Title
Disrupting Disease Phenotype in Dementia with Lewy Bodies with Neural Stem Cell Transplantation

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Disrupting Disease Phenotype in Dementia with Lewy Bodies with Neural Stem Cell Transplantation

DISSEPTION

submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Natalie Sashkin Goldberg

Dissertation Committee:
Assistant Professor Mathew Blurton-Jones, Chair
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2016
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<tr>
<td>αMT</td>
<td>α-methyl-p-tyrosine</td>
</tr>
<tr>
<td>α-syn</td>
<td>α-synuclein</td>
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<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
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<tr>
<td>AAV2</td>
<td>Adeno-associated virus serotype 2</td>
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<td>ASO</td>
<td>α-synuclein over-expressing</td>
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<td>ASOC</td>
<td>ASO NSC-transplanted</td>
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<td>ASOV</td>
<td>ASO vehicle-treated</td>
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<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
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<tr>
<td>CD3/4/8/19/49b</td>
<td>Clusters of differentiation 3, 4, 8, 19 and 49b</td>
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<tr>
<td>ChAT</td>
<td>Choline acetyltransferase</td>
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<tr>
<td>CNS10</td>
<td>Central nervous system cell line 10</td>
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<tr>
<td>CREB</td>
<td>cAMP response element B</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DCX</td>
<td>Doublecortin</td>
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<tr>
<td>DHK</td>
<td>Dihydrokianic acid</td>
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<tr>
<td>DLB</td>
<td>Dementia with Lewy bodies</td>
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<td>EAAC1</td>
<td>Excitatory amino acid transporter 1</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellular signal-regulated kinases 1 &amp; 2</td>
</tr>
<tr>
<td>FVM</td>
<td>Fetal ventral mesencephalic</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GCI</td>
<td>Glial cytoplasmic inclusion</td>
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<tr>
<td>GDNF</td>
<td>Glial-derived neurotrophic factor</td>
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<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GLAST</td>
<td>Glutamate aspartate transporter</td>
</tr>
<tr>
<td>GLT-1</td>
<td>Glutamate transporter 1</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of nuclear factor kappa-B kinase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
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<tr>
<td>MMSE</td>
<td>Mini-Mental State Exam</td>
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<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
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<td>mRNA</td>
<td>Messenger RNA</td>
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<td>MSA</td>
<td>Multiple systems atrophy</td>
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<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NOR</td>
<td>Novel object recognition</td>
</tr>
<tr>
<td>NPR</td>
<td>Novel place recognition</td>
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<tr>
<td>NSC</td>
<td>Neural stem cell</td>
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<td>Olig2</td>
<td>Oligodendrocyte transcription factor 2</td>
</tr>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PDD</td>
<td>Parkinson’s disease Dementia</td>
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<td>pS129</td>
<td>phosphorylated serine 129</td>
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<td>Rag</td>
<td>Rag2/Ii2ry double knockout</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>RET</td>
<td>“Rearranged during transfection” receptor tyrosine kinase</td>
</tr>
<tr>
<td>S100</td>
<td>S100 calcium binding protein</td>
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<tr>
<td>shRNA</td>
<td>short hairpin ribonucleic acid</td>
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<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>TrkB</td>
<td>Tropomyosin receptor kinase B</td>
</tr>
<tr>
<td>VGLUT1/2</td>
<td>Vesicular glutamate transporters 1 &amp; 2</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>WTC</td>
<td>WT NSC-transplanted</td>
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<tr>
<td>WTV</td>
<td>WT vehicle-treated</td>
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ACKNOWLEDGMENTS

CHAPTER ONE of this dissertation is a reprint of the material as it appears in *Can Stem Cells Be Used to Enhance Cognition?*. The co-author listed in this publication, Dr. Blurton-Jones, directed and supervised research which forms the basis for the dissertation.

CHAPTER TWO of this dissertation is a reprint of the material as it appears in *Neural stem cell transplantation rescues cognitive and motor dysfunction in a transgenic model of Dementia with Lewy Bodies by elevating BDNF and improving glutamatergic and dopaminergic function*. Goldberg NRS, Caesar JA, Park A, Sedgh S, Finogenov G, Masliah E, Davis J, Blurton-Jones M. 2015. *Stem Cell Reports* 5(5):791–804. The co-authors listed in this publication contributed to the design of and data collection for the manuscript. The senior co-author, Dr. Blurton-Jones, directed and supervised research which forms the basis for the dissertation.

CHAPTER THREE of this dissertation is a reprint of the material as it appears in *Human neural progenitor transplantation rescues behavior and reduces α-synuclein in a transgenic model of Dementia with Lewy Bodies*. Goldberg NRS, Marsh SE, Ochaba J, Shelley BC, Davtyan H, Thompson LM, Steffan JS, Svendsen CN, Blurton-Jones M. *Submitted to Science Translational Medicine*. The senior co-author, Dr. Blurton-Jones, directed and supervised research which forms the basis for the dissertation.

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Abstracts and Posters


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ABSTRACT OF THE DISSERTATION

Disrupting Disease Phenotype in Dementia with Lewy Bodies with Neural Stem Cell Transplantation

By

Natalie Sashkin Goldberg

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2016

Assistant Professor Mathew Blurton-Jones, Chair

Synucleinopathies are a group of neurodegenerative disorders sharing the common feature of misfolding and accumulation of the presynaptic protein α-synuclein into insoluble aggregates. Within this diverse group, Dementia with Lewy Bodies (DLB) is characterized by the aberrant accumulation of α-synuclein in cortical, hippocampal, and brainstem neurons, resulting in multiple cellular stressors that particularly impair dopamine and glutamate neurotransmission and related motor and cognitive function. The wide array of cellular stress and symptoms related to over-abundance of α-synuclein makes DLB and other synucleinopathies challenging to treat. Many currently approved therapies fall short, suggesting that effective approaches will require the targeting of multiple mechanisms in order to treat motor, cognitive and other non-motor aspects of these diseases.

The goal of this dissertation is to determine the contributions of neural stem cell (NSC) transplantation to targeting multiple mechanisms related to α-synuclein over-expression in transgenic models of DLB. NSCs are an ideal candidate for transplantation due to their ability to migrate throughout a region of injury, and produce high levels of neurotrophins or growth factors critical to the survival and plasticity of neurons within the brain.

I have determined that mouse allogenic transplantation of NSCs lead to dramatic and sustained behavioral improvement in transgenic models of DLB. Critically, this improvement is dependent on NSC production of the neurotrophic factor, BDNF, which
regulates downstream dopamine and glutamate neurotransmitter systems. Weighted Gene Co-Expression Network Analysis verified the involvement of multiple key networks at the transcriptome level, including neurotrophins, dopamine, glutamate and immune-modulating networks. In order to further investigate the therapeutic potential of this approach, I examined the contributions of human NSC (hNSC) xenotransplantation to improving these multiple mechanisms. I demonstrated that clinical-grade hNSCs also rescue motor and cognitive behavioral deficits in an immune-deficient DLB model, and that this improvement is sustained over time. Moreover, transplantation of hNSCs restored dopamine and glutamate systems through a non-BDNF dependent mechanism. Finally, in contrast to mouse NSCs, hNSCs likely achieve these behavioral and neurochemical benefits by altering α-synuclein conformers.
INTRODUCTION

A. Dementia with Lewy Bodies and Other Synucleinopathies

Protein misfolding and aggregation play a prominent role in many neurodegenerative disorders. Synucleinopathies are a diverse group of such disorders with a common pathogenic protein: the presynaptic protein α-synuclein. These disorders are particularly challenging to address in a therapeutically meaningful way due to the range of vulnerable neural cell populations and regions of the nervous system affected. In Parkinson’s disease (PD) for instance, α-synuclein is the primary component of aggregates called Lewy Bodies and Lewy Neurites that occur in the neuronal cell body and cell processes of primarily dopamine-producing neurons of the midbrain (Spillantini, Schmidt et al. 1997, Breydo, Wu et al. 2012). In contrast, Dementia with Lewy Bodies (DLB) features cortical Lewy Bodies and Neurites, with not only different localization but distinct morphology from those in PD. In contrast, α-synuclein aggregates occur almost exclusively in glial cells in cases of Multiple System Atrophy (MSA), and are referred to as glial cytoplasmic inclusions (GCI). Further confounding our understanding of how to target pathological α-synuclein is its frequent coincidence with other protein aggregates such as amyloid-β and tau (Marsh and Blurton-Jones 2012).

Also common among synucleinopathies is the disruption of multiple systems including neurotransmission, immune modulation and cell survival. Stem cell transplantation is arguably one of the most promising approaches to address these types of diseases that involve multiple mechanisms. Replacement of degenerated cells, integration into existing circuitry and induction of immunomodulatory and neuroprotective mechanisms
are only some of the ways stem cells can be used to target and potentially treat these neurodegenerative conditions. Importantly, stem cells can also be readily modified to target pathological proteins for degradation, or work in concert with immunization or small molecule therapies. While a veritable avalanche of research has examined the use of stem cells to replace lost neurons, the investigation of stem cell modification to target pathological α-synuclein has just begun to scratch the surface.

B. α-Synuclein: A Physiological and Pathological Protein

The first synuclein sequence was revealed in 1988 when Maroteaux and colleagues examined neurons from the electric organ of the Pacific electric ray (*Torpedo californica*) (Maroteaux, Campanelli et al. 1988). Because of its localization in presynaptic terminals, the protein was called synuclein. From this initial sequence, α-synuclein and its homologue β-synuclein were subsequently identified in brain tissue (Jakes, Spillantini et al. 1994), and three years later a protein highly abundant in breast cancer tissue was identified as a second homologue and is referred to as γ-synuclein (Ji, Liu et al. 1997, Buchman, Hunter et al. 1998). Although all three members of the synuclein family are abundant in the brain, their functions there continue to be mysterious. α- and β-synuclein are localized to the nerve terminals where they may regulate vesicular trafficking through lipid binding (Davidson, Jonas et al. 1998, Jo, McLaurin et al. 2000). More recently, specific roles for α-synuclein in phospholipid or SNARE complex interaction have also been elucidated (Diao, Burre et al. 2013). The biological functions of γ-synuclein, which is expressed throughout the nerve cell, is even more poorly understood (Clayton and George 1999). Although there may be relevant
redundancies in function (Vargas, Makani et al. 2014), it is clear that the α-synuclein contributes most significantly to associated disease phenotypes.

Two major findings brought α-synuclein to the fore in 1997. A missense mutation (A53T) in the α-synuclein was discovered in families with autosomal dominant inheritance of Parkinsonian phenotype (Polymeropoulos, Lavedan et al. 1997), and only months later the α-synuclein protein was first identified as a major component of Parkinsonian Lewy Bodies (Figure 1) (Spillantini, Schmidt et al. 1997). These findings ignited the idea that perturbation of α-synuclein may have a direct role in the pathogenic processes of PD and other neurodegenerative conditions now recognized as Synucleinopathies.

Figure 1. “Tissue from patients with DLB (from the tissue collection of the Department of Pathology and Laboratory Medicine, University of Pennsylvania) immunostained for alpha-synuclein” from Spillantini et al., Copyright 1997. Available at: http://www.nature.com/nature/journal/v388/n6645/full/388839a0.html
One year after Polymeropoulos and colleagues identified the A53T missense mutation in the α-synuclein gene in Italian and Greek families, another such mutation was reported. A German family with a pedigree of early onset PD exhibited a missense mutation at A30P (Kruger, Kuhn et al. 1998). In the years following, several familial α-synuclein mutations have been described in cases of PD and DLB (Zarranz, Alegre et al. 2004), including very recently identified H50Q (Appel-Cresswell, Vilarino-Guell et al. 2013) and G51D mutations (Lesage, Anheim et al. 2013). But despite the continually mounting discoveries of point mutations, the majority of cases of PD and DLB are idiopathic with or without multiplication of the wild-type gene (Pals, Lincoln et al. 2004, Mizuta, Satake et al. 2006, Ahn, Kim et al. 2008).

Structurally, α-synuclein is an exemplary member of the rapidly growing family of intrinsically disordered (natively unfolded) proteins. It is the flexible nature of this structure that makes α-synuclein’s amphipathic region (residues 1-95) optimal for lipid binding and membrane curvature functions, but also what makes it susceptible to misfolding and subsequent aberrant aggregation (Uversky 2011). This N-terminal region is where most identified familial mutations occur (Figure 2) (Barrett and Timothy Greenamyre 2015). The C-terminal tail (residues 96-140) on the other hand, is where the vast majority of protein-protein interactions and post translational modifications (PTM’s) occur (Oueslati, Fournier et al. 2010, Hejjaoui, Butterfield et al. 2012). These PTM’s include nitration, truncation, ubiquitination, transglutaminase cross-linking and
phosphorylation, most of which are associated with enhanced oligomer formation and decreased membrane binding. Nitration of C-terminal tyrosines diminishes lipid vesicle binding and increases fibril formation (Hodara, Norris et al. 2004). Dopamine modification through adduct oxidation products drive aggregation into SDS resistant oligomers (Lee, Baek et al. 2011, Follmer, Coelho-Cerqueira et al. 2015). Of the phosphorylation sites, serine 129 is one of the best characterized disease-associated α-synuclein post translational modifications, representing 90% of the total α-synuclein found in Lewy bodies (Fujiwara, Hasegawa et al. 2002, Schmid, Fauvet et al. 2013). However, despite its acquired reputation as potential biomarker for synucleinopathies, whether phosphorylation at S129 enhances or suppresses α-synuclein toxicity remains the subject of deliberation (Oueslati, Fournier et al. 2010, Oueslati 2016).
Although there are several hypotheses as to the toxic species or aggregate of the intrinsically disordered \(\alpha\)-synuclein protein, debate continues regarding which of its many conformers is particularly disruptive to cellular function (Cookson and van der Brug 2008, Lashuel, Overk et al. 2013, Poehler, Xiang et al. 2014, Forloni, Artuso et al. 2016). The older and predominant proposition is that the cytosolic Lewy Body inclusions themselves lead to inflammation and cell death by obstructing essential cellular processes. However, just as it remains unclear whether phosphorylation of \(\alpha\)-synuclein at S129 facilitates or inhibits toxic activity, it is difficult to dissect whether Lewy bodies are toxic or neuroprotective (Fahn and Sulzer 2004, Kramer and Schulz-Schaeffer 2007). Because the number of Lewy bodies was reported to be higher in Parkinson’s patients with lesser dopamine neurodegeneration than in patients with severe neuronal depletion, Lewy body-containing neurons have been assumed to be the neurons subsequently degenerated (Wakabayashi, Tanji et al. 2007). On the other hand, Parkinsonian neurodegeneration may occur in the absence of Lewy bodies (Libow, Frisina et al. 2009), and Lewy bodies may occur in the absence of cognitive or motor deficits (Jellinger 2004).
Figure 3. "Mechanisms of α-synuclein aggregation and propagation" from Lashuel et al., Copyright 2013. Available at: http://www.nature.com/nrn/journal/v14/n1/full/nrn3406.html
The hypothesized function of Lewy bodies and their formation has therefore shifted in recent years to a protective process of sequestering and compartmentalizing toxic oligomeric species. α-Synuclein is degraded and cleared from cells by both autophagy and the proteasome (Webb, Ravikumar et al. 2003), and the presence of both autophagic and proteasomal proteins in Lewy body composition are one argument in support of a protective function (Shin, Klucken et al. 2005, Tanji, Mori et al. 2011, Odagiri, Tanji et al. 2012). It has even been hypothesized that Lewy bodies form in an aggresome-like manner in which smaller α-synuclein oligomers are requisitioned from pre-synaptic terminals to the soma to aggregate in response to proteolytic stress (Kopito 2000, Olanow, Perl et al. 2004).

The corresponding contention to the neuroprotective Lewy body hypothesis is that the smaller α-synuclein oligomer species that are observed prior to fibril assembly are cytotoxic (Figure 3) reviewed in (Lashuel, Overk et al. 2013). The shifts in expression of these species is determined by many factors including mutations and PTM’s. Because of their particular insolubility, most α-synuclein oligomers and aggregates are notoriously challenging to isolate, visualize and characterize (Schulz-Schaeffer 2015). In turn, this leaves the determination of which species enhances or suppresses mechanisms of toxicity actively debated (Wang, Perovic et al. 2011, Dettmer, Newman et al. 2013, Pochapsky 2015). However, studies increasingly lean away from a pathogenic role for Lewy bodies and instead implicate the potential instigation of toxicity by small α-synuclein oligomers and fibrils (Cremades, Cohen et al. 2012, Chen, Drakulic et al. 2015) (Schulz-Schaeffer 2015).
Finally, the particular species of α-synuclein may not confer toxicity. Rather, the accumulation of oligomeric and fibrillary clearance or lysosomal-degradation intermediates (Lee, Suk et al. 2008) and subsequent exocytosis of those aggregates may lead to toxicity as well as stimulating the inflammatory response (Lee, Khoshaghideh et al. 2004).

The multifarious range of fibrillar, oligomeric, mutated and post-translationally modified forms of α-synuclein that have been identified within Lewy bodies suggests that the process of Lewy body formation is central to the pathogenesis of synucleinopathies. This diversity of means by which α-synuclein can shift from physiological to pathological indicates the need for therapeutic interventions that can target multiple mechanisms to bring about a return to homeostatic function.

C. Stem Cell Transplants in Parkinson’s disease

Parkinson’s disease (PD) is the most common synucleinopathy with the longest history of stem cell transplantation. Before α-synuclein was recognized as a component of Lewy Bodies in 1997 the hallmark pathology of PD was the loss of dopamine-producing neurons in the midbrain, largely in the nigrostriatal pathway (Spillantini, Schmidt et al. 1997). In humans, loss of nigrostriatal dopamine manifests most notably as motor impairment in the form of tremor, rigidity, postural instability and difficulty initiating movement. Nearly two decades before α-synuclein played a role in determining therapeutic approaches, neuronal replacement of these lost dopaminergic cells became an attractive approach to restoring motor function in PD patients.
In the early 1980’s, the first transplants in rodents demonstrated potential re-innervation of the striatum with dopamine-producing cells grafted there (Bjorklund, Dunnett et al. 1980, Freed, Perlow et al. 1980, Dunnett, Bjorklund et al. 1981). Shortly thereafter, a burst of clinical studies began grafting fetal ventral mesencephalic cells (FVM), the precursors to substantia nigra dopamine cells (Backlund, Granberg et al. 1985, Lindvall, Brundin et al. 1990, Kordower, Freeman et al. 1995). While potential for improving behavioral impairments seemed promising early on in open label studies, the first 20 years of stem cell replacement therapy in PD demonstrated modest and inconsistent efficacy. Well-controlled double-blind trials revealed little clinical motor benefit, and some even reported significant exacerbation of symptoms. Due to these inconsistent findings over the first decade of transplantation attempts, the method was abandoned in favor of new approaches like Deep Brain Stimulation, a way of controlling signaling in the remaining circuitry of the midbrain. However, after a long hiatus, clinical trials are currently recruiting through the TRANSEURO consortium (clinicaltrials.gov). This initiative includes teams across the United Kingdom, France, Sweden and the United States currently recruiting participants to receive FVM transplants, hypothesizing that advances in procedural techniques will dramatically improve outcomes from those previously observed (Lindvall 2015)( transeuro.org.uk).

In addition to identifying the potential of cell replacement therapy in PD, grafted cells yielded another tremendous discovery. In May of 2008, two groups reported that transplanted FVM cells in PD patients could acquire α-synuclein pathology after 5-24 years (Kordower, Chu et al. 2008). This finding led to a surge in examination of how α-
synuclein is transmitted, transported, transferred and templated between cells in the brain. It has even been hypothesized that pathological α-synuclein may have origins in the enteric nervous system, transmitting to and later through the brain (Braak and Braak 2000). But the ability of α-synuclein and Lewy bodies to transfer between cells raised a pressing concern for the safety and efficacy of cell transplantation in PD and other synucleinopathies. Some of the scientists who reported the discovery in 2008 hypothesize that the 3-7% of grafted cells that acquire pathology is not sufficient to negatively impact the therapeutic benefits of cell transplantation. Others suggest that this susceptibility can be overcome by cell modification techniques. Many hypothesize that cells other than FVM may be the best resource for achieving this through multiple homeostatic effects (Redmond, Bjugstad et al. 2007, Goldberg, Caesar et al. 2015, Gonzalez, Garitaonandia et al. 2015).

D. Potential of Neural Stem Cells to Impact α-Synuclein Pathology

A final limitation of FVM or other dopaminergic cell lineages is that their transplantation in the striatum does not help to slow or halt the progressive retrograde degeneration of nigrostriatal dopaminergic neurons. While it is possible that dopaminergic cell replacement will be optimized for effective therapeutic benefit in the future, we propose that other cell types may present a more accessible option with more widespread benefits.

A chief benefit of neural stem cell (NSC) engraftment is that cells are multipotent, capable of giving rise to neurons, astrocytes, and oligodendrocytes following transplantation (Blurton-Jones, Kitazawa et al. 2009). Thus, NSCs and their progeny
can potentially affect multiple mechanisms as opposed to solely replacing lost dopamine signaling. In addition, NSCs initially retain some of their proliferative and migratory capacity yielding a wider area of effect within the brain, whereas more mature dopaminergic precursors do not migrate significantly (Brundin, Strecker et al. 1988, Svendsen, Clarke et al. 1996, Sanchez-Pernaute, Studer et al. 2001). The lack of migration in dopaminergic precursors has not been of great concern since the ultimate goal of dopamine neuron replacement is to re-innervate the striatum, making functional connections with target medium spiny neurons. This has generally been achieved by transplanting cells into the striatum itself, however the ultimate goal of dopamine cell replacement is to guide cells transplanted into the substantia nigra to project to the striatum recreating the nigrostriatal pathway. Although this has recently been achieved

![Figure 4. Stem-cell based therapies in the mouse midbrain.](image)

Figure 4. Stem-cell based therapies in the mouse midbrain. Stem cell-based approaches could be used to provide therapeutic benefits in two ways: 1) using DA neuron precursors or modifying cells to produce DA and transplanting them either into the substantia nigra or the striatum, or 2) implanting cells into either region to release neurotrophic factors which would protect remaining neurons. Ideally, DAergic precursors could be transplanted into the substantia nigra and project to the striatum; although this has been achieved to a degree in murine models, it is far from feasible in humans.
to some extent in small animal models, such an approach will be far more challenging to achieve in a much larger non-human primate and human brain (Kauhausen, Thompson et al. 2013). In contrast to this approach, NSC transplantation could exert neuroprotective or neurorestorative effects in either the substantia nigra or striatum without needing to recapitulate the nigrostriatal pathway (Figure 4). Specifically, NSCs have the capacity to induce target-derived growth factor expression or immunomodulation which may not only support degenerating cells in the striatum, but might also impact α-synuclein pathology. The most promising evidence in support of this strategy comes from a 2007 study from Eugene Redmond and colleagues. The group showed that in male African green monkeys treated with MPTP, Parkinson’s factor scores improved up to 120 days following human NSC transplantation into the striatum and substantia nigra (Redmond, Bjugstad et al. 2007). Importantly, this coincided with normalization of MPTP-induced α-synuclein aggregation, and implicated hNSC-derived trophic factors as playing an important role in recovery.

E. Implication of Brain-Derived Neurotrophic Factor in Synucleinopathies

While glial-derived neurotrophic factor (GDNF) remains accepted as one of the best therapeutic candidates for PD and other synucleinopathies, BDNF is emerging as a promising alternative. Similar to dopamine replacement, one of the caveats of GDNF therapy in both clinical and preclinical studies is the absence of a positive impact on PD-related cognitive impairment. The first evidence implicating BDNF in cognition was reported by Phillips and colleagues in 1991. This group reported a reduction in BDNF mRNA in the post mortem hippocampus of Alzheimer’s disease patients (Phillips, Hains
et al. 1991). This was followed by reports of reduction in BDNF mRNA and protein in the neocortex and nucleus basalis of both human patients and animal models (Narisawa-Saito, Wakabayashi et al. 1996, Murer, Boissiere et al. 1999, Tapia-Arancibia, Aliaga et al. 2008). In the years following these reports, several studies have provided evidence for decreased BDNF expression in post mortem substantia nigra of PD patients (Mogi, Togari et al. 1999, Murer, Yan et al. 2001). This reduction is due, in part, to loss of nigral dopaminergic neurons that express BDNF, but importantly the surviving neurons also express less BDNF than is observed in controls (Howells, Porritt et al. 2000). These observations paralleled the reduced neurite outgrowth and subsequent nigral cell death in PD that would be expected in a BDNF deficient environment.

In the mouse midbrain, both heterozygous and homozygous knockout of BDNF leads to long-term reduction of DA nigral cells, accumulation of α-synuclein and compromised DA release in the striatum (Dluzen, Gao et al. 2001, Baquet, Bickford et al. 2005). These findings suggest that BDNF reduction in the nigra could contribute to the degeneration of these cells, although the evidence for this link is not overwhelming. In support of this association, it is of note that expression of Tropomyosin related kinase B (TrkB), the high-affinity BDNF receptor, is normal in post mortem PD nigral neurons (Benisty, Boissiere et al. 1998, Fenner, Achim et al. 2013), suggesting a continued capacity for plasticity and sprouting even in late stage PD. Other animal studies have found that increased dopamine uptake and axonal sprouting in compensatory response to surgical injury is associated with increased striatal production of BDNF (Fiandaca,
Kordower et al. 1988, Kordower, Cochran et al. 1991, Howells, Donnan et al. 1993, Wong, Liberatore et al. 1997 1990), and emphasize the potential of BDNF as an effective treatment for PD.

BDNF also appears to be implicated in the development of early cognitive deficits in PD and DLB. This hypothesis is corroborated by clinical reports that functional polymorphisms in the BDNF gene such as Val66Met may confer susceptibility to developing these synucleinopathies and be highly related to cognitive deficits (Hong, Liu et al. 2003, Lee and Song 2014). Although this association has not been confirmed in some populations (Hakansson, Melke et al. 2003, Liu, Walther et al. 2005), the Val66Met polymorphism may exert an ethnic and gender biased effect (Foltynie, Lewis et al. 2005).

The Braak hypothesis suggests that the stage of PD-related cognitive decline is based heavily on the cumulative load of α-synuclein or Lewy Body pathology, having demonstrated that Mini-Mental State Examination (MMSE) scores correlate highly with post mortem Lewy Body distribution (Braak, Rub et al. 2005, McKeith, Dickson et al., Braak, Rub et al. 2006). Indeed, a recent report has confirmed the correlation of cerebrospinal fluid α-synuclein with cognitive decline, but not motor deficit progression in PD patients who participated in the DATATOP study (Stewart, Liu et al. 2014). Several studies have proposed the influence of reduced BDNF levels on α-synuclein accumulation. Specifically, haplosufficiency for the TrkB receptor result in nigral cell loss and accumulation of α-synuclein in aged mice (von Bohlen und Halbach,
Minichiello et al. 2005). In vitro studies have corroborated this link showing that BDNF is reduced in PC12 cells and mouse neurons by over-expression of wild type α-synuclein (Saha, Ninkina et al. 2000, Yuan, Sun et al. 2010). These studies cumulatively demonstrate a functional relationship between BDNF and α-synuclein, and suggest that reduced BDNF may be important to the development of α-synuclein pathology and related cognitive decline, making BDNF a therapeutic target that could potentially influence motor, cognitive, and pathological aspects of PD and DLB.

**F. BDNF and Glutamate**

In addition to α-synuclein and dopaminergic pathology, dysfunction of glutamate transmission is known to play an important role in PD and DLB pathogenesis (Figure 5). Postmortem studies have revealed alterations in NMDA and AMPA expression throughout the basal ganglia (Ulas, Weihmuller et al. 1994, Gerlach, Gsell et al. 1996), reviewed in (Blandini, Porter et al. 1996), as well as changes in the glutamate transporters VGLUT1 and 2 (Kashani, Betancur et al. 2007, Francis 2009). The Kashani group study reports that VGLUT1 and 2 were increased by 24% and 29%, respectively, in the Parkinsonian putamen compared to controls. The elevation of these vesicular transporters reveal specifically that changes to the corticostriatal (VGLUT1) and thalamostriatal (VGLUT2) projections may contribute to motor and cognitive impairments. In addition, postmortem morphological analyses have revealed that the size of glutamate postsynaptic densities and number of perforated synapses increase by 24% and 88%, respectively, in the putamen of PD patients, suggesting corticostriatal
hyperactivity by end-stages of the disease (Anglade, Mouatt-Prigent et al. 1996).

Finally, one group has even reported selective accumulation of α-synuclein in VGLUT-1 positive corticostriatal nerve terminals (Nakata, Yasuda et al. 2012). Preclinical studies have corroborated that glutamate signaling is altered in Parkinsonian conditions (Carlsson and Carlsson 1989, Meredith, Totterdell et al. 2009) reviewed in (Shepherd 2013).

Although the relationship of BDNF and glutamate signaling has not been examined in patients, animal models have begun to characterize their interaction in the striatum. It is known that BDNF and glutamate show critical modulatory interactions that influence the

![Figure 5. Major glutamatergic inputs to the mouse striatum.](image)

Precisely how these inputs are altered in PD is likely phasic and determined by the stage of the disease, although hyperactivity has been suggested. Primary (M1) and secondary motor cortices (M2); primary somatosensory cortex (S1); striatum (Str); anterior commissure (a.c.); globus pallidus internal (GPI) and external (GPe); amygdala (Amyg); thalamus; hippocampus (Hipp); subthalamic nucleus (STN); substantia nigra pars compacta (SNpc) and pars reticulate (SNr); Pons; medulla (Med); and cerebellum.
development and activity-dependent formation of synapses, and this role remains important during synaptic changes in the mature brain (Lessmann 1998, Lu 2003, Tanaka, Horiike et al. 2008, Gottmann, Mittmann et al. 2009). This interaction is also key to regulating nigral DA neuron activity, and may be selective to DAergic and glutamatergic targets (Bustos, Abarca et al. 2004). In a single excitatory afferent to both glutamatergic and inhibitory GABAergic postsynaptic targets, BDNF selectively potentiated glutamatergic synapses (Schinder, Berninger et al. 2000). This suggests that the BDNF-glutamate association may be important to corticostriatal circuitry and related cognitive and motor integrity. In further support of this, it was reported that BDNF infusion normalized cocaine-induced dysfunction in corticostriatal glutamate transmission (Berglind, Whitfield et al. 2009). A recent study from D’Amore and colleagues assessed dorsal striatal glutamate dynamics using electrochemical recordings following BDNF application (D’Amore, Tracy et al. 2013). The group found that BDNF induced glutamate release by activating its receptor, TrkB, on glutamatergic terminals. This increased dorsal striatal glutamate induced cognitive enhancement in a strategy set-shifting task. Striatal glutamate signaling is also considered critical to cognitive flexibility and executive function, the earliest cognitive impairments observed in PD (Ragozzino, Ragozzino et al. 2002, Balleine, Delgado et al. 2007, Loving 2010).

G. Stem Cell-Mediated BDNF

In 1994, Frim and colleagues showed that fibroblasts modified to secrete BDNF were protective against MPP+ toxicity (Frim, Uhler et al. 1994). However, no behavioral outcomes were reported and the finding did not gain much traction in the field. This
approach was reported again in 2010 using modified mesenchymal cells in a 6-OHDA model, but once again no behavioral improvement was reported (Somoza, Juri et al. 2010). These two studies successfully utilized a cell-based approach to deliver trophic therapy and established a potential neuroprotective role for stem cell-mediated BDNF delivery.

![Diagram of neuronal signaling](image)

**Figure 6.** Glutamatergic inputs synapse onto the heads of striatal medium spiny neurons, and are acted on by BDNF.

It was during this time that Redmond and colleagues reported similarly successful neurorestoration and normalization of MPTP-induced α-synuclein aggregation using unmodified hNSCs, citing trophic factors expressed by hNSCs as key (Redmond,
Bjugstad et al. 2007). Shortly thereafter, pathogenic mutations of synuclein associated with early-onset PD were linked to loss of BDNF production in vitro (Kohno, Sawada et al. 2004, Karamohamed, Latourelle et al. 2005). These studies suggest not only that expression of neurotrophic factors make NSCs a promising cell type and vehicle for transplantation therapy, but that they might also impact pathology (Figure 6).

The evidence of a BDNF-α-synuclein interaction emphasizes the credibility of BDNF as a therapeutic target for not only supporting dopamine neurons but many other cell types attenuated by α-synuclein pathology. Surprisingly, BDNF remains largely uninvestigated in terms of cognitive or motor function in DLB models. *My dissertation therefore aims to address these areas through examining the role of BDNF in NSC-mediated changes in neurotransmission, α-synuclein and behavioral outcomes in transgenic DLB mice.*
CHAPTER ONE

CAN STEM CELLS BE USED TO ENHANCE COGNITION?

Abstract. Multipotent stem cells exist throughout the adult human body playing important roles in tissue maintenance and repair. Within the brain, growing evidence suggests that neural stem cells also play a critical role in cognition. These findings have led researchers to question whether strategies that increase neural stem cell populations within key brain regions could perhaps be used to enhance cognition. In this chapter, we review the brain’s capacity for plasticity and regeneration, and the potential role of both endogenous neurogenesis and stem cell transplantation to augment this capacity. We also explore the relationship between neural stem cells and cognition in the healthy, aging, and pathological brain, and discuss the molecular mechanisms by which stem cells may exert their effects on learning and memory.

Introduction

Stem cells are defined by two key attributes; they can self-renew, dividing to create near-perfect copies of themselves and can also differentiate to produce distinct mature cell types. Many different types of stem cells exist and can be classified based on their source and capacity for differentiation. For example, embryonic stem cells are derived from the inner cell mass of an early stage blastocyst and are considered pluripotent as they are able to give rise to every cell type within the body. In contrast, within the adult body multipotent stem cells exist in virtually every organ system and can give rise to a far more limited repertoire of cell types associated with that given organ. A growing
body of work continues to demonstrate the importance of multipotent stem cells in maintaining normal tissue function and homeostasis as well as playing critical roles in tissue repair following injury or disease (reviewed in) (Fuchs, Tumbar et al. 2004). The equilibrium between cell loss and replacement is well maintained by stem cells in most adult tissues with the exceptions of the pancreas, heart, and brain. With maturation and age, the brain’s capacity to produce new neurons is significantly diminished (Rossi, Jamieson et al. 2008). In addition to a reduction in stem cell turnover, the function of mature differentiated cells is also often diminished with age due to both intrinsic and environmental factors such as DNA damage, oxidative stress, or the accumulation of misfolded proteins (Welihinda, Tirasophon et al. 1999, Shetty, Hattiangady et al. 2013). These changes result in reduced integrity of tissue and age-associated impairments in organ function. As discussed below, one of the greatest functional correlates to the loss of cell homeostasis in the brain is a decline in cognitive function.

The role of Endogenous Neurogenesis in Cognition

Nearly fifty years ago, Altman and Das provided the first evidence that neurogenesis could occur in the adult mammalian brain (Altman and Das 1965) (Figure 7). Only decades later did the first reports of adult human neurogenesis first emerge. In a seminal study, Eriksson and colleagues demonstrated that neurogenesis occurs in adult humans by tracing the incorporation of the thymidine analog bromodeoxyuridine (BrdU) into DNA within the brains of deceased cancer patients (Eriksson, Perfilieva et al. 1998). These patients had had previously received BrdU to assess tumor progression during
treatment. Eriksson’s studies revealed that humans do indeed exhibit adult neurogenesis in two key areas; the dentate gyrus of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles. More recently a series of studies led by Dr. Jonas Frisen and colleagues has employed an inventive new approach to measure adult human neurogenesis (Spalding, Bergmann et al. 2013, Ernst, Alkass et al. 2014). Between 1955-1963, above-ground nuclear bomb testing led to high atmospheric levels of $^{14}$C that exponentially decreased after adoption of the Test-ban treaty in 1963. Frisen and colleagues found that levels of $^{14}$C within DNA closely paralleled atmospheric levels at the time of birth. Radiocarbon dating could therefore be used to retrospectively birth-date brain cells. Together, these studies confirmed that adult neurogenesis occurs within the hippocampus and SVZ of humans throughout life. Furthermore, adult hippocampal neurogenesis appears to be substantial; with roughly 700 new neurons being generated in each hippocampus per day and up to one third of all hippocampal granule cell neurons being replaced during one’s adult life. Most recently, this group has used the radiocarbon dating approach to provide compelling new data that SVZ-derived newborn neurons can also migrate into the adjacent striatum in humans giving rise to cholinergic interneurons (Ernst, Alkass et al. 2014). These profound discoveries continue to provoke the question: what are the functional consequences of adult neurogenesis?

The functional role of neurogenesis in the adult brain has been studied in its germinative pools of the SVZ adjacent to the striatum (Alvarez-Buylla, Herrera et al. 2000, Cheng, Pastrana et al. 2009), but our greatest understanding of the role of adult neurogenesis
in cognition has come from studies of the dentate gyrus of the hippocampus. The hippocampus plays a critical role in the encoding and retrieval of memories (Izquierdo and Medina 1997). A potential role for hippocampal neurogenesis in adult memory was first proposed by Shors and colleagues in 2001 (Shors, Miesegaes et al. 2001). Support for this finding came in 2006, when studies showed that newborn granule cells of the dentate gyrus are more highly activated by a novel exploration task compared to mature neurons of the same region (Ramirez-Amaya, Marrone et al. 2006). Possibly as a result of this increased excitability, newborn granule neurons also integrate more readily into memory-associated engrams than mature granule cells (Tashiro, Sandler et al. 2006, Ahn, Kim et al. 2008). Indeed, new granule cells have been shown to be preferentially integrated into spatial and temporal memory networks of the hippocampus compared to mature cells.

Over the last few years, several groups have adapted inducible transgenic approaches to directly test the necessity of adult hippocampal neurogenesis in learning and memory. For example, Imayoshi and colleagues used Tamoxifen-inducible Cre-LoxP technology to generate a mouse model in which new-born neurons could be selectively ablated (Imayoshi, Sakamoto et al. 2008). Using this approach, the group demonstrated that adult new-born neurons were critical for long-term spatial memory and performance in the Barnes Maze. In a similar study, Zhang et al. deleted the neurogenic gene Tlx (Tailess) in adult mice, leading to a dramatic reduction in hippocampal neurogenesis and impaired learning and memory in the Morris water maze (Zhang, Zou et al. 2008). The dentate gyrus has increasingly been implicated in
pattern separation, the process of transforming similar overlapping memories into distinct non-overlapping representations (Aimone, Deng et al. 2011). It follows that recent studies have shown an important role for adult neurogenesis in pattern separation. For example, deletion of the Bax gene leads to increased adult neurogenesis and mice that exhibit an improved capacity to distinguish between two similar contexts, suggesting enhanced pattern separation (Sahay, Scobie et al. 2011). Taken together these studies and others continue to show that procedures that abolish newborn neurons or progenitors such as gamma- and x-ray radiation or genetic ablation eliminate or significantly decrease learning, suggesting that these new neurons are indeed crucial to this form of memory encoding (Santarelli, Saxe et al. 2003, Garcia, Doan et al. 2004, Saxe, Battaglia et al. 2006, Imayoshi, Sakamoto et al. 2008, Yeung, Myczek et al. 2014) (Figure 7).
**Figure 7.** A historical timeline of neurogenesis and stem cell transplantation focused studies.
While there is significant evidence supporting a role for endogenous neurogenesis in rodent cognitive function, it has been far more challenging to study such relationships in humans. However, recent data suggest that hippocampal neurogenesis likely plays a similar role in the adult human brain. The phenomenon of “chemo brain” is one of the first observable correlations between human cognitive function and adult neurogenesis. During and following cancer chemotherapy and radiotherapy, patients commonly experience difficulty with memory, executive function, attention and visuospatial function (Staat and Segatore 2005, Raffa, Duong et al. 2006). In 2007, the hippocampi of 4 patients who had undergone cranial radiotherapy for various malignancies were examined post mortem, and showed significantly decreased neurogenesis (Monje, Vogel et al. 2007). In this case study, one patient who experienced greater radiation exposure in one hemisphere than the other had more severely decreased newborn neurons on that side compared to the less exposed hippocampus. Such correlations can of course be much more readily examined in animal models, indeed multiple recent studies have supported that there is a link between chemotherapy-related cognitive impairment and hippocampal neurogenesis in murine models (Yang, Kim et al. 2010, Hou, Xue et al. 2013) (Table 1 and Figure 8). As new potentially quantitative measures of adult neurogenesis are developed (Manganas, Zhang et al. 2007, Spalding, Bergmann et al. 2013, Ernst, Alkass et al. 2014) it is likely that our understanding of the role of adult neurogenesis in human cognition will continue to improve and evolve.
Table 1. Factors that influence in vivo neurogenesis and their association with cognitive function. (From left to right) Factors or pharmacological agents, whether those stimuli increase or decrease (arrows) in vivo neurogenesis, and the correlation of those changes with cognitive function. Check marks indicate that a change in that signaling factor has also been associated with improved cognitive function.

<table>
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<tr>
<th>Factor/Pharmacological Agent</th>
<th>Effect on Neurogenesis</th>
<th>Correlation with Cognitive Function</th>
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Improving Cognition with Stem Cell Transplantation

Our growing understanding of neurogenic mechanisms and their involvement in cognition could lead to novel therapeutic strategies for preserving or enhancing cognition. While many groups and several biotechnology companies are focused on the development of drugs that increase endogenous neurogenesis, stem cell transplantation may offer an alternative approach to either replace degenerated cells or provide support for dysfunctional neurons. This prospect has important clinical implications for numerous neurological disorders that are characterized by significant
neuronal loss and cognitive impairment. Yet, this is a daunting task given the seemingly infinite variation of structural and neurochemical phenotypes that comprise the neurons and synapses of the adult human brain. Fortunately, recent studies suggest that stem cell transplantation may provide meaningful benefit in animal models of neurodegeneration. Interestingly, these effects occur not necessarily via neuronal replacement, but rather by more indirect neuroprotective and plasticity promoting mechanisms. In the remaining portion of this chapter, we describe some of the key findings in this active area of neuroscience research, and make an argument for the continued pursuit of research into the effects of stem cell transplantation on cognition.
Neural Stem Cell Transplantation in Aging. There is growing evidence that neural stem cell (NSC) transplantation can improve cognition in both aging and pathologically-effected brains. There is even some indication that NSC transplantation can enhance performance in healthy brains. The mechanisms by which stem cells achieve these benefits likely vary across each of these three microenvironmental conditions, which speaks to the flexibility of these cells as a potential therapy. One of the first studies to examine the potential impact of NSC transplantation in aging, injected human NSCs into the lateral ventricle of aged rats, leading to improved performance in spatial learning and memory as measured in the Morris Water Maze task (Qu, Brannen et al. 2001).

**Figure 8. Stem cells can influence cognition via multiple mechanisms.** Considerable evidence supports the notion that both adult neurogenesis and neural stem cell transplantation can contribute to cognition. Factors such as exercise, SSRIs, and inflammation can modulate adult neurogenesis leading to enhanced or impaired cognition. Likewise, transplantation of neural stem cells can improve cognition in animal models of aging and neurodegenerative disease. While neurotrophic effects of stem cells are strongly implicated in this process, it is likely that many of the mechanisms shown here interact to modulate cognition.

**Neural Stem Cell Transplantation in Aging.** There is growing evidence that neural stem cell (NSC) transplantation can improve cognition in both aging and pathologically-effected brains. There is even some indication that NSC transplantation can enhance performance in healthy brains. The mechanisms by which stem cells achieve these benefits likely vary across each of these three microenvironmental conditions, which speaks to the flexibility of these cells as a potential therapy. One of the first studies to examine the potential impact of NSC transplantation in aging, injected human NSCs into the lateral ventricle of aged rats, leading to improved performance in spatial learning and memory as measured in the Morris Water Maze task (Qu, Brannen et al. 2001).
Interestingly, NSC transplantation can itself enhance endogenous hippocampal neurogenesis, suggesting that this likely plays a role in the effects of transplantation on cognition (Hattiangady, Shuai et al. 2007). Due to the minimal neurodegeneration that takes place in the aging brain relative to the diseased brain, it was important to determine whether such results could also be translated into animal models of brain injury and disease. Consequently, in the last decade multiple studies have emerged demonstrating that stem cell transplantation can provide cognitive benefits in a wide variety of disease states.

**Neural Stem Cell Transplantation in Neurological Disease.** Numerous preclinical studies have shown that stem cell transplantation can improve motor function in models of Huntington’s disease, Parkinson’s disease, stroke, and traumatic brain injury (TBI) (Lee, Lee et al. 2012). Until recently however, very few studies had assessed the potential effect of NSCs on cognitive function. One study did explore the combined effects of NSC transplantation and BDNF infusion in a unilateral fimbria-fornix transection model, finding improved performance in a Y-maze with BDNF+ NSC treatment (Xuan, Long et al. 2008). Our own studies also strongly implicate BDNF in stem cell mediated effects on cognition. For example, we found that murine neural stem cell transplantation in the triple transgenic (3xTg-AD) model of Alzheimer’s disease improves cognition in Morris Water Maze and Novel Object Recognition tasks via a BDNF-dependent mechanism (Blurton-Jones, Kitazawa et al. 2009). To confirm the importance of NSC-derived BDNF in functional recovery, we genetically modified NSCs with a shRNA to stably knockdown BDNF expression by 87%. Treatment of AD
transgenic mice with these BDNF-depleted NSCs no longer improved cognition, demonstrating the necessity of BDNF signaling in NSC-mediated recovery.

While most stem cell transplantation studies report some evidence of functional improvement, several note that transplantation improved some behaviors but not others. For example, one group reported that embryonic stem cell-derived neuronal and glial precursor transplantation could improve sensorimotor deficits in a model of TBI, but failed to restore cognitive function (Hoane, Becerra et al. 2004). A second group found contrasting results, reporting that human fetal neural stem cells could improve cognitive function in a rat model of TBI (Gao, Prough et al. 2006). Differences between these findings likely result from a number of factors including variability in the severity of TBI and the resulting neuronal loss. One potential way to avoid such variability is to use a genetic ablation approach. By pairing neuronal expression of the Diphtheria toxin A-chain (DTa) with tetracycline-regulated expression one can for example produce a much more consistent hippocampal injury. For example, we previously found that Tet-regulated expression of DTa under control of the Cam kinase 2α promoter can be induced for varying durations, allowing one to ablate more vulnerable CA1 neurons while sparing dentate granule and cortical neurons. Using such an approach, we have shown that neural stem cell transplantation can improve cognitive function following ablation of CA1 hippocampal neurons (Yamasaki, Blurton-Jones et al. 2007).
Most recently, we have observed a similar capacity of neural stem cells to improve memory in a transgenic model of Parkinson’s disease (Goldberg, Caesar et al. 2015) (Figure 9). To perform these studies we utilized a transgenic model of PD that over-expresses wild type human α-synuclein, a protein that accumulates in PD-associated Lewy bodies. These mice exhibit dysfunctional production and transmission of dopamine in primary motor pathways leading to deficits not only in motor function but also multiple cognitive domains (Lam, Wu et al. 2011). Syngeneic neural stem cells were transplanted bilaterally into the striatum of aged α-synuclein mice and one month later motor and cognitive behavior was examined. NSCs survive in the striatum and begin to differentiate into glia (glial fibrillary acidic protein) and neurons (doublecortin). In these initial studies we have found robust improvements not only in motor function but also cognitive function and BDNF again appears to be central to these improvements.

Figure 9. Stem cell preparation and transplantation into the brain. Neural stem cells (NSCs) were derived at post natal day 1 (P1) from GFP-expressing transgenic mice. NSCs were expanded for 15 passages, at which time cells are dissociated with Trypsin and neutralized in antibiotic-free growth media. NSCs are then resuspended and washed twice with transplantation vehicle (Hanks Buffered Saline Solution, 20 ng/mL EGF), then counted. Only cell populations with ≥90% viability are used, and concentration is adjusted to yield 50,000 cells/µL. NSCs or vehicle are then transplanted bilaterally into the striatum using a 30 gauge Hamilton microsyringe and stereotaxic apparatus (1 uL/site; anterior/posterior=+0.02, medial/lateral=±2.00, dorsal/ventral=−3.0 and -3.5).

Stem Cell Transplantation in the Healthy Adult Brain. Until very recently, no studies had reported an enhanced benefit of stem cell transplantation in the intrinsically
functioning adult brain. However, in 2013, Han and colleagues showed that human glial progenitor transplantation into the frontal cortex of immune-deficient neonatal mice lead to significant enhancements in adult and aged mouse cognitive function (Han, Chen et al. 2013). In contrast, transplantation of murine glial progenitors had no such effect. These researchers suggested that one potential explanation for the differential effect between human and murine progenitors may relate to species-specific differences in calcium wave propagation, a mechanism by which astrocytes communicate. Human glial progenitor calcium waves propagated at least 3 fold faster than did mouse cells, which may be attributable to their much larger size and structural complexity. These differences likely also contributed to the heightened basal level of excitatory transmission and enhanced long term potentiation that was also observed in this study. Another recent study examined the effect of NSCs that were modified to over-express choline acetyltransferase, an enzyme responsible for acetylcholine synthesis (Park, Yang et al. 2013). In this report, Park and colleagues transplanted these modified NSCs into the lateral ventricle of young and aged mice, and learning and physical activity were assessed one month later. Interestingly, this group found significant improvements in passive avoidance, Morris Water Maze, and spontaneous locomotor activity in aged mice receiving ChAT-expressing stem cells. The group suggests that the benefit of these ChAT-enhanced stem cells may be the secretion of either produced acetylcholine or the growth factors BDNF or NGF, which are commonly reported to be secreted from human stem cells. Although both the Han and Park studies as well as others return to the hypothesis of neurotrophin-based mechanisms of stem cell-induced change, the immune system continues to also be implicated. Both studies transplanted
human cells into immune-deficient mouse models, however Han et al acknowledged the role of the cytokine TNFα in glial progenitor transplant signaling, reporting that transplantation increased hippocampal TNFα levels leading to increased AMPA receptor GluR1 receptor expression, suggesting an important role for cytokine-regulated alteration of excitatory transmission. Although TNFα is primarily understood as proinflammatory and likely detrimental to endogenous neural stem cell proliferation, mounting evidence suggests a role for the cytokine in autophagic clearance mechanisms central to cell survival. TNFα is reported to induce activation of the IkappaB kinase (IKK), which then disinhibits the transcription factor NF-kB allowing it to translocate to the nucleus and bind to target gene promoter regions that contribute to selective autophagy, proliferation and cell survival (Bonizzi and Karin 2004, Baldwin 2012). The relative impact of TNFα and other cytokines on both endogenous and exogenous-derived neural stem cells and their influence on cognition clearly warrants further study.

As detailed above, several studies have now examined the effects of neural stem cells on cognition in various animal models of injury, aging, and disease. Likewise, some studies have explored the potential effects of glial precursors on cognition. Yet to date no reports have directly compared these varying cell sources. It therefore remains unclear whether a specific stem cell type is more appropriate for enhancing cognition and whether the ability of given stem cell population to improve cognition will vary with age or disease.
Contribution of Different Transplanted Cell Types to Cognition

Neuronal Replacement. The traditional goal of stem cell transplantation in the CNS was to replace dead or dysfunctional cells. In the substantial history of transplantation for Parkinson’s disease, this has meant trying to replace the dopamine-producing neurons of the Substantia Nigra where the disease pathology manifests in extensive neurodegeneration. To achieve this, studies have focused on the engraftment of fetal mesencephalic stem cells, which commonly develop a dopaminergic neuronal phenotype. Since the early 1980’s, dopaminergic precursor or dopamine-producing stem cells have been transplanted into the striatum of human patients, with some initial evidence of moderately improved motor control and decreased rigidity and tremor. However, there has been little to no reported impact of this approach on PD-associated cognitive deficits. In murine studies, transplantation of these cells into the substantia nigra often results in short term restoration of motor control, although effects on cognition have not been examined or reported (Barker, Barrett et al. 2013). Neuronal replacement clearly holds promise, but arguments can also be made for the transplantation of neural stem cells and progenitors of other specific cells types, such as astrocytes that might influence cognition via more indirect mechanisms (Figure 7).

Glial Precursor and Astrocyte Transplantation. The previously mentioned human glial progenitor transplantation study conducted by Han et. al. takes advantage of the supportive role of glia in the regulation of synaptic plasticity and neuronal function. Surprisingly, there have been few such studies that have explored the effects of glial stem cell transplantation on cognition or other brain function. Instead, most studies of
glial progenitor transplantation have focused on conditions that affect the spinal cord including amyotrophic lateral sclerosis (ALS) and spinal cord injury (Suzuki, McHugh et al. 2007); reviewed in (Nout, Culp et al. 2011, Toft, Tome et al. 2013). One exception is a study from over two decades ago that compared the capacity of fetal brain tissue and purified astrocytes to improve ethanol-induced cognitive deficits (Bruckner and Arendt 1992). This group found that astrocytes, but not fetal brain tissue grafts, could restore memory as assessed by the radial arm maze. Intracortical astrocyte grafts were associated with increased ChAT in the basal forebrain, which was thought to likely result from astrocyte-induced neurotrophic activity on cholinergic efferents. A few years later, it was reported that astrocyte transplantation into the cortex and hippocampus could also improve spatial memory in rats with basal forebrain lesions (Bradbury, Kershaw et al. 1995). However, this second group suggested that the effect of astrocytes were independent of cholinergic neurotransmission, as fetal brain tissue grafts were able to elevate ChAT activity but failed to improve memory. Instead, they proposed that astrocyte-induced cognitive improvements likely resulted from altered immune and trophic activity. Finally, recent developments in cell reprogramming research has identified that astrocytes may be a more useful parent cell type than the commonly used skin fibroblast (Tian, Wang et al. 2011). Tian and colleagues showed that reprogrammed mouse astrocytes retained a “memory” for their tissue origin, and a
greater potential for neuronal differentiation compared to fibroblasts. This same potential was identified in human astrocytes that were reprogrammed to neural stem cells and neurons (Corti, Nizzardo et al. 2012). Given the strong mechanistic evidence for the potential of transplanted astrocytes to improve cognition, astrocyte reprogramming may make these cells ideal candidates for IPSC transplantation studies (Table 2). Regardless of the precise mechanism by which astrocyte and glial precursor transplantation improves cognition, it seems that astrocyte and progenitor cells may provide a powerful approach to enhance cognition (Figure 8).

Table 2. Contribution of different stem cell lineages to cognitive function. For several stem cell lineages, impacted molecular mechanisms that correlate to changes in cognitive function have been reported. Only for some of these cell lineages has integration with endogenous circuitry been established. Check marks indicate that changes in the parallel signaling factors have been shown to correlate with cognitive function. n/d = not determined.

<table>
<thead>
<tr>
<th>Transplanted Stem Cell Lineage</th>
<th>Identified Signaling Mechanisms</th>
<th>Correlation with Cognitive Function</th>
<th>Circuitry Integration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neural stem cell</td>
<td>✓</td>
<td>✓</td>
<td>n/d</td>
</tr>
<tr>
<td>Astrocyte progenitor</td>
<td>✓</td>
<td>✓</td>
<td>n/d</td>
</tr>
<tr>
<td>Fetal mesencephalic</td>
<td>✓</td>
<td>n/d</td>
<td>✓</td>
</tr>
<tr>
<td>Embryonic stem cell</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>IPSC</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
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Neural stem cells and glial progenitors can also differentiate into oligodendrocytes, which generate myelin to insulate neuronal axons in the brain and promote the conduction of action potentials. The loss of oligodendrocyte precursor cells has been implicated in cognitive decline following ischemic injury in rats (Huang, Liu et al. 2009, Chida, Kokubo et al. 2011) and radiation therapy in humans (Monje and Dietrich 2012). Oligodendrocytes have also been proposed as a potential biomarker and therapeutic target for preventing cognitive decline in AD (Bartzokis, 2004). Specifically, it has been
noted that oligodendrocytic regulation of iron and membrane cholesterol in the brain may be disrupted by AD pathology, and predict cognitive decline (Kadish, Thibault et al. 2009). Though no oligodendrocyte fate-restricted transplant studies have been performed in the brain, several neural stem cell and glial progenitor transplant studies report that a large portion of these cells become oligodendrocytes which may contribute significantly to cell-mediated cognitive improvement (Kelly, Bliss et al. 2004, Blurton-Jones, Kitazawa et al. 2009). Therefore it is likely that these cells could also play an important role in enhancing cognition (Figure 8).

**Remaining challenges**

*Tumorigenesis.* Stem cell based strategies clearly offer a novel approach to enhance cognition in normal, aged, and disease brains. Yet these strategies are certainly not without risk. By definition, one of the key properties of stem cells is that they can replicate to create near-perfect copies of themselves. This property is not only inherent to stem cells but also to cancer cells. Indeed, there is growing evidence that cancer stem cells play a major role in tumorigenesis, metastasis, and recurrence (Baccelli and Trumpp 2012). Transplantation of stem cells or enhancement of endogenous stem cell populations therefore presents some risk of tumorigenesis. Once transplanted into the microenvironment of the brain, some stem cell populations appear to be unable to fully differentiate and instead continue to proliferate unchecked (Knoepfler 2009). This risk of tumorigenesis appears to be quite dependent on the type and maturation of a given stem cell. Pluripotent stem cells for example can readily form teratomas when transplanted into an immune-deficient host (Gutierrez-Aranda, 2010). Embryonic stem
cell derived neural stem cells can also form tumors although this is dramatically
influenced by their maturation state and more extensive pre-differentiation protocols
reduce tumorigenesis (Fukuda, Takahashi et al. 2006, Seminatore, Polentes et al.
2010). In contrast, there are very few reports of tumorigenesis following fetal-derived
neural stem cells transplantation (Geny, Naimi-Sadaoui et al. 1994). Transplantation
therapies that utilize more fully differentiated and restricted progenitors are therefore
likely to provide a safer approach.

Yamanaka’s groundbreaking work establishing the methods to generate induced
pluripotent stem cells (iPSCs) provided a novel source of pluripotent stem cells that
circumvented many of the ethical concerns regarding fetal and embryonic stem cell
research (Takahashi and Yamanaka 2006). However, it appears that iPSCs may be
more prone to tumorigenesis than other stem cells. Many of the genes used to induce
pluripotency are either established oncogenes (i.e., Myc and KLF4) (Wei, Kanai et al.
2006, Yamanaka 2007), or are variably associated with tumorigenesis (i.e., Sox2,
2008). The rapid adoption of non-integrating approaches for iPSC generation will likely
resolve many of these issues. However, iPSCs derived from aged cell sources will
carry increased numbers of de novo DNA mutations that could still promote
tumorigenesis. The majority of stem cell transplant studies reporting occasional
teratoma formation leave the issue largely unresolved. However, there are many
strategies that could likely mitigate the risk of tumorigenesis (reviewed in (Knoepfler
2009)). For example, introduction of a stem cell-specific suicide gene or specifically
targeted chemotherapeutic agent could be used to eliminate stem cells that fail to terminally differentiate. Pre-differentiation of cells toward more mature lineages will likely also greatly diminish risk.

**Delivering Stem Cells to the Brain.** One of the major challenges to the potential use of stem cell based therapies for neurological conditions is the difficulty of delivering stem cells to the brain. Several approaches have been examined including intravenous, intrarterial, intranasal, and direct intraparenchymal injection. Yet, the great majority of data suggests that of these; only direct intraparenchymal transplantation achieves meaningful engraftment of stem cells within the brain (Yamasaki, Blurton-Jones et al. 2007, Ebert, Beres et al. 2008, Blurton-Jones, Kitazawa et al. 2009, Andres, Horie et al. 2011, Redmond, McEntire et al. 2013). Venous delivery for example leads primarily to an accumulation of injected stem cells within the lungs with no cells reaching the brain (Pendharkar, Chua et al. 2010). As one might expect, injection into the carotid artery can increase the number of cells reaching the brain, although only very few cells transmigrate across the endothelial blood-brain barrier into the brain parenchyma (Pendharkar, Chua et al. 2010). Intranasal delivery offers an intriguing non-invasive approach to deliver stem cells to the brain. In theory, cells might be able to passage through foramina within the cribriform plate, a part of the skull that allows olfactory nerves to pass into the nasal epithelium. A few reports have tested this approach and while some stem cells may reach the brain via intranasal delivery, the numbers appear to be extremely low (Danielyan, Schafer et al. 2009, Reitz, Demestre et al. 2012). One promising new approach is to couple carotid artery stem cell injection
with MRI-guided focused ultrasound. The ultrasound temporarily disrupts the blood brain barrier within a specific brain region allowing neural stem cells to pass from a local cerebral artery into the brain parenchma (Burgess, Ayala-Grosso et al. 2011). These new methods may one day provide a promising less invasive approach to deliver stem cells to the brain. However, thus far direct stereotactic intraparenchmal injection remains the optimal approach to achieve robust stem cell engraftment within the brain (Yamasaki, Blurton-Jones et al. 2007, Ebert, Beres et al. 2008, Blurton-Jones, Kitazawa et al. 2009, Andres, Horie et al. 2011, Redmond, McEntire et al. 2013).

Conclusions

From decades of research across many subfields, key molecular mechanisms involved in the cognitive benefits of stem cells have been identified. Interestingly, one fairly consistent finding is that stem cells often influence cognition and disease via multiple mechanisms (Figure 8). Clearly, a great deal of additional research is needed to determine whether increasing endogenous neurogenesis or directly transplanting stem cells into the brain can be safely adapted to clinical use. It also remains to be determined exactly which aspects of human cognition may or may not be influenced by stem cell based therapies. Nevertheless, stem cell research may one day uncover promising new approaches to enhance human cognition.
CHAPTER TWO
NEURAL STEM CELLS RESCUE COGNITIVE AND MOTOR DYSFUNCTION IN A TRANSGENIC MODEL OF DEMENTIA WITH LEWY BODIES THROUGH A BDNF-DEPENDENT MECHANISM

ABSTRACT
Accumulation of α-synuclein (α-syn) into insoluble aggregates occurs in several related disorders collectively referred to as synucleinopathies. To date, studies have used neural stem cells (NSCs) to examine questions about α-syn propagation, but have overlooked the therapeutic potential of NSC transplantation to modulate cognition in disorders such as Dementia with Lewy bodies or Parkinson’s disease dementia. Here, we show that striatal transplantation of NSCs into aged α-syn transgenic mice significantly improves performance in multiple cognitive and motor domains. This recovery is associated with NSC-expression of brain derived neurotrophic factor (BDNF), which restores depleted levels and modulates dopaminergic and glutamatergic systems. Most importantly, transplantation of BDNF-depleted NSCs fails to improve behavior, whereas AAV-mediated BDNF delivery mimics the benefits of NSC transplantation, supporting a critical role for this neurotrophin in functional improvement. Thus, NSC transplantation could offer a promising new approach to treat the understudied yet devastating cognitive components of many synucleinopathies.

INTRODUCTION
Dementia with Lewy Bodies (DLB) is the second most common cause of age-related dementia, affecting over 1.3 million people in the US alone (Vann Jones and O'Brien
DLB is associated with the accumulation of insoluble aggregates of the presynaptic protein, α-syn within the cortex, hippocampus, and brainstem that leads to progressive neurodegeneration and impairments in cognition and spontaneous mild parkinsonism (Mayo and Bordelon 2014). DLB is closely related to a second disorder, Parkinson’s disease dementia (PDD) that develops in up to 70% of Parkinson’s disease (PD) patients (Dubois and Pillon 1997, Marsh and Blurton-Jones 2012). Current treatments for DLB and PDD are limited and provide only modest symptomatic relief, thus there is a pressing need to identify new and effective therapies.

Unfortunately, the impact of stem cell transplantation in models that develop α-syn pathology has thus far only been examined in terms of cell-to-cell transmission of pathology (Desplats, Lee et al. 2009, Hansen, Angot et al. 2011). In contrast, many studies have demonstrated promising improvements in motor function by transplanting dopaminergic precursors in neurotoxin models of PD (Lees and Smith 1983, Docherty and Burn 2010). However, cognitive deficits in DLB/PDD are strongly associated with α-syn and neurotoxin models fail to mimic this important phenotype. It therefore remains critical to examine the therapeutic potential of stem cell transplantation in the presence of α-syn pathology and to better understand the impact of cell transplantation in models of DLB and PDD.

In this context, neural stem cells (NSCs), which can migrate and produce high levels of neurotrophic factors, may offer a promising alternative to DA precursor transplantation. Here, we utilized a human α-syn expressing mouse model that recapitulates many of the salient features of DLB/PDD, including the progressive development of Lewy body pathology.
pathology and significant cognitive dysfunction (Masliah, Rockenstein et al. 2000, Amschl, Neddens et al. 2013). Using these mice, we investigated the migration and differentiation of transplanted NSCs within the striatum, and their impact on behavior, α-syn pathology, and dopaminergic and glutamatergic regulation. Our results reveal that NSC transplantation can dramatically improve both motor and cognitive function by elevating levels of brain-derived neurotrophic factor (BDNF), a protein implicated in DLB/PDD cognitive impairments (Leverenz, Watson et al. 2011). Pharmacological manipulations implicate both dopaminergic and glutamatergic circuits downstream of BDNF in this recovery. Furthermore, shRNA-mediated loss-of-function studies confirm the necessity of BDNF in this process, and AAV-mediated gain-of-function experiments demonstrate that BDNF can mimic the benefits of NSC transplantation. Collectively, our studies reveal that NSCs can improve both the motor and cognitive symptoms of DLB/PDD in a progressive transgenic model by elevating levels of BDNF and enhancing dopaminergic and glutamatergic function.

EXPERIMENTAL PROCEDURES

Animals. All procedures were performed in strict accordance with the University of California, Irvine animal use regulations and the NIH guide for the Care and Use of Laboratory Animals. ASO mice have been previously characterized, and are maintained on a purebred C57B6/J background by breeding heterozygous ASO mice with wild-type C57B6/J mice (Masliah, Rockenstein et al. 2000, Amschl, Neddens et al. 2013). All mice were housed on a 12 h light/dark schedule with ad libitum access to food and water.
Neural Stem Cell and AAV Preparation, and Stereotactic Surgery. Mouse NSCs: Hippocampal/cortical GFP-mNSCs were microdissected from GFP-transgenic syngeneic C57B6/J mice as previously described (Mizumoto, Mizumoto et al. 2003). GFP-mNSCs were isolated at age P1 in order to achieve a primarily gliogenic cell phenotype, promoting differentiation of these NSCs into support cells and not neuronal subtypes (i.e., dopaminergic, serotonergic). BDNF<sup>shRNA</sup> GFP-mNSCs were generated as previously described, using lentiviral delivery and stable selection of a shRNA construct targeting murine BDNF (Blurton-Jones, Kitazawa et al. 2009). Twelve month old ASO and WT mice were randomly assigned to a treatment group and either vehicle or 50,000 mNSCs/site (1μl volume, 2 sites per hemisphere) were transplanted bilaterally into the dorsal striatum (Bregma +0.03 AP, ± 2.0 ML, -3.0 and -3.5 DV). mNSCs remained on ice for the duration of the transplantation procedure and retained 89-94% viability. Mice were anesthetized with isoflurane, placed in the stereotax and injected with either 100,000 mNSCs per site (or vehicle (1x HBSS with 20 ng/mL hEGF) as a control treatment using a 5 μL Hamilton microsyringe (30-gauge) and an injection rate of 0.5 μL/min. Cells were delivered at two DV sites (1μl/site) per hemisphere to facilitate distribution of mNSCs throughout the dorsal and ventral medial striatum. The needle was retained within the injection site for 4 minutes before slowly removing the needle to reduce potential backflow of cells along the needle tract. For AAV experiments, AAV2-mCherry or AAV-mCherry-BDNF (human full length) were injected bilaterally at the same striatal coordinates (1μl of 1.5 x10<sup>15</sup> viral particles/site; Vector Biolabs). Accurate injection to the targeted region was confirmed by visualization of the needle tract and GFP cells or mCherry within coronal brain sections.
**Pharmacological Studies.** Mice were administered intraperitoneal injections of the GLT-1 inhibitor dihydromainic acid (DHK; 10 mg/kg/day; Tocris Bioscience, Briston, UK; (Gunduz, Oltulu et al. 2011, Gunduz, Oltulu et al. 2011). Mice recovered for 2 days following stereotactic surgery; thereafter, DHK was administered once daily until the time of sacrifice. Intraperitoneal α-methyl-p-tyrosine methyl ester hydrochloride (αMT; 100 mg/kg/day; Sigma Aldrich, St. Louis, MO, USA; (Kelly, Rubinstein et al. 1998, Sotnikova, Beaulieu et al. 2005, Rung, Rung et al. 2011) was administered daily beginning 10 days prior to sacrifice (i.e., during behavioral training and assessment). Assessment of safety αMT was conducted in our laboratory as described.

**Cognitive and Motor Behavioral Timeline & Assessment.** Thirty days following transplantation, behavioral assessments began lasting 10 days. On day 41, brains were harvested for histological and biochemical processing. All behavioral testing and analysis was performed blinded to treatment and genotype groups using an identification system decoded during statistical analysis. Cortical- and hippocampal-dependent memory tasks followed standard protocols for NOR and novel place recognition (NPR; (Bermudez-Rattoni, Okuda et al. 2005, Barker, Bird et al. 2007, Balderas, Rodriguez-Ortiz et al. 2008, McNulty, Barrett et al. 2012). Briefly, mice were allowed to explore the object-free recognition chamber with only bedding for 5 minutes, 3 consecutive days. On the 4th day, mice were trained for 10 minutes in the presence of 2 identical objects. Twenty-four hours later, one object was replaced with a novel one (NOR), or one object was moved to a novel location in the chamber (NPR) and animals were allowed to explore for 5 minutes. Object exploration was analyzed as degree of discrimination between novel and familiar objects \[\text{Time}_{\text{novel}} - \text{Time}_{\text{familiar}}\]
Time_{familiar}/(Time_{novel}+Time_{familiar})*100]. Importantly, it was determined that mean distance traveled, mean ambulatory speed and time spent investigating objects during the training period were not altered by either genotype or treatment for any of the reported studies. Animals that lacked exploratory behavior (< 4 seconds total exploration) were excluded. Mice were next tested on pole descent, Rotarod and beam traversal tasks. For Rotarod, mice were trained for 5 consecutive trials at 12 rpm to stay on the fixed speed Rotarod for 2 min (Ugo Basile, Italy). Twenty-four hours later, mice are tested at 12 rpm for 2 min and latency to fall off of the rotating rod is measured. The pole test has been used previously to assess basal ganglia-related movement deficits in mice, finding that Parkinsonian mice display slower performance on this task than controls (Matsuura, Kabuto et al. 1997, Sedelis, Schwarting et al. 2001, Fernagut, Chalon et al. 2003, Fleming, Salcedo et al. 2004). Briefly, mice are placed head-up atop a vertical pinewood pole (50 cm high, 1 cm diameter), which is based in a cage of bedding and then the time required to rotate into a head down position and descend the pole is measured. Finally, mice were tested on the beam traversal challenge as modified from Fleming et al 2004. Mice were trained for 2 days to traverse a narrowing Plexiglas beam to reach their home cages. On day 3, a mesh grid was placed over the beam, and mice were videotaped traversing from base to apex. The number of total steps and foot-faults (forelimb slipping through the wire grid) was counted by a blinded observer from slow motion video.

**Biochemical Analyses.** Six weeks after transplantation, mice were sacrificed by Euthasol and transcardial perfusion with 0.01 M phosphate-buffered saline (PBS). The left side of the brain was flash frozen for subsequent biochemical analysis. The right
hemisphere was post-fixed in 4% paraformaldehyde, and sectioned on a microtome (40 µm, coronal) for immunohistochemical analyses. Half brain dorsal striatum, were microdissected. Each region was then processed to isolate mRNA and protein via Trizol (Life Technologies, Inc., Carlsbad CA) extraction. Both soluble and insoluble (Sarkosyl soluble) protein fractions were collected. Protein concentrations were determined via Bradford assay and normalized samples compared via SDS-PAGE Western blot. Relative signal intensity of grayscale images were then be quantified by Image J and once all values were obtained sample identification was decoded.

**Immunofluorescent Labeling.** Fluorescent immunohistochemistry followed previously described protocols (Blurton-Jones, Kitazawa et al. 2009). Briefly, coronal brain sections were rinsed 3 times in 0.01 M PBS, then placed into blocking solution (0.01 M PBS+0.02% Tx100+5% Goat serum) for 1 hr at room temperature. Primary antibodies were then diluted in fresh blocking solution and applied overnight at 4°C. Sections were then rinsed in PBS and incubated for 1 hr at room temperature in appropriate highly-cross absorbed Alexa Fluor secondary antibodies (Life Technology). Following 3 additional rinses, sections were mounted on slides and cover-slipped using Fluoromount G (Southern Biotech). The following primary antibodies were used:

Dopaminergic antibodies: tyrosine hydroxylase (Millipore, #ab152), pTH ser31 (Cell Signaling, #3370), pTH ser19 (PhosphoSolutions, #p1580-19). Neurotrophin Antibodies: BDNF (Santa Cruz Biotech., # sc-546), TrkB (Cell Signaling, #4603), GDNF (Santa Cruz Biotech., #sc-328), phosphor-Erk1/2 (Cell Signaling, #9101).

Glutamatergic Antibodies: GLT-1 (Abcam, #ab106289), VGLUT1 (Synaptic Systems, #135303), VGLUT2 (Synaptic Systems, #135403), GLAST (Novus Biologicals, #NB100-
Differentiation Antibodies: GFAP (Millipore, #MAB260), DCX (Abcam, #ab18723), Olig2 (Millipore, #MABN50).

Confocal Microscopy and Quantification. Sections were imaged in a blinded manner using an Olympus Fluoview FV1200 confocal microscope. For analysis of human α-synuclein inclusions, sections were double-labeled for human and endogenous mouse α-synuclein. Relative signal intensity of grayscale images were then be quantified by Image J and once all values were obtained sample coding was revealed.

Statistical Analysis. All animals were randomly assigned to treatment groups based only on wild-type or transgenic genotype and sex. Animal identification codes were randomly generated in order to keep the researcher blind to genotype and treatment throughout the behavioral testing, scoring, and statistical analysis. All statistical comparisons were performed using Statview 5 software. Comparisons between multiple groups utilized two-way analysis of variance (ANOVA) followed by Fisher’s PLSD post hoc tests, with the exception of ASO-BKC studies which utilized one-way ANOVA due to the unmatched group design. Differences are considered significant when $p<0.05$ for both ANOVA and post hoc tests.

RESULTS

NSC transplantation rescues both motor and cognitive deficits in α-synuclein transgenic mice

In order to assess the impact of NSC transplantation on h-α-syn associated cognitive and motor dysfunction, we stereotactically injected haplotype-matched murine NSCs or vehicle, bilaterally into 12-month old h-α-syn over-expressing (ASO) mice or wild-type
(WT) littermates (100,000 cells/side, n=6-8). At this age, ASO mice exhibit widespread Lewy body-like pathology as well as substantial motor and cognitive impairments (Masliah, Rockenstein et al. 2000, Amschl, Neddens et al. 2013). In order to achieve the greatest impact of transplantation on both cognitive and motor systems that are altered by α-syn accumulation, we targeted the dorsal striatum (Figure 10A), widely known to be involved in learning and memory, and highly interconnected with multiple cortical regions. We used previously characterized GFP-expressing NSCs (Mizumoto, Mizumoto et al. 2003, Blurton-Jones, Kitazawa et al. 2009). One month after transplantation, mice were habituated, trained and tested on 3 motor tasks and 2 cognitive tasks, and engraftment was assessed.

As expected, vehicle-treated ASO (ASOV) mice showed significant impairments in all three motor tasks compared to vehicle-injected WTs (WTV). The ASOV mice took twice as long to reorient before descending on the pole task (Figure 10B, $F_{(3, 18)}=3.9; \text{ANOVA } p=0.03$). Likewise, ASOV mice fell of the Rotarod in half the time of WTV controls (Figure 10C, $F_{(3, 18)}=9.5; \text{ANOVA } p=0.0006$) and exhibited an 83% increase in foot faults in the beam traversal task (Figure 10D, $F_{(3, 18)}=12, \text{ANOVA } p=0.0002$). In contrast, ASO mice that received NSC transplants (ASOC) performed similarly to both WTV and NSC-injected WT mice (WTC) in all three motor tasks, demonstrating that NSC transplantation can dramatically improve motor function in transgenic α-synuclein mice.
Figure 10. Neural stem cell transplantation improves both motor and cognitive function in α-synuclein (ASO) transgenic mice. α-synuclein-overexpressing (ASO) and WT mice were aged to 12 months, then syngeneic GFP-NSCs were transplanted bilaterally into the striatum; one month later, motor and cognitive behavior were assessed (A). ASO vehicle treated controls (ASOV) show deficits in Pole reorientation (B), Rotarod performance (C), and beam traversal (D) tasks which were significantly ameliorated by NSC transplantation (ASOC). ASOV mice also showed significant deficits in cortical-dependent novel object recognition (NOR; E) and hippocampal-dependent novel place recognition (NPR; F), which were improved by NSC transplantation (ASOC). GFP-NSCs (green) migrated from their medial striatal injection sites and engrafted predominantly within the striatum (G). The majority of NSCs differentiated into astrocytes (H; GFAP, red; 28.9%) and immature oligodendrocytes (H; Olig2, red; 29.6%). Whereas, a smaller proportion of transplanted NSCs adopted an early neuronal phenotype (H; DCX, red; 7.9%). No differences in NSC engraftment or differentiation were detected between WTC and ASOC groups (I). Immunohistochemical analysis also revealed no differences in either cortical or striatal (shown) human α-synuclein inclusion number between ASOV and ASOC mice, and no inclusions were detected in WT mice (J; Fisher’s PLSD post hoc \textit{p}<0.003). Similarly, Western blot analysis of striatal monomeric h-α-syn (Syn211) was not significantly altered by NSCs (K). Data presented as mean ± S.E.M. ANOVA \textit{p}<0.05 and Fisher’s PLSD post hoc* \textit{p}<0.0001 compared to all other groups. Scalebar = 100µm (B), 10 µm (C,D).
To examine cognition in a model with motor impairments, it is critical to use tasks that are not heavily influenced by motor function. We therefore utilized Novel Object Recognition (NOR) and Novel Place Recognition (NPR) tasks; low-stress paradigms that quantify the proportion of time spent examining a novel object and provide data on cortical-dependent and hippocampal-dependent memory, respectively. All four groups were habituated, trained, and tested following standard protocols (McNulty, Barrett et al. 2012). Twenty-four hours after training, mice were exposed to a novel object or novel object placement, and the discrimination ratio between exploration time of old and new objects was calculated. As shown, ASOV mice exhibited significant impairments in NOR compared to WTV and WTC groups, demonstrating that \(h-\alpha\)-syn overexpression can model important aspects of DLB/PDD-related cognitive impairment (Figure 10E; \(F_{(3, 18)}=19.3\); ANOVA \(p=0.0001\)). Striatal transplantation of NSCs dramatically improved performance in this cortical-dependent task, as ASOC mice discriminated between novel and familiar objects to a greater degree than either WTV or WTC mice, indicating not only a benefit, but enhancement in this task. In the hippocampal-dependent NPR task, ASOV mice again showed significant impairments that were also rescued by NSC transplantation (Figure 10F; \(F_{(3, 18)}=21\); ANOVA \(p=0.0001\)). Because we found no differences in activity during training (data not shown), this confirmed that \(h-\alpha\)-syn expression and NSC transplantation alter memory performance independent of effects on motor function. Thus, we show for the first time that NSC transplantation can rescue both motor and cognitive deficits in a transgenic model of DLB/PDD.
NSCs migrate throughout the striatum and into the cortex, but do not reach the hippocampus

To begin to decipher the mechanism by which GFP-NSC transplantation ameliorates behavioral deficits, we examined the migration and differentiation of engrafted cells. Confocal microscopy demonstrated that 6 weeks following transplantation, GFP-NSCs had migrated throughout the striatum (Figure 10G). A modest number of cells were also observed within the motor and perirhinal cortices and amygdala, and in a few animals some cells even reached the substantia nigra (data not shown). However, no GFP-NSCs were detected within the hippocampus of any animals. Engrafted NSCs differentiated primarily into astrocytes and immature oligodendrocytes, with only a few cells exhibiting an early neuronal fate (Figure 10H, I and Figure 11). No examples of fully matured GFP/NeuN double-labeled neurons were detected (Figure 11). Taken together these findings are consistent with previous reports that murine NSCs predominantly acquire gliogenic phenotypes when transplanted into non-neurogenic regions, and with other studies that utilized these postnatal day 1-derived cells.
Figure 11. Transplanted GFP-NSCs survive and differentiate after 6 weeks. Low-magnification confocal images demonstrate that GFP-NSCs can adopt early oligodendrocytic (Olig2), glial (GFAP) or neuronal (DCX) phenotypes, but do not yet express a more mature neuronal marker (NeuN). Scalebar=50 μm.
Interestingly, no significant differences in NSC differentiation were detected between ASO and WT transplanted mice (Figure 10I) and each cell type migrated with similar distribution and distance from the injection site (Figure 11A). Finally, we examined markers of undifferentiated and proliferating cell types, finding that a small number of NSCs that had migrated beyond the dorsal striatum still expressed the mitotic marker Ki67, or immature NSC markers Nestin or Vimentin (Figure 14A-C).

Figure 12. Transplanted GFP-NSCs that express markers of multipotency and proliferation are infrequent and distal to the injection site. Endogenous cells near the lateral ventricle (LV) express the proliferation marker Ki67, but GFP-NSCs in the dorsal striatum do not (A). However rare examples of GFP cells that express the mitotic marker Ki67 are observed in areas that are distal to the injection site a (A; Scalebar=100 μm, 20 μm, 10 μm). Distally migrating NSCs also occasionally express Nestin (B) or Vimentin (C). Scalebar=10 μm B,C.
Human α-synuclein pathology is not altered by NSC transplantation.

Previous studies have shown that reduction of α-syn can improve motor function in α-syn transgenic mice (Masliah, Rockenstein et al. 2011). We therefore examined the effects of striatal NSC transplantation on α-syn pathology in multiple brain regions. Using a human-specific antibody we first confirmed that h-α-syn protein was expressed exclusively in ASO mice. Interestingly, we found no effect of NSC transplantation on h-α-syn as both ASOV and ASOC groups exhibited equivalent numbers of Lewy-body-like inclusions within the dorsal striatum (Figure 10J). Further quantification of both detergent-soluble and insoluble h-α-syn by Western blot in the striatum (Figure 10K), hippocampus, perirhinal, motor and prefrontal cortices, and the substantia nigra also confirmed that NSCs had no effect on monomeric h-α-syn (Figure 14A-F). Likewise, monomeric α-syn phosphorylated at serine 129 (pS129), a pathological associated epitope (Walker, Lue et al. 2013) was also equivalent between NSC and vehicle injected ASO mice (Post hoc Fishers’s PLSD, p=0.14; data not shown).

NSC-induced cognitive and motor improvements are accompanied by increased BDNF

Having found that NSCs had no impact on α-synuclein pathology, we began to explore other potential mechanisms that could be involved in NSC-mediated motor and cognitive improvements. We previously showed that these GFP-NSCs can produce high levels of specific growth factors including BDNF (Blurton-Jones, Kitazawa et al. 2009). Western blot analysis revealed that striatal BDNF expression in ASOV mice was half of that in WTV controls, but was fully restored to WT levels in ASOC mice by NSC transplantation (Figure 13A,B; \(F_{(3, 18)}=3.8\); ANOVA \(p=0.03\)). NSCs did not increase
Figure 13. NSC transplantation enhances striatal BDNF signaling with downstream effects on dopamine and glutamate systems. Western blot analysis revealed that striatal BDNF was significantly decreased in ASOV mice but restored to WT levels by NSC transplantation (A,B). Downstream of BDNF, phosphorylated Erk1 was significantly elevated by transplantation suggesting that NSCs enhance striatal neurotrophic signaling (A,C). Transplanted cells continue to express BDNF, primarily within GFAP or Vimentin co-labeled astrocytic cells (D). Activated forms of TH (p-ser31 and p-ser19) were significantly decreased in ASOV mice and elevated by transplantation (E-G). The DA receptor type D2 was also decreased in ASOV mice, and elevated in ASOC mice (E,H). Confocal optical densitometry confirmed significantly reduced expression of TH in ASOV striatum that was restored toward WT levels in ASOC mice (I,J). Striatal VGLUT-1 was also significantly decreased in ASOV mice and restored to WT levels by NSCs (K,L). NSC transplantation also elevated striatal levels of GLT-1, but not EAAC1 and GLAST (K,M). Data presented as mean ± S.E.M. All Western blot graphs presented as % of WTV group. Fisher’s PLSD post hoc *p<0.03 compared to all other groups, WTV, ASOV p<0.01, WTC, ASOC p<0.004. Scalebar=10 µm (D), 30 µm (I).
BDNF in WTC mice, likely due to neurotrophic self-regulatory mechanisms (Canossa, Griesbeck et al. 1997, Righi, Tongiorgi et al. 2000, Bambah-Mukku, Travaglia et al. 2014). Importantly, the BDNF receptor TrkB was unaltered by either genotype or treatment (Figure 14G,H), suggesting that increased BDNF could readily influence behavior via the existing TrkB receptor population. In contrast, no difference in expression of glial-derived neurotrophic factor (GDNF; Figure 14G-J) or its phosphorylated receptor RET were observed between any treatment groups, indicating that changes in BDNF specifically play an important role in α-syn transgenic mice. In addition to the detected changes in BDNF, phosphorylation of Erk1 (44 kDa), a major downstream effector of BDNF signaling, was also increased by NSC transplantation in both ASOC and WTC mice (Figure 13A,C; F(3, 18)=4.2; ANOVA p=0.02). We confirmed that transplanted NSCs continue to express BDNF primarily in cells co-expressing GFAP or Vimentin (Figure 13D), suggesting that glial-fated NSCs may contribute substantially to increasing BDNF levels. Thus, NSC-mediated changes in BDNF appear to influence growth and plasticity-associated signal transduction within the striatum.

**NSC transplantation alters Dopaminergic and Glutamatergic systems**

To further understand the mechanism by which NSC-derived BDNF ameliorates motor and cognitive behaviors, we examined changes in expression of the rate-limiting enzyme in DA synthesis, tyrosine hydroxylase (TH), as well as markers of glutamate transport and regulation. First, we utilized a well-established optical signal intensity analysis to examine TH terminals within the striatum (Fernagut, Hutson et al. 2007). As expected, we detected a significant reduction in TH-immunoreactive terminals within the
dorsal striatum of ASOV mice relative to WTV controls (Figure 13I,J). More importantly, there was a significant partial restoration of TH density in ASO mice that received NSCs. In contrast, we confirmed as previously reported that TH immunoreactive neurons in the substantia nigra pars compacta were not altered by h-α-syn in this model or by NSC transplantation (Masliah, Rockenstein et al. 2000, Rockenstein, Mallory et al. 2002, Fleming, Salcedo et al. 2006) (Figure 14L). To follow up on these findings, we performed biochemical analysis of TH as well as two phospho-epitopes of TH (serine-19 and serine-31) that are associated with increased activity and dopamine production. Consistent with our immunohistochemical data, TH phosphorylated at either site was significantly increased in ASOC mice, suggesting that NSCs elevate TH activity (Figure 13E,F,G; F(3, 18)=5.2; ANOVA p=0.01). In further support of this effect, expression of the DA receptor D2 was also significantly increased with NSCs (Figure 13E,H; F(3, 18)=6.4; ANOVA p=0.03). These data strongly suggest that NSC transplantation enhances the function and signaling of existing nigrostriatal dopaminergic neurons.

We next examined the expression of glutamate transporters within the striatum to determine whether α-syn expression or NSC transplantation might influence corticostriatal and hippocampal-striatal glutamatergic projections (Pennartz, Ito et al. 2011). BDNF is known to impact glutamate signaling and activity to enhance plasticity (Gottmann, Mittmann et al. 2009), and previous studies have strongly implicated glutamatergic dysfunction in PD (Greenamyre 1993, Albin, Young et al. 1995); yet, the
Figure 14. Effects of NSC transplantation on α-synuclein, neurotrophins, and tyrosine hydroxylase. Western blot analysis of h-α-syn (Syn211) in 5 microdissected brain regions was not significantly altered by NSCs (A-F). Complementary to NSC-induced BDNF expression in the striatum, expression its receptor TrkB was unaltered by genotype or treatment (G-H). Neither GDNF nor its activated receptor phospho-RET, commonly associated with changes in DA regulation, were affected by genotype or treatment (G,I,J). Representative images of TH demonstrate striatal downregulation due to h-α-syn (K), and confirm no loss of SNpc DA cells (L). Data are represented as mean ± S.E.M. All Western blot graphs are represented as % of the ASOV group. Fisher’s PLSD post hoc $^{WTW,WTC}p<0.0001$. 
potential therapeutic relevance of glutamatergic systems to synucleinopathies and especially DLB/PDD-associated cognitive dysfunction has been largely unexplored. Corticostriatal glutamatergic projections can be readily identified and examined via expression of vesicular glutamate transporter 1 (VGLUT-1). When levels of VGLUT-1 within the dorsal striatum were quantified, we found a significant 35% decrease in ASOV mice versus WTV controls. More importantly, this reduction was restored to normal levels by NSC transplantation (Figure 13K,L; $F_{(3,18)}=3.3$; ANOVA $p=0.04$). In contrast, thalamostriatal specific VGLUT-2 expression was unchanged, suggesting NSCs have specific effect on corticostriatal glutamatergic systems (Post hoc Fishers’s PLSD $p=0.1$, data not shown).

Another glutamate transporter implicated in PD is the glial-specific transporter GLT-1 (Massie, Goursaud et al. 2010, Salvatore, Davis et al. 2012), which plays an important role in multiple aspects of neuronal plasticity and can be up-regulated by BDNF (Huang, Sinha et al. 2004, Pita-Almenar, Zou et al. 2012). Although GLT-1 levels were unaltered between WTV and ASOV groups, we found that NSC transplantation significantly increased GLT-1 expression in both ASOC and WTC groups (Figure 13K,M; $F_{(3,18)}=7.6$; ANOVA $p=0.002$). By comparison, no changes were detected in the two other glutamate transporters expressed by both neurons and glia, EAAC1 or GLAST (Figure 13K). Together these data show that corticostriatal glutamatergic systems are indeed altered in α-syn transgenic mice and that NSC transplantation can modulate astrocyte-dependent corticostriatal glutamatergic systems.
Changes in BDNF, TH and GLT-1 correlate with distinct improvements in motor and cognitive tasks

The dramatic reduction in BDNF induced by h-α-syn expression and the corresponding elevation of BDNF following NSC transplantation suggest that this neurotrophin plays an important role in NSC-mediated behavioral improvements. Supporting this hypothesis, striatal BDNF expression in individual mice correlates well with NPR performance ($R^2=0.48; p=0.005$), and inversely to beam traversal faults (Figure 15; $R^2=0.60; p=0.0001$). Potentially downstream of BDNF, TH expression is also correlated strongly with beam traversal performance, but less so with hippocampal-dependent NPR. Conversely, GLT-1 was significantly associated with NPR but showed a less consistent

![Figure 15. BDNF expression correlates with both motor and cognitive function, whereas TH and GLT-1 show distinct associations with motor and cognitive function.](image-url)

To begin to ascertain the role of NSC-mediated alterations in behavioral recovery, BDNF, TH and GLT-1 were compared to cognitive (NPR) and motor (beam faults/step) performance in ASO and ASOC mice. BDNF was highly correlated with improvements in both behaviors. However, while TH was more closely correlated with motor behavior, GLT-1 was more significantly associated with cognitive function, suggesting that dopaminergic and glutamatergic systems play key roles in motor and cognitive recovery respectively. Data points represent within-subject means of individual animals $p<0.05$. X-axis: protein levels are represented as % of WTV; Y-axis: Beam Faults/Step are numerical ratios, NPR DI values are discrimination index ratios
association with beam traversal performance (Figure 15). To further examine the potential divergent role of these systems in motor and cognitive recovery we employed pharmacological loss-of-function approaches using the DA synthesis inhibitor α-methyl-p-tyrosine (αMT) (Brogden, Heel et al. 1981) and GLT-1 inhibitor dihydrokainic acid (DHK) (Bridges, Kavanaugh et al. 1999). Safe and optimal doses were determined by dose response studies (Figure 16). A new set of ASO mice were then transplanted with

![Figure 16. Safety and dose-response experiments for pharmacological studies.](image)

Prior to experiments, dose response studies determined safe and optimal doses of the TH inhibitor α-methyl-p-tyrosine (αMT, 100 mg/kg in 0.9% NaCl) (A). In addition, the safe dose of GLT-1 inhibitor dihydrokainic acid (DHK, 10 mg/kg in 0.01 M PBS) was determined based on lack of weight change (B). The appropriate DHK dose resulted in a significant compensatory change in expression of the glutamate EAAC1 transporter (C).

NSCs with or without co-treatment with αMT (ASOC-αMT) or DHK (ASOC-DHK) (Figure 17A). One month after transplantation, behavioral testing was again performed. In Rotarod and beam traversal motor tasks, αMT prevented NSC-induced recovery; however, ASOC-DHK mice still exhibited improved performance (Figure 17B,C; $F_{(3, 20)}=9.6$; ANOVA $p=0.0004$; $F_{(3, 20)}=3.9$; ANOVA $p=0.03$). These data further support the notion that while TH phosphorylation is clearly necessary for NSC-mediated motor improvements, changes in striatal glutamate reuptake via GLT-1 are not. Intriguingly, NSC-induced recovery of NOR was blocked by both αMT and DHK.
treatments in ASOC mice (Figure 17D; $F_{(3, 20)}=13.3$; ANOVA $p=0.0001$), indicating that both GLT-1 and TH activity are important for NSC-mediated improvements in cortical-dependent memory. In contrast, while DHK prevented NSC-mediated improvements in NPR, αMT co-treatment had no effect on this hippocampal-dependent task, as ASOC-αMT mice performed equivalently to saline-injected ASOC mice (Figure 17E; $F_{(3, 20)}=8.2$; ANOVA $p=0.009$). Taken together, these findings indicate that NSC-induced increase in GLT-1 is critical for both NOR and NPR, but that NSC-mediated DA regulation contributes primarily to motor improvements and NOR but not to hippocampal-dependent NPR.

Importantly, BDNF expression was unaltered by either αMT or DHK treatments, remaining elevated in all three NSC-transplanted groups (Figure 17F,G; $F_{(3, 20)}=6.3$; ANOVA $p=0.004$), suggesting that the observed changes in DA and glutamate systems occur downstream of BDNF signaling. In support of this, TH ser31 phosphorylation was decreased similarly in both ASOV and ASOC-αMT mice but remained elevated in saline- or DHK-injected ASOC mice (Figure 17F,H,I; $F_{(3, 20)}=5.4$; ANOVA $p=0.007$).

These experiments further confirmed the importance of NSC-induced changes in both DA and glutamate systems and their role in NSC-mediated motor and cognitive improvements. They also suggest that motor dysfunction and NSC-mediated recovery are more closely tied to dopaminergic systems whereas cognitive tasks are more influenced by NSC-mediated modulation of glutamatergic systems, and that both of these behaviors are strongly influenced by BDNF.
Figure 17. TH and GLT-1 function are necessary for NSC-mediated behavioral improvements. During the transplantation experiment, one group of ASOC mice received i.p. DHK injections daily following a post-transplantation recovery period of 2 days, and continuing throughout behavioral testing (A). Another group of ASOC mice received daily intraperitoneal injection of the TH inhibitor α-methyl-p-tyrosine (ASOC-αMT) over the ten days prior to and during behavioral assessment. αMT prevented NSC-mediated Rotarod (B) and beam traversal (C) recovery in ASOC mice, however, ASOC-DHK treated mice displayed similar recovery of motor function to the ASOC group in both tasks. Conversely, in cortical-dependent novel object recognition, NSC-mediated improvement was abolished by both αMT and DHK (D). NSC-mediated recovery in hippocampal-dependent novel place recognition was surprisingly not inhibited by αMT, but was prevented by DHK (E). Intriguingly, striatal BDNF was increased by NSCs regardless of αMT or DHK treatment (F,G), suggesting that αMT and DHK influence pathways that are downstream of BDNF signaling. As expected, TH levels were not significantly altered by NSCs and αMT co-treatment, however the activated form of TH (ser31) was reduced to ASOV levels by αMT despite NSC transplantation (F,H,I). Data presented as mean ± S.E.M. Western blot graphs presented as % of ASOV group. Fisher’s PLSD post hoc ASOC, ASOC-DHK p<0.02, *p<0.0004 compared to all other groups.
**NSC-derived BDNF is essential for stem cell-induced cognitive and motor benefits**

Given the strong correlations between striatal BDNF levels and both motor and cognitive function, we hypothesized that NSC-derived BDNF may be critical for NSC-mediated improvements. We therefore employed a loss-of-function approach to elucidate the role of BDNF in behavioral recovery (Figure 18A). NSCs were stably modified to express a shRNA targeting BDNF, leading to an 84% knockdown of BDNF protein (Figure 18B). Importantly, the resulting cells maintained multipotency as evidenced by unaltered expression of the NSC markers Sox 2 and Musashi (Figure 18B). The effects of transplantation with BDNF\textsuperscript{shRNA}-NSCs (BKCs) or unaltered NSCs (NSCs) were then examined in a new cohort of 12 month old mice.

As in our initial experiments, transplantation of NSCs increased the amount of time that ASO mice could stay on the Rotarod in comparison to vehicle-injected ASO mice. In contrast, transplantation with BKCs failed to improve performance in ASO mice (Figure 18C; \(F_{(3,16)}=8.3, 5.6; \text{ANOVA } p=0.006\)), indicating that NSC-derived BDNF is essential for NSC-mediated motor recovery. Performance on the beam traversal task further confirmed these exciting findings, showing that BKCs could not reduce the number of errors made by ASO mice (Figure 18D; \(F_{(3,16)}=6.2; \text{ANOVA } p=0.004\)). In NOR and NPR cognitive tasks, both WTV and ASOC mice again showed significant preference for the novel object or novel location equivalent to that observed in our initial studies; yet, transplantation with BKCs failed to improve memory for either the familiar object or location (Figure 18E,F; \(F_{(3,16)}=18.4; \text{ANOVA } p=0.0001\)). These results clearly
Figure 18. BDNF is necessary for NSC-induced motor and cognitive rescue. A new cohort of ASO and WT mice were aged to 12 months, then injected bilaterally with either NSCs or BDNF shRNA knockdown NSCs (BKCs); behavior was assessed as previously described (A). NSC BDNF expression was reduced 84% by shRNA in BKCs (B; \( p < 0.0001 \)). Importantly, multipotency of BDNF-shRNA-NSCs was not affected by BDNF knockdown as measured by Sox2 and Musashi expression (B). After four weeks, transplantation of BDNF-shRNA-NSCs into ASO mice failed to improve either Rotarod (C) or beam traversal performance (D). Similarly, NSC-derived BDNF proved to be critical for NSC-mediated improvements in cognitive function in NOR and NPR tasks performance in ASO mice (DE,F). As observed in the ASOV group, striatal BDNF was not increased in ASO-BKC mice (G,H). BDNF-shRNA-NSCs were also unable to significantly increase striatal TH ser31 or GLT-1 expression, again suggesting that TH activation and GLT-1 induction occur downstream of BDNF elevation (G,H,J). In ASO mice, transplanted NSCs express BDNF (K, red), whereas BDNF cannot be detected in transplanted, BDNF-shRNA-NSCs. Data presented as mean ± S.E.M. Western blot graphs presented as % of WTV group. Fisher’s PLSD post hoc WTV, ASOC \( p < 0.04 \), WTV \( p < 0.02 \), ASOC \( p < 0.04 \).
demonstrate that NSC-derived BDNF is necessary for both the motor and cognitive benefits of NSC transplantation.

We next performed biochemical analysis confirming that striatal BDNF levels were indeed increased in ASOC mice but not in ASO-BKC mice (Figure 18G,H; \( F_{(3,20)}=4.6; \) ANOVA \( p=0.01 \)). Likewise, striatal TH ser31 (\( F_{(3,20)}=4.4; \) ANOVA \( p=0.02 \)) and GLT-1 (\( F_{(3,20)}=1.7; \) ANOVA \( p=0.04 \)) were elevated by transplantation of NSCs in ASO mice, but were unchanged between ASOV and ASO-BKC mice, demonstrating that NSC-derived BDNF plays an important role in regulating dopaminergic and glutamatergic neurotransmitter systems (Figure 18G,I,J). Finally, we confirmed that one month following transplantation NSCs continue to produce BDNF, whereas BKCs produce far lower levels (Figure 18K), providing further evidence that NSC-derived BDNF is necessary to elevate striatal BDNF to behaviorally relevant levels. Together these results strongly implicate the necessity of NSC-derived BDNF for both the motor and cognitive benefits of NSC transplantation and the observed changes in dopaminergic and glutamatergic systems.

**Viral delivery of BDNF mimics the effects of NSCs**

Having shown that NSC-derived BDNF expression is essential for both motor and cognitive behavioral recovery, we next asked whether BDNF alone could provide a similar benefit. Both ASO and WT mice received bilateral striatal injections of either AAV2-mCherry or AAV2-mCherry-BDNF (1\( \mu l \) of 1.5 \( \times 10^{15} \) viral particles/side, \( n=7-9 \); Figure 19A,B). Four weeks later, mice were subjected to the same set of behavioral
tasks (Figure 19C-F). Similarly to NSC transplantation, AAV2-BDNF transduction improved both Rotarod (Figure 19C; \(F_{(3,27)} = 7.3\), ANOVA \(p=0.01\)) and beam traversal performance (Figure 19D; \(F_{(3,27)} = 7.8\), ANOVA \(p=0.009\)) in ASO-BDNF mice compared to ASO-mCherry mice, but had no effect in WT groups. In the cognitive tasks, although AAV2-BDNF increased preference for novel object and place in ASO-BDNF mice compared to ASO-mCherry mice, overall differences between all four groups via ANOVA were not significant (Figure 19E,F; \(F_{(3,27)} = 1.2\), ANOVA \(p=0.06\)). This suggests that AAV-BDNF leads to similar motor behavioral recovery to NSC-induced BDNF; however, cognitive function is not as effectively restored.

To understand why AAV2-BDNF leads to similar motor recovery as NSC-derived BDNF, but a diminished effect on cognition, we examined striatal BDNF expression. BDNF can undergo various forms of post-translational modification including phosphorylation and ATP-binding, which can in turn increase its biological activity (Konig, Hasche et al. 2008, Ferenz, Gast et al. 2012). Intriguingly, in addition to the typical mature 14kDa BDNF isoform observed in mouse brain lysates, we also detected a second 12kDa BDNF band in AAV2-BDNF groups. As this lower band may represent an alternatively processed or perhaps un-phosphorylated form of BDNF, we analyzed both bands individually. As expected, the 14 kDa BDNF band was decreased by 50% in ASO-mCherry mice compared to the WT-mCherry group (Figure 19G,H; \(F_{(3,27)} = 6.8\), ANOVA \(p=0.02\)). Furthermore, AAV2-BDNF resulted in a significant 37% increase in 14kDa BDNF levels in ASO-AAV-BDNF mice. In contrast, analysis of the 12 kDa band revealed a different pattern of expression, exhibiting an over five-fold increase in both
Figure 19. Viral delivery of BDNF partially mimics the effects of NSCs on motor and cognitive function. A new cohort of ASO and WT mice aged 12 months were bilaterally injected with either AAV2-mCherry-2A-BDNF (human full length) or AAV2-mCherry (A). AAV2-BDNF expression at the injection site (Scalebar= 100 μm), and subcellular localization of BDNF (Scalebar= 10 μm) were verified by immunohistochemistry (B). AAV2-BDNF reversed motor deficits in ASO mice for both Rotarod (C) and beam traversal (D) tasks. In novel object and place recognition tasks, AAV2-BDNF modestly improved performance, although these effects were not significant (E,F). Two bands representing mature BDNF were observed by Western blot in AAV2-BDNF-treated mice (G). The 14 kDa band which was decreased in AAV2-mCherry treated ASO mice was significantly increased by AAV2-BDNF (G,H). The protein at 12 kDa was dramatically increased by AAV2-BDNF expression in both WT and ASO mice (G,I). Monomeric h-α-syn (Syn211) remained highly expressed in both ASO groups, and was unaltered by AAV2-BDNF expression (G,J). Finally, BDNF expression levels were compared between ASO-AAV-BDNF and ASO-NSC mice (K). AAV2-BDNF resulted in slightly although not significantly lower levels of 14 kDa BDNF compared to NSC transplantation (K,L) but a 500% increase in 12 kDa BDNF relative to NSCs (K,M), suggesting potential differences in posttranslational modification of AAV versus NSC-derived BDNF. Data presented as mean ± S.E.M. Western blot graphs presented as % of the WTV group (H-J) or ASO-NSC (L,M). Fisher’s PLSD post hoc p<0.04, WT-BDNF, WT-CHerry p<0.03, WT-BDNF, WT-Cherry p<0.01, WT-Cherry, ASO-Cherry p<0.005, ASO-BDNF p<0.0004, ASO-NSC p<0.005, *p<0.05 compared to all other groups.
AAV2-BDNF groups versus AAV2-mCherry groups, (Figure 19G,I; F(3,27)=42.5, ANOVA p<0.0001). Despite this high elevation of BDNF by AAV transduction, there was no effect on monomeric h-α-syn expression (Figure 19G,J). In order to determine why AAV2-BDNF was not able to significantly restore cognitive function despite very high levels of the 12 kDa BDNF, we compared expression levels directly to NSC-transplanted ASO mice whose behavior was fully rescued. Interestingly, we find that in contrast to ASO-BDNF mice, NSC transplantation primarily increases levels of the 14kDa BDNF protein (Figure 19K). Furthermore, ASO-BDNF mice express modestly lower levels of the 14 kDa form and much higher levels of the 12kDa isoform compared to ASO-NSC mice (Figure 19L,M; F(3,27)=12.5, ANOVA p<0.005).

Taken together, these data suggest that differences in post-translational modification of BDNF provided by NSCs versus AAV likely explain the shift from the typical 14kDa endogenous BDNF signal to a predominant 12 kDa BDNF band in AAV-BDNF samples. The addition of a single phosphorylation could for example readily shift the electrophoretic mobility of a given protein by the observed 2kDa. These intriguing results could provide a potential explanation for why AAV-mediated BDNF delivery leads to a less robust effect on cognition, although future studies will clearly be needed to better understand the influence of post-translational modifications on BDNF-induced behavioral effects (Mowla, Farhadi et al. 2001, Liu, Walther et al. 2005, Konig, Hasche et al. 2008, Ferenz, Gast et al. 2012, Koppel, Tuvikene et al. 2015).
DISCUSSION

Here we show for the first time that transplantation of NSCs can dramatically improve both cognitive and motor function in a progressive transgenic model of DLB/PDD. Interestingly, the benefits occur without altering α-syn levels or inclusion number, instead revealing a critical role for BDNF-induced modulation of dopaminergic and glutamatergic systems in both the disease process and NSC-mediated recovery. We also demonstrate that although viral delivery of BDNF can mimic several outcomes of NSC transplantation, NSCs provide a greater benefit to cognitive function, suggesting that NSCs may be a more effective vehicle for BDNF-dependent recovery. Together, these studies demonstrate that transplantation of preferentially gliogenic NSCs could offer a promising new therapeutic approach to treat both the motor and understudied cognitive components of synucleinopathies (Righi, Tongiorgi et al. 2000, Bambah-Mukku, Travaglia et al. 2014).

Currently approved therapies for DLB/PDD primarily focus on strategies to replace or compensate for missing dopamine via L-DOPA therapy or electrical modulation of DA pathways (Freed, Greene et al. 2001, Olanow, Goetz et al. 2003, Bonelli, Ransmayr et al. 2004, McKeith, Dickson et al. 2005). While these approaches can provide meaningful relief of motor dysfunction, their effect on cognitive symptoms appears to be marginal and inconsistent (Aarts, Nusselein et al. 2014, Robbins and Cools 2014), and may even accelerate global cognitive decline (Jahanshahi, Ardouin et al. 2000, Kim, Jeon et al. 2014). Although DLB can include DA loss in the nigrostriatal pathway, it is modest (Piggott, Marshall et al. 1999, Colloby, McParland et al. 2012), and dopamine
transporter binding does not correlate to cognitive decline in DLB (Ziebell, Andersen et al. 2013). This collective evidence strongly argues for an alternative to DA therapy for DLB that is catered toward the specific consequences of synucleinopathy. The importance of α-synuclein in DLB/PDD is highlighted by the strong correlation between α-syn accumulation and cognitive dysfunction in patients (Kovari, Gold et al. 2003, Halliday, Leverenz et al. 2014). Indeed, a recent report confirmed that cerebrospinal fluid levels of α-syn correlates well with cognitive decline, but not with motor dysfunction (Stewart, Liu et al. 2014). The testing and development of treatments that could relieve the cognitive symptoms of DLB/PDD therefore likely requires the use of models that exhibit robust α-syn pathology with cognitive deficits, such as the ASO model employed here.

Interestingly, BDNF has also previously been implicated in the development of synucleinopathies. Specifically, haplosufficiency for the TrkB receptor results in nigral cell loss and accumulation of α-syn in aged mice (von Bohlen und Halbach, Minichiello et al. 2005). Changes in α-syn can in turn influence BDNF, as over-expression of wild type α-syn in primary neurons reduces BDNF production by suppressing the transactivation of CREB and NFAT, two transcription factors that can regulate BDNF expression (Saha, Ninkina et al. 2000, Yuan, Sun et al. 2010). It is likely that introduction of supplemental NSC or AAV2 derived BDNF was able to overcome this deficiency despite the persistence of α-syn expression (Cabeza-Arvelaiz, Fleming et al. 2011).
Although the ASO model does not exhibit substantial neuronal loss (Masliah, Rockenstein et al. 2000, Lam, Wu et al. 2011), the correlations between DA and glutamatergic proteins and behavioral function suggests that ASO mice nicely model the detrimental effects of α-syn accumulation on these transmitter systems. Perhaps more remarkable are the distinct associations of NSC-induced DA changes with motor improvement and glutamatergic changes with cognitive improvement. In support of our findings, it was recently shown that striatal DA transmission is disrupted in BDNF-deficient mice, thus the reductions in TH and phospho-TH we observe in ASO mice are likely influenced by the diminished levels of BDNF in these mice (Bosse, Maina et al. 2012). In the ASO model, changes in TH and phospho-TH induced by NSC transplantation likely reflect an enhancement of activity and function of existing nigrostriatal projections that in turn improves behavior (Hyman, Juhasz et al. 1994, Siuciak, Boylan et al. 1996). Supporting this notion, pharmacological inhibition of TH in the presence of NSC transplants prevented NSC-induced motor and cortical-dependent cognitive benefits. Fascinatingly, NSC-mediated changes in GLT-1 function were necessary for improvement in both cortical- and hippocampal-dependent cognitive tasks, but not motor performance. Although GLT-1 expression is altered by NSC transplantation regardless of α-syn overexpression, this marker may indicate an increase in NSC-derived astrocytes which are in turn stimulated to produce BDNF in ASO mice. Deficiency of striatal VGLUT-1 (Callaerts-Vegh, Moechars et al. 2013, Piyabhan and Wetchateng 2013, Granseeth, Andersson et al. 2015) or GLT-1 (Miller, Dorner et al. 2008, Hsu, Hung et al. 2015) can both lead to cognitive deficits; further supporting the hypothesis that NSC-induced increases in these transporters improves
cognition. Finally, expression and distribution of both VGLUT-1 (Bagayogo and Dreyfus 2009, Melo, Mele et al. 2013), and GLT-1 (Rodriguez-Kern, Gegelashvili et al. 2003) can be influenced by BDNF, further substantiating an upstream effector role for this neurotrophin in restoring balance to glutamate neurotransmission. The dissociation of the effects of NSCs on dopaminergic and glutamatergic systems and motor and cognitive function suggest that a balanced regulation of both striatal DA and corticostriatal glutamate could be key to rescuing α-syn related deficits.

The necessity of BDNF in NSC-mediated behavioral improvement in ASO mice is further confirmed by the inability of BDNF<sup>shRNA</sup>-NSCs to rescue either motor or cognitive function. In contrast, AAV2-BDNF mimics the effects of NSC transplantation on motor function and partially improves cognitive function. The less striking impact of AAV2-BDNF compared to NSC delivery may be explained by differences in the post-translational modification of BDNF (Liu, Walther et al. 2005, Garzon and Fahnestock 2007, Koppel, Tuvikene et al. 2015). Alternatively, it is plausible that additional NSC-derived factors might enhance the efficacy of NSC transplantation (Redmond, Bjugstad et al. 2007). In conclusion, our data strongly suggest that striatal NSC transplantation and the resulting elevation of BDNF could provide a promising therapeutic approach to restore dopaminergic and glutamatergic neurotransmission and motor and cognitive function in in DLB/PDD.
CHAPTER THREE

HUMAN NEURAL PROGENITOR TRANSPLANTATION RESCUES BEHAVIOR AND REDUCES α-SYNUCLEIN IN A TRANSGENIC MODEL OF DEMENTIA WITH LEWY BODIES

ABSTRACT

Synucleinopathies are a group of neurodegenerative disorders sharing the common feature of misfolding and accumulation of the presynaptic protein α-synuclein into insoluble aggregates. Within this diverse group, Dementia with Lewy Bodies (DLB) is characterized by the aberrant accumulation of α-synuclein (α-syn) in cortical, hippocampal, and brainstem neurons, resulting in multiple cellular stressors that particularly impair dopamine and glutamate neurotransmission and related motor and cognitive function. Recent studies have shown that murine neural stem cell (NSC) transplantation can improve cognitive or motor function in transgenic models of Alzheimer’s and Huntington’s disease, and DLB. However, the examination of clinically relevant human NSCs in these models is hindered by the challenges of xenotransplantation and the confounding effects of immunosuppressant drugs on pathology and behavior. To address this challenge, we developed an immune-deficient transgenic model of DLB that lacks T-, B- and NK-cells, yet exhibits a progressive accumulation of human α-syn (h-α-syn)-laden inclusions and cognitive and motor impairments. We demonstrate that clinically relevant human neural progenitor cells (specifically, line CNS10-hNPCs) survive, migrate extensively and begin to differentiate preferentially into astrocytes following striatal transplantation into this DLB model. Critically, grafted CNS10-hNPCs rescue both cognitive and motor deficits after 1- and 3-months and, furthermore, restore striatal dopamine and glutamate systems. These
behavioral and neurochemical benefits are likely achieved by reducing α-syn oligomers. Collectively, these results using a new model of DLB demonstrate that hNPC transplantation can impact a broad array of disease mechanisms and phenotypes and suggest a cellular therapeutic strategy that should be pursued.

INTRODUCTION

Dementia with Lewy Bodies (DLB) is thought to be the second leading cause of age-related dementia, and shares clinical features with both Alzheimer’s and Parkinson’s diseases, including memory loss, visual hallucinations and extrapyramidal symptoms (McKeith 2006, Vann Jones and O’Brien 2014). In DLB, these symptoms are closely associated with the accumulation of insoluble aggregates of the presynaptic protein, α-synuclein (α-syn) within neurons of the cortex, hippocampus, and brainstem. In turn, α-syn and Lewy body pathology can lead to widespread cellular stress and disruption of multiple neurotransmitter systems including dopamine and glutamate (Dalfo, Albasanz et al. 2004, Klein, Eggers et al. 2010). Although aggregated α-syn is a hallmark of several synucleinopathies, the physiological and pathological states of this protein remain unresolved. α-syn is an intrinsically disordered protein with a propensity for folding and oligomerization into multiple conformers and larger Lewy body-like aggregates (reviewed in (Lashuel, Overk et al. 2013). Hypotheses distinguishing physiological from pathological α-syn remain subject to debate, but increasing evidence suggests that small, soluble oligomers may contribute to cell toxicity while Lewy bodies may be part of a more neuroprotective response to cellular stress (Lee, Suk et al. 2008, Lashuel, Overk et al. 2013, Schulz-Schaeffer 2015). It is this variety of α-syn species
and lack of consensus as to the protein’s pathological states that makes it critical to examine multiple forms in disease and in response to therapeutic approaches.

The multiple mechanisms disrupted in DLB make this disease extremely challenging to treat. However, recent studies have shown that neural stem cell (NSC) transplantation can impact a broad array of disease mechanisms and phenotypes in related disease models, thus stem cell transplantation should be further examined in the context of DLB (Lee, Jeyakumar et al. 2007, Levy, Boulis et al. 2016). Recently, our group demonstrated that striatal transplantation of mouse NSCs (mNSCs) can ameliorate both motor and cognitive deficits in a transgenic model of DLB (Goldberg et al. 2015). Importantly, mNSCs achieved this benefit by elevating striatal levels of brain derived neurotrophic factor (BDNF), which in turn led to a restoration of dopaminergic and glutamatergic signaling. Transplanted mNSCs displayed a preferentially glial fate, suggesting that human NSC lines that are particularly gliogenic may provide an ideal cell type that could potentially target multiple disease mechanisms as a neuroprotective strategy for DLB. Some groups have also begun to show that glial transplantation may be more effective for rescuing cognitive deficits compared to other cell types (Bradbury, Kershaw et al. 1995, Ding, Tian et al. 2011, Han, Chen et al. 2013). In addition, glia distinctly express the glutamate reuptake transporter, GLT-1, whose cell surface expression can be reduced by accumulation of amyloid-β (Scimemi, Meabon et al. 2013) and may also be perturbed by α-syn (Marxreiter, Ettle et al. 2013). These attributes make glial progenitors an attractive candidate cell type for transplantation in these proteinopathies, particularly where glutamate neurotransmission is disrupted.
Clinically relevant human neural progenitor cells (hNPCs) have the potential to treat multiple neurodegenerative diseases (Levy et al. 2016). Importantly, hNPCs have been shown to survive transplantation and migrate throughout the striatum in both rodents and primates (Svendsen, Caldwell et al. 1997, McBride, Behrstock et al. 2004, Behrstock, Ebert et al. 2006). A well-characterized hNPC line derived from the fetal cortex, termed CNS10-hNPC, has been shown to expand in vitro for up to 50 passages with stable karyotype and to have high viability post-transplantation with no tumor formation (Gowing, Shelley et al. 2014, Shelley, Gowing et al. 2014). The bi-potent (neuronal and astrocytic) progenitors display preferential differentiation toward glial fate following transplantation (Gowing, Shelley et al. 2014, Das, Avalos et al. 2016).

Immune-rejection of transplanted xenograft cells is a major challenge to studying the safety and efficacy of CNS10-hNPCs in animal models (Mattis, Wakeman et al. 2014). Commonly used immune-suppressants such as cyclosporine A or tacrolimus (FK506) have been shown to modulate α-syn pathology, thereby complicating the potential design and interpretation of xenotransplantation studies (Gerard, Debyser et al. 2008, Agrawal, Dixit et al. 2015). To resolve this challenge, we generated an immune-deficient transgenic model of DLB by back-crossing with a Rag2/Il2rg double knockout line in order to permit survival and assessment of engrafted CNS10-hNPCs over time. Using this new model, we now examine the effects of clinically relevant human neural progenitor cells (specifically, line CNS10-hNPC) on multiple phenotypes in the DLB model. Preferentially gliogenic CNS10-hNPCs migrate throughout the striatum, leading
to a dramatic improvement in both motor and cognitive function by 1 month that is sustained for at least 3 months post-transplantation. Interestingly, these benefits are associated with changes in striatal dopamine and glutamate regulation and are accompanied by a reduction in soluble α-syn monomers and oligomers. Taken together, these data suggest that CNS10-hNPCs can effectively ameliorate motor and cognitive dysfunction and associated impairments in dopamine and glutamate neurotransmission in xenotransplantation-compatible DLB mice. Moreover, the ability of CNS10-hNPCs to alter α-syn oligomer and monomer levels indicates their potential to disrupt the underlying pathological phenotype and perhaps even slow disease progression.

MATERIALS AND METHODS

Generation of Immuno-Deficient Transgenic DLB Mice

All procedures were performed in strict accordance with the UC Irvine and NIH animal use regulations. The Rag-ASO immune-deficient DLB mouse model was created by backcrossing previously characterized hemizygous wild-type (WT) human-α-syn over-expressing (ASO) transgenic mice (PDGFβ promoter, line D) ((Masliah, Rockenstein et al. 2000) onto a Rag2/Il2rγ double knockout background (Strain #4111-Taconic; (Cao, Shores et al. 1995). This cross was repeated for 3 generations to create immuno-deficient DLB mice that are heterozygous for the ASO transgene and lack both copies of the Rag2 and Il2rγ transgenes. All treatment groups (Rag-WT and Rag-ASO) were sex- and age-matched, and were group housed on a 12h/12h light/dark cycle with access to food and water ad libitum.
**Cell Preparation and Striatal Transplantation**

Human NPCs (specifically, line CNS10-hNPC) derived from the fetal cortex are maintained as free-floating aggregates, termed neurospheres, in fibroblast growth factor and epidermal growth factor and are passaged by mechanical chopping in order to maintain cell-cell contact (Svendsen, ter Borg et al. 1998). Cells at passage 26 were thawed, dissociated to a single cells suspension, washed with vehicle transplantation media (1 part Liebowitz L-15 media, 1 part 0.6% glucose in sterile PBS supplemented with 2% B27), and re-suspended at a concentration of 100,000 cells/µl in transplantation media. Seven-month old Rag-ASO and Rag-WT mice were randomly assigned to a treatment group and either vehicle (transplantation media) or 50,000 cells/site (1 µl volume, 2 sites/side) were transplanted bilaterally into the dorsal striatum (Bregma +0.03 AP, ± 2.0 ML, -3.0 and -3.5 DV). Mice were anesthetized with isoflurane, placed in the stereotaxic frame and injected using a 5 µL Hamilton microsyringe (30-gauge) and an injection rate of 0.5 µL/min. Cells were delivered at two DV sites (1µl/site) per hemisphere to facilitate distribution of CNS10-hNPCs throughout the dorsal and ventral medial striatum. The needle was retained within the injection site for 4 minutes before slowly removing the needle to reduce potential backflow of cells along the needle tract. Cells remained on ice for the duration of the transplantation procedure and retained 89-93% viability as confirmed by post-surgery Trypan blue exclusion counts.

**Cognitive Behavior**
Thirty days (1 month group) or ninety days (3 month group) following transplantation, behavioral assessments began, lasting 10 days. All behavioral testing and analysis was performed blinded to treatment and genotype groups using an identification system decoded during statistical analysis as previously described (Goldberg, Caesar et al. 2015). Cortical- and hippocampal-dependent memory tasks followed standard protocols for novel object recognition (NOR) and novel place recognition (NPR) (Bermudez-Rattoni, Okuda et al. 2005, Goldberg, Caesar et al. 2015). Briefly, mice were allowed to explore the object-free recognition chamber with only bedding for 5 minutes on 3 consecutive days. On the 4th day, mice were trained for 10 minutes in the presence of 2 identical objects. Twenty-four hours later, one object was replaced with a novel one (NOR), or one object was moved to a novel location in the chamber (NPR) and animals were allowed to explore for 5 minutes. Object exploration was analyzed as degree of discrimination between novel and familiar objects or object locations $\frac{((\text{Time}_{\text{novel}}-\text{Time}_{\text{familiar}}))/((\text{Time}_{\text{novel}}+\text{Time}_{\text{familiar}}))\times100}$. Mean distance traveled, mean ambulatory speed and time spent investigating objects during the training period were not altered by either genotype or treatment. Different objects were used for each test at the 1- and 3-month time points. Animals with total exploration time <4 seconds were excluded.

**Motor Behavior**

To assess striatal-associated motor coordination and control, mice were tested on the fixed speed Rotarod (Ugo Basile, Italy) (Monville, Torres et al. 2006). At the 1-month time point, mice were trained for 4 consecutive trials at 12 rpm 20 min apart. Twenty-
four hours later, mice were tested at 16 rpm and latency to fall off of the rotating rod was averaged over 3 trials spaced 20 min apart. At the 3-month time point, mice were tested with no additional training trials. Subsequently, mice were tested on the beam traversal challenge as modified from (Fleming, Salcedo et al. 2004) in order to detect motor initiation and control. Mice were trained for 2 days to traverse a narrowing Plexiglas beam to reach their home cages. On day 3, a mesh grid was placed over the beam, and mice were videotaped traversing from base to apex. The number of total steps and foot-faults (forelimb slipping through the wire grid) were counted by a blinded observer from a slow motion video. At the 3-month time point, mice were tested on the grid with no additional training.

**Flow Cytometry**

Splenocytes were isolated following standard protocol. Briefly, spleens were mashed between two frosted slides and treated with standard ammonium-chloride-potassium lysis buffer to remove erythrocytes followed by filtration through 70μm nylon mesh. Cells were counted and 1x106 cells were resuspended in sterile 5 ml polystyrene tubes for blocking and labeling with cell surface marker antibodies. Isolated splenocytes were first incubated with anti-Fc CD16/32 block (1:100; BD Biosciences; San Jose, CA) for 10 minutes at 4°C. After blocking, cells were incubated with the following fluorescent-conjugated antibodies (1:200) against cell surface markers: FITC-CD3 (145-2C11; BioLegend; San Diego, CA), PE-CD4 (RM4-5; BioLegend), APC-CD8 (53-6.7; BioLegend), APC-CD19 (6D5; BioLegend), PE-CD49b (DX5; BioLegend). Following antibody incubation, cells were washed and resuspended for flow cytometry. Cells
were run on LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo (FlowJo LLC; Ashland, OR) by a blinded observer.

**Biochemistry**

Six weeks after transplantation and one day after the end of behavioral testing, mice were sacrificed by Euthasol® and transcardial perfusion with 0.01 M phosphate-buffered saline (PBS). The left hemisphere was post-fixed in 4% paraformaldehyde and sectioned on a microtome (40 μm, coronal) for immunohistochemical analyses. The right side of the brain was flash frozen for subsequent biochemical analysis. The dorsal striatum was then microdissected, homogenized in liquid nitrogen and split into two fractions for protein or mRNA isolation. The detergent-soluble protein fraction was extracted in TPER™ (Thermo Fisher Scientific, Carlsbad CA) with phosphatase (Thermo Fisher Scientific) and protease inhibitor cocktails (Roche, Risch-Rotkreuz, Switzerland), and supernatant was collected after centrifugation for 10 min at 1000 x g at 4°C to create a soluble fraction. The resulting insoluble pellet was washed 3x and resuspended in lysis buffer (10 mM Tris (pH 7.4), 1% Triton X-100, 150 mM NaCl, 10% glycerol and 4% SDS with protease and phosphatase inhibitors), sonicated 3x 10 seconds and boiled for 30 min. Formic acid extraction was then carried out as previously described (Sy et al. 2011). Briefly, detergent insoluble protein was resuspended in 70% formic acid and centrifuged at 15,000 x g for 10 min at 4°C. Formic acid fractions were neutralized by with 10N NaOH, and neutralizing buffer (1 mol/L Tris base and 0.5 mol/L Na2HPO) then neutralized and stored at -20°C, followed by washing with 10% trichloride in acetone. The resulting pellet was then resuspended
in TPER™. Protein concentrations were determined via Bradford assay, and normalized samples compared via 4-15% Tris-Glycine gel SDS-PAGE Western blot before transfer to .45 µm pore nitrocellulose membrane. Formic acid fractions were run on 4-12% Bis-Tris gel SDS-PAGE. For dot blot assays, 1 µg of soluble fraction in 4 µl was pipetted onto .45 µm pore nitrocellulose membrane. All blot membranes were incubated in StartingBlock™ (Thermo Fisher Scientific) for 20 min, then probed with primary antibodies (listed below) and secondary antibodies using standard protocols. Striatal tissue from an α-syn knockout mouse (Jackson Laboratories, Bar Harbor, ME; #016123) was prepared with experimental samples and is represented on every α-syn Western blot as a negative control. All electrophoresis was run under reducing conditions. Relative signal intensity of grayscale images was quantified by Image J, and sample identification was decoded once all values were obtained. The following primary antibodies were used: α-syn antibodies: human-α-syn (Syn211, Sigma #36-008), total a-syn (BD Transduction # 610787), oligomeric α-syn (ASyO2, Agrisera #AS13 2717B) (Brannstrom et al. 2014). Dopaminergic antibodies: tyrosine hydroxylase (Millipore, #ab152), pTH ser31 (Cell Signaling, #3370). Neurotrophin antibodies: BDNF (Santa Cruz Biotech., # sc-546), phosphor-Erk1/2 (Cell Signaling, #9101). Glutamatergic antibodies: GLT-1 (Abcam, #ab106289), EAAC1 (Alpha Diagnostic, #EAAC11-A).

**Histology and Confocal Microscopy**

Fluorescent immunohistochemistry followed standard protocols. Briefly, coronal brain sections were rinsed 3 times in 0.01 M PBS and were then placed into blocking solution.
(0.01 M PBS+0.02% Tx100+5% goat serum) for 1 hr at room temperature. Primary antibodies were then diluted in fresh blocking solution and applied overnight at 4°C. Sections were then rinsed in PBS and incubated for 1 hr at room temperature in appropriate highly-cross absorbed Alexa Fluor® secondary antibodies (Life Technologies, Inc.). Following 3 additional rinses, sections were mounted on slides and cover-slipped using Fluoromount G (Southern Biotech, Birmingham, AL). The following antibodies were used to detect human cells and assess differentiation: Stem121 (human cytoplasm, Clontech # Y40410), Ku80 (human nuclei, Abcam # ab80592), GFAP (Millipore #MAB260), Vimentin (Millipore # AB5733), S100β (Abcam #ab41548), Doublecortin (Abcam #ab18723), Olig2 (Millipore #MABN50), Nestin (Abcam #ab6142), Ki67 (Dako #M7249). Sections were imaged in a blinded manner using an Olympus FX1200 confocal microscope, with identical laser and detection settings across a given immunolabel. Immunoreactive cells and α-syn inclusions were hand-counted by a blind observer, and sample coding was revealed once all values were obtained.

RNA Isolation and Quantitative RT-PCR

mRNA was isolated using Trizol extraction (Thermo Fisher Scientific). Taqman quantitative real time PCR was modified from (Medhurst et al. 2000). In brief, first-strand cDNA was synthesized from 1 µg of total RNA by reverse transcription using a High Capacity RNA-to-cDNATM Kit (Thermo Fisher Scientific). Proprietary Taqman primer sequences to human α-syn (Hs01103383_m1) and mouse α-syn (Mm01188700_m1) were analyzed relative to mouse Rn18s (Mm03928990_g1), using a mixed WT-VEH sample as an internal reference control.
Statistical Analysis

All animals were randomly assigned to treatment groups based only on wild-type or transgenic genotype and sex. Animal identification codes were randomly generated in order to keep the researcher blind to genotype and treatment throughout the behavioral testing, scoring, and statistical analysis. All statistical comparisons were performed using Statview 5 software. Comparisons between multiple groups within one time point utilized two-way analysis of variance (ANOVA, in text) followed by Fisher’s PLSD post-hoc tests (within figures). Behavioral data was analyzed by repeated measures ANOVA as the same animals were assessed at 1 month and 3 months post-transplantation. Correlation analyses were within-animal comparisons, with behavior and protein of animals sacrificed at 1-month post-transplantation, and behavior and protein of animals sacrificed at 3-months post-transplantation. Differences are considered significant when p<0.05 for both ANOVA and post-hoc tests.

RESULTS

Validation of immuno-deficient ASO mice as a model for xenotransplantation

Immuno-deficient DLB mice were developed by backcrossing α-syn (ASO) transgenic mice onto a Rag2/il2ry double knockout background (Figure 20A). To confirm that the resulting Rag-ASO mice lacked B-, T- and Natural killer (NK) cells, flow cytometry was performed on splenocytes isolated from 6-month old mice and compared to immune-intact WT and ASO mice (n=6). Analysis verified that all immune-deficient Rag-ASO
animals lacked B cells, CD4 and CD8 T cells, as well as NK cells independent of WT or ASO genotype (Figure 20B).

Immune-deficient Rag-ASO mice develop equivalent h-α-syn (Syn211) inclusions to age-matched immune-intact ASO mice (Figure 21A). Likewise, Western blot analysis revealed similar levels of soluble h-α-syn and total (human and mouse) α-syn monomers (Figure 21B). Finally, Rag-ASO mice develop significant motor (Figure 21C; F(1, 9)=14.3; ANOVA p=0.004) and cognitive (Figure 21D; F(1, 9)=16.7; ANOVA p=0.0035) deficits compared to Rag-WT mice. Importantly for the interpretation of cognitive assessment, Rag-WT and Rag-ASO displayed similar total activity as measured by mean velocity (Figure 21E; F(1, 9)=0.43; ANOVA p=0.52) and total distance (Figure 21E; F(1, 9)=0.02; ANOVA p=0.9) during the training period.
Figure 20. Establishment and confirmation of immune-deficiency in the Rag-ASO transgenic mouse model. Abbreviated breeding diagram shows the strategy used to generate the Rag-ASO transgenic model and Rag-WT littermates (A). Flow cytometry performed on splenocytes of 6-month old animals confirmed that all immune-incompetent animals lacked T, B and NK cells independent of the h-α-syn transgene. In contrast, immune-intact animals expressed similar high percentages of these cell populations independent of h-α-syn transgene (B). n=6/group. All values are means ± S.E.M., expressed as percent of total live splenocytes.
Figure 21. Confirmation of the Rag-ASO mouse model disease phenotype. Human-α-syn (Syn211) inclusion number is equivalent between Rag-ASO and immune-intact ASO mice, and as expected Rag-WT mice show no inclusion bodies (A). Rag-ASO and ASO mice express similar levels of soluble monomeric h-α-syn and t-α-synuclein in the striatum, confirmed by the absence of signal in α-syn knockout mouse striatal tissue (B). Rag-ASO mice show significant deficits in Rotarod (C) and novel object recognition (NOR) (D) tasks. Overall mean velocity and distance traveled in the NOR arena were not different between Rag-WT and Rag-ASO mice (E). Scale bar=20 µm. n=4-6/group. All values are means ± S.E.M., expressed as percent of the WT group unless otherwise denoted. *p-values are significantly different from all other groups.
hNPC transplantation rescues motor and cognitive deficits in immune-deficient ASO mice

To assess the impact of hNPC transplantation on DLB-associated motor and cognitive impairments, the hNPC line CNS10-hNPC or vehicle control were delivered to 7 month old Rag-WT (hereafter, WT) or Rag-h-α-syn transgenic (hereafter, ASO) immune-deficient mice (n=6-8). Bilateral injections of 100,000 cells/site targeted the dorsal striatum, a region known to be involved in both motor function and learning/memory through connectivity within the basal ganglia and with multiple cortical regions. Behavioral outcomes were assessed 1-month or 3-months following transplantation (Figure 22A).

As predicted based on observations in immune-intact ASO mice (Goldberg, Caesar et al. 2015), immune-deficient ASO-VEH mice displayed significant impairment on the striatal-associated Rotarod (Figure 22B) and Beam Traversal (Figure 22C) motor tasks at both 1- and 3-month time points, suggesting deficits in motor coordination, fluidity and control. In contrast, CNS10-hNPC transplantation resulted in a robust and significant rescue of these deficits in ASO-CNS10 mice at both 1- and 3-months following transplantation. Significant interactions of genotype and treatment were observed for both motor tasks (Rotarod F(1,52)=33.7, p<0.0001; Beam F(1,52)=18.1, p<0.0001), with no main effect of time point, confirming a lasting positive impact of CNS10-hNPCs on these behaviors over time.

Examining cognition in a model with motor impairments requires the use of tasks that are not heavily influenced by motor function. Novel Object Recognition (NOR) and
Novel Place Recognition (NPR) tasks were therefore utilized, which are low-stress paradigms that measure cortical-dependent object and hippocampal-dependent spatial memory, respectively. All four groups were habituated, trained, and tested following standard protocols. Twenty-four hours after training, mice were exposed to a novel...
object or novel object placement, and the discrimination ratio between exploration time of old and new objects was calculated. A significant interaction of genotype and treatment was observed for both NOR (F(1,52)=17.4, p=0.0001) and NPR (F(1,52)=3.8, p<0.05), but these were independent of time point, suggesting a sustained positive impact over time. As shown, ASO-VEH mice exhibited significant impairments in both NOR (Figure 22D) and NPR (Figure 22E) compared to WT-VEH and WT-CNS10 groups at both 1- and 3-month time points, demonstrating that h-α-syn overexpression can mimic these important aspects of DLB-related cognitive impairment. In contrast, CNS10-hNPCs considerably improved both cortical- and hippocampal-related cognitive function at 1 and 3 months following striatal transplantation (Figure 22D,E). Although not significant, cortical NOR discrimination also appeared to improve from 1 to 3 months in WT-VEH and WT-CNS10 mice (Figure 22D). CNS10-transplanted ASO mice, however, did show significant further improvement in performance between 1 and 3 months. In addition, while hippocampal NPR discrimination slightly declined from 1 to 3 months in WT-VEH and WT-CNS10 mice, this was not evident in CNS10-transplanted ASO mice (Figure 22E). This may suggest early age-related decline in hippocampal function, resulting in compensatory function in the cortex which enhances NOR memory (Holdstock, Mayes et al. 2002, Oliveira, Hawk et al. 2010). Collectively, these results suggest that CNS10-hNPCs provided sustained cognitive improvement in both cognitive domains over time, even despite this age-related trend.

**hNPCs show robust engraftment and glial differentiation**
Whereas improved behavioral function in ASO mice was maintained at similar levels from 1 to 3 months following transplantation, CNS10-hNPC engraftment and migration increased considerably over time (**Figure 23A-C;** \( F_{(1, 25)}=5.4, p<0.03 \)). Importantly, CNS10-hNPCs showed robust engraftment independent of genotype, suggesting that h-\( \alpha \)-syn overexpression does not impair survival of transplanted human cells. Relevant to this result, h-\( \alpha \)-syn aggregates were not observed within transplanted CNS10-hNPCs at either 1 or 3 months (data not shown). The duration-dependent increase in hNPC number occurred both at the injection site (**Figure 23B;** \( F_{(1, 14)}=15.8; p<0.001 \)) and in the disperse regions to which CNS10-hNPCs migrated within the striatum (**Figure 23C;** \( F_{(1, 14)}=10.3, p<0.005 \)). While CNS10-hNPCs increased in number, the overall proliferative capacity of human cells, as assessed by Ki67 co-labeling, remained low at both 1 and 3 months in WT-CNS10 mice and was reduced in ASO-CNS10 mice by 3 months (**Figure 24A-C;** \( F_{(1, 25)}=3.5; p<0.03 \)). This result critically indicates decreased proliferation and thereby reduced tumorigenicity. Finally, CNS10-hNPCs that remained at the injection site expressed a more proliferative potential compared to the minimally proliferative phenotype of the disperse populations, which one might expect if the proximity of transplanted cells to one another affects proliferation rates (**Figure 24I**).

The progenitor state of CNS10-hNPCs, as assessed by nestin immunolabeling, declined dramatically from 1 to 3 months post-transplantation independent of genotype (**Figure 24A,B,D;** \( F_{(1, 25)}=78.6; p<0.0001 \)), indicating a shift from progenitor state toward fate commitment. The reduced numbers of progenitor cells suggest that CNS10-hNPCs are undergoing the expected fate commitment over time *in vivo*. Given that CNS10-
hNPC have been shown to preferentially differentiate toward an astrocytic fate \textit{in vivo} (Gowing, Shelley et al. 2014, Das, Avalos et al. 2016), we anticipated largely astrocytic differentiation profiles at both 1 and 3 months post-transplantation.

Immunohistochemical analysis confirmed that by 3 months post-transplantation, differentiating CNS10-hNPCs largely expressed the astrocytic intermediate filaments vimentin and glial fibrillary acidic protein (GFAP) (Figure 24A,B,E). Indeed, vimentin+ and GFAP+ human cells increased approximately 4-fold from 1 to 3 months post-transplantation ($F_{(1,25)}=15.1$; $p<0.0009$) as the transplanted cells matured into astrocytes in both genotypes. Furthermore, expression of the astrocyte-associated calcium binding protein, S100$\beta$, indicated early astrocytic maturation of hNPCs in WT and ASO
Figure 24. Human NPCs differentiate predominantly toward a glial fate independent of host genotype. Confocal micrographs demonstrate largely glial differentiation of human cells (green, SC121 cytoplasm or Ku80+ nuclei) near the injection site (A) and at dispersed locations (B). Counts of Ki67 revealed that ~20% of human cells remained mitotic at 3-months, although ASO mice exhibited a significant reduction in Ki67 CNS10-hNPCs between 1- and 3-month time points (C; 1-month black, 3-months green). Nestin-labeled hNPCs decreased significantly over time, independent of genotype (D). Whereas Vimentin/GFAP (E) and S100β co-labeling of human cells increased over time (F), but no significant changes in Doublecortin expression between 1- and 3-months (G; p=0.08). Further analysis of site-specific differentiation showed that S100β-labeled CNS10-hNPCs trended toward increased expression at disperse locations at both 1- and 3-month time points, and expression is significantly greater in ASO compared to WT mice at 3-month disperse locations (H). In contrast, Ki67-labeled CNS10-hNPCs are significantly decreased at disperse locations compared to the injection site at 3 months in WT and both time points in ASO mice (I). Scale bar=100 µm, inset scale bar=10 µm. n=6-8/group. All values are means ± S.E.M., expressed as percent of within-group total CNS10-hNPCs.
mice (Figure 24A,B,F). Site-specific analysis revealed a trend toward increased S100β+ hNPCs at disperse sites compared to injection sites in both WT and ASO mice (Figure 24A,B,H). Finally, an influence of genotype was observed as S100β+ hNPCs were significantly greater in number at 3-month disperse sites in ASO compared to WT mice (Figure 24A,B,F; F(1, 14)=4.5; p<0.04). This suggests that host h-α-syn over-expression may encourage glial differentiation in hNPCs that have migrated away from the injection site.

Doublecortin (DCX) labeling for early neuronal fated CNS10-hNPCs revealed a non-significant trend toward increased neuronal potential in ASO-CNS10 mice versus WT-CNS10 mice at both time points (Figure 24A,B,G), though no CNS10-hNPCs expressed the mature neuronal marker NeuN (data not shown). This elevated expression of DCX in ASO-CNS10 hNPCs is intriguing given reports that astrocytic fate in vitro decreases following over-expression of wild-type α-syn in CNS10-hNPCs (Schneider, Seehus et al. 2007). In the current report, h-α-syn aggregates were not observed within transplanted CNS10-hNPCs (data not shown), however extrinsic cues from host h-α-syn may have led to increased early neuronal differentiation of transplanted CNS10-hNPCs, but did not impair overall astrocytic differentiation of CNS10-hNPCs in ASO-CNS10 mice.

Finally, we assessed the host glial response to CNS10-hNPC transplantation in ASO and WT mice by quantifying the total endogenous glial response to vehicle or CNS10-
hNPC injection. Host Vimentin+/GFAP+ cells were evident in larger numbers in the CNS10-hNPC-transplanted striatum compared to vehicle-injected mice, independent of genotype (Figure 25A,B; $F_{(1, 25)}=55.6; p<0.0001$). Although no adaptive immune response to CNS10-hNPCs was possible in ASO and WT mice due to the homozygous Rag2/Ii2rγ knockout, resident microglial cells remain capable of mounting an innate response. Microglial recruitment as well as changes in morphology were therefore assessed at sites proximal to the injection site using confocal microscopy and IMARIS software analysis. Interestingly, no changes in total microglial number, process length or process branching were observed post-transplantation between ASO and WT mice (Figure 26A,B). This finding suggests that microglia played a minimal role in modulating the host response to CNS10-hNPC transplantation in Rag-ASO versus Rag-WT mice.
Figure 25. Endogenous glia are recruited toward the hNPC injection site. Confocal micrographs demonstrate that host astrocytes (blue and red) that are negative for human cytoplasm labeling (green, SC121) have migrated toward the injection site of vehicle and CNS10-hNPC transplanted mice, as well as disperse locations at 1 and 3 months post-transplantation (A). Vimentin/GFAP-labeled host cells are increased at the injection site in CNS10-hNPC-transplanted mice independent of genotype and time point (B). Scale bar=100 µm. n=6-8/group. All values are means ± S.E.M.
Striatal dopaminergic and glutamatergic regulation is restored by hNPC transplantation

ASO mice have previously been shown to exhibit impairments in both dopaminergic and glutamatergic systems that correlate with motor and cognitive dysfunction (Goldberg, Caesar et al. 2015). We therefore examined several protein markers associated with
these key striatal neurotransmitters (n=4). Dopamine regulation was assessed both by the rate-limiting enzyme in DA synthesis, tyrosine hydroxylase (TH), and its phospho-epitope (pSer31) that is associated with increased TH activity. The ratio of striatal pSer31/TH protein was then calculated, revealing a significant decrease in ASO-VEH mice that was rescued by CNS10-hNPC transplantation at 1- and 3-month time points (Figure 27A,B). This analysis demonstrated a significant interaction between genotype, treatment and time point in the striatal pSer31/TH protein ratio (F(1,24)=6.6, p<0.02), indicating that the main effects of CNS10-hNPC transplantation were greater in ASO mice compared to WT mice, and were further enhanced at 3 months post-transplantation compared to 1 month.

We next examined expression of glutamate transporters in the striatum in order to determine whether α-syn or CNS10-hNPCs influence corticostriatal and hippocampal-striatal glutamatergic projections. Significant main effects of CNS10-hNPCs were observed on expression of the glial glutamate re-uptake transporter, GLT-1, as transplantation was able to rescue expression at both 1 and 3 months post-transplantation (Figure 27A, C; F(1,24)=7.3, p<0.01). Corroborating the specific role of glial GLT-1 in CNS10-hNPC-mediated effects on glutamate regulation, the neuron-specific transporter EAAC1 was not altered by either genotype or treatment (Figure 27A,D).

Because we and others have reported that BDNF signaling plays an important role in the multiple homeostatic effects of murine NSC transplantation, we examined the
Figure 27. Human NPC transplantation enhances dopaminergic and glutamatergic markers. Detergent soluble striatal fractions were normalized to GAPDH (A) and the ratio of phosphorylated serine 31 tyrosine hydroxylase (pSer31) to total tyrosine hydroxylase (TH) protein was modestly increased by CNS10-hNPCs at 1 month (black) in ASO mice, and significantly elevated by CNS10-hNPCs after 3 months (B; green). The glial glutamate transporter GLT-1 was also significantly increased by CNS10-hNPCs at both 1 and 3 months post-transplantation in ASO mice (C). Distinctly, the neuronal glutamate transporter EAAC1 was unaltered by genotype or treatment (D). Mature BDNF was not altered by genotype or CNS10-hNPC transplantation (E). The most significant correlations to behavioral outcome in ASO mice were pSer31/TH and GLT-1, with pSer31/TH showing association with both cognitive (NOR) and motor (beam traversal) tasks whereas GLT-1 was only significantly correlated with cognitive performance (F). n=4/group. All values are means ± S.E.M., expressed as percent of within-time point WT-VEH group. *p-values are significantly different from all other within-time point groups.
neurotrophin BDNF (**Figure 27A,E**). Western blot analysis and quantification showed that mature BDNF expression was not altered by genotype or CNS10-hNPC treatment at this age, as was the case for the other neurotrophins glial cell line-derived neurotrophic factor (GDNF) and nerve growth factor (NGF) (data not shown). This suggests that increased expression of these mature neurotrophins is not required for CNS10-hNPC-mediated behavioral improvement.

Although neurotrophin signaling was unaffected, pSer31/TH and GLT-1 expression in individual ASO mice correlated with changes in motor and cognitive behaviors due to h-α-syn and CNS10-hNPC transplantation (**Figure 27G**). For example, pSer31/TH correlated with both cognitive ($R^2=0.52$, $F_{(1,13)}=13.9$, $p<0.003$) and motor ($R^2=0.43$, $F_{(1,13)}=9.9$, $p<0.008$) performance. In contrast, GLT-1 expression correlated with cognitive performance ($R^2=0.41$, $F_{(1,13)}=9$, $p<0.01$), but not with motor function ($R^2=0.13$, $F_{(1,13)}=1.9$, $p=0.2$). These associations indicate a CNS10-hNPC-dependent recovery of dopamine and glutamate neurotransmitter regulation that is associated with the observed improvements in behavior.

**hNPC transplantation does not impact α-synuclein inclusions or insoluble protein**

We next examined the role of α-syn pathology on the ability of CNS10-hNPC transplantation to modify behavior and neurotransmitter homeostasis using several approaches. We began by assessing whether insoluble forms of the protein might be affected by CNS10-hNPC transplantation. Quantification of h-α-syn inclusion bodies (Syn211) revealed that total inclusion number was not altered by CNS10-hNPCs, even
by 3 months post-transplantation (n=5-6) (Figure 28A,B). The absence of an effect on inclusion number, however, does not preclude potential influence on additional insoluble h-α-syn conformers that could affect behavior. Therefore, striatal samples extracted by formic acid treatment were examined by Western blot (n=4). A main effect of genotype was observed in insoluble h-α-syn ($F_{(1,24)}=43.3$, $p<0.0001$). However, there was no impact of CNS10-hNPC transplantation on insoluble monomeric h-α-syn or total human/murine (Figure 28C,D).
Figure 28. α-Synuclein inclusions and insoluble protein are unaltered by hNPCs.
Quantification of confocal micrographs of h-α-syn (Syn211)-labeled inclusions reveal no difference between VEH and CNS10-hNPC treated ASO mice (n=5-6/group; Scale bar=20 µm) (A,B). Immunoblots likewise indicate no effect of CNS10-hNPCs on insoluble human-α-syn or total α-syn at 1 (black) or 3 months (green) post-transplantation (n=4/group) (C,D). Dimer (E) and High Molecular Weight (HMW)(F) α-syn species were exclusively observed in total α-syn immunoblots, although CNS10-hNPC transplantation once again had no effect on these insoluble conformers. All values are means ± S.E.M., expressed as percent of within-time point WT-VEH group.
Although multimeric forms of h-α-syn (Syn211) were not observed in the insoluble fraction (Figure 28C,D), Western blots showed clear expression of a dimer (28 kDa) and a smear of high molecular weight (HMW) species in total α-syn, the specificity of which was verified by their absence in α-syn knockout samples (Figure 28C,E,F). A detected band at 12 kDa may represent a truncated form of monomeric α-syn (Games, Valera et al. 2014). Intriguingly, while expression of monomeric, dimeric and HMW conformers was significantly elevated in ASO mice at the 1-month time point, only the HMW species was significantly higher at 3 months, suggesting an effect of age on expression of this particular species (Figure 28F).

**hNPC transplantation alters soluble monomeric and oligomeric α-synuclein species**

The absence of CNS10-hNPC-induced changes in insoluble α-syn does not preclude alterations to the soluble protein. Therefore, we next assessed detergent soluble human-α-syn monomers (Figure 29A; n=4). As expected, a significant main effect of genotype confirmed that h-α-syn (Syn211) was expressed only in ASO mice ($F_{(1,24)}=107.6$, $p<0.0001$). However, we also observed a significant interaction between treatment and time point, indicating the increased ability of CNS10-hNPCs to reduce soluble h-α-syn over time ($F_{(1,24)}=5.8$, $p<0.02$). While monomeric h-α-syn (Syn211) was not reduced by CNS10-hNPCs at 1 month in ASO-CNS10 mice, CNS10-hNPCs did significantly reduce h-α-syn monomers by 3 months in the soluble fraction (Figure 29A,B). The ASO transgenic model expresses both human and mouse α-syn, therefore we examined whether either h-α-syn or CNS10-hNPCs impacted total levels of human
and murine α-syn. As would be expected, total levels of soluble α-syn were elevated in ASO mice (Figure 29A,C; F(1,24)=10.36, p<0.004). However, CNS10-hNPC transplantation had no impact on total α-syn levels, suggesting that CNS10-hNPC-induced changes in α-syn are specific to the over-expressed human form of this protein.

Next, to confirm that changes in monomeric α-syn were being driven at the level of protein accumulation rather than transgene expression, we conducted quantitative real-time PCR of both human and mouse α-syn. As predicted, h-α-syn transgene expression was unchanged between ASO-VEH and ASO-CNS10 groups at 3 months and undetectable in WT-VEH and WT-CNS10 groups, verifying that CNS10-hNPC driven changes occur at the protein level (Figure 29D). Further, mouse α-syn was also not affected by genotype or treatment between groups, supporting the explanation that total α-syn changes were likely driven at the protein level (Figure 29D).

Finally, we sought to address whether this change in monomeric h-α-syn was impacting larger soluble α-syn oligomers. We therefore assayed total α-syn oligomers by dot blot using an oligomer-specific antibody, ASyO2 (n=4-7) Agrisera (Brannstrom, Lindhagen-Persson et al. 2014). Staining with this antibody showed significant main effects of genotype (F(1,32)=53.8, p<0.0001) and time point (F(1,32)=14.9, p<0.0005), and an interaction of time point and treatment (F(1,32)=5.4, p<0.02) indicating that CNS10-hNPCs have a more substantial impact on oligomer expression over time. Total ASyO2 α-syn oligomers in ASO-VEH mice were elevated compared to their respective WT groups at both 1- and 3-month time points (Figure 29E). However, CNS10-hNPC
transplantation significantly reduced oligomeric α-syn to WT by 3 months post-transplantation (Figure 29E), suggesting that CNS10-hNPCs can reduce both monomeric h-α-syn and total oligomeric α-syn. Importantly, this pattern was confirmed using another oligomer-specific antibody (mOC 78, generously provided by Dr. Charles Glabe (UCI), Figure 29F), which detects fibrillar oligomeric conformations of several pathological proteins, with no crossover to monomers (Hatami, Albay et al. 2014). In contrast to ASyO2, M78 showed main effects of genotype ($F_{(1,32)}=6.3$, $p<0.02$), and an interaction of genotype and treatment ($F_{(1,32)}=4.1$, $p<0.05$), where oligomers increased in ASO-VEH groups at both time points but only significantly at the 3 month time point (Figure 29F). A significant reduction of α-syn oligomers by M78 was only observed after 3 months transplantation in ASO-CNS10 mice (Figure 29F). The modest differences in signal detection between ASyO2 and M78 antibodies in the 1-month groups demonstrates the importance of corroborating total oligomer expression across multiple antibodies. Together, these results suggest that CNS10-hNPC-induced reductions in soluble human α-syn monomers and oligomers plays a critical role in the observed improvements in neurotransmission and behavior.
Figure 29. Soluble human α-synuclein monomers and oligomers are reduced by hNPC transplantation. (A) Detergent soluble h-α-syn monomers are decreased by CNS10-hNPC transplantation only after 3 months in ASO mice (B), whereas total human and mouse-α-syn (t-α-syn) monomers are unaltered by CNS10-hNPCs (n=4/group) (C). Quantitative real-time PCR shows h-α-syn transgene expression only in ASO mice and unaltered by CNS10-hNPCs, while mouse-α-syn mRNA is present at similar levels in all four groups (n=4/group) (D). Dot blots probed with the oligomer-specific antibody ASyO2 show significant CNS10-hNPC-induced decreases in soluble oligomers at both 1-(black) and 3-month (green) time points in ASO mice (E), while the oligomer-specific M78 antibody reveals significant CNS10-hNPC-induced reduction only after 3 months (n=4-7/group) (F). All values are means ± S.E.M., expressed as percent of within-time point WT-VEH group. *p-values are significantly different from all other within-time point groups.
DISCUSSION

Dementia with Lewy Bodies (DLB) is one of many proteinopathies in which the aberrant behavior of pre-synaptic α-syn leads to cellular stress and neurodegeneration. In DLB, this pathology manifests in cortical and midbrain regions, which converge on the striatum through disruption of both nigrostriatal dopamine and corticostriatal glutamate signaling. Dysregulation of these neurotransmitter systems then leads to impairment of the motor and cognitive functions to which they contribute.

The potential of neural stem cell transplantation to alleviate motor and cognitive impairments associated with α-syn accumulation has been largely unexplored. However, our group recently found that allogeneic mouse NSCs can improve these deficits in immune-competent DLB mice (Goldberg, Caesar et al. 2015). In order to determine the translational potential of this approach using human-derived NPCs, we created a novel xenotransplantation-compatible model of DLB.

CNS10-hNPCs were utilized in the current study based on the long history using these cells in multiple models of neurodegeneration, including Huntington’s disease (HD), Parkinson’s disease (PD), retinal degeneration and amyotrophic lateral sclerosis (ALS) (Burnstein, Foltynie et al. 2004, McBride, Behrstock et al. 2004, Wang, Girman et al. 2008, Nichols, Gowing et al. 2013). The cells show robust survival, extensive migration and differentiation toward a glial phenotype (Gowing, Shelley et al. 2014, Das, Avalos et al. 2016). Critically, the cells do not display tumor formation or other adverse events in animals including rodents, primates and pigs (Behrstock, Ebert et al. 2006, Riley,
Federici et al. 2009, Gowing, Shelley et al. 2014). The CNS10-hNPCs have recently been manufactured under cGMP (Shelley, Gowing et al. 2014), making them now ready for clinical application.

In this current study, the data reveal that preferentially gliogenic CNS10-hNPCs can dramatically improve both motor and cognitive deficits in α-syn transgenic mice. Importantly, these benefits appear to be achieved by restoring dopaminergic and glutamatergic signaling and reducing soluble α-syn monomers and oligomers. Although we hypothesized that CNS10-hNPC transplantation might partially rescue behavioral deficits, motor and cognitive analyses instead yielded robust or nearly complete recovery. This is likely due to the multiple homeostatic mechanisms influenced by CNS10-hNPC transplantation, and the extensive migration of human cells throughout the striatum. CNS10-hNPCs engrafted and demonstrated impressive survival and migration by 3 months post-transplantation, likely due in part to their expression of the chemokine receptor CXCR4 which has been shown by several groups to mediate NPC migration within the brain (Imitola, Raddassi et al. 2004, Kelly, Bliss et al. 2004, Carbajal, Schaumburg et al. 2010). CNS10-hNPCs that remained near the injection site expressed a more proliferative potential compared to the minimally proliferative phenotype of the disperse populations. Concomitantly, CNS10-hNPCs that had migrated away from the injection site expressed a more glial phenotype. This is as one might expect as cells decrease in proliferative potential as they differentiate. This largely glial differentiation and enhanced expression of the glial glutamate transporter GLT-1 in striatal tissue suggests that CNS10-hNPCs may aid glutamate regulation
through a glial-dependent mechanism. It is unlikely that CNS10-hNPCs achieve changes in glutamate regulation through neuronal transport as evidenced by absence of change in EAAC1. This mechanism is also supported by reports that cortical α-syn can impair cognitive performance through disrupting striatal glutamate transmission (Lindgren, Tait et al. 2014). Cognitive performance correlated well with both glutamate and dopamine markers, substantiating the involvement of both neurotransmitter systems in object and spatial recognition (Frank and O’Reilly 2006, Watson, Loiseau et al. 2012). Motor recovery, in contrast, was clearly most strongly associated with dopamine regulation.

Based on our findings in ASO mice with allogeneic mouse NSC transplantation (Goldberg, Caesar et al. 2015), we did not anticipate an impact of CNS10-hNPCs on α-syn expression. However, our analyses revealed an unexpected decrease of soluble α-syn monomers and oligomers in response to long term (3 month) CNS10-hNPC transplantation. These results were contrasted by an absence of effects on insoluble α-syn protein and Lewy body-like aggregates. The finding that total (human and mouse) α-syn levels were not changed due to CNS10-hNPCs suggests that the human protein over-expressed in ASO mice is specifically targeted by CNS10-hNPCs. Although there are several hypotheses as to the toxic species of the intrinsically disordered α-syn protein, debate continues regarding which of its many conformers is particularly disruptive to cellular function (Cookson and van der Brug 2008, Auluck, Caraveo et al. 2010, Lashuel, Overk et al. 2013, Poehler, Xiang et al. 2014, Forloni, Artuso et al. 2016). One contention is that the cytosolic Lewy Body inclusions themselves lead to
inflammation and cell death by obstructing essential cellular processes. Another proposes that the toxic species are small soluble oligomers – wild-type or mutated -- which react with membranes to perturb vesicle trafficking, mitochondrial function and protein turnover. While we find that soluble monomeric and oligomeric α-syn is reduced by CNS10-hNPC transplantation, this does not suggest that these conformers are innately toxic. Rather, it is likely that an over-abundance of these particular α-syn conformers can have toxic effects. This hypothesis is supported by our observation of substantial α-syn oligomers in WT mice. Another argument proposes that the toxicity of α-syn is determined less by accumulation of a particular conformer, and more by the high extracellular concentrations of α-syn achieved in disease phenotypes that can stimulate inflammation and overwhelm protein degradation and clearance mechanisms (Lee, Suk et al. 2008). Although this proposition is beyond the scope of the studies reported here, characterization of the mechanisms by which hNPCs lead to reduced soluble α-syn in ASO mice warrants further investigation. The results we report herein suggest that both soluble monomeric and oligomeric α-syn contribute to the disruption of dopamine and glutamate neurotransmission that results in drastic cognitive and motor deficits in this model, and that CNS10-hNPC transplantation reduces these while having no effect on insoluble conformers.

Our finding that soluble human-α-syn but not insoluble species are exquisitely altered by CNS10-hNPC transplantation strongly suggests that these changes are most relevant to CNS10-hNPC mediated behavioral recovery. Complimentary to this is a recent report suggesting that multiple types of missense mutations in α-syn can promote a shift from
likely native tetramers to monomers (Dettmer, Newman et al. 2015, Dettmer, Newman et al. 2015). Excess soluble monomers are then free to assemble into β-sheet rich oligomeric intermediates which may participate in cytotoxicity (Breydo, Wu et al. 2012). Total murine and human soluble monomers were clearly not altered by CNS10-hNPC transplantation, and mouse specific mRNA was unaffected by either human-α-syn overexpression or CNS10-hNPCs. Therefore, it is likely that soluble mouse syn – specifically soluble monomers – continue to carry out native functions (Westphal and Chandra 2013), while the human-α-syn contributes to disease phenotypes in the Rag-ASO mouse model.

The absence of a transplantation effect on Lewy body-like inclusions and insoluble oligomeric species augments the evidence we report in soluble α-syn protein. The role of Lewy bodies in neurodegeneration is also debated, as their occurrence is not likely sufficient to be a major cause of degeneration (Duda, Giasson et al. 2002, Kramer and Schulz-Schaeffer 2007, Schulz-Schaeffer 2012). It has even been proposed that aggregation of α-syn into Lewy bodies is an adaptive response to primary insults such as inflammation due to small soluble α-syn oligomers and an attempt to sequester toxic levels of soluble species (Lee, Bae et al. 2014, Surendranathan, Rowe et al. 2015). Given these findings, further attention is warranted toward the shift in specific oligomeric species in models of DLB and the potential use of hNPC transplantation as a mechanism to alleviate cellular stress and improve both dopamine and glutamate neurotransmission and cognitive and motor function. In summary, the results demonstrate that transplantation of clinically relevant CNS10-hNPCs could offer a
promising new therapeutic approach to treat both the motor and cognitive components, along with the biochemical perturbations, of synucleinopathies.
CONCLUDING REMARKS

Stem cells are sources of trophic factor support, immune modulation, enzymatic regulation and protein delivery. These are the characteristics that make neural stem cells and specific cell type progenitors excellent tools for targeting multiple mechanisms that are perturbed in neurodegenerative disease, and particularly proteinopathies. My dissertation aimed to determine whether neural stem cells (NSCs) could be used to overcome and clear α-synuclein pathology, leading to improved neurotransmission and cognitive function.

My first approach to finding a candidate cell type was to transplant unaltered mouse NSCs into a syngeneic transgenic model of Dementia with Lewy bodies (DLB). Cells were transplanted into the striatum of ASO and WT mice. This region was chosen based on its exquisite degeneration in synucleinopathies such as Parkinson’s disease and DLB. The striatum is also a region of convergence for cortical glutamate and nigral dopamine projections, both of which are selectively perturbed in synucleinopathies (Dalfo, Albasanz et al., Klein, Eggers et al.). The robust connections of the striatum with these two regions which dominantly modulate multiple cognitive and motor behaviors also made it an ideal target region for transplantation of NSCs.

Having predicted that these cells would require genetic modification to heighten expression and secretion of certain growth factors or protein clearance enzymes in order to impact behavioral outcomes, I was surprised to find that unaltered mNSCs could drastically improve cognitive and motor function. These cortical/hippocampal NSCs derived at post-natal day 1 were chosen due to their elevated expression of brain-derived neurotrophic factor (BDNF) relative to other cell types, and preferential
glial differentiation, and they demonstrated both of those characteristics upon transplantation into immune-intact DLB mice. Paramount to the beneficial effects of mNSC transplantation and elevated BDNF on behavior was the NSC-induced modulation of dopamine and glutamate signaling markers. The regulating synthesizing enzymes and transporters of these neurotransmitters which were decreased in ASO mice were rescued to wild-type levels by transplantation. Critically, none of these restorations occurred if NSCs were modified not to express BDNF, and pharmacological inhibition of dopamine and glutamate also prevented parent mNSCs from improving behavior despite BDNF expression.

These observations led me to conclude that the important aspects of a candidate cell type for transplantation to disrupt DLB pathology were glial fate and neurotrophic production. Therefore, when choosing a human-derived stem cell type to begin translating this approach toward eventual clinical application, I decided on a glial-fated line that would likely produce higher levels of neurotrophic factors.

The clinical-grade CNS10-WT human NSC (hNSC) stem cell line derived and expanded in the lab of Dr. Clive Svendsen at Cedars Sinai is established for transplantation in several models of neural injury, including amyotrophic lateral sclerosis (Riley, Federici et al.), spinal cord injury (Ebert, Beres et al.) and stroke (Andres, Horie et al.). By back crossing a commercially available immune-incompetent mouse (Rag2/Ii2ry, Taconic #4111) to the ASO model, our lab developed the first xenotransplantation-compatible DLB model. This model provided an elegant approach to avoiding the complications of α-synuclein modulation by standard immunosuppressant drugs (Gerard, Debyser et al., Agrawal, Dixit et al.). Having established the candidate cell type and transgenic model,
I conducted short (1 month) and longer term (3 month) transplantation studies to examine early engraftment and differentiation as well as extended survival and engraftment of hNSCs. These studies demonstrated that hNSCs engraft, migrate throughout the striatum by 3 months, differentiate toward largely glial fate and rescue cognitive and motor function. Similarly to what I observed with allogenic mNSC transplantation, dopamine and glutamate regulatory markers were also returned to homeostatic expression levels. One major distinction between hNSC and mNSC transplantation studies was the absence of BDNF loss in Rag-ASO mice, and of elevation in hNSC transplanted Rag-ASO mice. The most illuminating finding from the hNSC study was the reduction of soluble α-synuclein monomers and oligomers 3 months following transplantation. A fascinating contrast was revealed in the null effect of hNSCs on α-synuclein inclusions and insoluble species. These findings point toward small soluble α-synuclein conformers as detrimental to dopamine and glutamate signaling in ASO mice, and that hNSC-mediated reductions in these conformers can conversely lead to substantial beneficial behavioral outcomes.

Importantly, although no α-synuclein pathology was observed within mNSCs or hNSCs, this does not preclude the possibility of transmission. Transmission of α-synuclein pathology to grafted cells in human trials have only been observed after 5 years of transplantation (Kordower, Chu et al., Li, Englund et al.). Although transmission of host α-synuclein to grafted cells has been shown short term in vivo, susceptibility of these cells may be explained by differences in isolation (E15-18 versus P1 in the present study) or transgenic mouse model (Thy-1 versus PDGFβ promoter driven α-synuclein in
the present studies), or the neuronal versus glial differentiation of transplanted NSCs. One study even reported that transplanted mesenchymal cells prevent propagation of α-synuclein in host cells (Oh, Kim et al.). The modulatory capacity of hNSC transplantation on α-synuclein warrants further investigation, as longer term engraftment and hNSC modification may amplify or accelerate α-synuclein clearance and associated improvement in cortical and striatal dependent behavioral function.

**The Way Forward**

Two concerns remain regarding the efficacy of stem cell transplantation for treating synucleinopathies: how to enable grafted cells to 1) resist α-synuclein accumulation, and 2) facilitate host Lewy Body and toxic oligomer degradation and clearance. The best evidence that this can be achieved comes from transplantation studies in Alzheimer’s disease models, where cells producing the degrading enzyme neprilysin (Hemming, Patterson et al., Blurton-Jones, Spencer et al.) or insulin degrading enzyme (Vekrellis, Ye et al.) have been able to reduce Amyloid-β plaques and tau tangles. Aberrant and reduced function of several protein degrading enzymes and protein clearance machinery have been specifically linked to α-synuclein aggregation, including neurosin (Iwata, Maruyama et al.), UCH-L1 (Liu, Fallon et al.), the S6 proteasome (Snyder, Mensah et al.) and the co-chaperone CHIP (Shin, Klucken et al. 2005). Transplanted cells could also potentially be modified to enhance chaperone mediated autophagy clearance of α-synuclein aggregates (Cuervo, Stefanis et al., Vogiatzi, Xilouri et al. 2008, Xilouri, Brekk et al. 2016). Enhancing such mechanisms via ex vivo
gene therapy are likely to confer both graft resistance to pathology, and reduction of host pathology.

Increased production and secretion of these proteins by transplanted cells would suggest a multi-pronged approach to disease modification. Several cell types produce and secrete other neuroprotective growth factors such as BDNF (Blurton-Jones, Kitazawa et al., Goldberg, Caesar et al.). Cell types like mesenchymal and neural stem cells are ideal candidates for modification due to their ability to migrate and impact widespread α-synuclein pathology (Chamberlain, Fox et al., Goldberg, Caesar et al.). Both migratory capacity of transplanted cells and further diffusion of secreted enzymes through the brain parenchyma contribute to the potential of such therapies to have widespread impact on α-synuclein pathology.

Together, my dissertation provides key insights into the multiple mechanisms modulated by NSC transplantation, and discrete benefits of both mouse and human cells. Further investigation is necessary to elucidate the best combination of cell type for pathology resistance, widespread migration and ex vivo delivery of clearance enhancing therapeutics. However, the promise of stem cell transplantation to ameliorate the behavioral impairments associated with synucleinopathies substantiates the continued pursuit of this approach.
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