However, the possible role of the LC NE system in these and other cognitive deficits of PD has not been examined. The role proposed for the LC NE system in regulating this flexibility dimension can be tested in animals using set-shifting tasks. We have developed cell type-specific optogenetic techniques to selectively stimulate LC NE neurons with high temporal precision in anesthetized and behaving rats (see Vazey et al., this meeting). We

expressed the light-sensitive cation channel channelrhodopsin-2 (ChR2) in LC NE neurons in order to photostimulate these cells physically or tonically in Long-Evans rats performing an automated, operant chamber-based attentional set-shifting task. We hypothesize that tonic stimulation on trials immediately following a rule change (extradimensional set-shift, EDS) will decrease perseverative errors and trials to criterion (TTC) for the new contingency, whereas such stimulation when the animal is approaching criterion will increase TTC. Conversely, phasic stimulation aligned with the original contingency but applied immediately following an EDS should increase perseverative errors and TTC, but decrease TTC when the animal is approaching criterion. Results of these studies will reveal the role of the LC NE system in cognitive flexibility and associated deficits in PD, and facilitate new treatments to offset these deficits.

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### **The Differential Regulation of the Transcriptional Repressor REST During Development Is Required for Proper Progression of NS/P Cells Along Different Neural Lineages**

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Nervous system development relies on a network of transcriptional repressors and activators to control the orderly acquisition and maintenance of neural cell fate. A potential key regulator in this network is the RE1-silencing transcription factor, REST, which represses a large number of neuronal genes. During development, the REST repressor complex plays important roles in mediating repression and chromatin plasticity in pluripotent embryonic stem (ES) cells and multipotent neural stem/progenitor (NS/P) cells. REST is highly expressed in ES cells, and downregulated to minimal levels in NS/P cells. As NS/P cells differentiate along the different neural lineages, REST remains present in glia but absent in neurons, allowing restricted expression of neuronal genes exclusively in neurons. However, why REST is regulated differentially at different stages of neural development remains unknown. To understand the importance of the differential regulation of REST, we manipulated REST expression using gain- and loss-of-function approaches *in vivo* and *in vitro*. We found that while ES cells maintained their pluripotency in the absence of REST and are able to generate the three germ layers, ES cell-converted as well as primary NS/P cells had reduced capacity to form neurospheres and self-renew. Upon withdrawal of growth factors and differentiation, we found a significant increase in the number and maturation of neurons and aberrant generation of glia. Our results suggest that while the high level of REST expression in ES cells is required for maintaining neuronal genes in a repressed state, is not essential for maintaining ES cell pluripotency. However, the low level of REST present in NS/P cells is critical for maintaining their full capacity to self-renew and to differentiate properly into the different neural lineages.

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### **Optogenetic Stimulation of Neural Stem Cell Grafts in Experimental Stroke Model**

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Stroke is one of the leading causes of death and long-term disability in the world. There are no effective treatments targeting the residual anatomical and behavioral deficits resulting from stroke. It is known that after stroke the brain undergoes limited self-repair to compensate for the lost structures. Changes in reorganization or neuroplasticity are thought to underlie the partial spontaneous functional recovery that often occurs over time following stroke. To understand this innate functional repair process, it is necessary to improve our comprehension of the mechanisms mediating stem cell functional recovery in stroke-damaged brain tissues. This understanding will help move forward the field of cell transplantation, a promising therapeutic strategy for brain repair. It has been shown that the transplantation of stem cell progeny from multiple sources ameliorates motor deficits after stroke. However, it is currently unknown to what extent the electrical activity of grafted neural stem cell progeny participates in the improvement of motor deficits and whether excitatory phenotypes of the grafted cells are beneficial or deleterious to motor performances. We first derived multipotent neural stem cells (NSCs) from human embryonic stem cells. The NSCs were then transduced with lentiviral vectors carrying the channelrhodopsin-2 (ChR2) gene fused with enhanced yellow fluorescent protein (ChR2-EYFP) under the elongation factor 1 $\alpha$  (EF1 $\alpha$ ) promoter. ChR2 is a transmembrane conductance regulator that, when expressed by a cell, can generate action potentials in response to blue light stimulation. The ChR2 expression was confirmed *in vitro*. To test the function of these cells in stroke model, Sprague-Dawley rats were subjected to 65-min middle cerebral artery occlusion. One week later, immunosuppressed rats were transplanted with NSCs ( $2 \times 10^6$ ) into the ischemic boundary zone in the striatum. Animals were tested biweekly for the use of their forelimbs in the cylinder test and for locomotor activity. After 12 weeks' survival time, the animals were perfused and brains were processed for histopathology and immunocytochemistry. Grafted NSCs, identified with a human-specific nuclear marker survived in the stroke-damaged peri-infarct tissue, expressed the ChR2 transgene and extended neurites into the host parenchyma. Our behavioral analysis demonstrated that light stimulation of animals grafted with the ChR2 optogenetically engineered NSCs increased their forelimb use in comparison to vehicle-treated animals. In addition, animals that received the ChR2-expressing NSCs increased their motor activity and total distance covered during light stimulation relative to vehicle-treated animals subjected to light stimulation. Our data suggested that excitatory influences of grafted neural stem cells may offer benefit in experimental stroke.

### **Biodistribution of Human Umbilical Cord Blood Cells in Infused PSAPP Mice**

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Pathological amyloidosis is a hallmark of Alzheimer's disease (AD), a dementia disorder affecting 20 million people worldwide. Most current strategies for the symptomatic and disease-modifying treatment of AD have met with disappointment. Consequently, a more effective treatment or prophylaxis is needed. Amyloid beta (A $\beta$ ) is a key protein in the regulation of AD. Furthermore, postmortem and basic research studies, demonstrating glial cell activation in close proximity to amyloid plaques (a recognized inflammation marker), have confirmed that inflammatory processes play a role in the pathology of AD. Previously, we have shown that human umbilical cord blood cells (HUCBC, U-CORD-CELL™) provide cognitive recovery in animal models of neurodegenerative disease. Moreover, we showed that multiple low-dose injections of HUCBC in AD mouse models caused a reduction in cerebral A $\beta$  levels/ $\beta$ -amyloid deposits, which further resulted in increased serum levels of A $\beta$ <sub>40,42</sub>. Similarly, there was a reduction of A $\beta$ -specific neurotoxic T-lymphocyte responses

and downregulated proinflammatory cytokines. HUCBC do not pose the ethical concerns associated with embryonic stem cells nor do they require full human leukocyte antigen (HLA) matching, or pretreatment for immunosuppression. They are devoid of major histocompatibility complex (MHC) II, major costimulatory molecules, and do not induce rejection of any kind. Further, HUCBC are easily accessible and inexpensive. While data suggest that HUCBC infusion mitigates AD-like pathology, no optimal dosing, response, and safety data have been elucidated. As part of safety characterization, the purpose of this study was to determine HUCBC presence and biodistribution in HUCBC-infused PSAPP mice (transgenic mice expressing specific presenilin and amyloid precursor protein mutations) after 24 and 72 h. HUCBC and PBS (control) were transplanted intravenously into the tail veins of PSAPP recipient mice. Presence and distribution in peripheral blood, liver, lung, spleen, heart, spinal cord, brain, gonad, and bone marrow of HUCBC CD45<sup>+</sup> cells were determined 24 and 72 h after transplantation by PCR and flow cytometry. We determined the presence of positive cells in bone marrow (BM) and blood through flow cytometry. Immunohistochemistry determined distribution of HUCBC CD45<sup>+</sup> cells. HUCBC CD45<sup>+</sup> cells mainly home into recipient mice bone marrow, not peripheral blood. Both PSAPP1<sup>+</sup> and PSAPP1<sup>-</sup> recipient mice had more HUCBC CD45<sup>+</sup> cells in the 24-h time point (TP) than those at 72 h (PSAPP1<sup>+</sup> 24 h, 3.7%; 72 h, 0.67% and PSAPP1<sup>-</sup> 24 h, 2.1%; 72 h, 1.6%). We report that while PSAPP1<sup>+</sup> mice had more positive HUCBC CD45 cells at 24 h than PSAPP1<sup>-</sup> recipients, at the 72-h TP, PSAPP1<sup>-</sup> had more HUCBC CD45<sup>+</sup> than their PSAPP1<sup>+</sup> counterparts. Positive HUCBC CD45 cells survive in bone marrow even after 72 h of transplantation in PSAPP mice.

#### Lateral Fluid Percussion Injury of the Brain Induces CCL20 Inflammatory Chemokine Expression in Rats

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Traumatic brain injury (TBI) evokes a systemic immune response including leukocyte migration into the brain and release of proinflammatory cytokines; however, the mechanisms underlying TBI pathogenesis and protection are poorly understood. Due to the increasing incidence of trauma, identification of the molecular signals involved in TBI progression is critical for the development of novel therapeutics. In this report, we used a rat lateral fluid percussion impact (LFPI) model of TBI to characterize neurodegeneration, apoptosis, and alterations in proinflammatory mediators at two time points within the secondary injury phase. Histological analysis of neurodegeneration by Fluoro-Jade staining showed mild injury in the cerebral cortex, hippocampus, and thalamus. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining confirmed the presence of apoptotic cells and CD11b<sup>+</sup> microglia, indicating initiation of an inflammatory reaction leading to secondary damage in these areas. Analysis of spleen mRNA by PCR microarray of an inflammation panel led to the identification of CCL20 (also known as macrophage inflammatory protein 3) as an important proinflammatory signal upregulated 24 h after TBI. Although CCL20 expression was observed in spleen and thymus after 24 h of TBI, it was not expressed in degenerating cortex or hippocampal neurons until 48 h after insult. These results demonstrate that the systemic inflammatory reaction to TBI starts earlier than the local brain response and suggest that spleen- and/ or thymus-derived CCL20 might play a role in promoting neuronal injury and CNS inflammation in response to mild TBI.

#### Transplanted Human Astrocytes Derived From BMP- or CNTF-Treated Glial Precursors Have Opposite Effects on Functional Recovery After Spinal Cord Injury

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Recent work from our laboratories on developing cell transplantation-based therapies for spinal cord injury (SCI) has demonstrated the benefits of utilizing predifferentiated astrocytic progeny of precursor cells over the use of multipotent precursors to promote repair. We have compared the effects of transplanting undifferentiated glial precursors and two distinct astrocytic populations that can be derived from the same precursor population by application of different pro-astrocytic inducers. Transplantation of glial precursor-derived astrocytes (GDA) generated using bone morphogenetic protein 4 (GDAs<sup>BMP</sup>) promoted robust functional recovery. Treatment of injury sites with the undifferentiated glial precursors, or a second type of astrocyte derived from the precursor population by induction with ciliary neurotrophic factor (GDAs<sup>CNTF</sup>), however, failed to promote locomotor recovery. As a first step towards translating the therapeutic benefits obtained with transplants of rodent GDAs<sup>BMP</sup> to use in humans, we have now successfully generated human GDAs<sup>BMP</sup> and GDAs<sup>CNTF</sup> from fetal cells, and have found that our previous studies with rodent GDAs were highly predictive of outcomes with human GDAs. Transplantation of hGDAs<sup>BMP</sup> promoted robust axon growth, neuroprotection, and functional recovery in adult spinal cord-injured rats. In marked contrast, human glial precursor cells and GDAs<sup>CNTF</sup> both failed to promote significant behavioral recovery or similarly robust neuronal survival and support of axon growth at sites of injury. This study is the first to show that two distinct subtypes of human astrocytes derived from a common fetal glial precursor can have opposite effects on functional recovery when transplanted into acute CNS injuries.

#### Cocaine Effects on Tumor Cell Proliferation

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We have previously reported that the AF5 neural progenitor cell line, normal neural progenitor cells, and A2B5<sup>+</sup> progenitor cells show decreases in cyclin A2 expression in response to cocaine, as well as inhibition of cell proliferation. In contrast, cyclin A2 is not changed in mature neurons and microglia, and is increased in astrocytes in response to cocaine (Lee et al., 2008). The purpose of the present study was to further explore the effects of cocaine on proliferation of immortalized neural progenitor cell lines in comparison to human tumor cell lines. A series of tumor cell lines and immortalized neural progenitor cells was tested for changes in cell proliferation in response to cocaine and related drugs. All cell lines tested showed inhibition of cell proliferation at 100  $\mu$ M. Smaller doses differentially affected the various cell lines. AF5 cells showed the strongest inhibitory effect of cocaine, with a decrease in cell proliferation of approximately 30% at 1  $\mu$ M ( $p < 0.001$ ), a dose that can be achieved in cocaine-abusing human subjects. Three other immortalized neural progenitor cell lines were tested. C17.2 showed an inhibition of approximately 11% at 1  $\mu$ M ( $p < 0.05$ ), while REN-CX and RTC2 cells did not respond to 1  $\mu$ M cocaine. Of the tumor cell lines, DAOY and MCIDX medulloblastoma cells were least responsive to cocaine, showing no response to 1–10

$\mu\text{m}$  cocaine, while D283 and SH-SY5Y were most responsive, showing significant inhibition of proliferation at 1  $\mu\text{m}$ . A number of local anesthetics and dopamine uptake inhibitors were tested for inhibition of proliferation. Procaine, benzocaine, bupivacaine, and lidocaine had no effect on cell proliferation. The dopamine transporter inhibitors GA2-50 and 4,4 diCl BZTP also did not affect cell proliferation, but were toxic at high doses (100  $\mu\text{m}$ ). Studies of the expression of cytochrome P450 isoforms CYP3A4, CYP3A5, CYP3A7, and CYP3A43 are currently in progress, but have not revealed any apparent relationship between cytochrome P450 expression and the proliferative response to cocaine. Studies on the relationship between the effect of cocaine on cell proliferation and generation of reactive oxygen species are currently in progress.

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### Combination of Nicotine and $\Delta 9$ -THC Reduces Microglial Activation and Proinflammatory Cytokine Production in PSAPP Mice

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Many neurodegenerative diseases are known to be associated with chronic inflammation. There exists a growing body of evidence suggesting that innate immunity is pathologically unregulated in various neurodegenerative diseases, such as Alzheimer's disease (AD). In these disease states, the activation of microglia releases proinflammatory cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), which have been implicated in causing neuronal cell death. The immune-suppressing properties of both nicotine and  $\Delta 9$ -tetrahydrocannabinol ( $\Delta 9$ -THC) have been well documented, but no previous studies have investigated their combined effects on the innate immune system function. In recent studies, we have discovered that nicotinic receptors are functionally expressed on microglia and that nicotine reduces microglial activation and enhances microglial uptake of amyloid beta<sub>1-42</sub> (A $\beta$ <sub>1-42</sub>), a peptide implicated in AD. The cannabinoid receptor, CB2, is also present on microglia and stimulation has been shown to result in reductions in proinflammatory cytokine production and alterations in microglial phenotype. Here we report findings from a lipopolysaccharide (LPS) mouse model and Alzheimer double transgenic PSAPP mice (expressing specific presenilin and amyloid precursor protein mutations) that were injected (IP) with a combination of nicotine and  $\Delta 9$ -THC. Treatment with these compounds result in reductions in microglial activation observed through expression of CD45, as well as reductions in A $\beta$  levels. Greater reductions in proinflammatory cytokines [TNF- $\alpha$ , interleukin (IL)-6] were also observed after treatment with nicotine and  $\Delta 9$ -THC. These findings suggest that the combination of  $\Delta 9$ -THC and nicotine clinically should have greater efficacy in reducing neuroinflammation with less side effects than either drug given alone.

### What Can Go Wrong During Convection Enhanced Delivery Into the Putamen Nucleus?

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Convection enhanced delivery (CED) is proposed as a way of optimizing viral vector infusions into the putamen nucleus for Parkinson's disease (PD) by increasing the area of distribution of infusate beyond the parameters of diffusion and, thus, minimizing the surgical risk by reducing the number of injections needed to cover the target. Viral vector transfection in nontargeted areas may induce side effects, implying that confinement of the infusate containing the viral vector suspension to the desired area becomes essential for patient safety. In that regard, CED is also hypothesized to produce a more predictable and even pattern of distribution. In this study, we decided to challenge this last concept and analyze: a) the maximal volume of infusate that remains in the desired area, b) the pattern of distribution beyond the putamen boundaries, c) the volume of infusion versus volume of distribution versus gadoteridol concentration, and d) the potential areas of leakage during the infusion procedure. Twenty-five adult macaque monkeys received bilateral intraputamenal infusions of a 100  $\mu\text{l}$  infusion of the MR contrast agent gadoteridol (ProHance, Bracco Diagnostics; 2 mM/L) and bromophenol blue (0.16 mg/ml), coinfused in a saline solution. Brain targeting to the postcommisural ventral putamen nucleus was achieved using intraoperative magnetic resonance imaging (MRI) intracerebral navigation combined with an adaptable, pivot point-based system (Emborg et al., 2010). Infusions were performed using an endpoint step catheter (0.64 mm OD) with or without a stylet and a pressure monitoring and infusion pump controller system (Engineering Resources Group Inc.) in combination with a MRI-compatible infusion pump (Harvard Apparatus). MRI (T1, angiographies and diffusion tensor imaging) was performed in a 3T GE scanner. Additional studies were performed in gels and ex vivo brains. Our results include: 1) at most 30  $\mu\text{l}$  can be infused into a single location in the macaque putamen before infusate begins to spread beyond the putamen boundary, 2) distribution beyond the putamen boundaries differs between white and gray matter due to different tissue expansion and defines the flow of infusate in the region, 3) during insertion, leakage is observed in catheters without a stylet, as the catheters get occluded and the fluid inside the catheter is displaced by tissue, 4) leakage of fluid in the catheter tract was observed after the extraction of the catheter 10 min after the infusion was completed, 5) if backflow from within the gray matter of the putamen reaches the dorsal boundary, infusate easily spreads into the surrounding corona radiata, and 6) blood vessels adjacent to a catheter's backflow region can redirect infusate to follow the vessel path. The use of a stylet, applying angiography data when planning infusions, monitoring in vivo infusions, and matching the volume of infusate to volume of target structure are possible methods to minimize leakage and maximize predictability. These results in nonhuman primates provide insight into the role that geometry and architecture of the target have to successfully achieving controlled, bounded infusions with CED.

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**Are Umbilical Cord Blood Cells Safe to Transplant for Neurodegenerative Disorders Without HLA Matching or Immunosuppression?**

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The mononuclear fraction of Human umbilical cord blood (hUCBCs) has been demonstrated by many researchers to have considerable potential as a treatment for a wide range of disorders including stroke, Alzheimer's disease (AD), amyotrophic lateral sclerosis, diabetes, and hematopoietic disorders based on animal models. However, there are some concerns about the ability of hUCBCs to be transplanted safely when they are used clinically due to the potential for rejection and graft-versus-host disease (GvHD). Studies in treatments for hematopoietic disorders suggest that the rate of rejection and GvHD is significantly less than observed with bone marrow cells, supporting the proposal that UCBCs are immune immature. However, delayed engraftment and graft failure are commonly observed in human transplants for the treatment of hematopoietic disorders. Whereas the use of multiple transplants has been advocated to improve the efficacy of the grafting, when considering their use for the treatment of neurodegenerative disorders such as stroke and AD, there is accumulating evidence that the cells do not need to engraft or survive long term for the transplanted cells to exert their benefit, suggesting that a lack of engraftment would not hinder therapeutic outcome. In this presentation we will summarize some of the latest studies, such as a recent clinical study performed in China (Yang et al., *J. Transl. Med.*; 2010) that showed that 4–5 allogeneic hUCBC transplants in 114 patients for a variety of nonhematopoietic degenerative disorders, including paraplegia, ataxia, multiple sclerosis, amyotrophic lateral sclerosis, cerebrovascular diseases and multiple system atrophy, were clinically safe, revealing no severe side effects such as rejection or GvHD with up to 4 years follow-up. No information on the efficacy of the cells in the treatment of the disorders was reported. No HLA matching or immune suppression were performed in this study, which would seem to suggest that they are not necessary for this type of treatment. A degree of efficacy and safety for multiple hUCBC transplants was also recently shown in an autologous study of a child with global ischemia, who could no longer be defined as being in a permanent vegetative state 6 months after treatment (Jozwiak et al., *Cell Med.*; 2010). Additional studies with more long-term follow-up as well as evidence of functional improvement following transplantation are required to fully advocate their use in the clinical setting without the need for matching or immunosuppression.

*C.V.B. and A.E.W. are consultants for, and P.R.S. is cofounder of, Saneron-CCEL Therapeutics. Saneron CCEL Therapeutics and Cryopraxis, Cell Praxis are biotech R&D companies researching the therapeutic use of cord blood and other stem cells.*

**The Importance of Gender**

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Recent studies in the fields of cerebrovascular and cardiovascular disease by Prof. Hurn (Oregon Health and Science University) and Prof. Taylor (University of Minnesota), respectively, have provided strong evidence that the gender of cells (recipient and donor in the case of cell therapies) is of paramount importance. For instance, there is a clear protection in females from cerebrovascular disease such as stroke for many years beyond menopause, suggesting that although reproductive hormones and the presence of androgen or estrogen receptors on the cells may play a role, they do not explain all the differences. There are a number of studies showing that male and female cells respond differently to ischemia and other types of injury by activation of distinct cell death signaling cascades, and the degree of damage is less severe in females. Males appear to be more susceptible to free radical-mediated damage such as nitric oxide mediated-toxicity, whereas females are more likely to exhibit caspase-dependent apoptotic cell death. However, not all cell types exhibit the gender differences because while female dopaminergic and hippocampal neurons are more resilient, female cerebellar Purkinje cells have no greater protection against experimental brain insults. In addition, some forms of injury, such as H<sub>2</sub>O<sub>2</sub>, appear to be gender neutral. Cellular therapy has considerable potential for a number of disorders, but the role of gender remains controversial. Mesenchymal stem cells (MSCs) from males have been shown to release significantly greater quantities of the proinflammatory factors tumor necrosis factor (TNF) and interleukin-6 (IL-6) and significantly less of the proangiogenic factor vascular endothelial growth factor (VEGF) than female MSCs. In addition, female cells are less likely to undergo apoptosis in response to hypoxia. This suggests that male MSC transplants may be less beneficial than female cell transplants and there is evidence of this with respect to endothelial progenitor cells and atherosclerosis. Female cells transplanted into females (or males) had a significantly greater benefit than male cells into males (or females). We are therefore advocating that in determining the optimal source and type of transplantable cells for use in a cell therapy, much consideration should be given to the gender of both the donor and the recipient. While many studies are performed only in males, the results may not translate well to the female population. It is tempting to speculate that autologous transplants may have the most benefit in females whereas an allogeneic transplant (of female cells) maybe better in males. Further studies are required to determine which disorders are affected by gender-specific transplants. Treatments may need to be tailor made to the gender of the patient.

**First-in-Human Spinal Cord Neural Stem Cell Transplantation Trial: Current Status**

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The current abstract describes the status on the first-in-human trial of neural stem cell transplantation for the treatment of amyotrophic lateral sclerosis (ALS). The primary objective of the current trial is to determine the feasibility, safety, and toxicity of direct spinal cord transplantation of human spinal stem cells in patients with ALS. The study was designed to balance risk to subjects with the acquisition of new knowledge regarding direct spinal cord transplantation. We have

coined the term “risk escalation” to describe the gradual increase in risk between cohorts. The trial begins with unilateral transplants (five injections spaced by 4 mm) into the segments of the lumbar cord supporting proximal limb function in nonambulatory patients ( $n = 3$ ). The second cohort receives bilateral lumbar transplants (five injections per side spaced by 4 mm,  $n = 3$ ). The third cohort receives unilateral lumbar injections but are ambulatory, hence bearing the potential to lose the ability to walk. The fourth cohort contains ambulatory patients that receive bilateral injections. The fifth cohort receives unilateral cervical injections (five grafts at 4 mm intervals from C3 to C5). The final group is ambulatory and receives staged unilateral cervical injections followed by bilateral lumbar injections. The FDA has approved the first stage of the trial, which consists of 12 patients. To date, we have completed the first and second cohorts ( $n = 6$ ). Surgeries on the third cohort (ambulatory patients) are ongoing. All patients remain alive.

#### Neuroprotective Effects of Bradykinin B2 Receptor Antagonist HOE-140 in Spinal Cord Injury Are Mediated Through Nitric Oxide-Related Mechanisms

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Bradykinin is a mediator of the blood–brain barrier (BBB) and edema formation. However, its role in spinal cord injury (SCI)-induced breakdown of the blood–spinal cord barrier (BSCB) and edema formation is still not well known. A previous report from our laboratory shows that bradykinin B2 receptor antagonist (HOE-140, Hoechst, Frankfurt, Germany) in low doses is able to thwart BSCB disruption, edema formation, and cell injury in a rat model of SCI. However, the mechanisms of HOE-140-induced neuroprotection in SCI are unclear. Recent studies suggest that bradykinin may stimulate endothelial nitric oxide synthase (eNOS) through B1 receptor-mediated events. However, the role of B2 receptors in NOS upregulation is not known. Thus, in the present investigation we sought to determine whether bradykinin B2 receptor is somehow involved in SCI-induced neuronal NOS (nNOS) activation. We used HOE-140, a specific B2 receptor antagonist, at a lower dose to ascertain the role of bradykinin in spinal cord trauma-induced alterations in nNOS expression, BSCB permeability, spinal cord blood flow (SCBF), edema formation, and cell changes in our rat model. Note that HOE-140 at higher doses may have some B2 receptor agonistic activity as well. SCI was produced in equithesin-anesthetized animals by making a longitudinal incision into the right dorsal horn at the T10–11 segment. The animals were allowed to survive for 5 h after the injury. A focal trauma to the rat spinal cord significantly increased the BSCB permeability to Evans blue, [<sup>125</sup>I]sodium in the five spinal cord segments examined (e.g., C4, T5, T9, T10–11, and T12). These spinal cord segments also showed marked upregulation of nNOS activity and exhibited profound reduction in the SCBF (mean –30%). Measurement of spinal cord water content showed a significant increase in all the above spinal cord segments. Profound nerve cell, glial cell, and myelin damage was seen in these spinal cord segments that are most pronounced around the vicinity of the lesion site. A close correlation between upregulation of nNOS and cell injury was clearly seen after SCI. Extravasation of lanthanum was largely present within the endothelial cell cytoplasm and also found occasionally in the basal lamina. Pretreatment with lower doses of B2 receptor antagonist HOE-140 (0.1 mg to 1 mg/kg, IV) 30 min prior to trauma significantly attenuated spinal cord pathology and upregulation of nNOS expression. The SCBF showed marked improvement in this group. This effect of HOE-140 was also seen when the compound was administered 10–15 min after SCI, but no neuroprotection was seen when the compound was

given 30 min after injury. Interestingly, increasing the dose of HOE-140 from 2 mg to 5 mg/kg, IV (either 30 min before or 10 min after injury) did not exhibit any neuroprotection. In fact, there was an exacerbation of the tracer’s extravasation, edema formation, cell injury, and nNOS expression. These observations are the first to suggest that bradykinin B2 receptors play an important role in SCI-induced nNOS expression and spinal cord pathology. An early blockade of bradykinin B2 receptors in the spinal cord following trauma is necessary to induce neuroprotection. Furthermore, the neurodestructive effects of bradykinin appear to be mediated through mechanisms involving nitric oxide, which has not been reported previously.

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#### Characterizing the Role of the Locus Coeruleus in Modulating Cognitive Deficits in Neuropathology and Down Syndrome: Novel Application of Designer Receptors Exclusively Activated by a Designer Drug (DREADDS)

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Individuals with Down syndrome (DS) will develop the neuropathological and cognitive deficits of Alzheimer’s disease (AD) by the age of 40. These neuropathological features include microglial activation, beta amyloid plaques, neurofibrillary tangles, and degeneration of both the basal forebrain cholinergic neurons (BFCNs) and the noradrenergic neurons of the locus coeruleus (LC-NE). These degenerative changes coincide with the onset of severe cognitive impairment beyond that of the syndrome itself and include impaired object recognition, working memory, short-term memory/recall, and visuospatial skills. The first sign of impairment is characterized by an early and progressive loss of LC-NE neurons. We have recently shown that by lesioning the LC in young, healthy DS mice we are capable of reproducing an early AD-like pathology that includes degeneration of BFCNs, increased microglial activation, elevated interleukin (IL)-1 $\beta$  mRNA levels in the hippocampus, and deficits in cognition. While this pinpoints LC degeneration as a starting point for propelling degenerative changes, the functionality of the LC has not been implicated. In an attempt to show a proof of principle while introducing a novel methodology in our mouse model of DS, the Ts65Dn, we used designer receptors exclusively activated by a designer drug (DREADDS) to directly stimulate the LC neurons at a time when the neurons have just started to degenerate. DREADDS target a specific promoter sequence via local viral delivery to target a select population of neurons, which allows for the transduction of a mutated acetylcholine (mACh) receptor that responds to the inert compound clozapine-N-oxide (CNO). The effects of CNO are acute and allow for temporal control; here they will be used to turn the LC “on” then “off” in aged (12 months old) Ts65Dn mice. We propose that turning on the LC in aged Ts65Dn animals will reverse cognitive impairment in the novel object recognition task. We also propose that long-term delivery of CNO will not only enhance cognition but will also reverse the degenerative changes observed in normal aging in DS as a result of increased LC-NE. These are the first studies of this kind to be evaluated in a mouse model of DS and therefore are a novel approach to understanding the mechanism by which neuropathology and cognitive decline develop in DS individuals.

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### Long-Term Treatment With a High-Fat/High-Cholesterol Diet in Young and Aged Rats: Inflammatory Response, Blood–Brain Barrier Breakdown, and Cognitive Impairment

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Consumption of mainly saturated fats and cholesterol in the diet has been proven to be damaging to organs such as the heart and liver, particularly through inflammatory mechanisms. However, the effects of a high saturated fat and cholesterol diet (HFHC diet) on the brain are not currently well understood. We hypothesize that long-term treatment with this type of diet may be damaging to the brain, especially the hippocampus, a vulnerable region to ischemia and hypoxia. We also hypothesize that aged subjects exhibit an enhanced response to this diet compared to young subjects due to increased inflammation and ensuing alterations to the blood–brain barrier (BBB) permeability. Previous work from our laboratory demonstrated that a 10% hydrogenated coconut oil and 2% cholesterol diet (HFHC diet) resulted in impaired performance on the 12-day water radial arm maze, increased circulating cholesterol and triglyceride levels, increased microgliosis, and decreased dendritic microtubule associated protein 2 (MAP2) staining in the hippocampus after only 8 weeks on the diet. Our current study has further evaluated long-term effects of the HFHC diet on cognition and hippocampal morphology using the same HFHC diet for 6 months starting at the age of 4 months (young) or 14 months (aged) in male Fischer 344 rats. In this long-term study, serum was collected in order to analyze triglyceride and cholesterol levels, water radial arm maze behavior was performed, and immunofluorescence studies on the hippocampus have been conducted. These experiments revealed impaired working and reference memory, a compromised BBB, increased microgliosis, and dendritic damage in HFHC-treated rats compared to control rats. Alterations in cognitive and morphological measures were exacerbated in aged versus young rats, suggesting enhanced sensitivity to this diet with aging. Together, these results provide novel evidence for a role of inflammation in diet-induced neurodegeneration.

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### Blood–Brain Barrier Impairment in a Mouse Model of MPS III B

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In Sanfilippo syndrome type B (MPS III B), a deficiency of alpha-N-acetylglucosaminidase (Naglu) enzyme leads to accumulation of heparan sulfate (HS), a glycosaminoglycan (GAG), within cells and to eventual progressive cerebral and systemic organ abnormalities. However, little is known about the competence of the blood–brain barrier (BBB) in MPS III B. BBB dysfunction in this devastating disorder could contribute to neuropathological disease manifestations. The aim of this study was to determine structural and functional integrity of the BBB in a mouse model of Sanfilippo type B at different stages of disease. The primary focus was analyzing BBB competence in various brain structures known to experience neuropathological changes. BBB structural characteristics were examined in microvessels from the cere-

bral cortex, hippocampus, striatum, and cerebellum of Naglu mice via electron microscopy (EM). BBB functional integrity was further investigated by tests for vascular leakage with Evans blue (EB) and albumin. EB dye was intravenously injected into Naglu mice at early, late, or end stage disease. Wild-type mice (controls) were also injected at the same ages. After 30 min, mice were euthanized and the brains examined for EB leakage. Immunohistochemical staining for albumin was also performed in serial brain sections. Also, immunohistochemical staining for GM3 ganglioside was performed to detect this secondary storage product in the brains of Naglu mice. Major findings of our study were: 1) capillary ultrastructure revealed endothelial cell damage and astrocyte degeneration, which compromised the BBB, resulting in vascular leakage; 2) endothelial cells showed endoplasmic reticulum swelling and formation of numerous large cytoplasmic vacuoles, degeneration of cytoplasmic organelles, abnormal microvilli formation, and shedding of fragments of cell membrane and microvilli; 3) edematous space around microvessels; 4) pericyte degeneration was observed in the large majority of damaged vessels, 5) highly vacuolated perivascular macrophages were found in contact with microvessels; 6) a microaneurysm was noted adjacent to a ruptured endothelium; 7) EB and albumin microvascular leakage was clearly indicated in multiple brain structures; 8) GM3 ganglioside accumulation was determined in brain microvasculature endothelium. These new findings of BBB structural and function impairment in MPS III B mice even at early disease stage may have implications for disease pathogenesis and should be considered in current and future development of treatments for MPS III B. Special attention should be given to the possibilities for cerebral hemorrhages in MPS III B.

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### Behavioral and Histological Analysis of a Partial Double Lesion Model of MSA-P

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Multiple system atrophy (MSA) is a neurodegenerative disease characterized by progressive autonomic failure, cerebellar ataxia (MSA-C), and parkinsonism (MSA-P) due to neuronal loss in multiple brain areas associated with oligodendroglial cytoplasmic  $\alpha$ -synuclein inclusion bodies. There is no effective treatment for MSA, and MSA-P patients are not even responsive to L-3,4-dihydroxyphenylalanine (L-dopa) due to the loss of striatal dopaminergic postsynaptic receptors. Rendering MSA-P patients sensitive to L-dopa administration following striatal tissue transplantation is now considered as a clinical option to manage the disease. The study describes the simple, skilled, and sensorimotor behavior deficits in a unilateral partial double lesion rat model of MSA-P. The double lesion combines a partial/terminal 6-hydroxydopamine (6-OHDA) lesion, followed by a striatal quinolinic acid (QA) lesion, and it aims to mimic the early stage of MSA-P. Animals received baseline training on the staircase and the corridor tests, and were additionally tested on the stepping, the cylinder, and the drug-induced rotation on multiple occasions following lesion surgery. Under the surgical and testing paradigms used, the behavioral data show robust lateralized deficits on all tasks, albeit the partial 6-OHDA and the double-lesioned animals were most impaired. Interestingly, the double lesion had an accumulative effect on the corridor test, partially on the staircase test, and altered deficit profile compared to the two other lesion groups on the rotation and the staircase tests. Histological analysis confirmed the ~40% dopamine loss from the striatum (6-OHDA and double lesion animals), as well as the similar loss of striatal neurones (QA and double lesion animals). In summary, the study generated the behavioral deficit profile of a partial double

lesion rat model mimicking an early stage of MSA-P. Using this platform, we will transplant embryonic ganglionic eminence cells into the double-lesioned striatum to investigate the degree and conditions under which L-dopa sensitivity can be reestablished.

### CX3CR1 Deficiency Leads to Impairment in Cognitive Function and Synaptic Plasticity

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The protective/neurotoxic role of fractalkine (FKN) and its receptor CX3C chemokine receptor 1 (CX3CR1) signaling in neurodegenerative disease is an intricate and highly debated research topic. It appears that the FKN/CX3CR1 axis plays a direct role in neurodegeneration and/or neuroprotection, depending upon the CNS insult. However, all studies to date focused on the role of FKN/CX3CR1 signaling in pathological conditions, ignoring the relevance of FKN/CX3CR1 signaling under physiological conditions. In the present study we used CX3CR1<sup>-/-</sup>, CX3CR1<sup>+/-</sup>, and wild-type mice to investigate the physiological role of CX3CR1 receptor in cognition and synaptic plasticity. Three-month-old CX3CR1<sup>-/-</sup>, CX3CR1<sup>+/-</sup>, and wild-type mice were exposed to contextual fear conditioning and Morris hidden platform water maze, to test hippocampus-dependent learning. Our results demonstrated for the first time that mice lacking CX3CR1 receptor show contextual fear conditioning and spatial memory deficits. To determine whether CX3CR1 deficiency also affects motor learning, all mice were tested on the accelerated rotarod. Wild-type mice performed significantly better on the last day of trial compared to the first day than did CX3CR1<sup>-/-</sup> and CX3CR1<sup>+/-</sup> mice, suggesting motor learning deficits in CX3CR1<sup>-/-</sup> and CX3CR1<sup>+/-</sup> mice. There were no significant differences on the first trial of testing between all experimental groups, which suggests that there is no difference in baseline motor skills. To investigate whether CX3CR1 deficiency leads to impairment in other forms of synaptic plasticity we analyzed the induction and maintenance of long-term potentiation (LTP) in the CA1 region of CX3CR1 null mice (3 months old). Acute hippocampal slices were made from CX3CR1<sup>-/-</sup>, CX3CR1<sup>+/-</sup>, and wild-type mice. Our results showed that deficiency in CX3CR1 causes a decrease in LTP. Mice lacking CX3CR1 receptor have increased levels of interleukin (IL)-1 $\beta$ , so we asked whether the IL-1 receptor antagonist (IL-1ra), which blocks IL-1 receptor I, might actually reverse the impairment in LTP that we observed in the CX3CR1-deficient mice. To determine if IL-1ra can improve LTP in CX3CR1-deficient mice, hippocampal slices from CX3CR1<sup>-/-</sup> mice were perfused with 100  $\mu$ g/ml of IL-1ra. We now show that blocking the function of IL-1 $\beta$  completely reverses the impairment in LTP. Taken together, our results reveal that, under physiological conditions, disruption in FKN signaling will lead to impairment in cognitive function and synaptic plasticity due to increased levels of IL-1 $\beta$ .

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### Comparison of Different Cell Sources for Neural Transplantation Into Regions of the Basal Ganglia in Experimental Epilepsy

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Up to 80% of patients suffering from temporal lobe epilepsy are pharmacoresistant despite adequate treatment with antiepileptic drugs. Neural transplantation into appropriate target regions within the brain is one promising approach to overcome this problem. The goal is to achieve long-term anticonvulsant effects and functional repair of brain regions via modulation of the epileptic network. In our previous transplantation studies using a rat model of temporal lobe epilepsy, we grafted different  $\gamma$ -aminobutyric acid (GABA)-producing cell types mainly into the substantia nigra pars reticulata (SNr), a basal ganglia structure known to be involved in the propagation and manipulation of different seizure types emanating from the limbic system. The different cell types we used for comparing their anticonvulsant efficacy included rat fetal striatal GABAergic cells and genetically engineered GABA-producing cells from conditionally immortalized mouse cortical neurons and from rat striatal neurons, respectively. All GABA-producing cell types showed clear but transient anticonvulsant effects after grafting (cf., Löscher et al., TINS 31; 2008). Our present studies mainly address the following questions: 1) Are specific subregions of the SNr or other basal ganglia regions such as the subthalamic nucleus (STN) more suitable for grafting GABA-producing cells? 2) Which other cell types will prove advantageous compared to previously used cells regarding long-term anticonvulsant efficacy after transplantation into basal ganglia regions? In our present studies we used local micro-injection of the GABA-potentiating antiepileptic drug vigabatrin as a strategy to identify optimum basal ganglia target regions for neural transplantation of GABA-producing cells in an acute seizure model (see Abstract by Backofen-Wehrhahn et al., this Meeting). With regard to the cell types, on the one hand we currently use human model neurons (Ntera-2 cells differentiated into a mixed population of mature neurons), which have previously been shown to integrate successfully into the nervous systems of both experimental animals and human basal ganglia infarct patients without evidence for tumor formation. Preliminary data did not reveal robust anticonvulsant efficacy of Ntera-2 cells after grafting into the STN. This is in line with previous data showing that increased inhibition of the SNr or the STN is necessary to inhibit seizures emanating from the limbic system. The differentiated Ntera-2 cells, however, comprise glutamatergic, GABAergic, and other phenotypes. On the other hand, we currently use rat striatal precursor cells pretreated with fibroblast growth factor-2 (FGF-2) and a caspase-1 inhibitor (protocol adapted from Hattiangady et al., Exp. Neurol. 212; 2008) and rat neural stem cells from the medial ganglionic eminence expanded as neurospheres and incubated with FGF-2 and epidermal growth factor (protocol adapted from Waldau et al., Stem Cells 28; 2010) for grafting into basal ganglia regions. First data from these transplantation studies will be presented.

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### Recovery of Spatial Memory and Hippocampal LTP in Homer1 Knockout Mice by Targeted Gene Delivery of Homer1c Via Recombinant Adeno-Associated Virus

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The Homer family of proteins has been implicated in the structure and function of the postsynaptic density in neurons. Homer1 knockout (KO) mice show deficits in sensory, social, and motor behavioral tasks, as well as those testing spatial learning and memory. Previous research in our lab implicates one of the long forms of Homer1, Homer1c, in learning and memory, specifically in cognitive aging. Here we used recombinant adeno-associated virus (rAAV) to deliver Homer1c to the hippocampus of Homer1 KO mice (rAAV-Homer1c), in an attempt to rescue the cognitive impairments found in these animals. We show that hippocampal expression of Homer1c in Homer1 KO mice resulting from a high dose of viral vector ( $1.2 \times 10^{11}$  viral genome copies) improves their performance in the radial arm water maze (RAWM), a test of spatial memory. A lower dose of rAAV-Homer1c ( $2.4 \times 10^{10}$  viral copies) resulted in a trend towards an improvement in performance. Although it has not been studied, we hypothesized that hippocampal learning impairments seen in Homer1 KO mice reflect deficits in the synaptic properties of these mice. Using two different long-term potentiation (LTP) stimulation paradigms in acute hippocampal slices, we found that the loss of Homer1 in the hippocampus results in a complete reduction of late-phase LTP upon stimulation with a high frequency stimulation (HFS) paradigm, and a significant reduction, but not total ablation, of both early and late-phase LTP with theta burst stimulation (TBS). The high-level dose of rAAV-Homer1c failed to rescue the LTP deficits found in Homer1 KO mice, but the lower viral dose resulted in complete recovery of late-phase LTP with HFS and an enhancement in both early and late-phase LTP with TBS. Finally, we show that rescue of Homer1c function in the Homer1 KO hippocampus leads to an upregulation of the metabotropic glutamate receptor 5 (mGluR5) in hippocampal astrocytes. As the serotype of rAAV used in this experiment does not infect glial cells (rAAV5), this result provides evidence for a complex role of Homer1c in hippocampal plasticity and learning and memory.

### Background Activity and Single Unit Firing of the Subthalamic Nucleus in Early Stage Parkinson's Disease

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Modulation of subthalamic nucleus (STN) output with deep brain stimulation (DBS) reduces the symptoms of advanced Parkinson's disease (PD), and analyses of microelectrode recordings (MER) collected during implantation have yielded invaluable data on the activity of the STN and surrounding structures. Such data have improved our understanding of the role of STN in ongoing degeneration, but are limited by the inability to compare with MER from normal patients or patients with early stage disease because the current FDA indication for DBS therapy only in severe PD precludes recording in these patients. Recent research indicates STN modulation may also protect dopaminergic neurons in the substantia nigra (SN), and these findings, plus evidence that DBS reduces motor fluctuations and provides better

symptom control than medicine alone, provide a rationale for testing DBS in early PD. Investigators at Vanderbilt are conducting the first randomized investigation of STN DBS in 30 patients with early PD (IDE#G050016). We present here intraoperative MER from the first 11 participants randomized to surgery. Patients in the single-blind, randomized, parallel-groups trial must be ages 50–75 with Hoehn & Yahr Stage II idiopathic PD, and on anti-PD medicine less than 4 years without development of fluctuations. Surgical and MER analysis methods were identical to standard of care for advanced PD. Briefly, merged CT/MRI and planning software (Waypoint planner, FHC) were used to plan the surgical trajectory and manufacture customized stereotactic miniplatforms (mT Platform, StarFix, FHC). During electrode implantation, 10-s MER were made using 0.3–1 M $\Omega$  tungsten electrodes at 0.5 mm intervals along the trajectory and then processed and stored in a four-channel Leadpoint recording system (Medtronic). Root mean square of each trace was calculated (MATLAB, The Math-Works) and the STN/SN ratio was used to assess background activity. Single units were extracted via automated cluster analysis (Spike Sorter 2, Plexon) and visual inspection with template matching, and characterized (Neuroexplorer 4, Nex Technologies). MER data from the first 11 patients to receive surgery were included. They are 10 males and 1 female, with an average age of 60.0 years (range 50–74) and untreated Unified Parkinson's Disease Rating Scale motor score of 27.0. STN was identified in 98 tracks in 22 hemispheres, across which STN background activity was  $11.9 \pm 0.6$  mV. Median firing rate of the 136 STN units isolated was 25.5 Hz (25–75th percentile; range 17.9–35.5 Hz). In this small analysis, STN showed both lower overall activity and firing frequency than the range of 33–42 Hz that is typically reported in advanced PD. These findings provide evidence that STN hyperactivity continues to increase with progression of clinical findings in idiopathic PD, and underscore the importance of applying potentially neuroprotective therapies that rely on STN activity modulation as early as possible in the disease course.

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### Aesthetic Microsurgical Postoperative Maneuver Using Skull Bone Flap and Bone Wax in Experimental Traumatic Brain Injury Model Exacerbates Cortical Tissue Damage

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Traumatic brain injury (TBI) is the signature wound of the Afghanistan and Iraq wars. Understanding the pathology and designing treatment strategies for TBI will require standardized animal models. Here, we investigated a widely used TBI rodent model of controlled cortical impact injury. A review of the literature reveals inconsistent postoperative surgical closure of the skull area overlying the targeted impact of injury. We hypothesized that this postoperative wound closure by replacement of skull bone flap and bone wax would allow aesthetic reconstruction of the TBI-induced skull damage. Moderate to severe TBI often results in malformations to the skull bone, creating an unpleasant physical image to the patient. An aesthetic surgical maneuver may offer normalized skull bone structure. However, whether such surgical reconstruction post-TBI affords any detrimental effects to the damaged cortical tissue remains to be determined. All surgical procedures were conducted under aseptic conditions. Under deep anesthesia, adult male Sprague-Dawley rats (8 weeks old) received a 4-mm craniectomy over the left frontoparietal cortex (center at  $-2.0$  mm

AP and +2.0 mm ML to bregma). A pneumatically operated metal impactor (diameter 3 mm) impacted the brain at a velocity of 6.0 m/s, reaching a depth of either 1.0 mm (moderate TBI) or 2.0 mm (severe TBI) below the dura mater layer and remained in the brain tissue for 150 ms. Postoperatively, animals were randomly assigned to skull bone flap replacement with or without bone wax or no bone reconstruction. At 5 days post-TBI, animals were euthanized, and brains were harvested and processed for triphenyltetrazolium chloride (TTC) staining. The replacement of the skull bone with bone wax proved to be aesthetically pleasing and provided normalized gross bone architecture. TTC results, however, revealed that TBI animals that received skull bone flap with bone wax had much larger cortical damage than those TBI animals that underwent skull bone replacement only or no aesthetic maneuver at all. Interestingly, following TBI, especially when the severe model was performed, we observed brain herniation that would benefit from therapies directed at releasing intracranial pressure (i.e., skull bone removal). Aesthetic repair of the skull bone after TBI, while able to reconstruct the general bone structure, may actually exacerbate TBI-induced cortical tissue damage.

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#### Identification of the Subpopulation of Human Umbilical Cord Blood That Alters Spleen Function

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Systemic treatment of cerebral ischemia with human umbilical cord blood (HUCB) mononuclear cells (MNC) 48 h after middle cerebral artery occlusion (MCAO) in rats decreases infarct volume, induces significant recovery of motor function, and decreases the inflammatory response. HUCB cells decrease spleen size and reduce T-cell proliferation after MCAO. The HUCB MNC are composed of heterogeneous cell populations including T cells, B cells, monocytes, and macrophage and a small population of stem cells. In this study we examined the effect of different HUCB cell fractions on the cellular profiles of the spleen and blood. Adult male Sprague-Dawley rats (Harlan), weighing 250–400 g, underwent MCAO. They were injected 48 h post-MCAO with HUCB MNC or HUCB MNC from which CD133<sup>+</sup> stem cells, CD14<sup>+</sup> monocytes/macrophage or CD19<sup>+</sup> B cells were depleted. At 72 h post-MCAO the animals were euthanized, blood samples collected, and spleens harvested and homogenized. The spleen cells were then sorted utilizing flow cytometry to isolate the CD4<sup>+</sup> T cells, CD4<sup>+</sup> macrophages, and CD45Ra<sup>+</sup> B cells. Only in the CD133<sup>-</sup> group was infarct size significantly smaller ( $p < 0.05$ ). The CD14<sup>-</sup> HUCB treatment significantly decreased the number of CD4<sup>+</sup> macrophages in the spleen while the CD133<sup>-</sup> HUCB treatment increased their number compared to the MCAO-only group ( $p < 0.001$ ). In the blood, there was a significant decrease in the number of CD4<sup>+</sup> macrophages in all groups except the CD133-depleted group ( $p < 0.05$ ). All HUCB treatments decreased the number of CD4<sup>+</sup> T cells in the spleen compared to the MCAO-only group ( $p < 0.05$ ), but had no statistically significant effect on circulating T cells. There were no significant differences between the experimental groups when the number of B cells in the spleen or blood was determined. These results suggest that the main population impacted by HUCB cell treatment regardless of the composition of the HUCB preparation is the macrophage in the blood and spleen. In addition, all HUCB cell preparations decreased the number of CD4<sup>+</sup> T cells in the spleen. Further analysis of splenocyte proliferation will be performed.

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#### The Role of Noradrenergic Innervation in Neurodegeneration

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Early and progressive degeneration of locus coeruleus noradrenergic (LC-NE) neurons has been reported in patients with both Alzheimer's disease (AD) and Parkinson's disease (PD). However, the role of LC-NE degeneration in cognitive impairment and neuropathology observed in both diseases is not well studied. It has been suggested that the central NE system might influence neuronal survival, as well as glial function, in the CNS by way of suppressing oxidative stress and inflammation. NE receptors are located on blood-brain barrier-associated endothelial cells, as well as on microglial cells, astrocytes, and neurons. In vitro studies of each of these cell types demonstrate that NE plays a crucial role for functional maintenance of several different elements in the CNS. Our recent studies show that an LC-NE lesion, caused by the selective LC neurotoxin DSP-4, leads to accelerated neuropathology and memory impairment in a mouse model for Down syndrome, Ts65Dn mice. We have demonstrated that a selective DSP-4 lesion of LC-NE neurons leads to accelerated neuropathology and memory impairment in a mouse model for Down syndrome (Ts65Dn mice). In addition to working memory impairment, the DSP-4 lesion increased activation of microglial cells and reduced levels of brain-derived neurotrophic factor (BDNF) in the hippocampus. Hippocampal and cholinergic cell loss were exacerbated by the NE neurotoxin, suggesting that the NE innervation modality supports survival of these neurons, at least during the aging process. In addition, we have observed that DSP-4 and 6-OHDA lesions have additive effects on dopaminergic cells, suggesting that LC-NE declines also affect other signaling systems that are critical for cognitive and motor function. The current presentation will demonstrate recent clinical and basic science work in this field and propose several plausible scenarios for biological mechanisms involved.

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#### Symposium: Repair of the Stroke-Damaged Brain

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Most patients who survive the acute phase of stroke recover at least partially over the ensuing weeks to months. Some of the underlying mechanisms are being identified, including changes in gene expression, altered neuronal excitability, axonal sprouting, synaptogenesis, somatotropic reorganization, and formation of new cortico-cortical connections. These phenomena, in turn, suggest a variety of therapeutic interventions that might help to hasten or enhance clinical recovery, some of which will be discussed by the symposium speakers. David Greenberg will provide an introduction. Justin Hill will describe the role of the glial scar in stroke repair. Ron Frostig will report on sensory stimulation and protection from stroke. Qi Wan will discuss electrical stimulation and brain recovery from stroke.

### Human Umbilical Cord Blood Cells Improve Motor Function After Middle Cerebral Artery Occlusion

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The mononuclear fraction of human umbilical cord blood (HUCB) is a mixed population of immune cells and stem cells. These cells can induce motor and cognitive recovery and decrease infarct size in a rat model of ischemic stroke. When the CD14<sup>+</sup> or CD133<sup>+</sup> HUCB cells were depleted from the HUCB transplant, the injected HUCB cells no longer induced recovery. This study tested the hypothesis that CD14<sup>+</sup> monocytes or CD133<sup>+</sup> stem cells from HUCB are the cells that induce recovery. Male Sprague-Dawley rats underwent permanent MCAO followed 48 h later by IV administration of either vehicle or HUCB-derived cell (mononuclear, CD14<sup>+</sup>, CD2<sup>+</sup>, CD133<sup>+</sup>, or CD19<sup>+</sup>) preparations from which selected immune cell populations had been depleted. Additional controls included CD14 antibody and CD14<sup>+</sup> cells isolated from adult human peripheral blood. A behavioral test battery was administered both prior to MCAO and 1 month after MCAO to assess functional recovery. Upon completions of the last behavioral test, the animals were euthanized and infarct volume determined. When we measured grip strength, there was a 52 ± 10% decrease in strength of the affected forelimb after stroke. In contrast, HUCB mononuclear, CD2<sup>+</sup>, and CD14<sup>+</sup> monocytes improved grip strength over baseline levels by 35 ± 15% ( $p < 0.01$ ) and 21 ± 12% ( $p > 0.5$ , n.s.), respectively. Consistent with previous studies, HUCB mononuclear cells and CD14<sup>+</sup> monocytes improved motor function in an experimental rat model of ischemic stroke. In contrast to earlier studies, CD2<sup>+</sup> T cells also significantly improved motor function while CD133<sup>+</sup> cells did not. Further studies are warranted to determine the mechanisms underlying these effects.

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### Six-Month Suppression of Wild-Type Huntingtin Via Viral Vector Delivery Does Not Cause Marked Pathology in the Adult Nonhuman Primate Striatum

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Huntington's disease (HD) is a fatal neurodegenerative disorder, with autosomal dominant inheritance pattern, caused by expression of a mutant form of huntingtin (htt) protein containing an expanded polyglutamine repeat. One possible treatment for HD may be to reduce the expression of mutant htt in the brain via RNA interference. Unless the therapeutic molecule is designed to be allele specific, both wild-type and mutant protein will be suppressed by an RNA interference treatment. While Htt is required for embryonic development, its normal function in the adult brain is unclear. To test the hypothesis that the adult primate brain could tolerate reduced levels of wild-type htt

protein, an adeno-associated viral vector encoding for a short hairpin RNA targeting rhesus htt mRNA was bilaterally injected into the caudate and putamen of four adult rhesus monkeys. Four additional monkeys received a comparable vector encoding for a scrambled control siRNA. General health and motor behavior were monitored for 6 months. Upon termination, brain tissues were blindly sampled for 1) htt mRNA knockdown by RT-PCR, 2) htt protein expression by immunohistochemistry, and 3) neuropathological changes. Widespread reduction in htt protein averaging ~30% was measured in the brains of monkeys receiving the active anti-htt shRNA 6 months postinjection. However, no adverse effects related to htt reduction were observed behaviorally, and no neurodegeneration was found on pathology versus controls. These results suggest that long-term suppression of wild-type htt may be safe, allowing suppression of both the wild-type and mutant protein to result in a net benefit for heterozygous HD patients.

*Supported by Medtronic, Inc.*

### Long-Term Results of a Double-Blind, Randomized, Sham Surgery-Controlled Trial of Intrastriatal Spheramine® Implantation in Patients With Advanced Parkinson's Disease

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Spheramine® consists of human retinal pigment epithelial (hrPE) cells attached to a microcarrier support matrix. hrPE cells produce L-3,4-dihydroxyphenylalanine (L-dopa) and could provide a more continual supply in the striatum compared with intermittent oral L-dopa. Following preclinical studies and a positive pilot clinical study suggesting a treatment effect, a randomized, double-blind, sham surgery-controlled trial was initiated to evaluate the efficacy of bilateral Spheramine implantation in patients with advanced Parkinson's disease (PD). Seventy-one patients with PD aged 36–70 years were randomized to receive either Spheramine ( $n = 35$ ) or sham surgery ( $n = 36$ ). Patients were symptomatic for ≥ 5 years, Hoehn and Yahr stage 3 to 4 (H&Y) OFF medication, and had parkinsonian symptoms responsive to oral L-dopa but insufficiently controlled by optimized pharmacotherapy. Spheramine patients received bilateral implantation (~325,000 cells/site) into the postcommissural putamen, whereas sham surgery patients received partial burr holes. The primary efficacy endpoint was a change in the Unified Parkinson's Disease Rating Scale (UPDRS) Part III (motor) score in the OFF medication state from baseline to 12 months after treatment. Patients were followed for up to 4 years under double-blind conditions, until the final patient reached the 12 month

follow-up point. The mean changes from baseline in UPDRS motor score OFF medication were not significantly different in the Spheramine and sham groups ( $-10.5$  and  $-10.1$ , respectively;  $p = 0.9387$ ). Secondary endpoints were similarly not significantly different between the two groups, including the UPDRS motor and activities of daily living (ADL) ON scores, and LL-dopa reduction. The improvement in the UPDRS motor OFF score achieved at 12 months was sustained through 24 months in both the Spheramine ( $n = 23$ ) and sham surgery ( $n = 27$ ) treatment groups. Additional long-term data were available, demonstrating persistent reductions in UPDRS motor OFF score at 36 and 48 months in both the Spheramine and sham surgery groups. At month 48, the mean  $\pm$  SD change from baseline was  $-14.8 \pm 13.88$  points for the Spheramine ( $n = 12$ ) and  $-11.0 \pm 8.26$  points for sham surgery ( $n = 14$ ) groups. While Spheramine did not provide antiparkinsonian benefits compared to a sham surgery control procedure, the data demonstrated a striking long-term change similar in magnitude in both the Spheramine and sham surgery groups under blinded conditions ("placebo effect").

#### **Transplantation of Schwann Cells With Embryonic Motoneurons Into Axotomized Tibial Nerve Promotes Neuron Survival, Axon Regeneration, and Muscle Reinnervation**

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Some spinal cord and peripheral nerve injuries cause flaccid paralysis because of motor neuron death. The absence of motor axons to distal muscles causes immediate skeletal muscle denervation atrophy and inexcitability for which there is no specific treatment. With time this denervation leads to muscle degeneration. One intervention strategy to restore muscle innervation is to transplant neurons into nerves connected to denervated muscles (Thomas et al., *J. Neurophysiol.* 84: 591–595; 2000). This local source of neurons results in muscle reinnervation, which curtails atrophy and reestablishes muscle excitability. However, the muscles are weak mainly because of incomplete reinnervation. Here we test the hypothesis that an exogenous neurotrophic supply for transplanted motoneurons provided via cotransplantation of Schwann cells improves motoneuron survival. Adult female Fischer 344 rats were transplant recipients. Denervation of hind limb muscles was by tibial nerve section. One week later, 1 million E14–15 ventral spinal cord cells ( $\pm 500,000$  Schwann cells) were placed into the distal tibial nerve stump. To test the importance of different kinds of neurotrophic support, Schwann cells were also transduced *ex vivo* with lentivirus (LV) vectors encoding the multifunctional neurotrophin, NT-3/D15A (Urfer et al., *EMBO J.* 13:5896–5909; 1994) or a modified version, NT-3/D15A/p75-2 [a mutation that reduces p75NTR (a neurotrophin/tumor necrosis factor receptor) affinity and possible p75NTR-dependent motoneuron death]. Ten weeks after transplantation there was greater cholinergic neuron survival in the NT-3/D15A/p75-2 cotransplants. The presence of local Schwann cells and neurons increased axon growth into the medial gastrocnemius muscle. Experimental muscles were weak compared to muscles of naive control rats despite significant reinnervation and reversal of muscle fiber atrophy. The increase in the mean myofiber size and muscle excitability introduces the potential to stimulate the transplanted neurons electrically to control muscle contractions and to initiate joint movements.

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#### **High Levels of Dopamine Transporter Immunoreactivity in the Reinnervated Putamen of Parkinson's Disease Patients Over a Decade After Transplantation With Fetal Dopamine Neurons**

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Transplanted human fetal ventral mesencephalic (VM) neurons have shown functional benefits in some patients with Parkinson's disease (PD). We have previously reported the clinical outcome and post-mortem analyses of five patients with advanced idiopathic PD, who received intraputamenal transplantation with fetal VM cells prepared as a cell suspension [Cooper et al., *J. Neurol.* 256(Suppl. 3):310–316, 2009; Mendez et al., *Brain* 128:1498–1510, 2005; Mendez et al., *Nat. Med.* 14:507–509, 2008]. Grafted cells survived for up to 14 years after transplantation. No side effects, such as off-period dyskinesias, were observed. Immunostaining for tyrosine hydroxylase (TH), G-protein-coupled-inward rectifying current potassium channel type 2 (Girk2), and calbindin revealed surviving dopaminergic grafts that were well-integrated with the host putamen and showed extensive neuritic outgrowth. The host microglial reaction was minimal and no T-cell infiltration in the host putamen was observed. To further understand the phenotypical characteristics of transplanted VM neurons, we have undertaken studies to assess expression of dopamine transporters (DAT) in transplanted neurons in our patient series. DAT are located presynaptically on dopaminergic terminals where they are responsible for the reuptake of dopamine after synaptic release. In PD, DAT expression in the striatum is diminished, reflecting the degeneration of midbrain dopaminergic neurons. We show, using immunofluorescence staining, that DAT is robustly expressed in transplanted dopamine neuron terminals in the reinnervated putamen and caudate, and that DAT expression is maintained for up to 14 years posttransplantation. In addition, the transplanted dopamine neurons show a healthy and nonatrophied morphology. Our postmortem examination of tissue from PD patients who have received fetal VM cell transplantation demonstrates that grafted fetal dopaminergic neurons survive for over a decade post-transplantation without significant signs of neurodegeneration. These findings are encouraging for the development of future fetal and stem cell replacement therapies for PD.

#### **Intravenous Administration of Human Multipotent Adult Progenitor Cells Provides Functional Benefit Through Immunomodulation in a Mouse Model of Multiple Sclerosis**

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Stem cell transplantation is emerging as a potential therapeutic treatment for autoimmune disorders such as multiple sclerosis (MS). However, little is known regarding the mechanistic interaction between the diseased tissue environment and transplanted cells. In the present study, we demonstrate sustained functional benefit in mice with experimental allergic encephalomyelitis (EAE) after IV administration of MultiStem®, an adherent human pluripotent adult stem cell product. EAE was induced via myelin oligodendrocyte glycoprotein (MOG) peptide and subsequent pertussis toxin injection. Three different doses of stem cells (1, 3, or 9 million cells), or vehicle, were adminis-

tered after symptom onset. Behavioral assessment was performed daily for 28 days after cell administration. Each cell dose level resulted in statistically significant improvement compared to vehicle treatment. Twenty-eight days after cell administration animals were sacrificed for postmortem analyses. Luxol fast blue and toluidine blue staining demonstrated decreased lesion burden within the spinal cord and a shift from complete to partial lesions in MultiStem-treated animals compared to controls. Electron microscopic analysis provided evidence of remyelination. Tissue microarray analysis was utilized to investigate the gene expression changes induced by EAE, and the subsequent effects of MultiStem administration upon EAE-induced gene expression changes. Animals treated with vehicle or 9 million cells were sacrificed and tissues harvested 3 days post-cell administration, to assay immediate gene expression changes. The paradigm was repeated at 28 days post-cell administration, to assess long-term gene expression changes. Spinal cord, spleen, and blood microarray analyses were performed using MouseRef-8 Illumina beadchips. Gene ontology and pathway analyses outlined a number of significant gene expression changes induced by EAE that were reversed by MultiStem administration. Within the spinal cord, significantly decreased expression of genes related to neuronal projection organization and synapse structure were seen in EAE animals and this was significantly reversed by MultiStem administration. Conversely, increased expression of a large number of immune-related genes within the spinal cord was seen in EAE and this was significantly reduced by MultiStem administration. Affected genes related to B- and T-cell-mediated immunity, antigen processing and presentation, and regulation of cytokine production. Blood and spleen microarray analyses showed similar immune-related changes induced by EAE and modulated by MultiStem administration. The results of these studies suggest that treatment of MS patients with MultiStem may provide clinical benefit through modulation of the autoimmune dysfunction that is thought to contribute to the disease.

#### **BMP7 Activates Gelatinase Activity and Alters Neuronal Morphology in Rat Primary Cortical Neurons**

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Previous studies from our group and others have demonstrated that bone morphogenetic proteins (BMPs) have neuroprotective and neuroregenerative properties in rodent models of stroke and Parkinson's disease. In primary cultures, BMP7 promotes neuritic outgrowth of rat sympathetic neurons. We explored the neuroregenerative effects of BMP7 using rat primary cortical cultures. Cultures were continuously exposed to BMP7 from DIV4 to DIV15 and exhibited a dose- and time-dependent change in axonal and dendritic morphology based on immunoreactivity to neurofilament (NF-H) and microtubule associated protein 2 (MAP2), respectively. BMP7-treated cultures contained large NF-H-immunoreactive fibers whereas untreated cultures showed a diffuse staining pattern of NF-H with small fibers. No changes were observed in glial fibrillary associated protein (GFAP) immunoreactivity, indicating that astrocyte morphology was unaffected by BMP7. These observed changes in neuronal morphology suggested alterations to extracellular matrix, so we next examined the effects of BMP7 on gelatinase and collagenase activity. Cultures were treated with BMP7 on DIV4 until DIV11 when significant changes in NF-H immunostaining were observed. BMP7 caused a dose-dependent increase in gelatinase activity as early as DIV6. Collectively, our data show that BMP7 increases gelatinase activity and alters NF-H and MAP2 staining in primary cultures. Our data suggest that the neuroregenerative effects of BMP7 may involve gelatinase activation and alterations to extracellular matrix.

#### **Synaptogenesis in the Interaction Between Astrocytes and Neurite Formation**

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Understanding the cross talk between astroglia and neurite formation is important for guiding the neurites to their targets, which has regenerative potentials for treatment of neurodegenerative diseases. This cross talk might occur through the integrin-associated protein CD47, which serves as a ligand for signal regulatory protein- $\alpha$  (SIRP $\alpha$ ). SIRP $\alpha$  takes part in synaptogenesis, and therefore the present study was focused on synaptophysin, a presynaptic protein, and its presence in relation to nerve fiber growth in organotypic tissue cultures from ventral mesencephalon (VM) of embryonic day (E) 14 and E18 fetuses. In E14 cultures from CD47 knockout mouse, tyrosine hydroxylase (TH)-positive nerve fiber growth was robust and independent of the presence of astrocytes, while in wild-type cultures nerve fibers were restricted to the astrocytes. Most of the astrocytes were immunoreactive for synaptophysin in the CD47 knockout cultures but not in the wild-type cultures. The presence of synaptophysin was then investigated in E14 and E18 cultures of rat VM. In E14 VM cultures, nerve fibers covered the most distally migrating astrocytes, independent of whether or not they were synaptophysin positive. In E18 cultures, neurites grew to a definite border within the migrating astrocytes, which reached much longer distances. Synaptophysin immunoreactivity was only present in astrocytes associated with neurites. Therefore, synaptophysin seems to play different roles during developmental stages and besides its presynaptic role, it appears to function as a permissive guiding molecule when associated with astrocytes. Thus, these data demonstrate the importance of the astrocytes as the main key player in guiding nerve fibers expressing synaptogenetic markers to modulate the neurite formation.

#### **Medial Ganglionic Eminence Precursor Cell Grafting Diminishes Spontaneous Seizures and Improves Memory Function in a Rat Model of Temporal Lobe Epilepsy**

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Many patients with temporal lobe epilepsy (TLE) display seizures that are resistant to antiepileptic drugs, and memory dysfunction. Hence, there is a need for novel therapies that both restrain spontaneous recurrent seizures (SRS) and improve memory function in TLE. Neural cell grafting approach has the potential to serve as an alternative therapy for TLE.  $\gamma$ -Aminobutyric acid (GABA)-producing cells from the medial ganglionic eminence (MGE) appear ideal for grafting into the hippocampus in TLE because the epileptic hippocampus exhibits an altered inhibitory function, and MGE cells have the ability to produce hippocampal-specific GABAergic interneurons. Therefore, we examined the effects of grafting of freshly harvested MGE cells from the gestation day 14 rat brains into the hippocampi (3 grafts per side, 100,000 live cells/graft) of rats exhibiting TLE typified by SRS and spatial memory dysfunction. The donor cells were labeled *in vivo* prior to harvesting via IP injections of 5'-bromodeoxyuridine into pregnant rats at gestation days 10–14. Induction of TLE in host rats was accomplished through graded IP kainic acid injections, which initially caused status epilepticus and later induced chronic epilepsy typified by SRS. A group of chronically epileptic animals exhibiting a similar frequency and intensity of behavioral SRS, and spatial memory dysfunction in a water maze test (WMT), was chosen for MGE cell grafting. The animals were implanted with epidural electrodes and

continuous video-electroencephalographic (EEG) recordings were done for 2 weeks during the second month after grafting to measure the effects of grafting on both behavioral and EEG SRS. The animals were next examined for spatial memory function in a WMT. The behavioral SRS were greatly reduced in epileptic rats receiving MGE precursor cell grafts. In comparison to their pregrafting scores, the reductions were 91% for the frequency of all SRS, 93% for the total percentage of time spent in SRS, and 100% for the frequency of stage V seizures. Additional comparison with the epilepsy-only group revealed that MGE precursor cell grafting greatly reduced both behavioral and EEG seizures. Analyses of spatial memory scores between the pre- and postgrafting periods showed that MGE cell grafting eased the memory dysfunction seen before grafting. Grafts demonstrated a survival that was equivalent to ~30% of injected cells and a migration pattern that was somewhat restricted to the immediate surrounding regions of the graft core. Furthermore, grafted cells differentiated into large numbers of GABAergic neurons including the subclasses that are positive for neuropeptide Y, somatostatin, parvalbumin, and reelin. Thus, grafting of MGE precursor cells into the hippocampi of rats exhibiting chronic TLE results in addition of substantial numbers of GABAergic neurons, reduction of both frequency and intensity of SRS, and an improved spatial memory function.

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#### **Elevation of Cyclic AMP After Spinal Cord Injury by Electrical Stimulation of Hindbrain Raphe Nuclei: A Role for Serotonin**

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A common strategy to improve recovery after spinal cord injury (SCI) is to support neuronal regeneration. Elevation of cyclic adenosine monophosphate (cAMP) is one of the more promising of these strategies. Many effects of cAMP are transcription dependent and promote the expression of regeneration-associated genes that could be beneficial after SCI. Methods that have been used to increase the intracellular level of cAMP include the administration of phosphodiesterase inhibitors such as rolipram and the cAMP analogue dibutyryl cAMP. The present study evaluates the effect of electrical stimulation (ES) of the nucleus raphe magnus (NRM) on the intracellular concentration of cAMP in various spinal cord segments after an injury. To determine the contribution of serotonin release to this modulation of cAMP, we treated animals with pimozone, a potent antagonist of stimulatory G-protein-coupled 5-HT<sub>7</sub> receptors. Following a moderate T8 contusion, rats were subjected to different treatments as follows: no ES, ES, pimozone (1.0 mg/kg) alone or in combination with ES. Control groups received no injury. Intermittent stimulation was applied to the NRM for 2 h 3 days postinjury. The intracellular concentration of cAMP in cervical, thoracic, and lumbar sections was measured by enzyme-linked immunosorbent assay. The contusion injury reduced cAMP concentration in thoracic tissue proximal to the site of impact and also in cervical and lumbar tissue. Application of ES significantly increased intracellular cAMP in cervical and thoracic tissue of injured animals when compared to the injured group that received no stimulation. Administration of pimozone led to a significant decrease in the levels of cAMP in all tissue in the injured nonstimulated groups compared to noninjured groups. This decrease was not overcome by ES. These results demonstrate that a few hours of ES in the NRM restores levels of cAMP to near normal in spinal cord tissue near and remote to the site of injury. Thus, the proposed beneficial activity of the cAMP second messenger system on SCI is at least partially under serotonergic control.

#### **Graft-Induced Recovery in an Automated Habit Learning Task**

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Near complete unilateral dopaminergic depletion via infusion of 6-hydroxydopamine to the ascending dopaminergic bundle has been shown to lead to an impairment in contralateral space when animals had to perform on a lateralized choice reaction time task. In the present experiment, naive rats were trained on an automated habit learning task conducted in the rat nine-hole operant chamber. The animals had to perform a sustained nose-poke into the illuminated center hole of an array of nine holes with the two holes to the left or right of the center hole being available on alternating days, while the remaining holes were covered. After a variable delay a brief light flash in either the near or the far response hole from the center hole indicated the required response. All animals were trained on this task on each side on alternating days before being matched into three groups. Whereas the untreated control group performed well on this task, animals that received unilateral lesions leading to near complete depletion of dopamine from one striatum displayed a bias towards the near hole when responses had to be made on the side contralateral to the lesion. Dopamine-rich dissociated cell suspension grafts were able to ameliorate some of the lesion-induced deficits on the operant task, including increasing the number of usable trials, a reduction in movement times, and a small increase in accuracy to far contralateral responses. These data confirm previous findings that this version of the lateralized choice reaction time task is useful for assessing terminal dopamine depletion, which causes a response bias to near contralateral space, and extend the findings to the unilateral bundle lesion model. The bundle lesion causes a stable deficit that does not show signs of spontaneous recovery up to 50 days postlesion testing. Furthermore, the task is sensitive enough to detect improvements by small dopamine-rich transplants and is therefore a valuable tool to assess cell replacement and repair strategies.

#### **Transplantation of Differentiated Human Embryonic Stem Cells Into a Huntington's Disease Model: The Challenges of Generating Neural Cells Suitable for Replacement Therapy in Neurodegenerative Disease**

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Therapies to replace the neuronal cell loss associated with neurodegenerative diseases to ameliorate the effects of diseases such as Huntington's disease (HD) are an attractive treatment paradigm. Our group in recent years has tasked itself with understanding and driving the neural differentiation of human embryonic stem cells (hESC) to neural progenitor cells suitable for HD cell replacement therapy. After the progress made by ourselves and others in producing candidate differentiation culture protocols, we have more recently begun to examine the challenges of downstream clinical applications such as further refining and converting our protocols to scale up while retaining cell phenotype and purity. Here we set out initial *in vivo* studies to determine efficacy and our ongoing strategy to further refine protocols, with the goal of suitable clinical grade cultures. To generate cells suitable for initial grafting experiments we built on previous neurosphere differentiation culture procedures, using two hESC lines (Hues9 and H9). *In vitro* quality control measures of cultures routinely include flow cytometry and immunofluorescence microscopic analysis, and RT-qPCR panels of cell fate markers [such as pluripotent markers (OCT4, NANOG), neural progenitor markers (PAX6, SOX1), and rostrocaudal neural identity (FOXP1, DBX1, EN2, and HOXB4)]. Cell

culture scale-up experiments were undertaken using a Novapod (Med-Cell) bioreactor, which produced a greater yield of more homogeneous neurospheres, compared to static dish cultures. During these initial scale-up studies morphological and gene expression analysis showed progression of neural progenitor differentiation, suggesting they are suitable for ongoing graft studies. Our protocol development strategy examined the effects of dose regimes of exogenous factors and animal product replacement substitutes upon phenotypic fate, and included the testing of Good Manufacturing Practice (GMP)-compatible media and supplements, together with the substitution of growth factors with small molecules. Derived cells were grafted into a unilateral quinolinic acid rat HD model and assessed for graft survival (HuNu), proliferation (Ki67), morphology, and differentiation (NeuN, FoxP1, DARPP32) using immunohistological investigation. We examined graft survival and morphology at 12 weeks postgraft, and further validated a desensitization protocol that will facilitate long-term graft studies in the rat model. Throughout these studies graft cell morphology and gene expression analysis showed progression of neural progenitor differentiation. While there is considerable way to go in refining the “neural cell replacement strategy” for transplantation in neurodegenerative diseases, these data demonstrate our continuing systematic approach to protocol development. In doing so, establishing standard procedures and quality assessments that are increasingly compatible with the needs of future clinical grade production of cells suitable for transplantation in HD and other neurodegenerative diseases.

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#### **The Master Negative Regulator REST/NRSF Controls Adult Neurogenesis by Restraining the Neurogenic Program in Quiescent Stem Cells**

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Transcriptional regulation is a critical mechanism in the birth, specification, and differentiation of newborn neurons in the adult hippocampus. One of the first negative-acting transcriptional regulators implicated in vertebrate development is repressor element silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF)—thought to regulate hundreds of neuron-specific genes—yet its function in the adult brain remains elusive. Here we report that REST/NRSF is required to maintain the adult neural stem cell (NSC) pool and orchestrate stage-specific differentiation. REST/NRSF recruits CoREST and mSin3A corepressors to stem cell chromatin for the regulation of proneuronal target genes to prevent precocious neuronal differentiation in cultured adult NSCs. Moreover, mice lacking REST/NRSF specifically in NSCs display a transient increase in adult neurogenesis that leads to the exhaustion of the pool of NSCs and eventually diminished newborn neurons. Our work identifies REST/NRSF as a master negative regulator of adult NSC differentiation and offers a potential molecular target for neuroregenerative approaches.

#### **The Use of Human-Induced Pluripotent Stem Cell-Derived Neural Precursors in the Treatment of Brain and Spinal Cord Injury**

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No treatment currently exists to restore lost neurological function after stroke or spinal cord injury. The transplantation of stem cells or progenitors may support neural repair. In this study, we used human-induced pluripotent stem cell (iMR90)-derived neural precursors (iPS-NPs) for transplantation in a rat middle cerebral artery occlusion (MCAO) model of stroke or a balloon-induced spinal cord compression lesion (SCI). Neural precursors were derived from iPS through the microaggregates stage using 300 ng/ml noggin and 20  $\mu$ M SB. Prior to *in vivo* experiments, *in vitro* analysis of iPS-NPs was performed. Subsequently, the percentage of cells expressing markers of neural precursors, including oct3/4, sox2, SSEA-4, SSEA-1, TRA-1-60, CD24, CD133, CD56,  $\beta$ III-tubulin, NF70, nestin, CD271, and CD29, was assessed by fluorescence activated cell sorting (FACS) in undifferentiated and predifferentiated [fibroblast growth factor (FGF) and epidermal growth factor (EGF) omitted from the culture medium (CM) for 7 days] NPs. Female Sprague-Dawley rats were subjected to focal cerebral ischemia by reversible right MCAO for 90 min, while male Wistar rats were used for SCI. A suspension of iPS-NPs (300,000 cells in 3  $\mu$ l of CM) was transplanted into the lesions 7 days after MCAO or SCI ( $n = 14$ ,  $n = 12$ ); the control groups ( $n = 8$ ) were injected with saline. Metabolic profiles in the striatal tissue of both hemispheres were assessed by magnetic resonance spectroscopy (MRS) in the MCAO model. Four months after MCAO, MRS revealed that the concentrations of brain metabolites (glutamate, glutamine, *N*-acetyl-aspartate, creatine, taurine, choline, and inositol) in grafted animals returned nearly to the values found in unlesioned animals. Functional recovery was assessed by the apomorphine-induced rotation, tape removal, rotating pole, and Basso-Beattie-Bresnahan (BBB) tests performed regularly after transplantation. The grafted animals in the stroke model displayed a decreased number of clockwise rotations in the apomorphine rotation-induced test, while animals with SCI significantly improved their BBB score when compared to control animals. iPS-NPs robustly survived in both models of injury, maintained their neural phenotype, and migrated toward the lesioned area during 2–4 months after transplantation. In addition, some of the cells differentiated into more mature and tissue-specific neurons (NSE-, MAP2-, and DARPP32-positive cells) in this period. No tumor formation was observed throughout the whole experiment. Taken together, these results suggest that iPS-NPs undergo further differentiation after transplantation, integrate into the neural tissue, partially improve functional outcome, and can serve as a safe tool for cell transplantation therapy.

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#### **Attenuation of Chronic Neuropathic Pain by Experimental Approach Using Recombinant Neuroprogenitor Cells in Rats**

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Hypothesized mechanisms underlying chronic neuropathic pain following injury to the peripheral or central nervous system include increased hyperexcitability of spinal dorsal horn neurons due to loss or dysfunction of inhibitory  $\gamma$ -aminobutyric acid (GABA)-ergic interneurons, and enhanced excitatory glutamate signaling through *N*-methyl-D-aspartic acid (NMDA) receptors. Restoration of inhibitory tone and/or reduction of hyperexcitability via transplantation of selected cell types is a potential long-term intervention. The use of a recombinant neuronal progenitor cell (NPC) approach targeting GABA and glutamatergic signaling was evaluated in these experiments. Predifferentiated rat GABAergic NPCs were intraspinally injected into rats with peripheral nerve injury-induced pain using the chronic constriction injury (CCI) model. An improvement in behavioral outcome was observed for mechanical hyperalgesia and cold allodynia. Concurrent intrathecal injection of serine-histogranin (SHG), an NMDA antagonist, enhanced the analgesic effect of grafted cells. In

accordance with behavioral data we found a decreased number of c-Fos-positive neurons in the superficial dorsal horn laminae in SHG-treated rats, suggesting attenuated injury-induced excitation of nociceptive spinal neurons. For site-specific release of SHG a recombinant NPC capable of releasing GABA and SHG was engineered and intraspinally transplanted into rats after peripheral nerve injury. A markedly enhanced attenuation of cold allodynia and modestly attenuated mechanical hyperalgesia were observed. Intraspinal injection of GABAergic NPCs was also used in the clip compression model of below-level pain after spinal cord injury. Beneficial effects in reducing tactile and cold allodynia were observed for several weeks postgrafting. Our results suggest that transplantation of GABAergic NPC can attenuate peripheral and central nerve injury-induced chronic pain and that enhancement of this approach could be achieved by genetic modification of grafted cells.

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### **Fate Determinant Expression in the Lesioned Brain: The Role of Dlx2 and Pax2**

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Compensatory replacement of neurons by endogenous subventricular zone (SVZ)-derived neural precursor cells (NPCs) has been demonstrated in the adult brain following striatal cell loss. Such cell replacement is associated with increased SVZ cell proliferation and neuroblast expansion in the rostral migratory stream (RMS), as well as directed migration of NPCs extending from the SVZ to the areas of striatal damage. However, the number of new neurons that migrate into the lesioned striatum and survive is low. SVZ-derived NPCs coexpress multiple transcription factors involved in lineage restriction and cell fate determination. We propose that compensatory neurogenesis in response to striatal cell loss will alter the temporal expression of transcription factors in discrete populations of SVZ-derived NPCs. We therefore examined the expression of mammalian achaete scute homolog 1 (Mash1), homeobox protein distal-less (Dlx2), paired box gene 6 (aniridia, keratitis) (Pax6), and oligodendrocyte transcription factor 2 (Olig2) in SVZ-derived NPC populations across a range of times following quinolinic acid (QA)-induced striatal cell death. We identified a heterogeneous population of SVZ-derived NPCs that respond independently to striatal cell loss. In both the anterior SVZ (aSVZ) and RMS we observed an increase in a subpopulation of Dlx2<sup>+</sup> transit amplifying precursor (TAP) cells and neuroblasts following QA lesioning when compared to controls. Subsequently, the number of Pax6<sup>+</sup> TAPs and neuroblasts in the QA-lesioned aSVZ and RMS was also increased. This suggests that Dlx2 and Pax6 may play an important role in directing NPC proliferation and neuroblast generation following striatal cell loss. Selective alteration of these factors in the SVZ in response to cell loss may predetermine the subsequent generation of specific neuronal subclasses for endogenous replacement. Retroviral green fluorescent protein (RV-GFP) tracing studies showed that, consistent with the bipotency of cells generated in the SVZ, both neuroblasts and oligodendrocyte precursor cells (OPCs) migrated into the damaged striatum. However, Dlx2<sup>+</sup> and Dcx<sup>+</sup> neuroblasts only made up a small percentage of migratory cells in the striatum, with the majority of GFP-labeled cells identified as Olig2<sup>+</sup> OPCs, NG2<sup>+</sup> precursor cells, or cells lineage negative (LN) for either neuronal or oligodendrocyte markers. This suggests there is a large capacity for enhanced neuronal induction in the damaged striatum. Our current studies are investigating whether overexpression of Dlx2 or Pax6 in SVZ-derived NPCs could redirect OPCs and/or LN cells towards a neuronal fate, therefore generating higher neuroblast numbers in the damaged striatum, and potentially enhancing the endogenous repair process. Identifying the temporal dynamics of specific transcription factors within the SVZ-RMS pathway in response to brain cell loss will expand our knowledge regarding the process of adult neurogenesis and may indi-

cate mechanisms by which endogenous neurogenesis can be enhanced for the treatment of brain injury or disease.

### **Alternative Neuroprotection Mechanisms of Cyclosporine A in Ischemic Stroke Via Mitochondrial Pathway**

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Oxidative stress-induced mitochondrial dysfunction plays a crucial role in the pathogenesis of a wide range of diseases, including ischemic stroke. Under hypoxic ischemia condition, the mitochondrial membrane potential, respiratory-related enzymes, and mitochondrial DNA (mtDNA) are deteriorated by radical oxygen species. Cyclosporine A (CsA), an immunosuppressant drug, has been investigated as a possible neuroprotective agent that ameliorates neuronal cell death. However, the molecular mechanism by which CsA interacts with mitochondrial membrane-associated proteins, which regulate mitochondrial membrane potential ( $\Delta\Psi_m$ ), and with neurotrophic factors remains an enigma. We examined CsA in an hypoxic-ischemia in vitro model in which we exposed primary rat neural cells to oxygen glucose deprivation (OGD), and found that pretreatment of primary rat neural cells with CsA increases neuroprotective effects, which increase cell viability and decrease oxidative stress during OGD periods, in comparison with the administration of CsA after ischemic insults. CsA prevented 1) depolarization of the mitochondrial membrane from OGD via blocking the formation of the mitochondrial permeability transition pore complex, 2) release of cytochrome c, which turns on the caspase-induced apoptotic signal pathway, and 3) induction of the mtDNA fragmentation. This efficacy occurred in a dose-dependent manner and over a longitudinal time scale. Interestingly, CsA does not significantly influence mitochondrial reductase activity, suggesting that CsA would not be neurotoxic but instead an alternative neuroprotective signal transduction. CsA selectively binds calcineurin, a Ca<sup>2+</sup>-calmodulin-dependent phosphatase that is highly enriched in the brain, and significantly inhibits its activity, which regulates plasma membrane depolarization by dephosphorylation of Na<sup>+</sup>/K<sup>+</sup>-ATPase. We found that CsA would cross talk with dopamine cAMP-regulated phosphoprotein-32 to maintain neuronal cell homeostasis and that, furthermore, CsA enhanced DJ-1, a Parkinson disease-associated protein, translocation in the mitochondria and secretion of DJ-1 into neighboring neuronal cells with reduced oxidative stress after OGD. This pilot study further supports the notion that CsA is intimately associated with early phases of disease progression in stroke.

### **Transplantation of Human Amniotic Mesenchymal Stromal Cells Via the Jugular Vein at Subacute Period of Neonatal Hypoxic-Ischemic Injury in Rats Promotes Long-Term Behavioral Improvement**

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Intravenous (IV) transplantation of human amniotic mesenchymal stromal cells (hAMSC) affords neurobehavioral benefits in an animal model of adult stroke (Kaneko et al., J. Pineal Res., in press). Here, we tested whether IV hAMSC also renders therapeutic effects in a neonatal rat model of cerebral palsy (CP). CP in the neonate occurs in about 10 of 1,000 full-term infants in the US. The current state of the

art for treating CP is hypothermia, but only neonates with moderate encephalopathy appear to exhibit the most favorable neurodevelopmental outcome. Hence, there is a clinical need to repair the already damaged regions requiring a cellular replacement approach that may be applicable for neonates with moderate to severe CP. CP and adult stroke share similar pathophysiological manifestations. Cell therapy has been explored in the clinic as an experimental treatment for stroke over the last decade and was recently initiated for CP patients. Whether functional recovery in transplanted CP is directly influenced by the dose of the transplanted cells remains to be fully determined. An ample supply of stem cells can be harvested from the amnion that should allow autologous transplantation in CP. We used the established Vannucci rodent model of CP, involving the ligation of a unilateral carotid artery in a postnatal day 7 rat followed by exposure to systemic hypoxia (8% oxygen) for 2.5 h. The model produces injury to the cerebral cortex, subcortical and periventricular white matter, striatum, and hippocampus on the side of the ligation, as well as behavioral deficits similar to that seen in human neonates with CP. At 7 days after CP, neonates received either IV transplants of hAMSC (0–500 K dose, supplied by Dr. Parolini) or vehicle. Weekly testing, up to 4 weeks post-CP, revealed that transplanted CP neonates displayed dose-dependent improvements in many of the behavioral indices, including general locomotor activity and sensorimotor functions, starting at 1 week posttransplantation, which were sustained throughout the 4-week testing period, indicating robust and stable efficacy of hAMSC. Histological analyses are ongoing. Cell therapy was shown to be beneficial in CP, advancing the concept of minimally invasive IV transplantation of hAMSC in neonatal brain injuries.

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#### **Etiopathogenic Events Leading to Cell Death in a Delayed and Progressive Parkinson's Disease Model**

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Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder and is clinically characterized by a battery of debilitating and painful motor and nonmotor symptoms. Pathologically, motor symptoms manifest when striatal dopamine (DA) levels are decreased ~80%, which correspond to ~60% loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). Currently, the only available treatments for PD patients temporarily suppress some symptoms but fail to slow the degenerative process. While the postmortem parkinsonian profile is well characterized, etiopathogenic events early in the disease process are poorly understood. Effectively modeling the pathogenic process leading to SNpc cell death in PD may be important for developing neuroprotective strategies. A recent series of studies using systemically injected lipopolysaccharide (LPS) have shown delayed and progressive decreases in SNpc DA neurons in mice (Qin et al., 2007; Liu et al., 2008). In this model, 2-month-old male C57BL/6J mice receiving 5 mg/kg IP LPS display a significant (23%) loss of tyrosine hydroxylase (TH) nigral neurons at 7 months that progresses to 47% loss over 10 months. Additionally, chronic neuroinflammation and increased  $\alpha$ -synuclein ( $\alpha$ -syn), the primary protein abnormally aggregated in pathogenic Lewy bodies, are observed. Using this model, initial studies from our laboratory revealed that striatal anterograde transport protein kinesin heavy chain levels are significantly decreased ( $20.81 \pm 0.91$ ;  $p < 0.05$ ) from saline-treated controls ( $25.55 \pm 1.96$ ) at 4 months, whereas no differences were observed at 3 months ( $15.19 \pm 1.23$  and  $15.36 \pm 1.34$  for LPS and saline, respectively). Additional

preliminary studies from our laboratory revealed that primate kinesin heavy chain levels are reduced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP;  $807.49 \pm 108.7$ ;  $p < 0.01$ ) and are not restored to control levels ( $1382.54 \pm 129.56$ ) despite intense dopaminergic marker expression by residual nigral neurons. Recently published work from our laboratory further demonstrated that lysosomal and proteasomal markers are significantly decreased in neurons displaying  $\alpha$ -syn-ir inclusions in a mutant human  $\alpha$ -syn nigral overexpression rodent model of PD (Chu et al., 2009). Our central hypothesis is that systemically administered LPS will cause changes in protein aggregation, protein clearance pathways, and axonal transport mechanisms that mimic those alterations seen in PD patients. It is our long-term goal to clarify the sequential underlying neuronal pathology leading to SNpc DA cell death in PD. The achievement of this goal might establish preclinical disease biomarkers leading to more effective pharmacotherapies and potential neuropreventative strategies.

#### **Inclusion of a 3'UTR Containing the Micro-RNA-7 Binding Site Reduces Toxicity of Human SNCA on Striatal-Projecting Dopamine Neurons in Rat Substantia Nigra**

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Viral vectors harboring the coding region of human  $\alpha$ -synuclein (hSNCA) have been used to model Parkinson's disease (PD). However, in the PD brain, the noncoding sequences of the gene are also expressed. Noncoding regions are typically enriched in micro-RNA binding sites, which are typically responsible for endogenous gene silencing processes. Recent in vitro studies have shown that micro-RNA-7 and micro-RNA-153 posttranscriptionally regulate SNCA expression by binding to the SNCA mRNA 3'UTR (3' untranslated region) (Doxakis, *J. Biol. Chem.* 285:12726–12734, 2010; Junn et al., *Proc. Natl. Acad. Sci. USA* 106:13052–13057, 2009). In this study, we examined the effects of different hSNCA forms containing or not containing a portion of the 3'UTR on striatal (ST)-projecting dopamine (DA) neurons in the substantia nigra (SN) in vivo. Adult, male Sprague-Dawley rats received a unilateral SN injection of adeno-associated virus (AAV)-wtSNCA, AAV-S129A SNCA, or AAV-S129D SNCA either with or without a portion of the 3'UTR that included the micro-RNA-7 binding site. DA neurons in SN that maintained projections to ST at the end of treatment were labeled using retrograde transport of fluorogold (FG). Rats received bilateral ST FG injections 5 days before perfusion at 5, 7, or 9 weeks postinjection, and FG-positive neurons in both SN were counted. At 5 weeks postinjection, rats injected with SNCA forms that did not contain the 3'UTR had reduced FG-positive neuron numbers in injected SN compared to uninjected SN (wtSNCA,  $p < 0.05$ ; S129A or S129D SNCA,  $p < 0.01$ ). In contrast, FG-positive neuron numbers were not significantly different between injected and uninjected SN when the 3'UTR was included for all SNCA forms. These data suggest that inclusion of the 3'UTR protects against the hSNCA-induced loss of ST-projecting DA neurons in the SN in vivo. When the percent of FG-positive cells (injected/uninjected SN) was examined, S129A SNCA (without the 3'UTR) resulted in the lowest percent of FG-positive neurons, but this was only statistically significant ( $p < 0.05$ ) when compared to S129D SNCA (without the 3'UTR). These data suggest that phosphorylated and unphosphorylatable hSNCA mimics both have a toxic effect on nigrostriatal-projecting DA neurons at 5 weeks, although S129D SNCA is less toxic than S129A SNCA. Analysis of effects on ST-projecting DA neurons at 7 and 9 weeks are currently in progress, but preliminary data suggest that wtSNCA and S129D SNCA-treated rats exhibit some recovery by 9 weeks compared to S129A SNCA-injected rats and that inclusion of the 3'UTR still protects against S129A SNCA-induced degeneration. These data support the hypothesis that micro-RNA dys-

regulation is involved in PD pathogenesis and suggest that delivery of micro-RNA-7 could be developed as a novel therapy for PD.

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#### **Skilled Reaching Behavior in a Rat Model of Huntington's Disease and the Effects of Neurorestorative Therapy**

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Skilled limb movements are severely impaired during the course of Huntington's disease (HD). Thus far, little attention has been paid to the qualitative and detailed analysis of reaching-for-food movements (i.e., skilled limb movements) in the context of the quinolinic acid (QA) lesion rat model and brain repair. In the present study we investigated rodent reaching behavior including a neurorestorative approach using fetal whole ganglionic eminence (wGE)-derived grafts. Twenty-one Lister-Hooded rats were unilaterally infused with QA into the dorsolateral striatum contralateral to their preferred paw. Eleven rats served as nonoperated controls. Six weeks after the lesion, 11 rats received 2  $\mu$ l of an E15 wGE cell suspension into their ipsilateral striatum (1 wGE/2  $\mu$ l). Ten rats were sham transplanted. Before and after the lesion and following transplantation, the rats' motor behavior was analyzed in the single pellet reaching test using a movement element score and the side-stepping/paw-placing paradigm. One week prior to perfusion the rats received a FluoroGold injection into the globus pallidus. The rats were transcardially perfused 19 weeks post-transplantation. The qualitative analysis of reaching behavior revealed that striatal grafts had a strong beneficial effect on the rats' performance in the single pellet reaching test. Interestingly, individual movement elements responded differently to the grafts. Particularly distal rotatory limb movements were most impaired, but also well restored; however, most movement elements returned to control levels after grafting, indicating a near complete restoration of reaching patterns. Reaching success of grafted rats nearly doubled compared to sham-transplanted rats, but still did not achieve performance equal to the control rats. Our results indicate that wGE grafts are an effective method to restore (at least partially) individual skilled limb movements in the QA-lesioned rat and support the continuing development of cell transplantation as a potential future therapy for HD.

#### **$\alpha$ -Synuclein in Colonic Submucosa in Early Untreated Parkinson's Disease**

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The diagnosis of Parkinson's disease (PD) rests on motor signs of advanced central dopamine deficiency. There is an urgent need for disease biomarkers. Clinicopathological evidence suggests that  $\alpha$ -synuclein ( $\alpha$ SYN) aggregation, the pathological signature of PD, can be detected in gastrointestinal tract neurons in PD. We studied whether we could demonstrate  $\alpha$ SYN pathology in specimens from unprepped flexible sigmoidoscopy of the distal sigmoid colon in early PD subjects. We also looked for 3-nitrotyrosine (3-NT), a marker of oxidative stress. Ten subjects with early PD not treated with dopaminergic

agents (7 males, median age 58.5 years; median disease duration 1.5 years) underwent unprepped flexible sigmoidoscopy with biopsy of the distal sigmoid colon. Immunohistochemistry studies for  $\alpha$ SYN and 3-NT were performed on biopsy specimens and control specimens from a tissue repository [23 healthy subjects and 23 subjects with inflammatory bowel disease (IBD)]. Nine out of the 10 PD samples were adequate for study. All showed staining for  $\alpha$ SYN in a pattern suggesting nerve fibers in colonic submucosa. A similar pattern was seen in one additional subject whose biopsy was taken 5 years prior to the diagnosis of PD. No control sample showed this pattern. A few showed light  $\alpha$ SYN staining in round cells. 3-NT staining was seen in 87% of PD cases, but was not specific for PD. This study suggests a pattern of  $\alpha$ SYN staining in PD that was distinct from healthy and IBD subjects. Absence of this pattern in IBD subjects suggests that it is not a sequelae of inflammation or oxidative stress because 3-NT immunostaining was common in all groups studied. These data suggest that  $\alpha$ SYN expression for biopsies derived from unprepped flexible sigmoidoscopy of the distal sigmoid colon may be an easy, inexpensive biomarker for the occurrence of PD.

#### **Plasticity in the Phrenic Motor System Following High Cervical Spinal Cord Injury in Adult Rats**

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Cervical spinal cord injury can compromise phrenic motor pathways, impairing diaphragm function and breathing, and such injuries in people often necessitate assisted ventilation. However, experimental studies have revealed plasticity within the phrenic motor system that can mediate functional recovery. Immediately following a lateral C2 hemisection (C2Hx) the ipsilateral diaphragm is paralyzed, but spontaneous recovery has been reported within weeks. While a number of studies have begun examining anatomical changes within the ipsilateral phrenic motor system, less attention has been given to contralateral pathways. The present work examines anatomical and functional changes in the ipsi- and contralateral phrenic pathways postinjury. Adult female rats received a lateral hemisection of the C2 spinal cord. Plethysmography was used to assess ventilation pre- and postinjury. Measurements were made under baseline (breathing normoxic, normocapnic air) and hypercapnic (7% CO<sub>2</sub>) conditions. All animals were then left to recover for 1–12 weeks postinjury. At the end of the study, a subset of animals was terminally anesthetized to assess diaphragm activity. Bilateral diaphragm electromyography (EMG) recordings were made in spontaneously breathing animals under baseline and hypercapnic conditions. Anterograde (biotin dextran amine delivered to inspiratory cells in the medulla) and transsynaptic retrograde tracing [pseudorabies virus (PRV) delivered to the diaphragm] was used to examine the changes in the phrenic circuitry following injury. Neuroanatomical tracing revealed a reduced connectivity within the ipsilateral phrenic motor system. While recovered diaphragm activity ipsilateral to injury persisted for 3 months postinjury, the extent of activity was much less than that seen contralaterally. Transneuronal tracing of the contralateral phrenic motor system revealed labeling in a subset of interneurons rostral to the injury. Alterations in the contralateral phrenic motor system may represent adaptive plasticity associated with compensatory activity in this circuit. Work is under way to determine how activity in each phrenic pathway may contribute to postinjury breathing. Collectively, these results reveal changes in the phrenic motor system contralateral to C2Hx that likely reflect compensatory plasticity and underscore its importance in maintenance of breathing postinjury. Studies aimed at enhancing phrenic recovery ipsilateral to C2Hx and optimizing restorative plasticity need to consider how such treatments also may affect compensatory plasticity.

### Nutritional Supplementation of NT-020 in Aged Rats Promotes an Environment Permissive for Stem Cell Genesis

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Neural stem cells have the ability to self-renew and replenish damaged neurons; these cells can be used in cell replacement therapy for neurodegenerative diseases. It has been clearly established that the systemic environment of an aged animal has significant impact on stem/progenitor cell function in many parts of the body. This has been demonstrated with the technique of heterochronic parabiosis where the circulations of a young and aged rat are combined together. Under these circumstances, it has been observed that aged progenitor cells in the liver, muscle, and many other tissues when exposed to the circulation of a young rat increased proliferation and regeneration index. Previous studies have shown that use of dietary supplements with distinct anti-inflammatory properties can restore deficits in adult neurogenesis. One such natural product formulation, termed NT-020, shows particular promise and our studies have shown effects of this supplement to increase neurogenesis in vivo. We hypothesize that NT-020 modulates systemic factors to enhance stem/progenitor cell genesis in the young host and decrease proliferation and regeneration in the aged host by using this parabiotic technique. To examine this, we used serum samples collected from young ( $n = 8$ ), aged ( $n = 8$ ), and aged-treated ( $n = 7$ ) male Fischer 344 rats. Aged rats were treated for 4 weeks with 135 mg/kg/day of NT-020 incorporated into the diet while young and aged control rats were given control NIH31 diet. Adult rat hippocampal neural stem cells were grown in neural basal media for 24 h, and then exchanged for fresh media with either 10% FBS or serum for 2 additional days. Proliferation of neural stem cells was assessed via bromodeoxyuridine (BrdU) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Serum samples from aged control rats on BrdU assay significantly decreased proliferation in comparison to aged rats treated with NT-020 ( $Y: 43.4 \pm 7.4$ ,  $A: 8.40 \pm 1.6$ ,  $A-NT: 30.1 \pm 5.0$ ; ANOVA:  $F = 11.6$ ,  $p < 0.05$ ). Using the MTT assay, serum samples from aged control rats again significantly decreased in comparison to aged rats treated with NT-020 ( $Y: 0.61 \pm 0.1$ ,  $A: 0.39 \pm 0.1$ ,  $A-NT: 0.87 \pm 0.1$ ; ANOVA:  $F = 8.92$ ,  $p < 0.0001$ ). In conclusion, our data suggest that NT-020 supplementation promotes neural stem/progenitor cell proliferation by modulating systemic factors in the aged rat brain as this may be a potential therapeutic target for combating age-related diseases. Future studies will address whether NT-020 acts on the brain's main immune cells, the microglia, or that circulating proinflammatory cytokines and/or chemokines mediate its effects, and identify signaling molecules that may be present in serum that effect stem/progenitor cell function.

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### hESC Differentiation to Midbrain Dopaminergic Neurons Is Enhanced in Lines With Amplified 17q21.31 Wnt Signaling Genes

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The self-renewing and differentiation capacities of human embryonic stem cells (hESCs) provide the foundation for numerous potential cell-based therapies, including dopaminergic (DA) amelioration of Parkinson's disease symptoms. Prolonged culture of hESCs can, however, lead to large chromosomal abnormalities including trisomies of chromosomes 12, 17, and/or X as well as more subtle genetic abnormalities. Five karyotypically normal hESC lines (BG01, 02, 03, ES02, 04) and one karyotypically abnormal trisomy 17 hESC line (BG01V2) were characterized for morphology, proliferation, cell survival, growth factor dependence, ability to differentiate into dopamine cell precursors, and copy number variation (CNV). All hESC lines displayed similar cell survival rates, normal morphology, and well-defined colony boundaries. BG01V2 and BG03, however, showed enhanced rates of proliferation. After withdrawal of fibroblast growth factor-2 (FGF-2), BG01V2 and BG03 lost pluripotency rapidly, displaying enhanced differentiation. DA neuronal differentiation was assessed using the protocol described by Yan et al. (Stem Cells, 2005). This method involves culture in the presence of FGF-2 and FGF-8 to yield neuroepithelial (NE) cells after 16 days, isolation and culture in the presence of FGF8 and sonic hedgehog (SHH) to yield mesencephalic DA (mDA) progenitor cells, and production of mature DA neurons by 45 days. BG01V2 and BG03 exhibited superior neuroectodermal specification, with more nestin-positive cells and fewer Oct3/4-positive cells at the NE stage. BG01V2 and BG03 also displayed more rapid DA differentiation than the other lines, as evidenced by the presence of DA progenitor cells that expressed *Lmx1A*<sup>+</sup>, *Otx2*<sup>+</sup>, and *Msx1*<sup>+</sup> as well as DA neurons that expressed tyrosine hydroxylase (TH) and *Tuj1* at the NE cell stage. The enhanced mDA differentiation of BG01V2, which had an extra copy of chromosome 17, suggested that enhanced differentiation of BG03 might also be linked to extra chromosome 17 genetic material. We thus assessed CNVs on chromosome 17 using Affymetrix 6.0 arrays. A genomic duplication located between 41.3 and 42.5 Mb at 17q21.31 was identified in BG03. This CNV encompasses the WNT3–WNT9 cluster of genes implicated in pluripotency, proliferation/survival, and differentiation. Additional copies of WNT3 and WNT9B in both BGO1V2 and BGO3 were confirmed by qRT-PCR. In addition, expression of both WNT3 and WNT9B mRNAs was increased during mDA differentiation in BG01V2 and BG03 cells. Using Western blotting, canonical WNT3 and noncanonical WNT9B signaling inhibitors (*Dkk-1* and *SP600125*, respectively), and a ChIP assay, we found that WNT9B promotes neuroectodermal fate through inhibiting hESC self-renewal. WNT3 stimulates both NE proliferation and mDA differentiation. Thus, amplification of the 17q21.31 WNT3–WNT9B cluster enhances mDA differentiation in hESC. Elucidating the roles that WNT signaling plays during mDA differentiation can help optimize strategies for inducing mDA differentiation of hESC.

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### Pulsed Electromagnetic Fields Induce Neurite Outgrowth in the MN9D Dopaminergic Cell Line

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Parkinson's disease is characterized by the loss of dopaminergic neurons in the substantia nigra. Unfortunately, current treatment strategies are only palliative, and long-term consequences of ablative surgery and deep brain stimulation are as yet unknown. Pulsed electromagnetic fields (PEMF), used to facilitate bone repair and reduce pain and swelling from soft-tissue injuries, have recently been configured to affect calmodulin (CaM)-dependent signaling, which will modulate biochemical cascades involving nitric oxide and cyclic nucleotides that lead to tissue regeneration and angiogenesis. Importantly, these molecules are also mediators of neuronal survival and differentiation. In this study, we tested the effects of PEMF signals on neuronal survival and plasticity in cell culture. The murine MN9D dopaminergic cell line was a gift from Alfred Heller (University of Chicago). Cells were plated at 100,000 cells/35-mm dish in Dulbecco's modified Eagle's medium (DMEM) in the presence or absence of 10% fetal calf serum and 1 mM dibutyryl cyclic adenosine monophosphate (Bt<sub>2</sub>cAMP). At 1 day, cultures were divided into groups and treated with a nonthermal radiofrequency PEMF signal (27.12 MHz carrier frequency, 0.05 Gauss, 4 ms burst width, 2 Hz), for either 2 × 30 min/day or 15 min/h for 3 days. Control cultures were exposed to the same conditions without PEMF signals. After treatment, cells were fixed and photographed for image analysis. Neurite length, total cells, and number of cells with processes were quantified in four consecutive fields under phase optics at 100× magnification. Data were analyzed and tested for statistically significant differences between treatment groups with the Student's *t*-test. Values of  $p \leq 0.05$  were considered significant. Removal of serum increased neurite length compared to cells maintained in 10% serum. Thirty-minute exposure to PEMF 2×/day for three days increased differentiated cells by 47% ( $p = 0.02$ ) and increased neurite length by 43% ( $p = 0.03$ ). No differences were found in total cell numbers. In 10% serum, PEMF treatments of 15 min/h for 3 days resulted in a 25% increase in neurite length ( $p = 0.04$ ). Results suggest that PEMF signals configured to modulate CaM-dependent signaling increase neurite outgrowth under conditions that both facilitate and inhibit differentiation using two different treatment regimens. Effects of PEMF signals were also compared with those of cAMP, a known inducer of neurite outgrowth. Addition of 1 mM Bt<sub>2</sub>cAMP significantly increased neurite length by 41% ( $p = 0.001$ ); however, PEMF treatment in the presence of cAMP did not further increase neurite length, suggesting that this was achieved through a common mechanism that reached its maximum effect with 1 mM cAMP. While neuritogenesis occurs during brain development, it may also represent the plasticity required to form and maintain synaptic connections throughout life.

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#### **Transplantation of iPS-Derived NES Cells in CD1 Mice: Development of Phenotype and Effects of Manipulating Postlesion Transplant Time**

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Given the ethical and logistical issues surrounding the use of fetal- and embryonic-derived cells in cell replacement therapies for neurodegenerative disorders, the advent of induced pluripotent (iPS)-derived neural cells may offer a safe and viable alternative. At present, however, little data exist regarding the ability of iPS-derived neuroepithelial (NES) cells to survive and differentiate in vivo. Furthermore, there exists experimental evidence demonstrating that the age of the transplant recipient influences the profile and function of the grafted cells. In the clinical setting, Huntington's disease (HD) patients would typically be considered suitable for cell replacement therapy several years after the onset of neurodegeneration. Despite this, grafts are typically assessed for survival and function in young rodent models of HD shortly after lesion. Thus, the aim of the current experiment was, firstly, to assess the time course of in vivo differentiation of "PKd"

NES cells and, secondly, to determine the viability and integration potential of the cells after grafting into the striatum of immunosuppressed mice at different time points postlesion. To this end, three groups of mice were given quinolinic acid lesions of the neostriatum and were grafted either 6 months postlesion (long term), 2 months postlesion (midterm), or 10 days postlesion (short term). As the experiment is currently ongoing, grafts will be harvested at two time points, 6 and 12 weeks, posttransplant and tissue will be evaluated by immunohistochemical analyses for a number of neuronal and glial markers, including HuNu, nestin, Tuj1, GFAP, Gad67, GalC, and Ki67. Given that transplant time postlesion has been shown previously to affect axonal growth and functional efficacy of dopaminergic cell transplants, it is expected that greater survival and integration of these GABAergic cells will be evident in cells transplanted 10 days postlesion, relative to those transplanted 6 months postlesion. Furthermore, it is anticipated that the in vivo differentiation profile of the PKd NES cells may yield information regarding the proliferation potential and the time course of GABAergic and glial cell differentiation and integration. These data are important for expanding knowledge of both iPS-derived cell lines for cell replacement therapies and optimizing parameters for transplantation of cells into the brain.

#### **The Effects of Hematopoietic Growth Factors on Clearance of Amyloid-Beta Deposition and Improvement of Cognitive Function in APP/PS1 Transgenic Mice**

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Alzheimer's disease (AD) represents the most frequent cause of dementia and the sixth leading cause of death in the US. Currently, there is no approved treatment with a proven disease-modifying effect for AD. Therefore, developing therapeutic strategies for AD is a critical need. Substantial evidence shows that abnormal accumulation of amyloid-beta (A $\beta$ ) in the brain is a direct cause of neurodegeneration and cognitive decline in AD. In our early study, we have demonstrated that the combination of two hematopoietic growth factors, stem cell factor (SCF) and granulocyte-colony stimulating factor (G-CSF) (SCF + G-CSF), decreases A $\beta$  deposition and improves cognitive performance in a mouse model of AD (APP/PS1 transgenic mice). The purpose of this study is to determine long-term therapeutic effects of SCF and G-CSF alone, or in combination in APP/PS1 mice. SCF, G-CSF, SCF + G-CSF, or equal volume of saline were subcutaneously administered for 12 days. A $\beta$  deposits in the brain were determined with live brain imaging, immunohistochemistry, and ELISA 7 days or 8 months after treatment. Cognitive function was evaluated with water maze test 3 and 7 months after treatment. Multiphoton images of the brains in live APP/PS1 mice showed a significant reduction in A $\beta$  deposition in the cortex 7 days after the initial treatment. At this point, the number of bone marrow-derived microglia and the colocalization of bone marrow-derived macroglial cells and A $\beta$  deposits in the cortex and hippocampus were significantly increased in SCF + G-CSF-treated mice. In addition, both escape latency and swimming distance in the mice treated with SCF + G-CSF were significantly shorter than those in the groups of saline, SCF, or G-CSF alone at 3 and 7 months after treatment. Furthermore, SCF + G-CSF-treated APP/PS1 mice displayed a significant reduction in the levels of A $\beta$  1–40 and A $\beta$  1–42 in brain tissue compared to saline controls, SCF, or G-CSF alone 8 months after treatment. These data suggest that SCF in combination with G-CSF has synergistic and long-lasting effects in removal of A $\beta$  deposits and improvement of cognitive function. This study provides new insights into the contribution of hematopoietic growth factors in the treatment of AD.

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### Precise MeCP2 Phosphorylation Affect Adult Neurogenesis

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Methyl CpG binding protein 2 (MeCP2) plays a central role in DNA methylation-dependent epigenetic regulation of the development and function of the nervous system. Recent studies suggest that MeCP2 may regulate neuronal differentiation and maturation through micro-RNAs. Our lab is interested in understanding the in vivo function of neuronal activity-induced MeCP2 phosphorylation. During the process of analyzing several lines of MeCP2 phosphomutant mice, we found that independent phosphorylation events on the MeCP2 protein had opposing effects of the proliferation and differentiation of adult neural stem/progenitor cells (NPCs) in the hippocampus. These opposing effects appeared to be mediated, at least in part, through the same micro-RNA. We are currently studying the mechanism of this convergence in NPCs isolated from various MeCP2 phosphomutant brains. Results from this series of experiments will further advance our understanding of how MeCP2 regulate neural differentiation, and may also be useful in understanding Rett syndrome, a severe neurodevelopmental disorder caused by mutations in the *MECP2* gene.

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### CEST Imaging Reveals Dynamic Changes of Implanted Hydrogel Scaffold In Vivo

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Hydrogel scaffolds have been proposed to be valuable in improving survival of transplanted cells by shielding the cells from a hostile microenvironment or promoting gas and nutrients exchange between graft and host. However, the microenvironment created by hydrogel scaffolds is amenable to changes due to interactions with encapsulated stem cells or surrounding host tissue, which may cause premature degradation and loss of their supportive property. To address this issue, we propose to use chemical exchange saturation transfer magnetic resonance imaging (CEST MRI), a powerful tool for the acquisition of molecular information, to monitor dynamic changes in hydrogel scaffolds in vivo. We first performed a phantom CEST MRI study on the molecular components of the hyaluronic acid (HA)-based hydrogel scaffold [i.e., HA, gelatin, and polyethylene glycol diacrylate (PEGDA)]. Interestingly, the highest CEST MRI contrast was detected from gelatin showing high magnetization transfer ratio (MTR) asymmetry values at 1.8 and 3.6 ppm. CEST MRI was then performed on mice implanted with scaffolds at 1 h (day 0) and 7 days after transplantation. Consistent with the phantom study, CEST signals at 1.8 and 3.6 ppm were easily identified in brains at day 0. Surprisingly, a dramatic drop in CEST MRI contrast was obtained at day 7, although the physical structure of the scaffold was maintained as detected histologically. According to the phantom study, CEST contrast was primarily derived from gelatin, and therefore the decay in those signals suggests degradation of gelatin in this scaffold in vivo. Although this hypothesis requires verification from other lines of evidence, it is consistent with the two following facts. One, given that gelatin provides anchoring sites for cells within the scaffold, the degradation of gelatin could hinder the ability of the scaffold to keep cells immobile, potentially leading to migration of encapsulated cells out of the scaffold, which we have confirmed by histological analysis of brains that have been implanted with cells encapsulated in this hydrogel scaffold. On the other

hand, the ubiquitously distributed matrix metalloproteinases with collagenase activity in living tissues including the brain may be possible culprits to degrade gelatin in vivo. To conclude, the use of biomaterials for stem cell encapsulation and improvement of cell survival is on the rise. By providing molecular information on the composition and degradation of scaffold materials, CEST MRI could become a valuable tool for studying dynamic changes in scaffolds in vivo and allow further optimization of implantation strategies aimed at improved stem cell therapy.

### Use of BLI Reporter Genes for Stem Cell Imaging: Comparison of Red-Shifted Firefly Luciferase Ppy RE9 and Conventional fLuc2

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Bioluminescence imaging (BLI) has been intensively used for in vivo tracking of transplanted stem cells, including hematopoietic stem cells, embryonic stem cells, mesenchymal stem cells, and neural stem cells. Induced mammalian expression of the firefly enzyme luciferase enables researchers to observe luminescence upon injection of its substrate, and to monitor the survival, migration, and differentiation of transplanted stem cells over time with high sensitivity at relatively low cost. However, one critical issue for noninvasive in vivo imaging of bioluminescent cells is that blood hemoglobin and myoglobin substantially absorb light with wavelengths below 600 nm, greatly attenuating the sensitivity of this imaging technique. As such, the development of red-shifted luciferase for BLI has been recently pursued. We explored a mutant of firefly luciferase Ppy RE9 (PRE9) with red-emitting spectrum, which provided 50- to 100-fold greater integrated light intensities than the conventional click beetle red BLI reporter. We compared PRE9 with the yellow luciferase fLuc2 gene, and evaluated its suitability for imaging of neural stem cells. Both BLI reporter genes were first inserted into lentivectors, which include a separately expressed enhanced green fluorescent protein (GFP), and were then used to transduce C17.2 cells. The two cell lines (PRE9-GFP and fLuc2-GFP) were fluorescence-activated cell sorted (FACS) for GFP, and cells with similar expression of both reporter genes were used to compare their light emission in living cells in vitro and after they were transplanted into immunodeficient mice. We found that luminescence from PRE9 was stable with a peak emission at 620 nm, shifted to red compared to that of fLuc2. In addition, the emission peak for PRE9 was stable in contrast to fluctuations in emission wavelength from fLuc2 in response to extracellular pH changes. In addition, the emission from PRE9 was much less affected by tissue absorbance compared to that of fLuc2. However, the total emitted light radiance from PRE9 was somewhat lower than that of fLuc2 both in vitro and in vivo, with a 50% in vivo reduction for PRE9 compared to Luc2. Nevertheless, we conclude that, taken as a whole, PRE9 has favorable properties compared to fLuc2 in terms of pH independence, red-shifted spectrum, and tissue light penetration, justifying further optimization of protein expression and enzymatic activity.

### Disease-Modifying Effects of Transplanted Fetal GABAergic Progenitors in Mice With Temporal Lobe Epilepsy and Spontaneous Recurrent Seizures

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Alterations in neuronal excitability are a common cause of seizure disorders and epilepsy. Decreased synaptic inhibition and increased

synaptic excitation in the dentate gyrus contribute to the development of spontaneous recurrent seizures in temporal lobe epilepsy (TLE). Adults with mesial temporal lobe epilepsy (MTLE), a common form of partial epilepsy, often have severe, drug-resistant focal seizures in limbic brain regions, including the hippocampus, amygdala, and entorhinal cortex, with concomitant cognitive impairments. The disease-modifying effects of  $\gamma$ -aminobutyric acid (GABA)-ergic progenitor transplants were investigated in mice with MTLE. Adult male mice were treated systemically with the cholinergic agonist pilocarpine to induce status epilepticus (SE) and subsequent development of spontaneous, recurrent seizures (SRS) over the course of several weeks. After the development of SRS, embryonic day 13.5 GABAergic progenitors were harvested from the medial ganglionic eminence (MGE) of fetal mice expressing enhanced green fluorescent protein (EGFP) in all tissues. Alternatively, some grafts consisted of MGE progenitors that were nucleofected with red fluorescent protein (RFP). High levels of RFP expression in axons and dendrites of the transplanted cells allowed us to visualize at the single cell level how they formed connections within host brain neural circuits. Between 100,000 and 200,000 cells were stereotactically transplanted bilaterally in either the hilus of the dentate gyrus or the lateral entorhinal cortex. Spontaneous seizures were then monitored using continuous video-electroencephalographic recordings (v-EEG) for periods of up to 100 days. A gradual suppression of SRS over the course of the recording period was observed in the group of mice receiving hilar MGE grafts. There was a statistically significant reduction in the mean number of seizures in the MGE transplant group compared with media-injected controls. The effects were observed by 6 weeks posttransplantation and lasted up through 100 days of continuous v-EEG monitoring. To determine whether the size of the grafts could account for their ability to suppress SRS, we reconstructed the transplants and measured graft volumes. Despite the fact that the cells showed robust survival and extensive connections with the host brains, there was not a strong correlation between overall graft size and degree of seizure suppression. The molecular properties of the cells were also examined and we determined that over 80% of the cells were GABAergic interneurons and a majority of these expressed the neuropeptide somatostatin. Although the percentage of GABAergic interneurons was comparable in the dentate and entorhinal grafts, only the mice with dentate gyrus transplants showed marked seizure suppression, suggesting that cell type and anatomical site for transplanting MGE progenitors may be a critical parameter for stem cell therapies designed to treat MTLE.

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#### **Electrical Stimulation of Embryonic Transplants in Peripheral Nerve Improves Axon Regeneration, the Percentage of Muscle Reinnervation, and the Number of Functional Muscles**

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Motoneuron death following spinal cord injury or disease results in muscle denervation, atrophy, and elimination of voluntary movements. To minimize the amount of muscle atrophy after complete denervation, ventral spinal cord cells from Fisher rat embryos have been transplanted into the tibial nerve near denervated muscles as a source of neurons for muscle reinnervation (Thomas et al., *J. Neurophysiol.* 84:591–595, 2000). However, muscle function was limited by incomplete muscle reinnervation and poor motoneuron survival. The aim of this study was to determine whether acute electrical stimulation of the cell transplant changes motoneuron survival, axon regeneration, muscle reinnervation, and function because neural depolarization is crucial for the survival of embryonic motoneurons (Goldberg et al., *Neuron*

33:689–702, 2002) and it may promote activity-dependent axon growth (Ben-Ari, *TINS* 24:353–360, 2001). One week after denervation by sciatic nerve section, embryonic day 14–15 cells were injected into the tibial nerve of adult Fischer rats. The transplanted cells were then stimulated at high frequency (20 Hz), low frequency (1 Hz), or not at all for 1 h. Groups controlling for stimulation frequency, pattern, and pulse number were included. Assessments were made 10 weeks later. Acute electrical stimulation of transplanted cells for 1 h at 1 Hz: 1) increased the number of myelinated axons in the tibial nerve compared to no stimulation; 2) improved muscle reinnervation because higher proportions of muscle fibers had large areas. All groups that received stimulation for 1 h had more functional muscles and motor units. These data suggest that acute electrical stimulation of embryonic neurons in nerve enhances axon regeneration, muscle reinnervation, and function, resulting in reduced denervation-induced muscle atrophy.

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#### **Incorporating Regenerative Medicine Techniques is an Outpatient Orthopedic Setting**

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In today's healthcare environment, we are met with rapid changes. Managing acute and chronic painful conditions often employs a variety of treatment options; however, the natural degenerative cascade continues. Embracing regenerative therapies to treat degeneration is discussed, with a look at how platelet-rich plasma (PRP), stem cells, nutraceuticals, and telomerase activators may impact our lives.

#### **Regulation of Neural Differentiation by Ryk-Mediated Wnt Signaling**

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Neural stem cells are characterized by their ability to generate both self-renewing and differentiating daughter cells. We are interested in how the different daughter cells' fates are determined using mouse cortex development as a model. Self-renewal and differentiation of neural stem cells are regulated by extrinsic signaling in combination with intrinsic transcription and epigenetic factors. Our research focuses on the noncanonical Wnt receptor Ryk. We have demonstrated that Ryk is cleaved at its transmembrane region in the differentiating neural stem cells and the intracellular domain (ICD) of Ryk moves to the nucleus during neuronal differentiation. Cleavage of Ryk and nuclear translocation of Ryk ICD is required for neuronal differentiation. Inducible expression of Ryk ICD regulates expression of some master transcription factors that control neural differentiation. Using Mass spectrometry analysis, knockout mice analysis, and neural stem cell culture, we reveal how Ryk ICD is stabilized in the cytoplasm, how Ryk ICD moves to the nucleus, and how it regulates gene expression to induce neural differentiation.

#### **Molecular Logic of Neocortical Projection Neuron Development, Degeneration, and Repair**

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Given the heterogeneity of CNS neuronal subtypes, and the complexity of their connections, detailed understanding of molecular con-

trols over differentiation, connectivity, and survival of specific neuronal lineages will contribute not only to 1) understanding of the development, evolution, organization, and function of CNS circuitry, but also to 2) support or regeneration of vulnerable populations in neurodegenerative [e.g., amyotrophic lateral sclerosis (ALS), Hereditary Spastic Paraplegia and Primary Lateral sclerosis (HSP/PLS), Huntington's disease (HD), and Parkinson's disease (PD)] or acquired disease [e.g., spinal cord injury (SCI)], to 3) enabling accurate models of neuron type-specific disease, to 4) identification of disease genes, and to 5) attempts to functionally repair CNS circuitry. For example, data from our lab and others demonstrate that new neurons can be added to adult neocortical circuitry via manipulation of transplanted or endogenous precursors in situ [including induction of limited neurogenesis of clinically important corticospinal motor neurons (CSMN) in adult mice], indicating that cellular repair of cortical and cortical output circuitry is possible, if controls over specific lineage differentiation are understood. Using fluorescence-activated cell sorting (FACS)-purified CSMN and other neocortical projection neuron populations (including callosal projection neurons, corticothalamic projection neurons, corticotectal projection neurons, and target-specific subsets of these populations) at critical stages of development in vivo, we identified a broad set of combinatorially interacting developmental controls—both novel and largely uncharacterized transcriptional regulators and other genes, and cell-extrinsic controls—that are instructive for development of specific neuron subtypes as they develop in vivo (in particular, for CSMN, corticothalamic, callosal, and related projection neuron populations). These control key developmental processes from progenitor parcellation and progenitor subtype restriction, to subtype-specific differentiation, to acquisition of precise areal identity, to axonal outgrowth. Loss-of-function and gain-of-function analyses for multiple identified genes and molecules reveal a nested “molecular logic” of progenitor stage and postmitotic, areally specific, combinatorial molecular genetic controls over the precise development of key forebrain projection neuron populations that might enable directed control of neural progenitors/“stem cells” [or embryonic/induced pluripotent stem (ES/iPS) cells] toward accurate disease models, neuronal support or regeneration, or functional CNS repair.

#### Endogenous–Exogenous Neural Precursor Cell Synergy in the Parkinsonian Rat

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Current neural stem/precursor cell (NPC)-based therapeutic strategies for Parkinson's disease (PD) focus either on cell transplantation or endogenous cell stimulation. Future PD cell therapies most likely will involve combining these two approaches. Our previous studies in a 6-hydroxydopamine (6-OHDA) rat model of PD suggest a “synergy” between transplanted (exogenous) and endogenous NPC actions (Madhavan et al., *J. Comp. Neurol.* 2009; Madhavan et al., *Neuropharmacology*, 2010). In particular, our work demonstrated a significant endogenous NPC response (proliferation, migration, and neurogenesis) to transplantation of exogenous NPCs prior to a 6-OHDA insult. These events were associated with protection of the host nigrostriatal system along with amelioration of behavioral deficits (spontaneous paw placement in cylinder task). In addition, the transplanted NPCs expressed certain factors [glial-derived neurotrophic factor (GDNF), sonic hedgehog (SHH), and stromal-derived factor 1 $\alpha$  (SDF1 $\alpha$ )], providing a potential molecular basis for the observed phenomenon. We have begun to investigate mechanisms underlying the phenomenon by examining

the roles of (a) endogenous NPCs and (b) above-mentioned graft-expressed factors. To determine the role of endogenous NPCs, experiments in which host NPC proliferation and neurogenesis was inhibited using cytosine- $\beta$ -D-arabinofuranoside (Ara-C) have been conducted. Analyses indicate that endogenous NPCs do in fact contribute to transplantation-induced neuroprotection by influencing donor and mature host cell function in many ways. More specifically, with regards to neuroprotection, Ara-C-infused animals that were subsequently grafted with NPCs performed significantly better on the cylinder task than sham controls, but were also significantly worse than NPC-grafted animals that had received no prior Ara-C infusion. Stereological tyrosine hydroxylase (TH) cell counts through the substantia nigra support the behavioral data. With respect to graft behavior, it was observed that endogenous NPC inhibition altered the differentiation and growth factor expression pattern of grafted NPCs. Also, the host immune response was perturbed in grafted animals lacking endogenous NPCs. Presently, we are investigating the roles of graft-expressed GDNF and SHH using RNA interference (RNAi) techniques. Specifically, NPCs in which either GDNF or SHH, or both, have been silenced have been transplanted into host rats to determine whether or not they contribute to the observed NPC-mediated neuroprotection and endogenous response to transplantation. Overall, our studies will help determine some of the microenvironmental signals fundamental to the exogenous–endogenous stem cell synergism and neuroprotection, and will contribute towards the development of novel stem cell-based therapies for PD.

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#### Testing of Candidate Neuroprotective Genes in Traumatic Brain Injury

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The trkB ligand neurotrophin-4/5 (NT-4/5) offers neuroprotection for selectively vulnerable CA3 hippocampal neurons following experimental lateral fluid percussion traumatic brain injury (LFP-TBI) in rats (Royo et al., 2006). Acute administration of recombinant NT-4/5 (5  $\mu$ g/kg/day) prevented up to 50% of the hippocampal CA pyramidal cell death following LFP-TBI in rats. To determine the mechanism by which NT4/5 offered neuroprotection we have now identified the genes regulated by NT4/5 treatment in the CA3 area of hippocampus in a rat model of TBI (Malik et al., 2010). To test them, our lab has developed a method for highly efficient, nontoxic, stable long-term transduction of primary hippocampal neurons using different adeno-associated viruses (AAV) serotypes (Royo et al., 2008). Our lab has used this method in concert with a glutamate toxicity assay in cultured hippocampal neurons at 2 weeks in vitro. To date, we have tested the neuroprotective efficacy of the first four genes, transthyretin (TTR), thyrotropin releasing hormone (TRH), chemokine (C-C) motif ligand 7 (CCL7) and chemokine (C-C) motif ligand 2 (or monocyte chemoattractant protein-1; CCL2) (Malik et al., 2010). Each protein protected at a different concentration, and some exhibited a ceiling effect while others were not effective at higher doses. We have now completed the cloning of 11 candidate neuroprotective genes into AAV backbones that also contains a green fluorescent protein (GFP) reporter gene. These 11 constructs have been screened for GFP expression in cell culture; all were positive, indicating the upstream genes of interest are also expressed. These AAV genome constructs are ready for packaging in the AAV1 capsid, which we previously identified as a highly neurotropic serotype of AAV (Royo et al., 2008). We will use these packaged vectors containing potentially neuroprotective genes in our neuronal cell culture model for neurotoxicity and neuroprotection, before selecting the most efficacious ones for in vivo testing. These

genes identify new molecular cascades in TBI neuroprotection and hold the potential to be neuroprotective hours to days after TBI in humans.

**The Effect of Preinjury Fitness Level on Prognosis and Clinical Outcome After a Single Moderate-to-Severe TBI: A Retrospective Case Study**

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Clinical experience suggests that people with active lifestyles tend to recover better from traumatic brain injury (TBI) than more sedentary people. Surprisingly, however, there are no published studies about the effect of preinjury fitness level on prognosis or recovery in humans after TBI. Our study aims to evaluate how the preinjury physical fitness level affects the clinical outcome after a single moderate-to-severe TBI. The enrollment of the subjects will be done through the University of Pennsylvania Health System (UPHS) trauma and clinical databases for consecutive patients who suffered a single moderate-to-severe TBI over the course of the study and/or during the past 1 year. Several data points, including the Glasgow Coma Scale (GCS), Acute Physiology and Chronic Health Evaluation (APACHE), Marshall Score, functional independence measure (FIM) on discharge, and Modified Rankin Scale, Occupational/Physical Therapy (OT/PT) assessment, will be extracted from the databases for preinjury fitness and postinjury outcome assessment. In addition to the retrospective chart review the patients and/or the caregivers will be contacted telephonically and/or via postal mail and asked a standard set of questions [Physical activity questionnaire and activity measure for post acute care (AMPAC)] to determine preinjury physical fitness and postinjury outcome. Informed consent will be obtained at the time of enrollment either in person or through their caregivers during their initial hospital stay or via telephone if subject was identified from the database established in the past year. The results of this study will help clinicians advise their patients about prognosis, investigate the role (if any) of preinjury fitness level on the recovery from TBI, and suggest molecular and biochemical pathways for future laboratory investigations and translational research.

**rAAV-Mediated shRNA Knockdown of  $\alpha$ -Synuclein in the Rat Substantia Nigra Results in Aberrant Dopamine Handling**

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Parkinson's disease (PD) is a relatively common progressive neurodegenerative disorder generally affecting the aging population. PD patients generally present with unilateral motor symptoms, such as resting tremor, which progresses bilaterally over time with increasing severity. Motor symptoms arise from the progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc) resulting in the loss of dopaminergic innervation in the terminal fields of the caudate/putamen. Although a majority of PD cases are sporadic, several familial forms of PD have been identified.  $\alpha$ -Synuclein ( $\alpha$ -syn) was the first such gene to be identified where it was shown that different mutations in the encoding gene results in PD.  $\alpha$ -syn is an abundant protein in the CNS, which has been associated with the pathogenesis of several neurodegenerative disorders. Although the function of  $\alpha$ -syn is largely unknown, the protein has been shown to

be associated with synaptic vesicles and is thought to play a role in vesicle maintenance and neurotransmitter release. Paradoxically,  $\alpha$ -syn knockout mice have revealed no overt pathogenesis. However, evidence exists for altered dopamine (DA) release, altered DA levels, and changes in DA vesicle number in these animals. Clinical and experimental data have shown that overexpression of wt or mutant  $\alpha$ -syn can induce PD-like neurodegeneration in humans and animals. This has led to the suggestion that downregulation of  $\alpha$ -syn might provide a therapeutic modality for synucleinopathies. However, recent data show that virally delivered short hairpin RNAs (shRNAs) targeting  $\alpha$ -syn in the rodent substantia nigra result in significant dopaminergic cell loss and resultant motor behaviors corresponding to levels of knockdown. In this project we sought to further characterize the effects of  $\alpha$ -syn knockdown in the rodent basal ganglia. Recombinant adeno-associated virus type 2/5 expressing either  $\alpha$ -syn shRNA and green fluorescent protein (GFP) as a transduction marker, or GFP alone, was stereotactically injected in the substantia nigra (SN) or globus pallidus (GP) of adult rats. A subset of animals was sacrificed at 7 and 14 days following the injections. Histological analysis of these animals indicates the presence of apoptosis in the SNc of  $\alpha$ -syn shRNA-treated animals as early as 7 days following injection. In addition, evaluation of low-dose amphetamine-induced rotational behavior at later time points demonstrated significant contraversive behavior of animals treated with  $\alpha$ -syn shRNA in the SN, an indication of increased levels of cytosolic DA. However, high concentration amphetamine rotational behavior resulted in significant ipsiversive behavior of the SN-injected animals when compared to the GP group. Together, these behavioral data indicates that the effect of  $\alpha$ -syn shRNA is specific to the DA neurons of the SN. Our data, in concordance with previously published reports, agree with the fact that some level of  $\alpha$ -syn is required for the survival of dopaminergic neurons, and is consistent with the hypothesis that loss of  $\alpha$ -syn results in aberrant dopamine handling.

**High Content Screening to Identify and Evaluate Remyelination Therapeutics for Multiple Sclerosis**

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Multiple sclerosis (MS) is the most common inflammatory demyelinating disease of the central nervous system (CNS) and is the major cause of nontraumatic neurological disability in young adults in North America. Current MS therapies are anti-inflammatory or immunomodulatory in nature, but these therapies are partially preventive (delay the disease progression) and not restorative. Replacement of oligodendrocytes and the subsequent remyelination of demyelinated axons have the potential to halt and reverse axonal loss and neurological decline. One approach to increasing remyelination is through therapeutic stimulation of endogenous oligodendrocyte progenitor cells (OPCs). This approach is feasible because generation of new oligodendrocytes and spontaneous remyelination occur in many MS lesions during early stages of the disease, and even in some chronic lesions, although endogenous remyelination eventually fails. The hypothesis is that small molecules can be used as therapeutic agents to stimulate differentiation of oligodendrocytes and promote endogenous remyelination. Two recent technological developments enable the identification and evaluation of such small molecules. First, we have developed a standard operating protocol to generate a relatively homogeneous (>85% pure) and consistent population of OPCs that can be used as a reliable starting material for high-content and relatively high-throughput screening. Second, we developed a high-content and relatively high-throughput cell-based phenotypic screening method that can automate identification and analysis of differentiated cells in these cultures. The phenotypic screen is employed in two phases: primary screen to identify potential candidates that promote OPC differentiation based on cell morphology, and secondary screen to confirm and qualify compounds

by immunocytochemical staining for oligodendrocyte-specific markers. A set of compounds was used to validate this approach. Ciliary neurotrophic factor (CNTF), which is known to promote OPC differentiation, has been identified by the primary screen and later qualified by the secondary screen (O4-positive staining), demonstrating the utility of our *in vitro* high-content and relatively high-throughput assay to identify lead candidates that promote OPC differentiation and potentially promote remyelination. This model system can revolutionize the identification and development of new therapeutics for MS and other demyelinating diseases.

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### Functional Reorganization in the Medulla Following High Cervical Spinal Cord Injury

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Primary respiratory drive arises from neurons within the ventral respiratory column (VRC) of the medulla. The VRC is comprised of neurons within the Bö complex, pre-Bö complex, rostral ventral respiratory group (rVRG), and caudal VRG (cVRG). The rVRG and cVRG innervate respiratory spinal motoneurons controlling inspiratory and expiratory activity, respectively. Whether this supraspinal respiratory organization changes following cervical spinal cord injury (SCI) is unknown. Given the extent of reorganization of supraspinal activity (e.g., cortical) that has been reported following SCI, we hypothesized that a high cervical lateralized hemisection (C2Hx) will result in reorganization of respiratory activity in the medulla. The present study used stereotaxic coordinates and neurophysiological recordings for mapping the distribution of neurons active during either inspiratory or expiratory phases of spontaneous breathing in anesthetized, adult female Sprague-Dawley rats. Electrophysiological recordings from normal (uninjured) animals were compared with animals 12 weeks following C2Hx. Once anesthetized the dorsal surface of the caudal brain stem was surgically exposed. Extracellular recordings within the medulla were then made at multiple locations 200  $\mu\text{m}$  apart, using a grid pattern, and at depths  $\sim$ 500  $\mu\text{m}$  apart from the dorsal surface of the medulla. The phasic respiratory activity was amplified and passed through an audio monitor, and digitized for Spike2 software on a PC computer, to correlate activity with the animals observed breathing pattern. 3D maps of inspiratory and expiratory activity for each animal were created using Adobe Illustrator. Preliminary results have revealed a dramatic shift in the pattern of inspiratory and expiratory activity following injury. The number of sites at which inspiratory activity could be located was reduced. In contrast, expiratory phase activity was now more widely detected and was frequently observed within the regions normally shown to have inspiratory phase activity. Neuroanatomical studies are being pursued to visualize the distribution of neurons with inspiratory versus expiratory activity. What role the expansion of expiratory phase activity may play in postinjury breathing function remains undefined at present, but the decreased number of cells active during the inspiratory phase may limit the potential for recovery of inspiratory drive following SCI.

### The Evolution of Alzheimer's Disease: Neuroplastic and Neurodegenerative Changes Within the Human Cortical Connectome

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The neuronal "connectome" represents the network of elements and connections underlying the neurostructural substrate of cognition and memory. Disruption or reduction of the connectome (e.g., changes in dendritic branching and/or spines) appears to play a key role in the onset and progression of dementia. Mild cognitive impairment (MCI), which is associated with subtle memory loss, is regarded as a prodromal stage in the development of Alzheimer's disease (AD). Here we characterized, first, the earliest alterations in the cortical connectome associated with MCI, and secondly, additional connectome changes associated with the progression of MCI into frank AD. Connectome changes were compared from three cortical regions: the inferior parietal cortex (Brodmann areas 39, 40), the inferior temporal cortex (area 21), and the superior frontal cortex (area 9). Formalin-fixed tissue blocks were harvested from individuals diagnosed as noncognitively impaired (NCI), MCI, or AD. The blocks were Golgi impregnated and coded slides prepared for analysis of branching and spines. Assessment of randomly selected layer II–III pyramids from the three diagnostic categories and the three cortical brain regions showed that neurons from both parietal and temporal cortices displayed a comparable loss of connectome circuitry:  $\sim$ 30% and  $\sim$ 50% in MCI and AD, respectively. However, in the frontal cortex, in MCI there is a massive neuroplastic enhancement in branching and spines that amounts to a 75% increase in the connectome for these neurons. In the subsequent progression from MCI to AD, there is a 68% reduction of the connectome in the frontal cortex. These results indicate that in the evolution of AD, layer II–III neurons of the temporal and parietal regions undergo a progressive loss of branching and spines, initially in MCI and, additionally, in AD. However, by contrast, neurons in the frontal cortex show an initial neuroplastic response in MCI. This could be a compensatory mechanism—unique to the frontal cortex—which is helping to maintain circuitry and minimize cognitive dysfunction before being overwhelmed by the subsequent further progression of AD. It also suggests that if the molecular mechanisms associated with the neuroplasticity seen in the frontal cortex in MCI can be identified, they may provide a unique prophylactic or therapeutic approach to prevent, minimize, or reverse the loss of the neuronal networks associated with AD-related cognitive dysfunction.

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### Preclinical Efficacy Testing of a Human Neural Stem Cell Line (CTX0E03) in a Rat Model of Stroke

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Stroke causes severe neurological deficits. Transplanting human neural stem cells can alleviate some of these deficits. We here compared the effect of intraparenchymal and intraventricular grafts (450,000 CTX0E03 cells) in a rat model of stroke, on behavioral recovery and in vivo anatomical changes by MRI, as well as histological correlates. Intraparenchymal, but not intraventricular, CTX0E03 implants recovered deficits on the bilateral asymmetry test, the foot fault test, amphetamine-induced rotations, but no improvement was observed in the water maze. Serial T2-weighted MRI (pre, 1, 4, 12 weeks) did not exhibit any significant anatomical changes. Only animals with intraparenchymal grafts had a mean graft survival of 10,000 cells, although two animals (out of 15) did not have any cells surviving. These stereological results were corroborated by Alu-PCR. Survival was correlated with the spread of cells, the volume of the graft, and the density of the graft. However, there was no correlation between lesion volume and graft survival, although animals with stroke in the striatum and cortex had more cells surviving than those with a purely striatal lesion. Of the transplanted cells, 16% differentiated into glial fibrillary acidic protein (GFAP)<sup>+</sup> astrocytes. Animals with intraparenchymal grafts also exhibited an upregulation of collagen IV in the ipsilateral striatum (outside the lesion scarring), reflecting a potential effect of transplanted cells on angiogenesis. These results for a clinical grade human neural stem cell line are encouraging and support an initial clinical trial using these cells.

### Chronic LPS-Induced Disruption of FKN-CX3CR1 Signaling Is Associated With Hippocampal-Dependent Cognitive Dysfunction

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Neuroinflammation is associated with a variety of neurological and pathological conditions, such as Alzheimer's disease (AD) but also normal aging, and is reliably detected by the presence of activated microglia, the resident immune cells of the brain. AD is characterized by progressive memory loss and cognitive dysfunction and it is due, in part, to a deficit in synaptic plasticity that precedes the neurodegenerative changes; this early deficit may be exacerbated by activated microglia. Activated microglia and their products are key mediators of neuroinflammation and contribute to neuronal damage. In early AD, before neuronal loss occurs, the highest numbers of activated microglia are observed in the hippocampus and entorhinal cortex. Neuroinflammation can be reproduced by chronically infusing lipopolysaccharide (LPS) into the fourth ventricle of rats, resulting in region-selective microglia activation in the hippocampus, elevated tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ), and impaired hippocampal-dependent functions. Furthermore, this treatment results in altered expression of the plasticity-related behaviorally induced immediate early gene Arc only within these regions showing activated microglia, suggesting altered network activity. Recent findings indicate that neurons are not passive targets, but rather control microglial activity and modulate neuron-glia communications. The chemokine fractalkine (FKN)

is a neuronally derived signal that has been shown to regulate the neurotoxic effects of microglia through its receptor CX3CR1. Disruption of FKN-CX3CR1 signaling has been shown to exacerbate neurotoxicity in animal models of many neuropathological disorders, including AD. In the present study we investigated if chronic inflammation alters neuron-microglia signaling via the FKN-CX3CR1 signaling pathway. Rats ( $n = 6$ ) were chronically infused for 28 days with LPS (0.25  $\mu\text{g}/\text{h}$ ) or artificial cerebrospinal fluid (aCSF,  $n = 6$ ). On day 29–31 animals were tested for novel object and novel place recognition. LPS-induced chronic neuroinflammation resulted in impairment of hippocampus-dependent novel place recognition but not of hippocampus-independent novel object recognition. Analysis of hippocampus protein lysates revealed no significant changes in FKN levels between aCSF- or LPS-infused animals. Interestingly, there was a significant reduction in the amount of CX3CR1 protein levels in LPS-infused animals compared to aCSF controls. The LPS-induced reduction in CX3CR1 paralleled a significant induction of the proinflammatory enzyme phospho-p38 mitogen-activated protein kinase (pp38 MAPK). These results suggest that FKN/CX3CR1 signaling is involved in the modulation of LPS-induced chronic neuroinflammation. The FKN/CX3CR1 signaling pathway may mediate the altered coupling of neuronal activity with macromolecular synthesis implicated in plasticity and memory (Arc expression) during LPS-induced neuroinflammation, further studies are ongoing to test this hypothesis.

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### Young, But Not Adult, Primate Dopamine Neurons Are Protected From Methamphetamine- or MPTP-Induced Toxicity: Role of GDNF and Uncoupling Protein-2

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Oxidative stress occurs in Parkinson's disease (PD) and is linked to damage of dopamine (DA) neurons. Both methamphetamine and MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) are toxic to nigrostriatal DA neurons in adult humans and adult nonhuman primates through mechanisms involving oxidative stress. We have found that in young monkeys DA neurons are markedly resistant to either methamphetamine or MPTP. Methamphetamine or MPTP reduced striatal DA concentration and tyrosine hydroxylase expression (by 70–95%) in adults, while causing no significant loss of either measure in young monkeys. Altered pharmacokinetics do not provide an explanation for these differences, as the same dose of methamphetamine results in higher striatal and plasma drug levels in the young, compared with the adult, monkey. We are examining mechanisms that would provide an explanation for the resistance of young monkeys to methamphetamine and MPTP, as this may shed light on the plasticity and regenerative capacity of the DA system at different stages of primate development, and provide targets to enable the early-in-life protection from damage to DA neurons to be reinstated later in life. Oxidation of cell-permeable dihydroethidium (DHE) to red fluorescent cell-impermeable ethidium provides a way to detect intracellular reactive oxygen species (ROS). Following injection of DHE very little ethidium was observed in the young nigrostriatal system, compared with the adult striatum, indicating a relative lack of ongoing ROS formation in the younger brain. This low background of oxidative stress may contribute to the protection from oxidative stress elicited by methamphetamine or MPTP. Elevated production of the neurotrophic factor, glial-derived neurotrophic factor (GDNF), is a potential mechanism that developing DA neurons could utilize to avoid the toxic effects of methamphetamine. Young monkeys demonstrated a 2.5-fold increase in striatal

GDNF after exposure to methamphetamine. Untreated adult monkeys had a similar level of striatal GDNF as untreated infant monkeys. The same treatment with methamphetamine did not, however, result in any change in striatal levels of GDNF in the adult. The ability of the young brain to induce overexpression of GDNF in response to oxidative stress could mitigate the toxic effect of methamphetamine. Uncoupling proteins (UCPs) are mitochondrial membrane proteins that regulate ROS production, so a differential expression of UCPs is another potential mechanism that might protect developing DA neurons. A significant two-fold higher expression of UCP-2 mRNA occurred in substantia nigra of young compared with adult monkeys, suggesting that this might be a protective mechanism in the developing DA system that is suppressed later in life. Our recent work (Andrews et al., *J. Neurosci.* 29:14057–14065, 2009) has shown that the hormone, ghrelin, protects against MPTP toxicity by a UCP-2-dependent mechanism. Thus, ghrelin may be a novel UCP-dependent strategy to reduce DA neurodegeneration that is associated with PD and aging.

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#### **Engineered Nanoparticles From Metals Ag, Cu, and Al (50–60 nm) Induce Oxidative Stress, Blood–Brain Barrier Disruption, Neuronal Nitric Oxide Synthase Upregulation, and Cell Injury in the Rat Brain. Neuroprotective Effects of Insulin Like Growth Factor-1**

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The possibility that chronic exposure of nanoparticles leads to breakdown of the blood–brain barrier (BBB) and brain pathology by inducing oxidative stress and increased nitric oxide production was examined in a rat model using biochemical and morphological approaches. Separate groups of rats (male Sprague-Dawley, body weight 200–250 g, age 18–22 weeks old) were treated with silver (Ag), copper (Cu), or aluminium (Al) nanoparticles (50 mg/kg, IP) once daily for 7 days, whereas, the control group received saline under identical conditions. These rats were tested on the 8th day for sensory and cognitive dysfunction using Rota rod performance, grid walking, inclined plane angle tests, and stride length test using standard procedures. After that the BBB permeability was measured using Evans blue albumin and radioiodine tracers in several brain areas. Brain edema formation was examined by measuring brain water content, and oxidative stress parameters were determined using brain oxidants levels [e.g., myeloperoxidase (MP), malondialdehyde (MD), and glutathione (GT) were measured in various brain regions]. Using immunohistological methods, upregulation of neuronal nitric oxide synthase (nNOS) immunoreactivity was examined on paraffin sections under light microscope. In these serial sections, cell changes were seen at light microscopy using Nissl or hematoxylin and eosin. Rats treated with Cu and Ag nanoparticles exhibited mild to moderate sensory motor dysfunction as seen by reduction in time for staying on the Rota rod (16 rpm, 120 s to 80 s), decline in the angle of inclined plane (from 60° to 40°), placement error of forepaws during grid walking (from 0% to 34%), and increase in stride length between two hind limbs while walking (from 45 to 85 mm). These changes were only mildly affected by Al treatment. We observed a significant increase in MP and MD levels (MP  $4 \pm 1$  to  $9 \pm 2$  U/g,  $p < 0.01$ ; MD  $24 \pm 4$  to  $56 \pm 8$  nM/g,  $p < 0.01$ ) in the brains of Cu- and Ag-treated rats, whereas, GH showed a significant decline (from  $1.8 \pm 0.04$  to  $0.8 \pm 0.04$   $\mu$ M/g,  $p < 0.01$ ) in Cu- and Ag-treated rats. Changes in Al treatment were not significant in any of these oxidative stress parameters. A significant increase in brain water and BBB breakdown to Evans blue and radioiodine tracers

were observed in Cu- and Ag-treated but not in Al-treated rats. The number of dark and distorted neurons in various brain regions was significantly increased in Cu- and Ag-treated but not in Al-treated rats. A significant increase in the number of nNOS positive neurons was seen in the cortex, hippocampus, cerebellum, thalamus, and hypothalamus in these nanoparticle-treated rats compared to normal rats. Interestingly, the occurrence of nNOS-positive neurons was seen into areas showing BBB disruptions to Evans blue. Intravenous administration of insulin like growth factor-1 (IGF-1) in high doses (1  $\mu$ g/kg, once daily for 7 days) commenced at the same time as nanoparticle administration, significantly reduced the oxidative stress parameters and nNOS upregulation. The cell changes and the BBB breakdown caused by the nanoparticles were also considerably reduced. Taken together these novel observations demonstrate that nanoparticles, depending on their type (Cu and Ag and but not Al), are able to induce severe oxidative stress and nNOS expression, leading to BBB disruption and cell injury. Furthermore, IGF-1 has profound neuroprotective effects on nanoparticle-induced brain damage, not reported earlier.

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#### **Live Brain Imaging: Neural Network Remodeling by Hematopoietic Growth Factors in Aged Mice With Chronic Stroke**

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Stroke with high incidence in the elderly is a leading cause of long-term disability worldwide. Currently, there is no treatment available for chronic stroke other than physical therapy. We have recently demonstrated the therapeutic effects of two hematopoietic growth factors, stem cell factor (SCF) and granulocyte colony-stimulating factor (G-CSF), in animal models of chronic stroke. However, the mechanisms underlying SCF + G-CSF-induced functional improvement during chronic stroke remain to be explored. Neural network remodeling including apical dendritic spinogenesis of pyramidal neurons in the peri-infarct cortex has been thought to play a critical role in functional recovery after stroke. Here we aimed to determine the effects of SCF + G-CSF in apical dendritic spinogenesis in a mouse model of chronic stroke. Aged male transgenic mice (16–18 months old) genetically expressing yellow fluorescent protein (YFP) in the layer V pyramidal neurons were subjected to cortical brain ischemia through permanent occlusions of the right common carotid artery and middle cerebral artery. Four to 5 months after induction of brain ischemia, mice were randomized to receive subcutaneous injection of saline or SCF + G-CSF for 7 days. Two thinned skull windows (~200  $\mu$ m in diameter) were produced adjacent to the infarct cavities before starting treatment. Live imaging of the apical dendritic spines of layer V pyramidal neurons in layer I/II was acquired with a Zeiss multiphoton microscope (920 nm to excite YFP) through the thinned skull windows before treatment or 2 weeks after the final injection. High-resolution image stacks were taken 20  $\mu$ m deep from the brain surface with 1- $\mu$ m intervals. Live brain imaging data revealed that the number of dendritic spines was not different between the two groups before starting treatment. However, significant increases in the number of the thin or mushroom-like spines on the dendrites were observed in SCF + G-CSF-treated mice 2 weeks after treatment. These data suggest that SCF + G-CSF can enhance neural network remodeling in the aged brain of chronic stroke. This study provides new insights into the role of hematopoietic growth factors in brain repair during chronic stroke and would aid in developing a new therapeutic strategy for chronic stroke.

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### Effect of a HDAC Inhibitor on Neural Stem Cell Regulation and its Application to Spinal Cord Injury

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Neural stem cells (NSCs) possess the ability to self-renew and to differentiate into the three major cell types found in the central nervous system (CNS): neurons, astrocytes, and oligodendrocytes. Recent studies have shown that epigenetic gene regulation events such as DNA methylation and histone modification play important roles in regulating NSC fate specification. In this context, we have previously shown that the histone deacetylase (HDAC) inhibitor and well-known antiepileptic drug, valproic acid (VPA), enhances neuronal differentiation of NSCs. Perhaps because patterns of NSC differentiation are exquisitely controlled during normal embryonic development, restoration of damaged neural networks in the injured adult CNS is severely limited. Here, using a mouse model of spinal cord injury (SCI), we show that administering VPA and transplanting NSCs (Hdac Inhibitor and Neural stem cell Transplantation; HINT) enhances the functional recovery of their hind limbs. Neuronal differentiation of transplanted NSCs was promoted in VPA-treated mice. Anterograde corticospinal tract tracing revealed that transplant-derived neurons partially reconstructed the broken neuronal circuits, most likely in a "relay" manner. Ablation of the transplanted cells abolished the recovery of hind limb motor function, indicating that transplanted cells contributed directly to the improvement of motor function. These data raise the possibility that epigenetic regulation in transplanted neural stem cells can be exploited to provide treatment for SCI.

### Use of Viral Vectors Encoding Endomorphins and Histogranin for Pain Alleviation in a Rodent Spinal Cord Injury Model

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Pain is a frequent consequence of spinal cord injury (SCI), which profoundly impairs the patient's quality of life. SCI pain is clinically difficult to treat and, despite many decades of drug development, effective therapies still remain elusive. Ionotropic glutamate receptors contribute to maintained pain following damage to the nervous system in rats. Serine-histogranin (SHG) is a synthetic 15-amino acid peptide that exhibits *N*-methyl-D-aspartate (NMDA) receptor antagonist activity. Previous studies in our lab showed that intrathecal coadministration of *m*-opioid peptide receptor agonists, endomorphins and SHG, resulted in robust antinociceptive effects in SCI rats. Because continuous administration of pharmacological agents is often associated with undesirable side effects, the aim of this experiment was to investigate the recombinant potential of these genes in an SCI neuropathic pain model. In order to accomplish this, lentiviruses that encode endomorphins 1 and 2 (EM1 and EM2) and SHG were generated. SCI pain was produced by clip compression of the spinal cord at the level of T6–T8. Rats were randomly assigned to different treatment groups with a minimum of eight rats per group. At 2 weeks following SCI, separate groups of animals received intraspinal injections of lenti-EM1, lenti-EM2, mixed SHG/EM1/EM2 (3:1:1 ratio), or control vector. Injections were made stereotaxically into the dorsal horn at the lumbar enlargement in order to target below-level SCI pain. Animals were tested for heat hyperalgesia and mechanical and cold allodynia using the Hargreaves method, Von frey filament, and acetone tests, respectively. At the end of the experiment, the contribution of EMs and SHG to analgesic effects was assessed by intrathecal injection of either opioid antagonist naloxone or anti-SHG antibody, respectively. Clip compression SCI resulted in neuropathic pain symptoms by 2

weeks postinjury, which were sustained for at least 6 weeks in control-injected animals. Compared with the control injection group, mechanical and cold allodynia were reduced significantly by recombinant analgesic peptides. The injection of lenti-EM1, lenti-EM2, or the SHG/EM1/EM2 combination resulted in reduced neuropathic pain lasting at least 1 month after injection. The antinociceptive effects of EM1 or EM2 alone were comparable to the mixture. Intrathecal injection of naloxone reversed the allodynic effects of the lenti-EMs, and both naloxone and the anti-SHG antibody attenuated cold allodynia in animals receiving the mixed vectors. In conclusion, intraspinal administration of recombinant constructs encoding EMs and SHG can produce significant analgesic effects on neuropathic pain after SCI in rats and suggests a promising therapeutic approach in the management of intractable clinical SCI pain.

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### Levodopa Affects Potassium-Evoked Corticostriatal Glutamate Release in Dyskinetic Rats

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The symptoms of Parkinson's disease are pharmacologically counteracted with levodopa to increase the striatal dopamine levels. Unfortunately, long-term treatment causes levodopa-induced dyskinesia in the majority of patients. Levodopa-derived dopamine release from the serotonergic neurons has been pointed out as the main contributing factor for the onset of dyskinesia. At present, several studies indicate that apart from uncontrolled dopamine signaling, increased corticostriatal glutamate transmission may contribute to the pathophysiology of dyskinesia. Therefore, in the present study, in vivo amperometry was employed to study glutamate release in the striatum of chronically levodopa-treated dyskinetic and normal rats. Glutamate release was potassium evoked before and after local levodopa administration in anesthetized animals, and the response was monitored second-by-second with electrodes highly selective for glutamate. Hence, it was possible to observe how long-term levodopa treatment and acute application of the drug affect the extracellular glutamate release and reuptake. In the normal levodopa-naive striatum, the amplitude of potassium-evoked glutamate release was 20–30  $\mu$ M, which was sustained upon levodopa loading. In the chronically levodopa-treated dyskinetic animals, glutamate release was significantly reduced to approximately 5  $\mu$ M, and it was further attenuated after local levodopa administration. This was seen in both the dopamine-lesioned and intact striatum of dyskinetic animals, thus not affected by the presence of dopaminergic nerve fibers. Furthermore, the levodopa injection itself provoked a glutamate release in all animals, regardless of treatment. This release was several-fold higher than the potassium-evoked glutamate release. To conclude, chronic levodopa treatment seems to depress the glutamate release in dyskinetic animals, possibly due to elevated basal extracellular glutamate levels.

### Validating Exogenous Markers of Transplanted Cells

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Postmortem evaluation relies on being able to identify transplanted cells within the host brain, weeks and potentially years after transplan-

tation. Bromodeoxyuridine (BrdU), PKH26, and Hoechst are often used to label cells, but their validity and reliability to unequivocally identify transplanted cells is potentially compromised due to label transfer from grafted to host cells. Labels were assessed in vitro to analyze their labeling efficiency of human neural stem cells (hNSC), as well as their effects on viability, proliferation, maintenance of NSC markers, and differentiation towards neural and glial lineages. Hoechst and PKH26 efficiently labeled cells in vitro, with BrdU being the lowest, at ~90%. However, all three affected cell proliferation. To establish the temporal evolution of the reliability of these markers in vivo, hNSC were grafted into rat brains and postmortem analyses were conducted at 1, 7, 28, and 64 days posttransplantation. The label was assessed on the extent to which it colocalized with specific endogenous markers of human cells, such as human nuclear antigen (HNA) and Y chromosome. The total number of cells identified by each label was also compared to Alu-PCR. BrdU labeling was generally colocalized with HNA, although at day 7, there were also a large number of HNA/BrdU<sup>+</sup> cells. This suggests a potential label transfer to host cells, and thereby an overestimation of the total number of transplanted cells. In contrast, at 64 days, reliance on BrdU as the sole indicator of transplanted cells would have led to an underestimation of transplanted cells. Finding a reliable marker to detect transplanted cells is paramount to assessing the neurobiological basis of their efficacy. This might not only depend on their labeling efficiency, but also their effect on cell viability, proliferation, differentiation, and retention of the label within transplanted cells in vivo.

**The Brahma Related Gene 1 (Brg1)-Containing Chromatin Remodeling Complex Interacts With the Transcription Factor Pax6 to Maintain the Neurogenic Potential of Adult Neural Progenitors**

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Neural stem cells (NSC) persist in the adult subependymal zone life-long and have the capacity to produce both olfactory bulb (OB) interneurons and oligodendrocytes of the corpus callosum. The intrinsic fate determinants Pax6 or Olig2 influence this fate decision and are regulated by extrinsic signals, such as bone morphogenetic protein (BMP) inhibiting Olig2 expression (Colak et al., 2008; Jablonska et al., 2010). However, the molecular mechanisms of how Pax6 endows cells with a neurogenic fate are still elusive. Here we show that Brg1, an ATP-dependent chromatin remodeling factor, is required for the neurogenic fate maintenance. In the absence of Brg1 function, adult-generated neuroblasts convert to glial cells expressing the hallmarks of glial progenitors such as NG2 and Olig2. We further showed that Brg1 directly interacts with Pax6 in the migrating neuroblasts at the late stage of their differentiation. Similar to Brg1 loss-of-function, the loss of Pax6 in neuroblasts, even when already migrating along the rostral migratory stream (RMS) towards the OB, results in the fate conversion of these neuroblasts towards an NG2<sup>+</sup> glia fate. Interestingly, Pax6 expression is not altered in the Brg1 mutant and Pax6 cannot mediate neurogenesis in the absence of Brg1. These data suggest that Brg1 and Pax6 form a functional complex necessary for the maintenance of neuroblast fate in the adult RMS. We shall further present data demonstrating a transient role of this complex in neuronal differentiation, as this complex is no longer needed in mature OB neurons, such as the dopaminergic periglomerular neurons that do not alter their identity upon loss of either Pax6 or Brg1. In summary, our data reveal the functional complex of the transcription factor Pax6 with the Brg1-containing chromatin remodeling machinery as a key mechanism to maintain neuronal fate in migrating neuroblasts, thereby

identifying for the first time a mechanism necessary for the active maintenance of commitment in adult neurogenesis.

**Exposure to the Peripheral Toxin MPP<sup>+</sup> Enhances MPTP-Induced Dopamine Neuron Loss and Neuroinflammation**

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Our laboratory has shown that different dopamine (DA) neurotoxins [1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP), 6-hydroxydopamine, rotenone, and lipopolysaccharide] produce blood-brain barrier (BBB) disruption in DA-rich regions such as the substantia nigra (SN) and striatum. Areas of BBB leakage colocalized with  $\beta$ 3 integrin expression, a marker of angiogenesis. This finding was not surprising as newly created angiogenic vessels are often leaky and are unlikely to have a fully developed BBB. We have also found evidence of  $\alpha$ v $\beta$ 3 integrin expression in postmortem human brain tissues from patients with Parkinson's disease, suggesting ongoing angiogenesis and BBB compromise. We propose that a dysfunctional BBB would allow entry of peripheral toxins that further contribute to DA neuron loss and neuroinflammation. The objective of this study is to determine if MPTP treatment makes the mouse susceptible to subsequent systemic treatment with MPP<sup>+</sup>, the polar active metabolite of MPTP that normally does not cross the BBB, enhancing inflammation and DA neuron loss. Middle-aged male C57BL/6 mice were treated with either MPTP (10 mg/kg q 2 × 4) or saline (SAL). On day 5, animals were systemically given either MPP<sup>+</sup> (120  $\mu$ g) or SAL. On day 10, the animals were sacrificed and assessed for DA neuron loss [tyrosine hydroxylase-immunoreactive (TH-ir) cell counts], BBB disruption [FITC-labeled albumin (FITC-LA) leakage and tight junction protein ZO-1 expression], and neuroinflammation [Iba-1-immunoreactive (Iba1-ir) microglia density]. MPTP/saline treatment reduced TH-ir cell counts by 33% [ $F(3, 24) = 7.892, p < 0.001$ ] and increased Iba-1-ir microglia density [ $F(3, 15) = 6.620, p < 0.05$ ] compared with SAL/SAL-treated mice. MPTP/MPP<sup>+</sup> treatment further reduced TH-ir cell counts to 50% ( $p < 0.01$ ) and enhanced microglia density compared with SAL/SAL-treated mice. The TH-ir cell counts and microglia density in SAL/MPP<sup>+</sup>-treated mice were unchanged compared to SAL/SAL-treated mice. MPTP/SAL- and MPTP/MPP<sup>+</sup>-treated mice exhibited punctate areas of FITC-LA leakage in the SN, suggesting BBB compromise. The tight junction protein, ZO-1, was redistributed in the vessels of the SN of MPTP/MPP<sup>+</sup>-treated mice, suggesting that these vessels lack the characteristic tight junctions of a functional BBB. Exposure to a peripheral water-soluble toxin, MPP<sup>+</sup>, exacerbates DA neuron loss, neuroinflammation, and BBB permeability, but this effect is limited to animals previously treated with MPTP. These results are consistent with the hypothesis that MPTP induces BBB dysfunction that then allows peripheral toxins to enter the parenchyma and enhance inflammation and DA neuron loss. Ongoing studies using longer protocols and multiple exposures to MPP<sup>+</sup> will determine if soluble toxins can induce a progressive loss of DA neurons in the MPTP mouse model.

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### Generation of an Age- and Region-Specific Map of the Developing CNS to Characterize Cells for Neurotransplantation

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Detailed analysis of the composition of neural precursors for cell transplantation is a critical process because contamination of cells from other brain regions is undesirable and might mitigate the functional benefits. Here we report the establishment of a marker-based map that allows the analysis of the composition and developmental age of cultured neural precursors by using immunocytochemistry. E14 rat embryos as well as human fetuses between the 6th and 11th week of pregnancy were dissected into cortex (dorsal telencephalon), whole ganglionic eminence (WGE; ventral telencephalon), diencephalon, ventral mesencephalon (VM), dorsal mesencephalon (tectum), and rhombencephalon (hindbrain). Cells were enzymatically dissociated and cultured under differentiation conditions for 24 h and 7 days. Afterwards wells were processed for immunocytochemistry, using fetal brain region-specific transcription factors to analyze neural precursors and neurotransmitter phenotype-associated markers to analyze mature neurons. In addition, well-characterized glial and general neuronal markers as well as markers of the VM and WGE were used. We identified markers for the fetal and adult cortex, WGE, thalamus, and VM and hindbrain that did not cross-stain with other brain regions, or their expression patterns clearly differed from the profile in other regions. Additionally, tissue inclusions from adjacent brain regions of the target region can now be identified with certainty. Extension of this marker tool for the application on human fetal neural precursors is currently in progress, but the data are comparable with the results obtained from fetal rat tissue. In summary, we established a set of markers to analyze differentiation stage and region specificity of cultured rodent and human neural precursors, which will provide an essential tool in future applications of cell-based therapies in clinical neurotransplantation studies.

### Experiences From Fetal Neurotransplantation in Huntington's Disease

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Whole ganglionic eminence (WGE)-derived cells are a potential source for the replacement of striatal projection neurons by transplantation in neurodegenerative diseases, like Huntington's disease (HD). The ongoing European multicenter trial Multicentric Intracerebral Grafting in Huntington's Disease (MIG-HD) currently investigates beneficial effects of neurotransplantation in HD patients. We here present experiences from the German branch of the trial on safety, side effects, and functional parameters. Risk assessment included pharmacological safety, involving immune rejection, tumor formation, disease transmission, and side effects of immune suppression. Functional assessment comprised motor scoring, neuropsychiatric evaluation, and cognitive tests. Fetal WGE grafts were processed mechanically and injected by stereotactic implantation into up to six trajectories of the striatum bilaterally. Tissue was obtained from elective abor-

tions. Blood samples from women undergoing abortions were analyzed for viral infections. Testing for bacterial contamination was performed after implantation. Fourteen HD patients were transplanted bilaterally and two unilaterally, receiving tissue from 1–2 donors/side. Age at enrollment ranged from 31 to 53 years. No transmission for human immunodeficiency virus (HIV), hepatitis B (HBV), hepatitis C (HCV), or human T-cell lymphotropic virus (HTLV) was detected. Fetal neurotransplantation was generally safe and not associated with severe side effects in most patients. The most common side effects were associated with immune suppression. There was no case of graft overgrowth. One patient deteriorated closely after withdrawal of immune suppression and improved after administration of high-dose steroids. By assessing functional parameters, the clinical course after transplantation was assessed. While cognitive parameters showed a slight worsening initially after transplantation and a slow improvement, individual patients had marked stabilization of cognitive parameters and other parameters continued to deteriorate. Different types of clinical developments have also been identified when regarding the motoric part of the HD rating scale. Clinical WGE transplantation in HD has been shown to be generally safe and feasible, contrasting with results from solid organ transplantation. The potential immunogenicity of the grafts and the ongoing degeneration of the host brain might inhibit more substantial clinical beneficial effects in the long-term course of the disease. Our data indicate general safety and promote further refinements of the method and upcoming trials using alternative cell resources in HD. However, the complete evaluation of the multicenter MIG-HD trial will help gain substantial information on the effectiveness of this method.

### Phase I/II Randomized Controlled Trial of Autologous Bone Marrow-Derived Mesenchymal Stem Cell Therapy for Chronic Intracranial Hemorrhagic Stroke

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Intracranial hemorrhagic (ICH) stroke is a common neurological disorder and associated with permanent neurological deficits. Currently, there is no effective treatment for restoring the lost neurological functions. The discovery of stem cell plasticity and neurogenesis in the adult brain has raised the hope that stroke might become amenable to cellular therapy to replenish the loss and degeneration of functional neural cells. Mesenchymal stem cell (MSC) therapy has been shown to be safe and effective in animal stroke models and a phase I/II trial for acute stroke. In March 2007 to January 2009 we conducted a double-blind, randomized, controlled phase I/II trial to examine the safety and efficacy of MSC therapy in a cohort of nine patients (four females and five males) with a mean age of 52 years (range 41–59 years) who had undergone ICH stroke for a year. MSCs were *ex vivo* expanded from 29 ml (17–42 ml) autologous bone marrow. Patients with severe disability were randomized to have two intravenous injections of autologous MSCs or placebo in 4 weeks. Neurological functions and clinical outcome were monitored pretreatment and at 4, 12, 24, and 48 weeks posttreatment. In the treatment group of three female and two male patients,  $8.67 \times 10^5$  ( $2.65 \times 10^5$ – $1.31 \times 10^6$ ) and  $8.41 \times 10^5$  ( $2.65 \times 10^5$ – $1.45 \times 10^6$ ) MSCs up to passage 8 per kg body weight were administered on two occasions. The control group of one female and three male patients received placebo in an identical manner. The cell viability was 95.3% (88.5–99.0%). Derived cells were immunophenotypically positive for CD29, CD44, CD73, CD90, CD105, and CD166, but negative for human lymphocyte antigen (HLA)-DR, CD45, CD33, CD38, CD3, CD19, CD16, CD34, and CD133. No adverse event was noted. Significant or trend of improvement in motor and cognitive

functions was only observed in the treatment group [Functional Independence Measure (FIM) of motor components:  $p = 0.068$ ,  $0.068$ , and  $0.043$  at 8, 16, and 28 weeks after treatment; cognitive functions:  $p = 0.038$ ,  $0.041$ ,  $0.042$ , and  $0.066$  at 8, 16, 28, and 52 weeks; modified Barthel index:  $p = 0.043$  and  $0.068$  at 28 and 52 weeks]. However, no difference in the Extended Glasgow Outcome Scale (GOSE) score was found. The study findings suggest that MSC therapy is safe and may improve both cognitive and functional recovery after ICH stroke, but beneficial clinical outcome was not apparent. Further phase III trials may need to confirm the findings.

### Survival and Migration of Neural Progenitor Cells After Neonatal Implantation in a Mouse Model of Down Syndrome

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Down syndrome (DS) is the most common genetic cause of intellectual disability, affecting approximately 1 in every 766 live births. While some atypical development occurs in the fetal period, much of the aberrant development associated with DS occurs in largely postnatally developing structures, such as the hippocampus. Thus, the brain provides a "window of opportunity" for intervention. Recent successful outcomes in the application of neural progenitor cells (NPC) for other neurodegenerative disorders indicate that their application in DS may be a viable option by preventing or ameliorating the postnatal degenerative changes and aberrant development. Additionally, the neonatal brain may be uniquely equipped to accept and be influenced by implanted NPC due to endogenous cues present at this stage of development. To explore the feasibility of early postnatal treatment in a mouse model of DS, we bilaterally implanted 100,000 C17.2 murine NPC (mNPC) into the dorsal hippocampus of postnatal day 2 (PND 2) Ts65Dn pups. This immortalized cell line is labeled with green fluorescent protein (GFP) and lacZ for identification after implant. Disomic littermates provided karyotype controls for DS modeling trisomic pups. Control litters were either not treated or sham implanted with saline. The implanted C17.2 mNPC had robust survival and had widely migrated 7 days after implantation. In addition to mNPC found at the hippocampal site of implantation, cells were found to have migrated to the somatosensory cortex and to the hypothalamic area surrounding the third ventricle. Analysis of long-term migration and survival of implanted mNPC revealed that at 16 weeks of age, 8 of 14 (57%) of the trisomic mice and 50% of the disomic mice had positive GFP-labeled mNPC. However, the number of surviving cells was small and primarily limited to the hippocampus in both karyotypes. mNPC were found as granule cells in the dentate gyrus, in the pyramidal cell layer of Ca1 and Ca3, and in the oriens and molecular layer and morphologically appeared to be interneurons. No needle track was observed as a result of the implantation procedure in either karyotype. Long-term implanted cell survival varied greatly even within litters, as often littermates that received the same cell preparation had widely variable mNPC survival. Overall, the number of implanted cells found in adult animals was low and did not affect the density of cells found in the dentate gyrus, which showed no significant difference between any group ( $p > 0.05$ ). This study confirms that implanted mNPC can survive and migrate in the trisomic environment present in DS. While the implanted mNPC can migrate from the hippocampal site of implantation, long-term cell survival appears to be limited to this region, which is preferentially compromised in DS. Future studies will focus on the potential ability of these cells to prevent age associated tau accumulations and behavioral degenerative changes found in these mice.

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### Pulsed Electromagnetic Field Therapy: Applications to Neuronal Survival After Traumatic Brain Injury

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Traumatic brain injury (TBI) is a major cause of morbidity and mortality in civilian and military populations throughout the world. In the US alone, approximately 2 million cases occur annually with often dismal prognostic outcomes among survivors. Researchers have long searched for a TBI biomarker that could shed light into the degree/severity of head injuries. Ubiquitin carboxyl-terminal esterase L1 (UCH-L1) is a neuron-specific enzyme that is released into cerebrospinal fluid (CSF) after head injury and has demonstrated potential as a TBI biomarker. However, despite advances in being able to better characterize and detect TBI, few therapies currently exist for TBI patients. High-frequency, low-amplitude pulsed electromagnetic fields (PEMFs) have been shown to exert medically relevant, biologic effects through the activation of calmodulin-dependent nitric oxide (NO) synthase. PEMF therapy has demonstrated anti-inflammatory properties through its ability to attenuate postsurgical inflammatory cytokine expression. Interleukin-1 $\beta$  (IL-1 $\beta$ ) is an inflammatory cytokine and important mediator of the body's immunologic and systemic response to injury, especially in the context of TBI. Similar to UCH-L1, the degree of IL-1 $\beta$  expression after head injury has been shown to correlate with the severity of TBI. In this study, we report the ability of PEMF therapy to reduce IL-1 $\beta$  expression and microglial activation after invasive and noninvasive TBI. The effects of PEMF on TBI-induced inflammation and neurodegeneration were investigated in an animal model. Adult Sprague-Dawley rats were subjected to a 4-Newton head impact and analyzed for IL-1 $\beta$  expression 6–7 h later via enzyme-linked immunosorbent assay (ELISA) of brain homogenates and CSF. A second cohort of rats was subjected to a bilateral, invasive stab injury, and similarly analyzed for IL-1 $\beta$  expression over the course of 5 days. Histopathologic evidence of microglial infiltration and astrogliosis was obtained by immunostaining for CD11b and glial fibrillary acidic protein. UCH-L1 was characterized with Western blot analysis. PEMF treatment was associated with a 30–40% reduction in IL-1 $\beta$  levels compared to control ( $p < 0.05$ ) in CSF and brain homogenates for both types of injury. PEMF signals also appeared to decrease histopathologic evidence of microglial infiltration and astrogliosis in the invasive-injury group compared to control. Taken together, results suggest that PEMF therapy can attenuate molecular and cellular inflammation after TBI. Because inflammation is directly related to edema and subsequent neuronal loss, we predict that this modality will reduce mortality and neurological deficits.

### Effect of Spinal Cord Tissue Preservation by Bone Marrow Stromal Cells on Functional Recovery After Spinal Cord Contusion in Rats: More Is Better

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In a rat model of spinal cord injury, the effects of an allogeneic bone marrow stromal cell (BMSC) graft on hind limb locomotor and sensory function and on tissue volume and axon numbers were investigated. The T9 spinal cord was contused using the Infinite Horizon Impactor at a force of 200 kdyn(e) and injected 3 days later with 5  $\mu$ l DMEM with  $1 \times 10^6$  BMSCs or DMEM alone into the injury epicen-

ter. Two months after BMSC transplantation, rats exhibited a fourfold increase in Basso, Beattie and Bresnahan (BBB) subscore, a 26% smaller angle of hind paw rotation, and a 70% improvement on the horizontal ladder compared to controls. Also, rats with a BMSC transplant exhibited a 52% decrease in mechanical allodynia and a 25% decrease in thermal allodynia compared to controls. BMSC transplantation resulted in a 66% increase in spared tissue volume compared to controls. Correlative analysis revealed that larger volumes of intact spinal cord tissue corresponded with better motor and sensory function outcomes. The number of serotonergic and catecholaminergic axons caudal to the contusion was similar in both groups. BMSC-treated animals showed increased numbers of fast blue back-labeled brain stem neurons with extensions caudal to the injury site. Together the results indicate that BMSC exert their beneficial effect on motor and sensory function by preserving spinal cord tissue. Our results underline the importance of BMSC-mediated neuroprotective mechanisms for spinal cord repair. The data support further investigations of BMSC as a possible future cell-based therapy for the injured spinal cord.

#### Basement Membrane Collagen Accumulation in Brain and Spinal Cord Vessels of ALS Patients

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Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by progressive loss of motor neurons in the brain and spinal cord, leading to muscle atrophy, paralysis, and death. Recent studies show that impairment of the blood-brain/spinal cord barrier (BBSCB) in both animal models of ALS and ALS patients occurs due to compromised capillary endothelium integrity followed by vascular leakage. Additionally, alterations of basement membrane components are noted. The endothelial basement membrane in the vasculature of the central nervous system (CNS) is composed mainly of collagen type IV and laminin, followed by smaller proportions of collagen types III, VII, XV, and XVIII, entactins, and proteoglycans. However, little information exists regarding vascular collagen of ALS patients. The aim of this study was to evaluate the basement membrane collagen in brain and spinal cord vessels from ALS patients. Microvascular basement membrane was examined in gray matter postmortem tissue of the brain stem (medulla) and spinal cords (cervical and lumbar) from ALS patients ( $n = 12$ ) and age-matched controls ( $n = 8$ ), 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), via electron microscopy. Collagen expansion was measured in vessel quadrants ( $n = 5$ ) in the medulla, cervical, and lumbar spinal cord from each ALS patient and control via electron micrographs. Tissue samples were also stained with 0.1% Sirius Red, confirming collagen in the vascular basement membrane. Our results showed a significant increase ( $p < 0.0001$ ), 2–2.5 times, of basement membrane collagen accumulation in the majority of medulla, cervical, and lumbar spinal cord microvessels from ALS patients ( $0.9273 \pm 0.06$ ,  $2.5832 \pm 0.13$ , and  $2.1386 \pm 0.10$   $\mu\text{m}$ , respectively) compared to controls ( $0.5807 \pm 0.049$ ,  $0.9538 \pm 0.103$ , and  $0.9560 \pm 0.095$   $\mu\text{m}$ , respectively). Histopathological staining for Sirius Red confirmed collagen basement membrane expansion in ALS patients, corroborating the ultrastructural

observations. This extensive vascular basement membrane collagen accumulation in ALS patients suggests impairment of capillary endothelium integrity, probably leading to BBSCB dysfunction in ALS as a major pathophysiological mechanism. Furthermore, it is possible that abnormal buildup of basement membrane collagen alters barrier transport functions and thus impairs CNS homeostasis in ALS. Although the mechanism(s) of basement membrane collagen accumulation is unclear, we hypothesize that the vascular basement membrane collagen increase in ALS results from a metalloproteinase/inhibitor imbalance. Currently, we are investigating this possibility.

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#### Effect of the Sources and Passage Numbers on Efficacy of Mesenchymal Stem Cells Transplants in the R6/2 Mouse Model of Huntington's Disease

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Mesenchymal stem cells (MSCs) are defined as self-renewing and multipotent cells capable of differentiating into multiple cell types, such as neurons. MSCs represent an attractive source for cell replacement therapy in neurological disorders, such as Huntington's disease (HD). However survivability and efficacy of MSC transplants has been a source of recent controversy. To address the discrepant findings, we focused our work on characterizing MSCs from various sources and following a different number of cell passages. Our previous work (Rossignol et al., JCM, 2009) suggested that reducing the number of cell passages may increase transplant survivability in rats and increase their efficacy in reducing behavioral deficits in the 3-nitropropionic acid rat model of HD (Rossignol et al., BBR, 2010). The two major goals of the present study were to: 1) compare the expression of markers in vitro for both a low and high number of cell passages from MSCs derived from bone marrow (BM) and from umbilical cord blood cells (UCB); 2) assess the efficacy of these cells when transplanted into the striata of R6/2 mice. Characterization of the cells was done using a BD-LSRII flow cytometer (FACS) for different markers, including CD90, stem cell antigen (Sca-1), nestin, CD105, and CD45 on mice MSCs at different passages. High passaged ( $\geq 40$ ) and low passaged ( $\leq 7$ ) MSCs from both BM and UCB were transplanted bilaterally into the striata (400,000 cells/striatum) of 5-month-old R6/2 mice. Following surgery, all mice were tested in the Morris water maze (MWM) and rotarod prior to sacrifice at 7 weeks after transplantation, at which point their brains were processed using immunohistochemistry (IHC). Our in vitro results indicated that CD90 expression decreased, and Sca-1 increased as the number of passages increased. This indicates that there are significant changes in cell characterization over passages. Our in vivo results revealed that only R6/2 mice receiving high passaged BM-MSCs had longer latencies to fall off the rotarod, while all transplant groups had decreased latencies to find the hidden platform in the MWM task. Our IHC results indicated greater survival of BM-MSCs, but less neuronal differentiation (as quantified by NeuN labeling), relative to transplanted UCB-MSCs. Our results suggest that high passaged BM-MSCs provided increased behavioral efficacy and transplant survivability, while UCB-MSC transplants provided more neuronal differentiation. As such, our results indicate that the source of MSCs and the number of cell passages are critical variables to be considered for transplantation therapies.

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### Anatomical Integration of Transplanted Neural Tissue Following Cervical Contusion Injury in Adult Rat

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Debilitating respiratory deficits frequently result from trauma at or above the level of the phrenic nucleus (C3–C5). Such injuries involve both an upper and lower motoneuron components due to damage to descending bulbospinal inspiratory fibers and portions of the spinal phrenic circuitry, respectively. While interrupted communication between brain stem respiratory centers and spared phrenic circuitry associated with diaphragm function is likely to be of greatest importance, a contribution of gray matter injury cannot be arbitrarily dismissed. Using a midcervical midline contusion model, the present study explored whether respiratory outcomes could be improved by intraspinal tissue transplantation directed at gray matter repair. Adult female Sprague-Dawley rats were deeply anesthetized and a laminectomy was performed to expose the dorsal surface of the C3–C4 spinal cord. A midline contusion was subsequently performed at a predetermined force of 150 kilodynes (Infinite Horizon pneumatic impactor, Lexington KY; mean resulting force =  $154 \pm 2$ ). This injury has been shown to impair the animal's ability to increase diaphragm activity in response to respiratory challenge (in this case hypercapnia/hypoxia). One week postinjury, transplant recipients were anesthetized and the injury site reexposed. Embryonic day 13–14 rat spinal cord tissue was dissociated and the resulting cell suspension injected into the lesion epicenter. Diaphragm activity ipsi- and contralateral to injury was assessed terminally via electromyography (EMG) recordings at 5 weeks postinjury (4 weeks posttransplant). Pseudorabies virus (PRV; transsynaptic retrograde tracer) was used to examine the phrenic circuitry in each experimental group and determine the extent of host–transplant integration. Initial results have revealed some restoration of diaphragm responses to respiratory challenges in transplant recipients. Electrophysiological recordings of diaphragm activity have revealed that the response to respiratory challenge is improved in treated animals. PRV delivered to the diaphragm resulted in phrenic motoneuron (PhMN) and interneuronal labeling in all animals. In addition, infection of donor neurons was also observed, suggesting synaptic integration with the host phrenic circuit in those animals. To further investigate host–graft neuronal integration, PRV was injected directly into grafts of a subset of transplant recipients. This labeling approach demonstrated putative second-order or later-order labeling of host neurons in the cervical spinal cord. Our results offer anatomical evidence for a host–transplant–host relay circuit that may modulate phrenic activity ipsilateral to injury.

### Loss of Nogo-A-Expressing Neurons in a Rat Model of Parkinson's Disease

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The myelin associated protein Nogo-A is among the most potent growth inhibitors of the adult CNS. Nogo-A is mostly expressed on the surface of oligodendrocytes and has been described to limit neuronal regeneration and plasticity particularly after injury. More recently, Nogo-A expression was demonstrated in a number of neuronal subpopulations of the adult and developing CNS, suggesting that Nogo-

A bears other functions beyond the inhibition of axonal regeneration and plasticity. At present, only little is known about the expression of Nogo-A in the midbrain, a brain structure severely affected in Parkinson's disease (PD). For that purpose the present study aimed at characterizing the expression pattern of Nogo-A-immunoreactive (-ir) cells in the adult midbrain of control rats and in a 6-hydroxydopamine (6-OHDA) rat model of PD. One week and 1 month after unilateral striatal injections of 6-OHDA, rats were perfusion fixed and the brains processed for histological analyzes. We found that Nogo-A-ir cells were predominantly distributed in the substantia nigra pars compacta (SNc) and in the ventral tegmental area. Interestingly, a substantial number (about 50%) of tyrosine hydroxylase (TH)-ir neurons in the SN also expressed Nogo-A. Moreover, semiquantitative analyzes showed that about 70% of the Nogo-A-positive cells coexpressed TH, hinting to the idea of a predominant neuronal expression of Nogo-A. In line with this notion, no colocalization was observed for Nogo-A and the astrocytic marker glial fibrillary acidic factor (GFAP). Our preliminary data revealed that 1 week after 6-OHDA injection animals displayed a significant loss of dopaminergic neurons as well as Nogo-A-ir cells in the SNc of the lesioned compared to the unlesioned side (by 40% and 50%, respectively). The number of both TH-ir neurons and Nogo-A-ir cells was observed to be further decreased after 1 month (by 90% and 70%, respectively). Interestingly, by means of double-immunofluorescence stainings we detected that cell loss was predominantly seen in the subpopulation of SNc neurons expressing Nogo-A and TH. In particular, we found that the percentage of Nogo-A-ir cells also expressing TH was significantly lower both at 1 week (with 40% colocalization) and 1 month after the lesion (with 30% colocalization) compared to control sides (with 70% colocalizations). Based on these results we hypothesize that the subpopulations of dopaminergic neurons expressing Nogo-A are particularly vulnerable to insults. In sum, our findings strongly suggest a function of Nogo-A in the nigrostriatal system and that Nogo-A may play a substantial role in PD.

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### Brain Infiltration of Peripheral Cells of the Monocyte/Macrophage Lineage in Response to Chemokine Overexpression

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The role of glial cell activation in Alzheimer's disease is multifaceted; evidence exists demonstrating detrimental neurodegenerative activity as well as neuroprotective actions. Activated microglia are increasingly being phenotyped into classical (M1) or alternative (M2) activation states. Moreover, the contribution of peripherally derived macrophages infiltrating into the brain to the outcomes of glial-activating stimuli is an area of considerable interest. Chemokines and their receptors can regulate macrophage trafficking during development, cell surveillance and inflammation. In this study we investigate the hypothesis that delivery and overexpression of C-C chemokine such as CCL2 (also known as monocyte chemoattractant protein 1) via adeno-associated virus serotype 9 (AAV-9) injections into brain will increase the infiltration of peripheral cells of monocytic-macrophage origin. AAV9-CCL2 expression is driven by the chicken  $\beta$ -actin promoter. The vector was injected unilaterally in the mouse frontal cortex and hippocampus via convection-enhanced delivery and expressed for 6 weeks. Our previous studies have shown that maximal expression levels of the chemokines are achieved at this time point. Then bone marrow-derived macrophages (BMDM) isolated from donor mice ubiquitously expressing green fluorescent protein (GFP; positively selected for the CD11b monocytic marker) were injected via intracardiac puncture into nontransgenic mice 24 h prior to euthanizing the animals.

Flow cytometry and immunohistochemistry data indicate that more GFP<sup>+</sup>/CD11b<sup>+</sup> BMDM cells infiltrate the brain parenchyma following CCL2 overexpression compared with the contralateral hemisphere of AAV9-CCL2-injected or control animals ( $p < 0.001$ ,  $t$ -test). CCL2 overexpression led to significant activation of brain resident microglia in both brain regions as shown by CD45 staining of the brain tissue with threefold higher activation in hippocampus and twofold in cortex compared to control. Using major histocompatibility complex II (MHCII) staining, we confirmed the same level of activation of microglia, especially around the region of injection. Interestingly, we observe that overexpression of CCL2 in the brain can drive the expression of alternative activation state markers, YMI and arginase-1, as well as markers associated with classical activation of microglia such as calgranulin B (S100B) and IL-1 $\beta$ . We also measured the cytokine/chemokine levels in the treated brain and found significant increases in interleukin-6 (IL-6), granulocyte colony-stimulating factor (G-CSF), and eotaxin levels, but not in tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) or interferon- $\gamma$  (IFN- $\gamma$ ). Moreover, the real-time PCR analysis confirmed increases in gene expression of arginase-1 (2.4-fold), YMI, (2.7-fold), and signal regulatory protein beta (Sirpb, 17-fold), but a decrease in macrophage receptor with collagenous structure (MARCO) gene expression (0.6-fold) compared to controls following CCL2 overexpression. Monocyte chemoattractant protein (CCL2) overexpression led to GFP<sup>+</sup>/CD11b<sup>+</sup> BMDM infiltration into the brain as well as recruitment and activation of CD45- and MHCII-positive macrophages. Phenotypic characterization together with gene/protein expression profile of brain tissue by using multiple microglial markers demonstrated both M1 and M2 activation markers of microglia in response to CCL2. Overall, the results from this study present both a challenge and an opportunity for targeting peripheral monocyte/macrophage cells and evaluating their role in modifying the pathogenesis of AD and other inflammatory diseases.

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#### Human Umbilical Cord Blood (HUCB) Cells Protect Neurons Following Oxygen Glucose Deprivation Through Activation of the Akt Pathway

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The Akt pathway plays a key role in mediating neuronal cell survival. Here we test the hypothesis that the neuroprotective effect of HUCB following oxygen glucose deprivation (OGD) is mediated through activation of the Akt signaling pathway. Neurons were isolated from E17 Sprague-Dawley rats and cultured in Neurobasal media for 6 days. After 6 days, the cultures were then exposed to one of eight experimental conditions: normoxia, normoxia + HUCB cells, normoxia + Akt inhibitor (AktI), normoxia + HUCB + AktI, OGD, OGD + HUCB, OGD + AktI, OGD + HUCB + AktI. The HUCB cells were cocultured with a filter insert partitioning them from the neurons. AktI was added to the cultures just prior to OGD exposure. After 20 h, a fluorescein diacetate/propidium iodide (FDA/PI) assay was performed and the number of viable cells in each group was determined using Image Pro software. Significant differences were determined using ANOVA [ $F(7, 32) = 96.84$ ,  $p < 0.001$ ]. Survival in the normoxic culture was  $67 \pm 3\%$  compared to  $80 \pm 2\%$  in the normoxia neuron-HUCB coculture ( $p < 0.01$ ) and  $40 \pm 2\%$  after OGD ( $p < 0.001$ ). The Akt inhibitor significantly abolished the neuroprotective effect of HUCB both in normoxia ( $14 \pm 2\%$ ,  $p < 0.001$ ) and following OGD ( $23 \pm 2\%$ ,  $p < 0.001$ ). Immunoblot analysis using phosphoAkt antibodies will be employed to determine the activation of Akt protein

present in neurons under normoxic or OGD conditions. We have previously observed that expression of peroxiredoxin 5 (Prdx5), vascular cell adhesion molecule 1 (Vcam1), chemokine ligand 1 (Cxc11), paired box 6 (Pax6), doublecortin-like kinase 1 (Dclk1), phosphatase 3 regulatory subunit B (Ppp3r1), and stathmin-like 4 (Stmn4) genes were induced in the neurons by the presence of HUCB under normoxic or OGD conditions. We will further examine whether Akt signaling is critical for the induction of these genes by HUCB cells. These results demonstrate that the activation of the Akt protein is essential for the HUCB-dependent survival of neurons exposed to OGD.

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#### Chronic Exposure of Silica Dust (SiO<sub>2</sub> Nanoparticles) Exacerbate Stress Symptoms, Cognitive Dysfunction, and Brain Pathology Following Hyperthermic Brain Injury

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Exposure of silica dust (SiO<sub>2</sub> nanoparticles, 20–80 nm) to humans living in desert areas could influence their mental health function as SiO<sub>2</sub> nanoparticles may get access into the human body through inhalation. Thus, it appears that humans exposed to desert environment may have altered physiological conditions due to the presence of SiO<sub>2</sub> nanoparticles and thus react differently to heat stress or trauma biologically compared to nonexposed subjects. This idea was examined using a rat model of hyperthermia simulating these environmental conditions and exposure to SiO<sub>2</sub> (40–50 nm) nanoparticles. Rats were exposed to SiO<sub>2</sub> nanoparticles suspended in Tween 80 (10, 20, 30 mg/kg, IP) once daily for 10 days. On day 10 nanoparticles or vehicle (Tween 80) treated animals were subjected to 4-h heat stress in a biological oxygen demand incubator (BOD) maintained at 38°C (relative humidity 45–50% and wind velocity 20–25 cm/s). After heat exposure, the animals were examined for cognitive function using a Rota rod, walking on mesh grid, inclined plane angle test, and stride length. In addition, their blood-brain barrier (BBB) function was examined using Evans blue albumin and radioiodine tracers. Brain edema was measured using water content and cell injuries were examined using Nissl staining. Rats treated with nanoparticles (30 mg) when exposed to 4-h heat stress exhibited greater hyperthermia ( $42.21 \pm 0.14^\circ\text{C}$ ; normal animals  $40.67 \pm 0.21^\circ\text{C}$ ,  $p < 0.001$ ) and behavioral dysfunctions. Thus, these rats could not maintain themselves on a Rota rod for more than  $60 \pm 3$  s (normal animals  $80 \pm 6$  s,  $p < 0.001$ ). The inclined plane angle test was also lower for these nanoparticle-treated animals ( $38 \pm 6^\circ$ , normal  $45 \pm 6^\circ$ ,  $x\text{lmp}0.001$ ) and placement error was significantly increased ( $48 \pm 5\%$ , normal  $34 \pm 6\%$ ,  $p < 0.001$ ) in these rats compared to untreated heat-stressed rats. The nanoparticle-treated rats exhibited higher magnitude of BBB breakdown to Evans blue ( $2.85 \pm 0.12$  mg%; normal  $1.56 \pm 0.11$  mg%,  $x\text{lmp}0.001$ ) and radioiodine ( $3.57 \pm 0.21\%$ , normal  $2.34 \pm 0.18\%$ ,  $x\text{lmp}0.001$ ) tracers and brain edema formation (water  $82.38 \pm 0.23\%$ , normal  $80.44 \pm 0.21\%$ ,  $x\text{lmp}0.001$ ) following heat stress. The number of damaged nerve cells in nanoparticle-treated rats was also significantly higher (cortex  $183 \pm 4$ ; hippocampus  $105 \pm 8$ ; cerebellum  $234 \pm 12$ ; thalamus  $156 \pm 12$ ; hypothalamus  $87 \pm 12$ ; spinal cord  $86 \pm 8$ ) than in normal animals (cortex  $88 \pm 6$ ; hippocampus  $65 \pm 4$ ; cerebellum  $89 \pm 6$ ; thalamus  $77 \pm 6$ ; hypothalamus  $44 \pm 6$ ; spinal cord  $36 \pm 4$ ,  $p < 0.001$ ). Taken together these observations suggest that nanoparticle-treated animals are more susceptible to heat-induced sensorimotor functions, mental abnormalities, and brain disorders.

### The Role of Serotonergic Neurons in the Expression of Graft-Induced Dyskinesia

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Parkinson's disease is a neurodegenerative disorder characterized by dopaminergic cell loss in the substantia nigra. Transplantation of fetal ventral mesencephalic (VM) cells is a therapeutic approach aimed at restoring striatal dopamine (DA) levels in parkinsonian patients. This approach has been shown to be able to reduce L-dopa-induced dyskinesia (LID) both in animal models and patients. However, OFF drug dyskinesias, referred to as graft-induced dyskinesia (GID), have emerged as a serious complication in transplanted patients. An increasing body of evidence points to the serotonin (5-HT) system as an important player in the appearance of dyskinesias induced by L-3,4-dihydroxyphenylalanine (L-dopa) treatment. Although the mechanism of GID is unknown, in a recent study the partial 5-HT<sub>1A</sub> agonist buspirone markedly diminished GID in two patients (Politis et al., 2010). Moreover, positron emission tomography (PET) imaging of the two patients' brains showed excessive serotonergic innervation in the grafted striatum several years after transplantation. Therefore, we hypothesized that dysregulation of 5-HT release may be responsible for GID and aimed to find possible underlying mechanisms (amphetamine-like and false transmitter-like hypothesis as suggested in Politis et al., *Sci. Trans. Med.* 2:38–46, 2010). Amphetamine-induced dyskinesia has been proposed as a possible model for GID in 6-OHDA-lesioned rats (Carlsson et al., *Neurobiol. Dis.* 21:657, 2006). In this study, young adult rats have been lesioned with 6-OHDA in the medial forebrain bundle to achieve complete DA lesion. The degree of lesion was confirmed with amphetamine-induced rotation (more than 6 turns/min) and L-dopa (6 mg/kg) was injected daily to induce LID. Once stable LID was achieved, different embryonic VM cell suspensions were prepared and transplanted into the lesioned rat striatum to the following five groups: 1) DA narrow (DA cells mainly), 2) DA wide (a large portion of DA and some of 5-HT cells), 3) 5-HT mainly, 4) sham, and 5) lesion only plus drug naive after transplantation. Preliminary results appear to confirm a role for the 5-HT system in the expression of amphetamine-induced dyskinesia, and pharmacological manipulations are now ongoing to unveil the precise mechanism. A better insight into the mechanism of GID will help in understanding the interaction between host and grafted cells, and optimizing transplantation protocols to avoid appearance of this side effect in the future transplantation trials.

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### *Rhodiola rosea* Increases the Proliferation of Adult Bone Marrow Stem Cells and Improves Mitochondrial Function in SweAAP N2a Neuronal Cells

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As we age, adult stem cells are known to have a reduced restorative capacity and are more vulnerable to oxidative stress, resulting in a reduced ability of the body to heal itself. We have previously reported that a proprietary nutraceutical formulation, NT-020, promotes proliferation of human hematopoietic stem cells in vitro and protects stem cells from oxidative stress when given chronically to mice in vivo. Used for centuries, *Rhodiola rosea* L. is one of the most popular immune- and endurance-enhancing medicinal plants in European and Asiatic traditional medicine. We report here that an extract of *Rhodiola rosea* (Rrx) dose-dependently increased the proliferation of human adult bone marrow cells in culture (CyQUANT) and enhanced the stem cell proliferative action of NT-020 when the two were combined (MTT assay). In addition, Rrx and NT-020 both significantly increased ATP levels and mitochondrial membrane potential while significantly reducing reactive oxygen production in N2a neuronal cells transfected with the human SweAAP Alzheimer's gene. This pilot preclinical study suggests that NT-020 plus Rrx may act to promote healing by stimulating stem cell populations and by improving mitochondrial function.

P.C.B. and P.R.S. are founders of, and R.D.S. is a consultant of, Natura Therapeutics, Inc., a USF spin-out company.

### Developments in Graft-Induced Dyskinesia

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Clinical trials of fetal cell transplantation in the treatment of Parkinson's disease (PD) hit two major roadblocks. Functional efficacy in double blind trials appeared less than anticipated from open label trials and importantly a significant number of patients were found to develop abnormal involuntary movements (graft-induced dyskinesia; GID) in the "off" L-3,4-dihydroxyphenylalanine (L-dopa) state in the months or years following transplantation. Resolving these issues has been critical to the future of cell transplantation of fetal and stem cell-derived tissue sources. We have explored parameters that influence the development of GID, and the underlying pharmacology and pathophysiology in an animal model of the disorder. Previous studies have demonstrated that dyskinesia induced by L-dopa (LID) may be a risk factor for the development of GID and our studies have examined the changes in the pharmacology of LID posttransplantation and the regulation of amphetamine-induced dyskinesia in grafted animals (an animal model of GID). Female Sprague-Dawley rats lesioned with 6-OHDA in the median forebrain bundle were treated with L-dopa for 3 weeks until they demonstrated stable expression of LID. Rats then received intrastriatal transplants of a cell suspension of dissociated E12 or E14 ventral mesencephalon. A minimum of 12 weeks following transplantation, rats were reassessed for LID and amphetamine-induced dyskinesia. Manipulations of this outlined protocol have enabled us to evaluate the pharmacology of amphetamine-induced dyskinesia, the effect of graft storage, and the role of environmental enrichment on graft development and dyskinesia. Postmortem we have

also used immunohistochemistry, radioligand binding assays, and non-radioactive in situ hybridization to assess the striatal biochemistry of rats that do or do not express dyskinesia in response to amphetamine challenge posttransplantation. Our results demonstrate that amphetamine-induced dyskinesias are pharmacologically distinct from L-dopa-induced dyskinesia. Furthermore, we can find no biochemical correlate of amphetamine-induced dyskinesia; dopamine receptor and fosB expression is normalized by the transplant, and there is no apparent relationship between their expression and the development of abnormal movements in response to amphetamine. Environmental enrichment does not appear to influence the expression of GID, nor does storage of the graft tissue or the embryonic age of the tissue, but we have demonstrated that grafts transplanted into the lateral striatum increase the risk of amphetamine-induced dyskinesia. In summary we have a model that—despite requiring amphetamine—shows clear similarities with the spontaneous occurrence of clinical GID. Importantly, these studies support the transplantation protocol proposed for future clinical trials in fetal cell transplantation for PD.

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#### **Graft-Induced Dyskinesia in Transplanted Hemiparkinsonian Mice and Rats: A Pharmacological Manipulation**

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The neural transplantation of embryonic dopamine-rich cells into Parkinson's disease (PD) patients has led to symptomatic improvements. However, clinical trials have shown that a proportion of patients develop debilitating dyskinesias in response to the graft, irrespective of L-3,4-dihydroxyphenylalanine (L-dopa) treatment. These hyperkinesias can be mimicked experimentally in rats but it is unclear whether these can be modeled in the mouse due to practical difficulties, high mortality rates, and small graft sizes. Also the mechanistic similarity between graft-induced dyskinesia (GID) and L-dopa-induced dyskinesia (LID) is currently unknown. This may be elucidated by the pharmacological modulation of GID with substances known to affect LID in rodent models. Thirty-two rats and 27 mice were unilaterally lesioned with 6-hydroxydopamine (6-OHDA), targeted to the median forebrain bundle and primed with L-dopa (3 weeks). All animals received ventral mesencephalon tissue transplant (E14 rats and E13 mice) into the denervated striatum. Abnormal inhibitory movements were subsequently triggered by amphetamine (2.5 mg/kg) and scored based on a modified version of the Cenci and Lundblad rating scale. A subset of rats were coadministered with a pharmacological challenge of either: raclopride (0.5 and 2 mg/kg), SCH-22390 (0.05 and 0.2 mg/kg), nafadotride (0.6 and 1 mg/kg), yohimbine (10 mg/kg), naloxone (4 and 8 mg/kg), amantadine (20 and 40 mg/kg), WIN55,212-5 (1 and 2.5 mg/kg), MK-801 (0.03 and 0.3 mg/kg), MTEP (1.25 and 6.25 mg/kg), IEM-1460 (1 and 3 mg/kg), 8-OH-DPAT (1.5 and 3 mg/kg), or CP94253 (1.5 and 3 mg/kg). Our results show for the first time that GID can be modeled in the mouse to a moderate degree of success. Furthermore, GID is significantly increased in response to high doses of MK-801 and MTEP and decreased in response to SCH-22390, raclopride, and 8-OH-DPAT. This work shows that GID can be modeled efficiently in the mouse and this has much promise for the in vivo testing of other cell-based therapies in PD. Also, dyskinesia caused by L-dopa in lesioned models and methamphetamine in grafted models is differently modulated by pharmacological agents, initiative of an alternate mechanism of AIM development. Furthermore, the dopamine, glutamate, and serotonin systems are likely to have a fundamental role in the development of GID.

#### **“Disease-in-a-Dish”: The Ability of Stem Cells to Model Pathophysiology In Vitro and Facilitate Drug Discovery May Represent an Underrecognized Contribution to Neural Therapeutics**

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Stem cells are typically viewed as potential therapeutic agents based on their ability to be transplanted into injured, diseased, or dysfunctional organs and promote recovery of function. An underrecognized—and perhaps even more tractable—use of stem cells is their potential to emulate pathophysiological processes in culture, a concept termed “Disease-in-a-Dish.” In this paradigm, “normal” stem cells (either undifferentiated or differentiated) can be stressed or perturbed in prescribed ways (including via the knock-in of genes), or “diseased” stem cells (either undifferentiated or differentiated) can be obtained from patients with particular diseases under the assumption that the cells will continue to behave abnormally in vitro in a disease-specific manner. The latter option is particularly appealing for conditions in which authentic and/or predictive animal models do not exist. The stem cells can be used to (a) study underlying molecular mechanisms of disease; (b) to identify drug targets, diagnostic, or prognostics; (c) to identify protective agents and/or mechanisms; (d) to identify and test drugs or “leads”-to-drugs. Drug discovery is typically carried out through the unbiased high-throughput screening of small drug-like molecule libraries. When stem cells are used in such paradigms, it is the drugs—rather than the cells themselves—that are used to treat patients. The stem cells to be studied may be obtained from three sources: (a) from somatic stem cells isolated directly from the organ of interest (e.g., brain, spinal cord, tumor); (b) from the inner cell mass of embryos diagnosed as bearing a disease following preimplantation genetic diagnosis (PGD) (i.e., embryonic stem cells); (c) from end-differentiated somatic cells (e.g., skin fibroblasts) biopsied from patients with a particular disease and reprogrammed back to pluripotency (i.e., induced pluripotent stem cells). The latter strategy has received the most attention of late because of its relative ease and accessibility. This symposium will feature investigators at the forefront of Disease-in-a-Dish technology. Importantly, the following unresolved questions will be addressed. 1) Can a disease process be faithfully modeled in vitro, in isolation from the rest of the body, or does disease expression require the complete in vivo environment (an intact immune and vascular system as well as multiple lineages and cell types, etc.)? 2) Can a phenotype be observable in vitro that reliably distinguishes normal from “diseased” cells in a meaningful manner and that is attributable to the disease's pathophysiology? 3) Can the phenotype account for the disease manifestations in a patient and can it be a useful drug target against which therapeutic molecules can be screened? 4) Does the reprogramming or culturing process change the cells such that the disease is no longer represented? 5) Which cells model a disease best: pluripotent stem cells or somatic/tissue-resident/tissue-derived stem cells?

#### **Neuroprotective Effects of Bone Marrow Stromal Cells in Cocultures of Fetal Dopaminergic Neuronal Cultures**

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Bone marrow-derived stromal cells (BMSC) or mesenchymal stem cells (MSC), in vivo, have been reported to be effective in hastening

recovery from neurologic deficits in rodent models of stroke and several neurodegenerative diseases. Initially, it was hypothesized that BMSC could be transdifferentiated into neuron-like cells or fusion with injured neurons. Another mechanism for the beneficial effects of BMSC (or MSC) is attributed to their capacity to generate a number of soluble growth factors, cytokines, and brain natriuretic peptide (BNP). The primary goal of this in vitro cell culture study was to determine if direct contact of BMSC with fetal midbrain (MB) neuronal cultures is necessary to confer neuroprotection against the neurotoxicant 1-methyl-4-phenylpyridinium iodide (MPP<sup>+</sup>). Results presented here demonstrate that diffusible growth factors, and not direct cell contacts, were responsible for mitigating neurotoxicity. When compared to the effects of MPP<sup>+</sup> on MB monolayer cultures, the neurite length and functional activity indicated by [<sup>3</sup>H]DA uptake of dopaminergic (DA) neurons was significantly greater in bilayer cultures where there was no contact between the green fluorescent protein-expressing (GFP<sup>+</sup>) BMSC layer and the MB cell culture layer. Mixed cocultures (with MB and GFP<sup>+</sup> BMSC in direct contact) were also protected against MPP<sup>+</sup> to the same extent as in the bilayer cultures. There was no evidence of fusion of tyrosine hydroxylase expressing (TH<sup>+</sup>) neurons with GFP<sup>+</sup> BMSC despite careful search for doubly labeled DA cells in the mixed cultures. A small fraction (7–10%) of neurons coexpressed GFP<sup>+</sup> and neuron-specific nuclear protein (NeuN), suggesting possible fusion of GFP<sup>+</sup> BMSC with non-TH<sup>+</sup> neurons, or transdifferentiation of GFP<sup>+</sup> BMSC into non-TH<sup>+</sup> neurons, a finding that does not explain the neuroprotective effects observed in the bilayer cultures. In summary, the neuroprotection conferred by BMSC in monolayer and bilayer cultures can be attributed to elaboration of neurotrophic and other unknown factors that enhance neurite sprouting and improve DA neuron function.

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#### Subthalamic Nucleus Stimulation Increases Brain-Derived Neurotrophic Factor in the Nigrostriatal System and Primary Motor Cortex

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High-frequency deep brain stimulation (DBS) of the subthalamic nucleus (STN) has become a popular neurosurgical treatment strategy for Parkinson's disease. However, the mechanism by which DBS exerts its benefits is unclear. We have previously demonstrated in our rodent model that 2 weeks of continuous STN DBS halts dopamine (DA) neuron loss in the substantia nigra (SN). The present study examined whether functionally effective, long-term STN DBS may function, in part, by modulating neurotrophic factors important for dopamine neuron viability: glial cell line-derived neurotrophic factor (GDNF) and brain derived neurotrophic factor (BDNF). Examination of BDNF levels was of particular interest as high-frequency stimulation of glutamatergic synapses results in the release of BDNF in vitro. The STN has glutamatergic projections to the globus pallidus externa (GPe) and interna (GPi), the SN, the motor cortex, and the striatum. Rats that received 2 weeks of continuous unilateral STN DBS exhib-

ited significant improvements in parkinsonian motor behaviors in tests of forelimb akinesia and rearing activity. No changes in GDNF were observed with STN DBS. However, unilateral STN DBS increased BDNF protein two- to threefold bilaterally in the nigrostriatal system with the location (SN vs. striatum) dependent upon lesion status. Further, BDNF protein was bilaterally increased in primary motor cortex (M1) by as much as twofold regardless of lesion status. STN DBS did not impact cortical regions that receive less input from the STN. STN DBS also was associated with bilateral increases in BDNF mRNA in the SN and GPi. The increase observed in GPi was completely blocked by pretreatment with 5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801), suggesting that the activation of N-methyl-D-aspartate (NMDA) receptors was involved in this phenomenon. The upregulation of BDNF associated with long-term STN DBS may play a role in the therapeutic effects of this therapy as well as support the neuroprotective effect of stimulation documented in this rat model. Our finding that long-term STN DBS increases BDNF indicates that this therapy may exert pronounced and underappreciated effects on brain plasticity.

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#### Can the Parkinsonian Striatum Be "Remodeled" With Calcium Channel Antagonists or Is the Aged Brain Refractory to Target Repair: A Study on Dendritic Spine Recovery of Medium Spiny Neurons in the Parkinsonian Striatum of Young and Aged Rodents

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Medium spiny neurons (MSN) comprise approximately 90% of neurons in the intact striatum. Dendritic spines located on striatal MSN are the main site of synaptic connection of nigral dopamine (DA) and cortical glutamate neurons, and function to integrate locomotor signals coming into the basal ganglia. It is well documented in postmortem Parkinson's disease (PD) brains that there is a significant atrophy of dendrites and dendritic spines on striatal MSNs with advancing disease. Similar pathology is observed in rodents and nonhuman primates with severe DA depletion. It is reasonable to anticipate that an absence of these critical input sites in the PD striatum would make it difficult for: 1) standard pharmacotherapy to recapitulate normal physiological responses, and 2) "new" DA terminals introduced following grafting or growth factor therapies to reestablish normal connections. Dendritic spine loss secondary to severe DA depletion can be prevented by pharmacologically blocking aberrant activity of Cav1.3 calcium channels (Day et al., Nat. Neurosci., 2005). Our lab, and others, recently demonstrated that preventing spine loss with pharmacological inhibition of Cav1.3 channels in severely parkinsonian rats can enhance therapeutic efficacy of intrastriatal DA grafts (Soderstrom et al., EJN, 2010) and reduce dyskinesia severity (Soderstrom et al., EJN, 2010; Schuster et al., Biol. Psychiatry, 2009), suggesting that pharmacologic manipulation of this process may be a useful therapeutic tool in PD. For this potential therapy to offer clinical benefit, several factors remain to be

investigated. First, by the time PD is diagnosed, significant striatal DA depletion and associated spine pathology exists. Thus, for Ca<sup>2+</sup> channel blocker therapy to have an impact, particularly in patients with advanced disease, it will be necessary to reverse existing spine loss. Second, spine dynamics are classically associated with neuroplasticity, which decreases with advanced age, a factor associated with idiopathic PD. In the current study, we tested whether normalizing intraspine Ca<sup>2+</sup> with the Ca<sup>2+</sup> channel blocker, nimodipine, in severely parkinsonian rats after the initial spine loss has occurred would allow for recovery of spine density. A small pilot study indicated that there is the capacity to recover spine density when nimodipine is administered after spine loss in young parkinsonian rats. A larger study was undertaken to examine whether a difference in ability to recover spines exists between young (3 months) and aged (22 months) subjects. The ability of MSN to regain a normal complement of spines following cessation of aberrant calcium signaling and the ultrastructural integrity of remodeled synapses is being compared between age groups. Changes in Cav1.3 channel protein with age are also being examined. The ability to reestablish normalized target morphology may allow for improved symptomatic therapy for patients with PD.

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#### Serum Metabolite Biomarkers for the Diagnosis of Ischemic Stroke

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Stroke is one of the leading causes of death and disability in the US. Presently, a definitive, quick diagnosis of stroke in the clinical setting has yet to be developed to exclude conditions that can mimic a stroke. In addition, current methods of stroke diagnosis are often time consuming and costly, thus affecting the short time window in which treatment of stroke is most beneficial to the patient. There is therefore a great need for a quick, noninvasive diagnostic tool that will accurately diagnose stroke in its acute phase in order to facilitate timely treatment and improved outcome for the patients. We postulated that metabolites in serum could be used as biomarkers to aid in the diagnosis of ischemic stroke. To test this hypothesis we have analyzed the blood of Sprague-Dawley rats that have undergone ischemic stroke to identify changes in the metabolomic profile that are indicative of stroke. Serum was analyzed using <sup>1</sup>H nuclear magnetic resonance spectroscopy (<sup>1</sup>H-NMR) to obtain a high-throughput screening of the metabolites present in the serum of animals 24 h following stroke. Our preliminary analysis has shown a distinct change in the overall metabolomic profile in the serum of rats following stroke. Specifically, we have noted downregulation in the expression of 18 metabolites, including acetate, citrate, creatine phosphate, and glucose. Analysis of changes in the metabolome allows us to look at the functional changes that are occurring systemically following stroke, and may provide further insight into the pathophysiology of stroke. Studying the metabolome could provide potential biomarkers for the diagnosis of stroke in early stages. If translated to the clinic, this information could allow prompt and effective treatment of this devastating disease.

#### GDNF Is Required for Maintenance of Dopamine and Striatal Neurons in the Nigrostriatal System

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Glial cell line-derived neurotrophic factor (GDNF) is a potent factor for ventral mesencephalic (VM) dopamine neurons. GDNF im-

proves survival and nerve fiber growth in grafted dopamine neurons. In VM tissue culture, dopamine nerve fiber production is hampered in cultures derived from *Gdnf* knockout (K/O) compared to wild-type; however, little is known about the long-term maintenance of the nigrostriatal dopamine system in the absence of GDNF. Therefore, this study was undertaken to investigate the long-term survival of intraventricular cografts of VM and lateral ganglionic eminence (LGE) from *Gdnf* K/O. The results revealed that a similar number of tyrosine hydroxylase (TH)-positive neurons were counted in VM from all *Gdnf* genotypes at 3 months postgrafting. At longer time points (6 and 12 months), the number of TH-positive neurons was significantly reduced in *Gdnf* heterozygous (Hets) transplants, and the entire VM/LGE cografts had degenerated in *Gdnf* K/O-derived grafts. The TH-positive innervation of the striatal cograft was patchy and dense in wild-type transplants, while it was less dense and widespread in *Gdnf* K/O grafts. The dense TH-positive zones in wild-type-derived striatal cografts overlapped with areas demonstrating dense clusters of DARPP-32 (dopamine cAMP-regulated phosphoprotein of 32,000 kDa)-positive neurons, while no dense DARPP-32-positive clusters were found in *Gdnf* K/O transplants. To further investigate whether the poor development of the striatal tissue affected the survival of VM, single VM grafts from the *Gdnf* genotypes were investigated, and magnetic resonance imaging (MRI) scans were performed to follow the volume of the transplants. At 2 months, the volume of the *Gdnf* K/O transplants was reduced while the wild-type grafts had grown. At 6–7 months, degenerating areas were found in the MRI scans of grafts from *Gdnf* Hets, which also was confirmed by histological evaluations. Thus, the nigrostriatal system develops in the absence of GDNF, although the striatal organization appears malformed, and both the striatum and the VM dopamine neurons need GDNF for maintenance during adulthood.

#### Human Retinal Pigment Epithelial Cell (hRPEC) Cografts Improve Survival of Xenografted Dopaminergic Human Embryonic Stem Cells (hES), Diminish Host Inflammation, and Immune Response

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Human embryonic stem cell (hES) striatal grafts without immunosuppression in adult animals is accompanied by a cell-mediated chronic immune response and graft rejection. hES transplants into the striatum demonstrate massive cell death even with cyclosporine (CSA) immunosuppression. In contrast, human retinal pigment epithelial cell (hRPEC) striatal transplants survive without any need for immunosuppression and these cells appear to secrete immunosuppressive substances that promote graft survival. We tested the hypothesis whether hRPEC cografted with hES is capable of avoiding a deleterious host immune response and whether hRPEC/hES cografts can provide trophic support to the hES graft using two cohorts. In cohort 1 histological studies were performed after 18-day survival postbilateral striatal transplants in three groups of animals: group 1, hRPEC cografted with hES ( $n = 4$ ); group 2, hES transplants with daily cyclosporine (CSA) treatment ( $n = 2$ ); and group 3, hES transplants without any immunosuppression ( $n = 4$ ). In addition, we directly compared the effects of systemic immunosuppression to cografts of hRPEC/hES in the 6-hydroxydopamine (6-OHDA)-lesioned hemiparkinsonian rat and the differential effects in any parkinsonian behavior over a period of 3 months. This second cohort consisted of group 4, hRPEC cografted with hES ( $n = 6$ ), and group 5, hES with daily CSA treatment ( $n = 4$ ). Each hemisphere received a total of 480,000 hES cells alone or in conjunction with 60,000 hRPEC cells divided equally into three targets. All animals had excellent placement of striatal grafts and cellular TH expression. Estimation of TH-positive cell bodies using systematic random sampling and the optical fractionator method yielded a mean of 78,738 cells/hemisphere ( $CE = 0.13$ ) for the hES-alone cohort. In

the cografed cohort the mean estimate of TH-positive hES cells was 209,592 cells/hemisphere (CE = 0.08). hRPEC/hES cografes were easily distinguished from hES grafted cells via the enhanced green fluorescent protein (EGFP) expression. All animals that had hES-alone grafted showed evidence of host immune reaction and graft rejection, whereas animals that received cografes of hRPEC/hES showed good survival of grafted with minimal inflammation or activation of MHC class II antigens and no evidence of active graft rejection. Unbiased counts using the optical fractionator demonstrated more than double the number of inflammatory class II-expressing microglial cells in and around the hES xenografes when compared to identical xenografes that contained cografes of hRPEC/hES. Comprehensive behavioral testing using a rodent behavioral battery of tests showed that both group 4 and group 5 animals had comparable statistically significant behavioral improvements compared to baseline pretransplant behavioral assessments. Our findings indicate a very powerful anti-inflammatory and survival-promoting effect of hRPEC/hES cografes on hES dopaminergic cells. These studies suggest that hRPEC/hES cografes provide powerful immunomodulatory and growth-promoting effects that could have significant impact on the field of CNS transplantation, neuroimmunology, and cell-mediated immune therapies.

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#### **DJ-1 Ameliorates Neuronal Cell Death in Ischemic Stroke Via Mitochondrial Pathway**

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DJ-1 is an important redox-reactive neuroprotective protein implicated in regulation of oxidative stress after ischemia. However, the molecular mechanism, especially the mitochondrial function, by which DJ-1 protects neuronal cells in stroke remains to be elucidated. The aim of this study was to reveal whether DJ-1 translocates into the mitochondria in exerting neuroprotection against oxidative stress. In particular, we examined DJ-1 secretion from primary rat neurons exposed to experimental stroke. Stroke is characterized by neural tissue death due to deprivation of oxygen, glucose, and other nutrients that results from a reduction in blood flow to the brain. The hallmark pathological milieu of stroke primarily involves an infarcted core, and subsequently the formation of an ischemic penumbra, which, over a subacute period remains, as salvageable neural tissue, thereby amenable to therapeutic intervention. Secondary cell death processes, including oxidative stress, can endanger the penumbra, limiting neurorestoration. Exploring the DJ-1 mitochondrial functions against oxidative stress may pose as a novel stroke therapy. Primary rat astrocyte/neuron cell cultures were exposed to oxygen glucose deprivation (OGD), an established *in vitro* stroke model. After this acute injury period, DJ-1 translocation was measured by immunocytochemistry and its secretion from the primary neuronal cells detected by ELISA. In order to further establish the neuroprotective mechanism of action of DJ-1, subsequent studies involved DJ-1 antibody blocking experiments, which were designed to show that sequestration of DJ-1 would abolish the therapeutic benefits of DJ-1 upregulation following OGD. In addition, to further define the mitochondrial function of DJ-1, the mitochondrial-acting cyclosporine-A (CsA) was dose-dependently manipulated under OGD to begin to understand the interaction of DJ-1 and its mitochondrial translocation in affording neuroprotection. Consistent with our previous findings in human neuronal progenitor cells, DJ-1 was secreted by rat primary neuronal cells and translocated into the mitochondria after the experimental stroke insult. Employing the anti-DJ-1 antibody paradigm, we confirmed that capturing extracellular DJ-1 secreted by rat primary neuronal cells intrinsically increased the vulnerability of the

cells to OGD-mediated oxidative stress. Furthermore, CsA dose-dependently enhanced DJ-1 translocation in the mitochondria, which positively correlated with reduced oxidative stress after OGD. Altogether, these results suggest that DJ-1 participates in neuroprotection against stroke. This study further supports the notion that DJ-1 is intimately associated with early phases of disease progression in stroke. That DJ-1 is detected immediately after stroke, efficiently translocated into the mitochondria, and its expression enhanced by the mitochondrial-acting CsA, but effectively blocked by DJ-1 antibody, offer a new venue for developing neuroprotective and/or neurorestorative strategies against ischemic stroke.

#### **Moving Cell Therapy From Basic Research Into the Clinic: SB623 Cells for Stroke Disability**

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Transplantation of SB623 cells, which are genetically modified human bone marrow stromal cells, promotes recovery following experimental stroke and neurodegenerative disorders (e.g., Parkinson's disease, retinal degeneration). SB623 cells aid damaged tissue through secretion of beneficial soluble and insoluble factors that support host neural cell survival and regeneration and have immunomodulatory functions. SB623 cells are currently in phase I/IIa clinical trials to treat stroke disability in the US. We will describe the path taken to move this cell therapy into the clinic with respect to demonstrating efficacy and safety in animal models, meeting production requirements, and designing the clinical study.

#### **Effects of Carbamylated Erythropoietin Fusion Protein on Parkinson's Disease Model of Rat**

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Erythropoietin (EPO) has neuroprotective effects on Parkinson's disease (PD) model of rats, but there are thromboembolic complications. To avoid them, we evaluated the neuroprotective and hematopoietic effects of CEPO-Fp (carbamylated erythropoietin fusion protein) and EPO-Fp on this model. SD rats were divided into three groups: PBS, EPO-Fp, and CEPO-Fp. Each group of rats received the respective drug via intraperitoneal (IP) injection for 3 days. On the second day, 6-hydroxydopamine (6-OHDA) was injected into the right striatum. Behavioral tests were performed at 1, 2, 3, and 4 weeks after lesion. Blood sampling was collected before the lesion and at 3 days, 1 and 4 weeks after for hematocrit and hemoglobin measurements. CEPO-Fp showed effectiveness in terms of improvement on behavioral tests. The scores for forelimb asymmetry ameliorated on both CEPO-Fp and EPO-Fp groups while PBS group showed no significant improvement. Amphetamine-induced rotation for the EPO-Fp and CEPO-Fp groups was significantly lower than the PBS group. Correspondingly, significant preservation of tyrosine hydroxylase (TH) fibers in striatum and TH-positive neurons in the substantia nigra pars compacta were demonstrated in both the CEPO-Fp and EPO-Fp groups, compared to the PBS group. There were no hematological adverse effects in the CEPO-Fp group, because hemoglobin and hematocrit levels remained stable. On the other hand, those values in the EPO-Fp group increased, showing the possibility of adverse effects. These results suggest that CEPO-Fp is a hopeful drug for PD.

### Molecular Context of Human Mesenchymal Stromal Stem Cells in Injured Spinal Cord

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Mesenchymal stromal stem cells (MSCs) isolated from adult tissues such as bone marrow or lipoaspirate offer tangible potential for regenerative medicine due to their availability, and capacity for autologous transplantation. MSCs have been studied in the settings of stroke, amyotrophic lateral sclerosis, and spinal cord injury (SCI), but the molecular and cellular bases for their putative therapeutic effects remain largely unknown. Our previous observation that neural stem cells can repair neurological injury without cell replacement led us to hypothesize that MSCs, like other types of stem cells, may exert neurotherapeutic effects through multimodal molecular interactions with the nervous system. We tested this hypothesis by using an established in vitro organotypic system where human MSCs (hMSCs) were physically separated from cocultured adult rat dorsal root ganglia (DRG) explants. hMSC exposure resulted in axonogenic (i.e., 45% increase in average growth cone extension,  $p = 0.03$ ; 22% increase in average neurite length,  $p = 0.03$ ) effects. We additionally induced neuroinflammation to mimic post-SCI pathology by application of bacterial lipopolysaccharide (10 ng/ml); hMSCs treatment decreased DRG mRNA expression of the proinflammatory cytokines tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) by 62% and 65%, respectively; DRG expression of IL-6 was lessened by 72%. We subsequently used real-time PCR for hMSCs and DRGs in conjunction with mass spectrometry of culture supernatants for multilevel screening of potential molecular mediators such as brain-derived neurotrophic factor (BDNF), IL-10, etc. Based on these findings, we next designed in vivo studies in which hMSCs seeded in PLGA scaffolds were transplanted into the rat spinal cord with T9–T10 hemisection. Pilot outcomes ( $n = 4$ ) showed that the treated rats did not develop at-level or below-level allodynia. We are now validating these findings with a systematically controlled formal in vivo study, and anticipate that our findings would enrich the field's understanding on molecular events underlying the neuroimpact of MSCs, and help build a molecular context-based approach to investigate potential roles of hMSCs for treating neurotrauma.

### Optogenetic Modulation of Locus Coeruleus Noradrenergic Function In Vivo

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The nucleus locus coeruleus (LC) projects widely throughout the CNS and is the primary source of norepinephrine (NE) in the brain. Early and progressive degeneration of LC NE neurons is a prevalent feature of both Alzheimer's and Parkinson's diseases. However, the role of LC NE degeneration in cognitive impairment and neuropathology observed in these disorders is not well studied. Specific electrical and pharmacological stimulation studies of the LC have been challeng-

ing and many LC lesion studies have proved inconclusive. The development of pharmacogenetic and optogenetic technology now allows unprecedented specificity and precision for direct modulation of LC NE neurons to investigate their functions. Using electrophysiologically guided viral injections of the light sensitive cation channel channelrhodopsin2 (ChR2) we targeted expression to NE LC neurons using the synthetic dopamine beta hydroxylase (DBH) promoter PRSx8. We saw robust colocalization of the mCherry marker with tyrosine hydroxylase within LC neurons. Using in vivo photostimulation and single unit recording under isoflurane anesthesia, we entrained ChR2-expressing LC NE neurons to light pulses ranging from 1 to 15 Hz. In vivo photostimulation of LC under isoflurane produced electroencephalography (EEG) arousal in both cerebral cortex and hippocampus. In freely moving animals, photostimulation produced EEG arousal and acute sleep-to-wake transitions within seconds. These results show that optogenetic stimulation of the LC has widespread impact on forebrain function. These results indicate that cell type-specific optogenetics is a valuable tool for investigating functions of the LC system, and for developing novel treatments to offset behavioral deficits in neurodegenerative disorders.

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### Macrophage/Microglial Phenotype Modulation After Traumatic Brain Injury: Effects of MultiStem®

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Over the past 10 years a growing body of literature has developed supporting the use of various progenitor cell types to treat acute neurological injuries. Neural stem cells (adult and embryonic), mesenchymal stromal cells (MSC), multipotent adult progenitor cells (MAPC), human umbilical cord blood cells (hUCB), and bone marrow mononuclear cells have all shown efficacy in preclinical models of neurological injury and disease through numerous proposed mechanisms. However, few groups believe that true neural replacement and integration are the major mechanisms involved in cell-mediated efficacy observed. It is becoming more evident that the major therapeutic benefit of the infused cell populations is through modifying the regional response to injury (inflammatory/repairative vs. replacement). MultiStem® is a MAPC product manufactured under strict specifications and release criteria approved by the Food and Drug Administration for use in humans in the treatment of heart attack, ischemic stroke, inflammatory bowel disease, and prophylaxis of graft-versus-host disease. In this study, we have sought to determine if intravenous infusion of MultiStem provides benefit in two rodent models of traumatic brain injury (TBI) by specifically addressing how cell administration modulates the inflammatory component of secondary brain injury. Two rodent models of controlled cortical impact TBI were used: Sprague Dawley (SD) rats were used in the first study (Results 1) and C57B6 mice in the second studies (Results 2). MultiStem was delivered intravenously at 2 and 24 h after injury, and animals were euthanized at 72 h postinjury (or as delineated). In the first study, the effects of splenocyte–MultiStem interactions on blood–brain barrier (BBB) permeability (Evans blue dye extravasation), splenic mass, and splenocyte cytokine output were studied. In the second study, the effect of MultiStem administration on T-regulatory cell appearance in the spleen and blood was examined, followed by characterization of the status of microglial/macrophage phenotypes in the injured brain. Our results suggest that MultiStem infusion alters the innate immune response directly via interactions with splenocytes to increase anti-inflammatory cytokine output and therefore change the splenocyte phenotype and increase T-regulatory cell output from the spleen. This, in turn, pro-

motes a Th2 type response with an increase of “alternatively” activated (M2) macrophage/microglial phenotypes, and increased neuroprotection following the injury in the brain.

### T-Lymphocytes Influence Behavioral Deficits in the 6-OHDA Unilateral Lesion Model for Parkinson’s Disease

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Parkinson’s disease (PD) is a neurodegenerative disorder characterized by a loss of dopaminergic neurons in the substantia nigra (SN), leading to motor impairments. Recently, T lymphocytes have been implicated to protect dopaminergic neurons in the SN from induced cell death. Furthermore, changes in the blood–brain barrier (BBB) following brain injury or inflammation have been suggested to promote extravasation of immune cells into the central nervous system. Nonetheless, the role of T cells in neurodegeneration and alterations of the BBB have not been fully elucidated. Here we have examined the role of T lymphocytes on motor behavior in the 6-hydroxydopamine (6-OHDA) unilateral striatal lesion rat model for PD. Athymic, Rowett nude (RNU<sup>-/-</sup>) rats, which are deficient in T lymphocytes, and RNU<sup>+/-</sup> rats, which are phenotypically normal, were given 6-OHDA injections into the right striatum, resulting in hemiparkinsonian lesions. Lesion progression in the substantia nigra was analyzed by immunohistochemistry and motor skills were assessed by the cylinder and *d*-amphetamine sulfate-induced rotational behavioral tests. Here, the cylinder test showed that unilateral lesioned RNU<sup>-/-</sup> and RNU<sup>+/-</sup> rats favored the use of the limb ipsilateral to the lesion. However, amphetamine-induced rotational test revealed greater rotational asymmetry in RNU<sup>-/-</sup> rats compared to RNU<sup>+/-</sup> rats at 2 and 6 weeks postlesion. Immunohistochemistry (IHC) was performed on RNU<sup>-/-</sup> and RNU<sup>+/-</sup> rat brain sections to determine the loss of substantia nigral tyrosine hydroxylase (TH)-positive cells and BBB changes. IHC at 2 weeks postlesion showed a decrease in glial fibrillary acidic protein (GFAP)-immunopositive cells and a reduced interaction of astrocytic end-feet (AQP4) contacting blood vessels (laminin) in the lesioned striatum compared to the contralateral striatum. These results suggest that T lymphocytes may protect dopaminergic neurons from 6-OHDA neurodegeneration as observed through motor behavior test and that BBB alterations play a role in 6-OHDA-induced neurodegeneration.

### Sigma Ligands as Potential Treatments for Cerebral Ischemia

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The sigma agonist 1,3-di-*o*-tolylguanidine (DTG) decreases infarct size when administered 24 h after a stroke in a rat model of permanent middle cerebral artery occlusion (MCAO). The DTG appears to induce this “neuroprotective” effect by modulating the peripheral immune system to decrease inflammation. DTG binds to both the sigma-1 re-

ceptor, implicated in neuroprotection after ischemia, and the sigma-2 receptor, implicated in both neuroprotection and immune modulation. The goal of this project is to identify new high-affinity sigma-1/sigma-2 agonists developed from the DTG molecule but that have better efficacy than DTG in improving functional recovery. Male Sprague-Dawley rats underwent permanent MCAO and were then injected SC with DTG analogue [bromo-DTG (8.2 mg/kg/day) and meta-chloro-DTG (8.5 mg/kg/day)] daily for 3 days beginning 24 h post-MCAO. Control animals were injected with vehicle. Motor function was measured prior to MCAO and at 1 month post-MCAO with a battery of behavioral tests including spontaneous activity, step, elevated body swing, corner, grip, and cylinder tests. Learning and memory were assessed with a passive avoidance task. There were no significant differences between bromo-DTG and vehicle-treated animals in survival or body weight. Infarct volume was similar between the groups. There were no improvements in motor or cognitive function on any of the tests employed even though short-term *in vivo* studies had indicated that Bromo-DTG was neuroprotective, significantly decreasing infarct volume. Initial indications are that the results are similar for meta-chloro-DTG. These results suggest that the short-term neuroprotective effects of DTG-based sigma receptor analogues may not be maintained long term, but only delay development of the infarct and functional deficits and highlight the necessity of performing long-term functional studies early in the development of new potential treatments for stroke.

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### Adeno-Associated Virus-Mediated Vascular Endothelial Growth Factor Gene Expression Attenuates Ischemic Brain Injury After Focal Cerebral Ischemia in Rat

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Vascular endothelial growth factor (VEGF) has been shown to display neuroprotective effects in hypoxic-ischemic injury. Here, we investigated the neuroprotective capacity of VEGF in a rat model of stroke. To increase the *in vivo* transduction efficiency in the brain, we compared seven serotypes (2, 5, 7, 8, 9, rh8, and rh43) of double-stranded adeno-associated virus (dsAAV) and decided to use serotype 2 to deliver VEGF (dsAAV2-VEGF) due to its infection efficiency. Next, we explored whether dsAAV2-VEGF protected primary cultures of cortical neurons from hypoxia. After infection of dsAAV2-VEGF in primary cortical neurons for 3 days, the number of surviving cells was significantly increased and the activation of caspase 3 was reduced. We further examined the signaling pathway of VEGF’s neuroprotective activity, the expressions of Akt, extracellular signal-regulated kinases (ERK)-1/-2, and p38 mitogen-activated protein kinase (p38) were analyzed. Cortical neurons infected with dsAAV2-VEGF showed increased phosphorylated Akt in hypoxia while the expressions of phosphorylated ERK-1/-2 and p38 were without differences. *In vivo* data show that a single administration of dsAAV2 vector carrying VEGF 7 days before the onset of ischemia increases phosphorylated Akt and effectively decrease the infarct volume, and the number of TUNEL-positive, cleaved caspase3-positive and fluoro-Jade B-positive neurons. These results revealed that exogenous expression of VEGF by dsAAV2 vector could attenuate ischemic brain injury through activation of Akt.

### Human Albumin Protects Against 6-Hydroxydopamine-Induced Dopaminergic Cell Death Via MAPK Pathway Followed by Anti-Ros Formation and Antiapoptosis

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Human albumin has recently been demonstrated to protect brain neurons from injury in rat ischemic brain. However, there is no information available about whether human albumin can protect dopaminergic neurons from the neurotoxicity of 6-hydroxydopamine (6-OHDA), which is most commonly used to create a rat model of Parkinson's disease (PD). In the present study, we tested the hypothesis that human albumin would protect dopaminergic neurons and improve neurobehavioral outcome in a rat model of PD, and examined the mechanisms underlying neuroprotection of human albumin. Two microliters of 1.25% human albumin was stereotaxically injected into the right striatum of rats 1 day before or 10 days after the 6-OHDA lesion in the same side. D-Amphetamine-induced rotational asymmetry was measured 7 days, 3 and 10 weeks after 6-OHDA lesioning. After the last behavioral test, rats were sacrificed, and the brains were prepared for immunocytochemistry. We observed that intrastriatal administration of human albumin significantly reduced the degree of rotational asymmetry. Tyrosine hydroxylase (TH)-immunoreactive neurons of the substantia nigra were protected from 6-OHDA-induced degeneration in the human albumin-treated rats. TH immunoreactivity in the 6-OHDA-lesioned striatum was also significantly increased in the human albumin-treated rats. To examine the mechanisms underlying neuroprotection of human albumin, we challenged PC12 cells with 6-OHDA as an in vitro model of PD. Incubation with human albumin prevented 6-OHDA-induced reduction of cell viability in PC12 cell cultures, as measured by MTT assay. Furthermore, human albumin reduced 6-OHDA-induced formation of reactive oxygen species (ROS) and apoptosis in cultured PC12 cells, as assessed by flow cytometry. Western blot analysis showed a dose-dependent activation of c-jun N-terminal kinases (JNK), c-Jun, extracellular signal-regulated kinases (ERK), and p38 mitogen-activated protein kinases (MAPK) signaling in PC12 cultures challenged with 6-OHDA. Human albumin inhibited 6-OHDA-induced activation of MAPK signaling in PC12 cell cultures. Our results suggest that intrastriatal injections of human albumin can protect nigral dopaminergic neurons from extensive cell death and improve neurobehavioral outcome in a rat model of PD. Human albumin may protect against 6-OHDA-induced dopaminergic cell death via MAPK pathway followed by anti-ROS formation and antiapoptosis.

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### Embryonic Dopamine Neurons Genetically Modified to Enhance Akt/PKB Activation Provide Functional Improvements When Transplanted Into the Striata of the MitoPark Mouse Model

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Transplants of fetal dopamine (DA) neurons have been used to restore DA neurotransmission in animal models of Parkinson's disease (PD), as well as in patients with advanced PD. However, poor survival rates of grafted DA neurons remain a primary factor that limits cell replacement from becoming a viable treatment for PD. Numerous factors contribute to the survival rate of DA neurons during the posttransplantation interval, including growth factor withdrawal, hypoxia, and oxidative stress. Akt/PKB is a serine/threonine protein kinase that promotes cell growth, and is inhibited by the lipid phosphatase and tensin homolog (PTEN). A series of recent studies has demonstrated that the intrinsic inhibition of Akt/PKB and its downstream targets significantly limit the capability of neurons to regenerate after injury and disease. In accordance with these results, we have demonstrated that PTEN ablation in DA neurons lead to Akt/PKB activation and this is followed by a remarkable reversal of apoptosis in DA neurons during developmental pruning and injury, resulting in significantly larger number of DA neurons surviving from these events. We are therefore particularly interested in determining whether it is possible to prevent apoptotic pathway activation and increase the regenerative capabilities of embryonic DA neurons intended for transplantation, by enhancing the activity of this signaling growth pathway. In this study we have transplanted ventral mesencephalic tissue from conditional DA-PTEN KO transgenic mice bilaterally into the striata of mice with a selective deletion of mitochondrial transcription factor A (TFAM) in DA neurons (MitoPark mice). The locomotor activity of MitoPark mice declines over time because of respiratory chain dysfunction, development of intraneuronal inclusions, and eventual DA cell death. In the DA-PTEN KO transplants, Cre recombinase expression through the dopamine transporter (DAT) promoter induces *Pten* deletion prior to transplantation. Control grafts express Cre recombinase in DAT-positive cells but do not contain the floxed *Pten* gene. Preliminary results suggest that PTENless DA neurons, when transplanted into the striata of 20-week-old MitoPark mice, are able to reduce behavioral deficits. In addition, morphological analyses performed 2 months after grafting demonstrate the survival of numerous tyrosine hydroxylase-positive neurons in the DA-deficient striatum, accompanied by the presence of thick neurites extending away from the grafted area. These results indicate that manipulation of the Akt/PTEN pathway may provide an avenue for the enhancement of cellular replacement therapies intended for the treatment of neurodegenerative disorders.

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### Hematopoietic Growth Factors Induce Neurite Outgrowth Through the Regulation of PI3K Signaling and Neurotrophic Factors

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Recent studies have drawn attention to the role of hematopoietic growth factors in the central nervous system. It has been shown that

stem cell factor (SCF) and granulocyte colony-stimulating factor (G-CSF), two hematopoietic growth factors, have neuroprotective effects in acute stroke. We have recently demonstrated that SCF in combination with G-CSF (SCF + G-CSF) improves sensorimotor functional recovery in chronic stroke, suggesting an effect of SCF + G-CSF treatment in neuronal plasticity rather than their neuroprotective effects. It remains unclear, however, whether SCF and G-CSF have direct effects in regulation of neural network remodeling and how SCF and G-CSF regulate it. In this study, we have determined the contribution of SCF and G-CSF on neurite extension in primary cortical neuron culture system by using the approaches of neurite outgrowth quantification assay, cellular signal detection, RNA interference, quantitative RT-PCR, and live neuron imaging. We observed that SCF + G-CSF has a synergistic effect in promoting neurite outgrowth. In addition, the PI3k/Akt/NF- $\kappa$ B signaling pathway is required for SCF + G-CSF-induced neurite outgrowth. Further, SCF + G-CSF dramatically increases the gene expressions of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) through the regulation of NF- $\kappa$ B. Moreover, SCF + G-CSF-induced neurite outgrowth is prevented when knocking down BDNF and NGF gene expression. These data suggest that SCF and G-CSF can directly promote neurite extending through the regulation of PI3k/Akt/NF- $\kappa$ B/BDNF and NGF. This study provides evidence in support of the contribution of hematopoietic growth factors in neuronal plasticity and also gives insights into developing new therapeutic strategies for neurological disorders and neurodegenerative diseases.

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#### Cross Talk Between micro-RNA and Epigenetic Pathways in Regulating Neural Stem Cells

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Methylated-CpG (MeCP) binding proteins are central players of epigenetic regulation. Mutation of MeCP2 results in impaired neuronal maturation and Rett Syndrome, and mutation in methyl-CpG binding domain protein 1 (Mbd1) results in reduced neurogenesis and autism-like behaviors. We have employed a combination of genetic, molecular, cellular, and behavioral biology methods to investigate how MBDs regulate brain development and function. We have identified both protein coding and noncoding small RNAs that are epigenetically regulated by MBDs. In the absence of either Mbd1 or MeCP2, there is altered expression of these coding and noncoding RNAs in both neural progenitor cells and brain tissues. Several of these RNAs could regulate neural stem cell fate specification and neuronal maturation both in vitro and in vivo. These results demonstrate that cross talk between noncoding RNAs and epigenetic regulation are important modulators of neural stem cells and neurogenesis.

#### DNA Methylation Diversification Signals Neural Stem Cell Differentiation

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Transition of quiescent neural stem cells (NSC) into neural differentiation is preceded by a major shift in gene expression. The mechanism

implementing such a large and coordinated shift is unknown. Emerging information indicates that epigenetic modification plays a major role in regulating gene expression. We previously reported that epigenetic marks were dynamic within NSC through differentiation. Here we demonstrated a genome-wide DNA methylation program of NSC from quiescence to differentiation. First, we found, during quiescence, a genome-wide DNA methylation landscape featuring an evolutionarily conserved "methylation dip," in which DNA methylation level reduced when approaching the coding region, and sharply elevated ~150 bp 5' to the coding region. As NSCs differentiate, the methylation landscape is altered by reducing the methylation dip, particularly of those genes with high CpG density. Second, a diversification of DNA methylation occurred—a large number of moderately methylated genes became hyper- and hypomethylated, favoring silencing and activation of gene populations. Microarray analysis indicated that many genes showed collectively altered expression in six major canonical pathways in neural development: neural specification and patterning, cell cycle–apoptosis, Wnt1, bone morphogenetic protein (BMP), sonic hedgehog, and retinoic acid pathways. The programmed DNA methylation changes were closely associated with the gene expression in the pathways; for example, *Bmp4*, *Notch (2&4)*, *FGF (11&12)*, and *Wnt7b* are key to NSC renewal, increased methylation, and decrease expression, whereas *Msh1* and *FGF2* are key to neural specification, decreased methylation, and increased expression. In addition, DNA methylation binding and modification genes, *MBD1*, *MBD3*, *DNMT3*, and histone modification gene *HDAC1* also had altered methylation. The DNA methylation program is closely associated with gene expression in the key gene pathways that are essential for differentiation.

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#### Functional Restoration After Transplantation of Human Fetal Ventral Mesencephalon in a Rat Model of Parkinson's Disease

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Improvement of graft survival and surgery techniques and identification of the optimal target area are imperative for the further success of neural transplantation in Parkinson's disease (PD). In the present study, human primary fetal ventral mesencephalon (VM)-derived tissue from 7–9-week-old human fetuses was transplanted into 6-hydroxydopamine-lesioned adult Sprague-Dawley rats. Graft survival, fiber outgrowth, and drug-induced rotational behavior were compared between different intrastriatal transplantation techniques (single cell suspension vs. tissue pieces suspension infused by glass capillary or metal cannula) and the intranigral transplantation of a single cell suspension by means of a glass capillary. The results demonstrate a higher survival rate of dopamine neurons, a higher reduction in amphetamine-induced rotations, and more extensive fiber outgrowth for the intrastriatally transplanted tissue pieces suspension compared to intrastriatal single cell suspension grafts. Apomorphine-induced rotational bias was significantly reduced in the intranigraly grafted rats, which also displayed solid graft survival. However, striatal target areas remained denervated in this group. These data indicate that the target area as well as modifications of the transplantation protocol substantially influence the survival and functional recovery of human VM-derived grafts (different from the results from rat VM grafts) and should be taken into consideration also for the further optimization of the clinical transplantation approach in PD.