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Autologous Peripheral Nerve Grafts to the Brain for the Treatment of Parkinson's Disease

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Andrew Welleford, Student

Dr. Greg A. Gerhardt, Major Professor

Dr. Wayne Cass, Director of Graduate Studies

Autologous Peripheral Nerve Grafts to the Brain for the Treatment of Parkinson's Disease

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DISSERTATION

A dissertation submitted in partial fulfillment of the Requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
Andrew Stoll Welleford

Lexington, Kentucky

Co-Directors: Dr. Greg Gerhardt, Professor of Neuroscience
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Lexington, Kentucky

2019

ABSTRACT OF DISSERTATION

Autologous Peripheral Nerve Grafts to the Brain for the Treatment of Parkinson's Disease

Parkinson's disease (PD) is a disorder of the nervous system that causes problems with movement (motor symptoms) as well as other problems such as mood disorders, cognitive changes, sleep disorders, constipation, pain, and other non-motor symptoms. The severity of PD symptoms worsens over time as the disease progresses, and while there are treatments for the motor and some non-motor symptoms there is no known cure for PD. Thus there is a high demand for therapies to slow the progressive neurodegeneration observed in PD. Two clinical trials at the University of Kentucky College of Medicine (NCT02369003, NCT01833364) are currently underway that aim to develop a disease-modifying therapy that slows the progression of PD. These clinical trials are evaluating the safety and feasibility of an autologous peripheral nerve graft to the substantia nigra in combination with Deep Brain Stimulation (DBS) for the treatment of PD. By grafting peripheral nerve tissue to the Substantia Nigra, the researchers aim to introduce peripheral nerve tissue, which is capable of functional regeneration after injury, to the degenerating Substantia Nigra of patients with PD. The central hypothesis of these clinical trials is that the grafted tissue will slow degeneration of the target brain region through neural repair actions of Schwann cells as well as other pro-regenerative features of the peripheral nerve tissue.

This dissertation details analysis of the peripheral nerve tissue used in the above clinical trials with respect to tissue composition and gene expression, both of injury-naive human peripheral nerve as well as the post-conditioning injury nerve tissue used in the grafting procedure. RNA-seq analysis of sural nerve tissue pre and post-conditioning show significant changes in gene expression corresponding with transdifferentiation of

Schwann cells from a myelinating to a repair phenotype, release of growth factors, activation of macrophages and other immune cells, and an increase in anti-apoptotic and neuroprotective gene transcripts. These results reveal in vivo gene expression changes involved in the human peripheral nerve injury repair process, which has relevance beyond this clinical trial to the fields of Schwann cell biology and peripheral nerve repair. To assess the neurobiology of the graft post-implantation we developed an animal model of the grafting procedure, termed Neuro-Avatars, which feature human graft tissue implanted into athymic nude rats. Survival and infiltration of human graft cells into the host brain were shown using immunohistochemistry of Human Nuclear Antigen. Surgical methods and outcomes from the ongoing development of this animal model are reported. To connect the results of these laboratory studies to the clinical trial we compared the severity of motor symptoms before surgery to one year post-surgery in patients who received the analyzed graft tissue. Motor symptom severity was assessed using the Unified Parkinson's Disease Rating Scale Part III. Finally, the implications and future directions of this research is discussed. In summary, this dissertation advances the translational science cycle by using clinical trial findings and samples to answer basic science questions that will in turn guide future clinical trial design.

KEYWORDS: Neurodegeneration, Neuroprotection, Neuroregeneration, Schwann cell, Clinical trial

Andrew Stoll Welleford

2019

AUTOLOGOUS PERIPHERAL NERVE GRAFTS TO THE BRAIN FOR THE TREATMENT OF PARKINSON'S DISEASE

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07/14/2019
Date

Dedicated to my parents, who gave all of their children the love and support they needed to set out into the world,

And to Lauren, my partner through this chapter of life.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF COMMONLY USED ABBREVIATIONS

BDNF: Brain-Derived Neurotrophic Factor

CNS: Central Nervous System

DBS: Deep Brain Stimulation

GDNF: Glial cell line-Derived Neurotrophic Factor

GFAP: Glial Fibrillary Acidic Protein

GPi: internal Globus Pallidus

HNA: Human Nuclear Antigen

MR: Magnetic Resonance

PD: Parkinson's Disease

PN: Peripheral Nerve

PNS: Peripheral Nervous System

SC: Schwann Cell

SN: Substantia Nigra

STN: Subthalamic Nucleus

UPDRS: Unified Parkinson's Disease Rating Scale

VEGF: Vascular Endothelial Growth Factor

Chapter One: Introduction

Parkinson's Disease and Peripheral Nerve Grafting to the CNS[1]

Parkinson's Disease and Its Current Therapies

Parkinson's disease (PD) is a disorder of the nervous system that causes problems with movement (motor symptoms) as well as other problems (non-motor symptoms). The motor symptoms of PD are the most widely-known complications of the disease and include slow movement (bradykinesia), rigidity, and/or a tremor in body parts at rest (resting tremor). The non-motor symptoms of PD include cognitive impairment, autonomic dysfunction, sleep disorders, constipation, depression, olfactory dysfunction, and pain.

PD primarily affects the elderly. The average age of PD diagnosis is in the late fifties, and the risk of being diagnosed with PD increases with age. (Pringsheim, Jette, Frolkis, & Steeves, 2014) PD affects over 630,000 people in the United States (US), leading to the total economic burden of 14.4 billion per year in 2010 (Kowal, Dall, Chakrabarti, Storm, & Jain, 2013). Because PD primarily affects the elderly, and because average age of the US population is rising, these numbers are projected to increase to an estimated 1.34 million people in the US alone by 2050 (Kowal et al., 2013).

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[1] Andrew S. Welleford, Nader El Seblani, George E. Quintero, Craig van Horne, Greg A. Gerhardt
Illustrations by Tom Dolan
University of Kentucky

The severity of PD symptoms worsens over time as the disease progresses, and while there are treatments for the motor and some non-motor symptoms there is no known cure for PD. PD is classified as a neurodegenerative disorder, meaning that it is caused by the progressive degeneration and death of cells of the nervous system. In PD, loss of dopamine-producing cells in the substantia nigra region of the midbrain leads to decreased function of the nigrostriatal pathway and dysregulation of the basal ganglia motor circuit, leading to the motor symptoms of PD (Kordower et al., 2013). Other regions of the nervous system also degenerate in PD and contribute to the non-motor symptoms of the disease. The Braak Staging hypothesis states that neurodegeneration in PD is first observed in the enteric nervous system (as well as the olfactory bulb) (Braak et al., 2003) and progresses in an anatomically connected pattern to affect the dorsal motor nucleus of the vagus, basal forebrain, substantia nigra, mesial temporal cortex, and in late-stage disease the cerebral cortex (Halliday et al., 2010). Progressive degeneration of these brain regions is correlated with the progression of clinical symptoms (De Pablo-Fernandez, Lees, Holton, & Warner, 2019; Wolters, 2006).

Unlike most other neurodegenerative disorders, the motor symptoms of PD can be effectively treated pharmacologically. The primary medical treatment for PD is levodopa (LeWitt, 2008), which is often given in combination with other medications to increase its effectiveness. Levodopa, or L-DOPA, is the biosynthetic precursor to dopamine in the human body. Levodopa administered orally in pill form can cross the blood-brain barrier and enter the striatum, where it is converted to dopamine. This supplements the level of dopamine in the striatum which would be provided by an intact

nigrostrial pathway but which is reduced in PD. In doing so levodopa reduces the severity of motor symptoms in PD. Other medications given in combination with levodopa can increase this effectiveness. For example, carbidopa is an inhibitor of the enzyme aromatic-L-amino-acid decarboxylase (DOPA decarboxylase or DDC), which converts levodopa to dopamine. Carbidopa does not cross the blood-brain barrier, so it inhibits the conversion of levodopa in the periphery but not in the CNS, leading to more levodopa being available in the brain. This increases the effectiveness of levodopa medication (Koller, Hutton, Tolosa, Capilldeo, & Group, 1999). Because of this synergistic action, carbidopa + levodopa (brand name Sinemet) combination therapy is the first-line treatment for PD (Yeh et al., 1989).

However, the severity of motor symptoms in PD worsens over time even if PD is medically treated (De Pablo-Fernandez et al., 2019). Because of this worsening of symptoms, higher doses of medication are required to manage the symptoms of PD over time (LeWitt, 2008). This leads to negative complications of medical therapy such as dyskinesia; or uncontrolled, involuntary movement (Kumar, Mishra, Dwivedi, & Subramaniam, 2015). Fortunately, more effective therapies for PD now exist.

Parkinson's disease is also distinct from other neurodegenerative diseases in that it can be effectively treated using a surgical approach. Deep Brain Stimulation (DBS) is an FDA-approved surgery for the treatment of PD (Benabid, Chabardes, Mitrofanis, & Pollak, 2009). This surgery involved the implantation of stimulating electrodes to targeted brain regions. In the case of PD, the target are the basal ganglia regions of either the internal segment of the globus pallidus (GPi) or the subthalamic nucleus (STN) (Williams, Foote, &

Okun, 2014). By stimulating these brain regions, the functional signaling of the basal ganglia is altered and the severity of motor symptoms is reduced (Perlmutter & Mink, 2006). These electrodes are connected through a subcutaneous lead wire to a programmable stimulator + battery usually placed below the skin of the chest. This allows for the frequency and pattern of stimulation to be remotely programmed in the clinic to fine-tune the control of a patient's motor symptoms and to adjust the programming as PD progresses and the severity of motor symptoms increases (Volkmann, Moro, & Pahwa, 2006). For this reason DBS has primarily replaced surgical lesioning of basal ganglia structures as the surgical treatment of choice for the treatment of PD (Marsden & Obeso, 1994), and has been shown to be more effective than the best medical therapy for the treatment of PD (Weaver et al., 2009).

While effective, the medical and surgical treatments for PD have limitations. Medication and surgery can treat the motor symptoms of PD, they are not known to slow or reverse the degenerative process of the disease (Hilker et al., 2005). Furthermore, most non-motor symptoms of PD are not addressed by either of these therapies. Thus there exists a high demand for disease-modifying therapies to slow or reverse the progressive neurodegeneration observed in PD.

The DBS+ Clinical Trials

Two clinical trials at the University of Kentucky College of Medicine (NCT02369003, NCT01833364) are currently underway that aim to develop a disease-modifying therapy that slows the progression of PD. These clinical trials are evaluating the

safety and feasibility of an autologous peripheral nerve graft to the Substantia Nigra in combination with DBS for the treatment of PD. Figure 1.1 illustrates the nerve graft procedure. By grafting peripheral nerve tissue to the Substantia Nigra the researchers aim to introduce peripheral nerve tissue, which is capable of functional regeneration after injury, to the degenerating Substantia Nigra of patients with PD. (van Horne et al., 2017) The central hypothesis of these clinical trials is that the grafted tissue will slow degeneration of the target brain region through neural repair actions of Schwann cells as well as other pro-regenerative features of the peripheral nerve tissue.

The combination of DBS with delivery of an additional therapy is termed “DBS+” by the investigators. DBS+ utilizes DBS surgeries as a platform for the delivery of therapeutics to the CNS. The same stereotaxic hardware used to implant DBS electrodes is also effective in delivering other treatments to deep brain regions. In addition, these therapies can be delivered during the same surgical and anesthesia events as DBS alone. In these trials, PD patients receive both the standard of care DBS surgery as well as an experimental disease-modifying therapy (i.e. peripheral nerve tissue), but the delivery of other therapeutics using DBS+ is possible as well.

A core practice of this clinical trial is the pre-lesioning of nerve graft tissue two weeks prior to implantation. This pre-lesioning practice is also known as a conditioning injury, and has been the subject of research in the field of peripheral nerve regeneration. (Neumann & Woolf, 1999) The reasoning for this practice is that peripheral nerve tissue undergoes a repair program following injury, during which Schwann cells transdifferentiate to a repair phenotype, growth factors such as Glial-Derived

Neurotrophic Factor (GDNF) are secreted, and infiltrating macrophages release pro-regenerative cytokines; all in a coordinated fashion which promotes functional regeneration of the injured nerve. (Cattin & Lloyd, 2016) Figure 1.2 summarizes this process. The clinical trial researchers hypothesize that by pre-lesioning the nerve tissue before grafting they will induce this repair program and create a pro-regenerative tissue for implantation. The rationale for this approach is further discussed in the following section.

Peripheral Nerve Grafts to the Central Nervous System

Peripheral nervous system (PNS) axons are capable of functionally regenerating, while central nervous system (CNS) axons cannot (Cajal, 1928). The stark dichotomy between PNS and CNS regenerative potential suggests an approach to develop therapeutics for PD and other neurodegenerative diseases: make use of the regenerative properties of PNS to slow or reverse the progression of the disease by providing the existing neurons in the CNS with increased ability to regenerate their projections.

The capability of peripheral nerves to regenerate, and to support PNS repair, is already in use in medical applications. Some microsurgical techniques use peripheral nerve scaffolds to bridge large gaps in damaged nerve, improving motor and sensory recovery after injury (for review see (Pabari, Yang, Seifalian, & Mosahebi, 2010)). Other research focuses on creating synthetic extracellular matrix scaffolds for the same purpose, however these artificial scaffolds are far less effective than autologous peripheral nerve for facilitating regeneration of nerve tissue and restoring function (for

review see (Gu, Ding, Yang, & Liu, 2011). This is the first of several pieces of evidence discussed that the regenerative properties of peripheral nerve are derived from the interaction of multiple tissue components.

The ability of extracellular matrix to promote axonal regeneration is not seen in the CNS. Research has demonstrated the opposite effect: factors such as Nogo and MAG in the CNS extracellular matrix regulate and limit axonal growth (Yiu & He, 2006). Thus, a axonal growth-inhibiting extracellular matrix distinguishes the CNS from the PNS. However, CNS neurons have been shown to be capable of growing through peripheral nerve tissue (P. M. Richardson, McGuinness, & Aguayo, 1980). Seminal work from Richardson, David, and Aguayo demonstrated that peripheral nerve bridges grafted onto the spinal cord and brainstem cause CNS axons to grow into and through the entire length of the nerve. It was this finding that led to the first attempts to use PNS tissue to treat CNS disorders. Demonstration of spinal cord axon growth into peripheral nerve grafts drew much attention for its potential to treat spinal cord injury. The goal of such experiments was to bridge injured areas of the spinal cord and re-establish connections with distal neurons, restoring motor and sensory function below the lesion. Such applications were found to be infeasible when it was demonstrated that spinal cord neurons did not grow much beyond the peripheral nerve graft (David & Aguayo, 1981), and that functional recovery was unsatisfactory (P. M. Richardson, McGuinness, & Aguayo, 1982). More studies examining the use of peripheral nerve are discussed in the following section.

One prominent difference between PNS and CNS tissue is the presence of Schwann cells (SCs) within peripheral nerves. These cells provide mechanical protection and electrical insulation to the axons associated with them. SCs also provide trophic support for existing axonal populations and facilitate the regeneration of a peripheral nerve after injury via the release of soluble neurotrophic factors (Jessen, Mirsky, & Lloyd, 2015). In response to nerve injury, SCs change their phenotype from a myelinating form to a regenerative form (Jessen & Mirsky, 2016; Kidd, Ohno, & Trapp, 2013). Regenerative SCs in cutaneous sensory nerve, for example, release soluble growth factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), neurotrophin-3 (NT-3), and glial cell line-derived neurotrophic factor (GDNF) (Bunge, 1994; Henderson et al., 1994; Hoke et al., 2006; Park & Hoke, 2014). In doing so the SCs of the distal end of an injured peripheral nerve guide new axonal growth from the proximal nerve ending (Fu & Gordon, 1997).

The conversion of SCs from a myelinating phenotype to a repair phenotype is controlled by the transcription factor c-Jun. c-Jun induces the activation of genes involved in promoting repair (Arthur-Farraj et al., 2012). Trans-differentiation of Schwann cells consists of autophagy of myelin (Jung et al., 2011), inhibition of myelin-related gene expression (reviewed in (C. J. Chen et al., 2007; Jessen & Mirsky, 2008)), and expression of markers for immature Schwann cells such as GFAP and p75 (C. J. Chen et al., 2007; Jessen & Mirsky, 2008). In addition to these changes analogous to phenotypic reversion, repair Schwann cells also exhibit a phenotype unique from both immature SCs and

uninjured myelinating Schwann cells (Arthur-Farraj et al., 2012). Markers that distinguish repair Schwann cells include growth factors such as GDNF and Artemin, which promote axonal growth and survival of peripheral nerves. (Arthur-Farraj et al., 2012; Fontana et al., 2012; Jessen & Arthur-Farraj, 2019).

There is also evidence to suggest that factors produced by regenerative Schwann cells also promote growth of CNS axons. Peripheral nerve grafts lacking SCs are not as effective in promoting CNS axon growth as nerve grafts with viable SCs (Smith & Stevenson, 1988). Addition of many of the individual growth factors released from SXs (GDNF, BDNF, NGF, NT-3, and VEGF) have been shown to promote CNS neuron survival, for example NGF (Kromer 1987). Direct trophic factor delivery has been demonstrated to be therapeutic in animal models of disease such as Alzheimer's disease (Tuszynski, U, Amaral, & Gage, 1990) and Parkinson's disease (Gash et al., 1996). Direct delivery of trophic factors to the CNS has also been evaluated for treatment of these disease is humans, with some degree of success (S. S. Gill et al., 2003; Lang et al., 2006; Slevin et al., 2005; Tuszynski et al., 2005; A. Whone et al., 2019). These findings demonstrate a less explored application of peripheral nerve grafts to the CNS: supplying trophic factors. Peripheral nerve cells engineered to overexpress growth factors have the potential to serve as living trophic factor delivery systems in a new generation of CNS disorder therapeutics. However, details about which specific growth factors are effective in which neuronal populations, what dose of growth factors is effective, the logistics of growth factor delivery, and coordination with other pro-repair processes have not yet been fully explored in the scientific literature. The staggering complexity of the growth factor

activity response of peripheral nerve to injury is illustrated by the transcriptomic changes after injury described in Chapter Two of this dissertation. Thus there is an elegant appeal to using pre-existing autologous peripheral nerve tissue which has the demonstrated capacity to promote nervous system repair.

Trophic factors working in concert with the extracellular matrix, immune cells, and other repair SC properties are the pro-regenerative features that led this clinical trial to revisit peripheral nerve as a potential disease-modifying therapy for PD. The following sections will discuss previous pre-clinical and clinical studies investigating the use of peripheral nerve grafts or peripheral nerve cells for grafts to the CNS.

Pre-Clinical Studies of Peripheral Nerve Grafts to the CNS

A discussion of peripheral nerve grafting to the CNS would be incomplete without acknowledging the work of Richardson, Issa, David, Benfey, and Aguayo. These researchers laid the foundation of modern investigations into the ability of PN tissue to stimulate CNS axon growth through their multiple animal studies (Albert Aguayo, David, Richardson, & Bray, 1982; AJ Aguayo, Vidal-Sanz, Villegas-Perez, & Bray, 1987; Albert J Aguayo, Benfey, & David, 1983; A. J. Aguayo, Björklund, Stenevi, & Carlstedt, 1984; Benfey & Aguayo, 1982; David & Aguayo, 1981, 1985; P. Richardson & Issa, 1984; P. Richardson, Issa, & Shemie, 1982). One study particularly relevant to this work is when Benfey and Aguayo (Benfey & Aguayo, 1982) showed that basal ganglia neurons grow into peripheral nerve, with axons from the striatum showing the most largest number of projections into the graft. Another foundational paper showed that fetal mesencephalic

dopamine implanted in the CNS and connected to the striatum of a 6-OH-dopamine-lesioned rat with a peripheral nerve bridge led to the growth of fetal mesencephalic dopamine neurons into the striatum. This approach combines two themes of research into PN grafting to the CNS: using peripheral nerve as a bridge and replacing specific neuronal populations lost in human neurodegenerative disease.

A previous preclinical study highly relevant to the human DBS+ procedure is when Watts and colleagues (Ray L Watts, Mandir, & Bakay, 1995) implanted co-grafts of adrenal medulla and sural nerve tissue into the striatum in a MPTP-lesioned macaque model of parkinsonism. These implanted animals showed a favorable safety and feasibility of the procedure as well as improved motor performance versus controls. This study was a continuation of previous research investigating adrenal medulla tissue grafts, which contain catecholaminergic chromaffin cells, for the treatment of PD. Peripheral nerve was added to this grafting approach in the hopes of improving the chromaffin cell survival and efficacy. The continuation of this line of research in humans is discussed in the following section.

Other studies have previously studied the use of isolated peripheral nerve cells as grafts to the CNS. Guest and colleagues (Bastidas et al., 2017; Guest, Rao, Olson, Bunge, & Bunge, 1997) implanted purified and expanded human SCs with or without a solid polymer guidance channels into athymic nude rats given methylprednisolone with a T9-T10 spinal cord resection. This study observed that the grafts without channels showed more myelinated axons and larger cross-sectional area with growth of propriospinal neurons 2.6 mm beyond the grafts. A small increase in motor function was

observed in the grafted animals without caps on the channels. This study is also particularly relevant due to its use of athymic nude rats, the model organism used in this dissertation work and discussed in Chapter Three.

A later study (Kohama et al., 2001) demonstrated that cultured human SCs implanted into the demyelinated spinal cord of Wistar rats immunosuppressed with cyclosporine A improved the conduction velocity of the demyelinated rat spinal cord. Similarly, Bastides and colleagues (Bastidas et al., 2017; Guest et al., 1997) showed the survival, repair properties, and lack of tumorgenicity of cultured human SCs implanted into the contused spinal cord of athymic nude rats. The described lack of tumorgenicity, or the formation of tumors from the peripheral nerve cells, is supportive of the safety of using grafts containing peripheral nerve cells.

Clinical Trials of Peripheral Nerve Grafts to the CNS

The history of grafting peripheral nerve tissue to the CNS in humans for the treatment of neurodegenerative disease is limited. Adrenal medulla tissue plus peripheral nerve grafted to the striatum (R. L. Watts et al., 1997) established the safety of peripheral nerve grafts for the treatment of PD. However, follow-up at two years showed no significant motor improvements. This study differed in three significant ways from the current clinical trial discussed in this dissertation: the grafts were not implanted in combination with DBS, the grafts contained adrenal medulla tissue, and the grafts were targeted to the striatum

A second study of note is the use of PN grafts serving as tissue bridges plus bulbar olfactory ensheathing cells showed some efficacy in inducing functional regeneration in one patient with a transected spinal cord. (Tabakow et al., 2014). This work highlights one the most compelling possibilities of the use of peripheral nerve tissue grafting to the CNS: serving as a bridge to guide regenerating CNS axons across blocking lesions. The addition of bulbar olfactory ensheathing cells, and promising results, perhaps indicates the untapped potential of peripheral nerve as a therapeutic for spinal cord injury.

Expanded discussion of peripheral nerve grafts to the CNS can be found in the upcoming review article "Peripheral Nerve Tissue Grafting for Repair of the CNS in Trauma and Degenerative Disease" (El Seblani and Welleford et al., in preparation).

Conclusions

The primary research aim of this clinical trial evaluate whether PN tissue grafts to the brain can reduce neuronal loss and slow the clinical progression of neurodegenerative diseases like Parkinson's disease. There are several unanswered questions about this approach, and its implementation in these clinical trials, that this dissertation project aimed to address. What is the exact cellular and molecular composition of the PN grafts used in these trials? Does the conditioning injury approach induce the predicted changes to nerve phenotype? In order to assess these questions, the second dissertation chapter details analysis of the peripheral nerve tissue used in the above clinical trials with respect to tissue composition and gene expression, both of injury-naive human peripheral nerve as well as the post-conditioning injury tissue used in the grafting procedure. Histology is

used to survey the structure of the peripheral nerve grafts used in the clinical trials. RNA-seq analysis of sural nerve tissue pre- and post-conditioning injury show significant changes in gene expression corresponding with transdifferentiation of Schwann cells from a myelinating to a repair phenotype, release of growth factors, activation of macrophages and other immune cells, and an increase in anti-apoptotic and neuroprotective gene transcripts. These results reveal *in vivo* gene expression changes involved in the human peripheral nerve injury repair process, which has relevance beyond this clinical trial to the fields of Schwann cell biology and peripheral nerve repair.

Next, this dissertation project aimed to answer the question: how does the graft tissue respond after implantation in the brain? And does injury-naïve PN tissue differ from injury-conditioned tissue in this response? The third chapter of this dissertation details the development of an animal model of the grafting procedure aimed to answer these questions. This animal model, termed Neuro-Avatars, features human peripheral nerve tissue implanted into athymic nude rats. Survival and infiltration of human graft cells into the host brain were shown using immunohistochemistry of Human Nuclear Antigen. The logistics, implementation, and findings of these experiments are discussed.

Lastly, this dissertation addresses the question: how does the neurobiology of the graft correlate with clinical outcomes? The fourth chapter of my dissertation includes a published report of the safety and feasibility of the ongoing clinical trials with further details about the human surgical procedure. To connect the results of these laboratory studies to the clinical trial, fifth chapter compares the severity of motor symptoms before surgery to one year post-surgery in patients who received the analyzed graft tissue. Motor

symptom severity was assessed using the Unified Parkinson's Disease Rating Scale Part III. Finally, the fifth chapter discusses correlates between the laboratory and clinical data collected by these clinical trial as well as the implications of this dissertation work for future clinical trial decisions.

This dissertation project aims first and foremost to be translational: to connect basic science and clinical research in a meaningful way. Taken together, the findings discussed in this dissertation answer many questions that arose in the course of clinical trials investigating peripheral nerve grafts to the brain for the treatment of Parkinson's disease. This work also identifies new basic science and clinical research questions that can be addressed by future translational research. In summary, this dissertation advances the translational science cycle by using clinical trial findings and samples to answer basic science questions that will in turn guide future clinical trial design.

Chapter 1 Figures

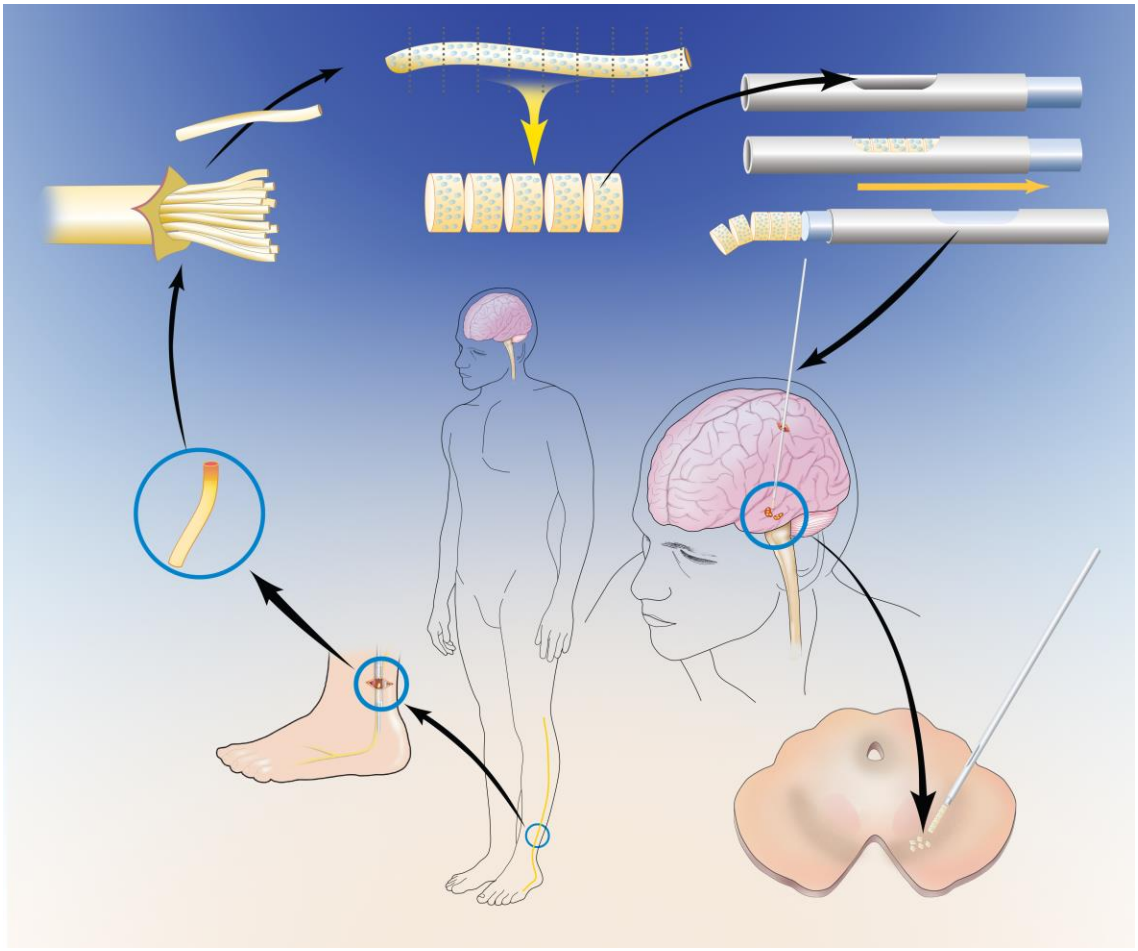

Figure 1.1: Implantation of autologous peripheral nerve graft in the substantia nigra of a human subject with Parkinson's disease.

During Stage I of DBS surgery a conditioning injury is introduced to the sensory sural nerve to activate the repair phenotype of the Schwann cells. The distal segment of the conditioned sural nerve, which contains the repair SCs, is harvested after two weeks during Stage II of DBS surgery. The epineurium is removed, the fascicles are stripped and sectioned into 5 segments and loaded into a custom-made graft cannula. The grafts are stereotaxically deployed in the degenerating substantia nigra.

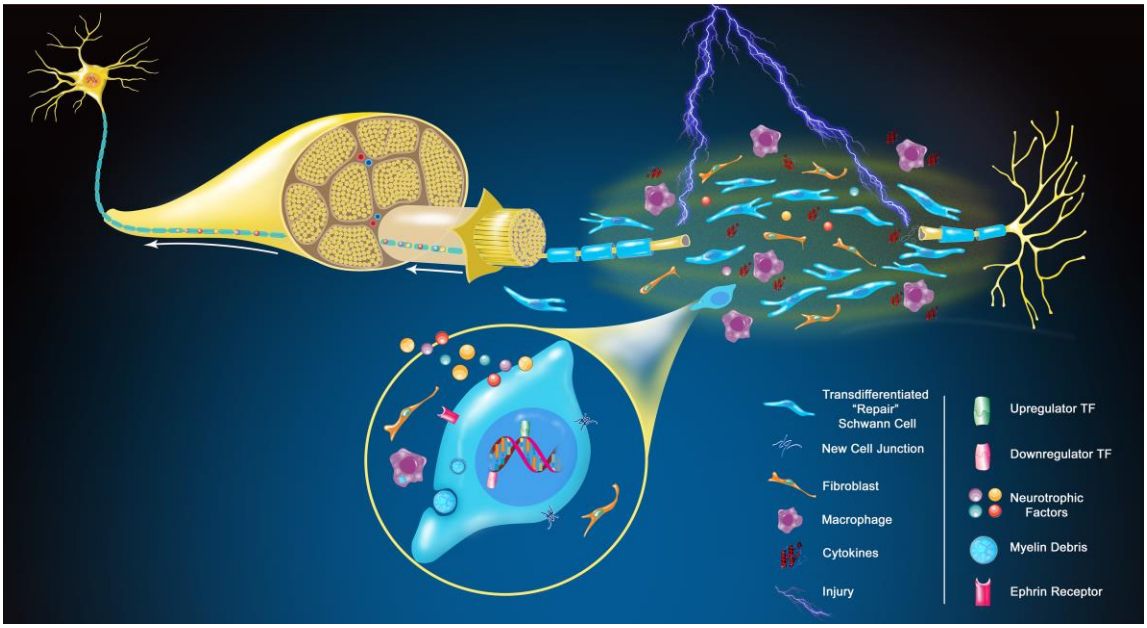

Figure 1.2: Schematic of the Repair Schwann Cell

After a nerve injury, distal SCs shed myelin, lose SC differentiation markers, re-express precursors markers, alter their morphology, and migrate within regenerating tracks (Büngner bands). These tracks guide the regenerating axons back to their functional targets. SCs crosstalk with the macrophages and they interact with the released cytokines to activate different inflammatory pathways. The axonal debris and the myelin are cleared up by both the repair SCs and the macrophages to promote the regeneration of the new axons. The pro-myelinating genes are downregulated by c-Jun, Sox-2, Pax-3, Id2, and Egr-1/3 transcription factors. Repair SCs release various neurotrophic factors including NGF, BDNF, NT-3, NT-4/5, CNTF, trkB, trkA, GDNF, and Neuregulin. These factors induce neurite growth locally and transported retrograde towards the perikaryon to upregulate the anti-apoptotic pathways and enhance the injured neuron.

Chapter Two: RNAseq Characterization of Peripheral Nerve Tissue

RNAseq Analysis of Human Sural Nerve Fascicles Pre- and Post-Injury: Evidence for Trans-differentiation of Human Peripheral Nerve[2]

Introduction

It has long been known that peripheral nerve tissue is capable of functional repair after injury (Cajal, 1928). In contrast to central nervous system (CNS) axons, the axons of the peripheral nervous system (PNS) are capable of regenerating after injury (Brushart, 2011) and re-establishing functional connections with their distal target (Sebille & Bondoux-Jahan, 1980). This regeneration is enabled by the interaction between axons, the nerve extracellular matrix, immune cells such as macrophages, and Schwann cells (Oldfors, 1980; Pellegrino, Politis, Ritchie, & Spencer, 1986). These nerve components individually contribute to peripheral nerve repair and interact with each other to further support functional regeneration (Jessen & Arthur-Farraj, 2019).

Following peripheral nerve injury, axons distal to the lesion undergo a process known as Wallerian degeneration. These distal portions of the axon undergo a controlled process of myelin, axon membrane, and axon cytoskeletal breakdown followed by clearance by Schwann cells and macrophages (Gaudet, Popovich, & Ramer, 2011). Under

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favorable conditions the proximal segment of the neuron can sprout new processes that re-associate with Schwann cells and regenerate through the remaining extracellular matrix of the distal nerve. In this way, the regenerating peripheral nerve can re-establish communication with its distal target and restore some degree of function.

Schwann cells are the primary supporting cells (glia) of the PNS and normally surround PNS axons and insulate them with myelin. In response to peripheral nerve injury Schwann cells change their phenotype from a myelinating form to a regenerative, or trans-differentiated form (Kidd et al., 2013). They shed their myelin, become mobile, and secrete growth factors and other signaling molecules to promote clearance of myelin debris and axonal regrowth (Jessen & Mirsky, 2016). For example, Schwann cells in injured cutaneous sensory nerve release soluble growth factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), neurotrophin-3 (NT-3), and glial cell line-derived neurotrophic factor (GDNF) (Hoke et al., 2006). These growth factors play a role in guiding the distal end of an injured peripheral nerve and guide new axonal growth from the proximal nerve ending (Fu & Gordon, 1997).

The genes and gene products involved in successful recovery of peripheral nerve after injury are of particular interest in the basic science fields of neural protection, regeneration, and repair. Promoting successful regeneration in the central nervous system is the subject of clinical and translational research in neurodegenerative diseases, traumatic brain injury, stroke, epilepsy, and others. Understanding the process of peripheral nerve repair requires consideration of all peripheral nerve components as well

as the interaction between these components. In addition, the most clinically relevant research must take place in humans.

Whole-transcriptome responses to peripheral nerve injury can now be feasibly studied using RNA-seq. RNA-seq combines molecular biology approaches for RNA amplification with bioinformatics approaches for measuring and validating large RNA sequencing datasets. This technique allows for quantitative measurements of thousands of gene transcripts using small (10-30 mg or less) quantities of tissue. For review of the many applications of RNAseq see (Han, Gao, Muegge, Zhang, & Zhou, 2015).

This study was conducted in conjunction with an ongoing clinical trial (clinicaltrials.gov: NCT#02369003), which involves the surgical collection of human peripheral nerve from patients with Parkinson's disease for use in an experimental nerve graft surgery in combination with Deep Brain Stimulation (DBS) (van Horne et al., 2018). The goal of this ongoing clinical trial is to slow the progression of motor symptoms of Parkinson's disease (PD) by providing a source of neuroprotective and/or pro-regenerative factors to the basal ganglia, the brain area causing the prominent motor deficits seen in PD. Peripheral nerve tissue was chosen because it has been successfully transplanted to the CNS in both animal models (Albert Aguayo et al., 1982) and humans (Tabakow et al., 2014; Ray L Watts et al., 1995). In addition, peripheral nerve tissue has been shown to release neurotrophic factors such as GDNF, which has been evaluated for use as a PD therapeutic and the nerve can be collected intraoperatively from the same patient. This surgery, performed as a part of a Phase I clinical trial, involves the collection of two samples of peripheral nerve: one initial sample that is cut from the participant's

sural nerve, and a second sample taken two weeks later from the distal end of the same nerve for implantation into the substantia nigra (van Horne et al., 2018). The samples collected are surgically isolated fascicles of peripheral nerve after the epineurium has been removed. This two-stage approach corresponds with the two stages of DBS surgery and was designed with the objective of inducing pro-regenerative changes in the peripheral nerve following injury as described in the literature (Cattin & Lloyd, 2016; Fu & Gordon, 1997). To evaluate whether this two-stage approach is successful in inducing pro-regenerative changes in peripheral nerve tissue we used a transcriptomic approach to characterize both sets of tissue. This surgical protocol enables the analysis of human peripheral nerve tissue response to transection injury from the same human subject, which is to the best of our knowledge the first study of this type. The results provide insights into changes in human peripheral nerve tissue in response to injury, which may guide therapies and molecular target development for repair of both the peripheral and central nervous system.

Methods

Subjects

Six participants in the clinical trial, which met DBS criteria for the DBS therapy for PD (2 female, 4 male) aged 63.8 $\pm$ 6.9 SD years (range 53-70 years), were selected for RNASeq analysis. The study was approved by the University of Kentucky institutional review board, and informed consent was obtained from all study participants.

Tissue collection

Human sural nerve samples were collected during DBS surgeries. DBS at our institution is carried out in two stages: Stage I is followed by Stage II two weeks later. (van Horne et al., 2017) For the Stage I tissue collection, the surgeon made a transverse incision through the skin of the lateral ankle, 2 cm superior to the lateral malleolus. The surgeon then identified the neurovascular bundle containing the sural nerve and associated artery and vein. The sural nerve was then separated from the bundle using blunt dissection. Next two black silk sutures were tied around the nerve roughly 1 cm apart to mark the nerve. The section of the sural nerve between these sutures was then cut, a piece removed, and the surgical area sutured closed.

The removed section of nerve was then stripped of its epineurium using microsurgical dissection in sterile saline. Individual fascicles of nerve fibers were separated using jeweler's forceps and the perineurium was discarded. These fascicles were placed in conical micro-centrifuge tubes and snap-frozen on dry ice before long-term storage at -80°C until assayed. For Stage II tissue collection, the surgical area from Stage I was reopened at the original incision. The surgeon located the suture markers on the sural nerve and removed a new piece of tissue from the distal stump of the nerve. This tissue was kept on sterile saline ice before implantation. Tissue was prepared for study intervention as in (van Horne et al., 2017). Once the study intervention was completed, the remaining fascicles were placed in conical micro-centrifuge tubes, snap-frozen on dry ice, and stored at -80°C until assayed.

Preparation for Histology

Surgical tissue preparation was performed as above to the point of fascicle isolation. Fascicles were then placed in 4% paraformaldehyde solution. The tissue was then embedded in paraffin blocks for histological evaluation.

Histology

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RNA Extraction

RNA isolation was performed by homogenizing sural nerve fascicles in 1ml of TRI Reagent Solution (ThermoFisher AM9738) using a Fisher Scientific Power Gen 35 homogenizer with a microtip homogenizing probe. The homogenized lysate was transferred to a pre-pelleted 5Prime Phase Lock Gel – Heavy 2 mL tube (ThermoFisher NC1093153) and incubated at room temperature for 5 minutes. 200ul of chloroform was added and the tube was shaken vigorously by hand 15 seconds. Phase separation was performed by microcentrifugation at 12,000 x g for 10 minutes. The RNA containing aqueous phase was taken from the top of the Phase Lock Gel layer and transferred to a 1.7 mL microfuge tube. RNA was precipitated by adding 0.5 ml isopropyl alcohol, mixed by repeated inversion and incubated at room temperature for 10 minutes. RNA was pelleted by microcentrifugation at 12,000 x g for 10 minutes at 4°C. RNA pellet was washed two times with 80% ethanol using a 7,500 x g microcentrifugation for 5 minutes

at 4°C to pellet RNA between washes. RNA pellets were air dried 5-10 minutes at room temperature and resuspended in 25ul of nuclease free water. RNA purity was assessed by OD260/OD280 ratio calculation using a ThermoFisher NanoDrop 1000. RNA Integrity was assessed by Agilent Bioanalyzer 2100 using the Eukaryotic Total RNA Nano assay.

RNA-seq Analysis of Sural Nerve Fascicles

RNA-Sequencing was performed at a strand specific 100 cycle paired-end resolution, in an illumina HiSeq 2500 sequencing machine (Illumina, San Diego, CA). In a repeated measure design, mRNA from six individual samples were sequenced before and after surgery resulting in 12 samples in total. The 12 samples were multiplexed in two lanes of a flow-cell, resulting between 25 and 34 million reads per sample. The read quality was assessed using the FastQC software. (Andrews, 2010) On average, the per sequence quality score measured in the Phred quality scale was above 30 for all the samples. The reads were mapped to the human genome (GRCh38) using the STAR software, version 2.3.1z. (Dobin et al., 2013) On average, 96.4% of the sequenced reads mapped to the genome, resulting between 24.3 and 32.8 million mapped reads per sample, of which on average 89% were uniquely mapped reads. Transcript abundance estimates were calculated using HTSeq (version 0.6.1). (Anders, Pyl, & Huber, 2015) Expression normalization and differential gene expression calculations were performed in edgeR (release 2.14) (Robinson, McCarthy, & Smyth, 2010) to identify statistically significant differentially expressed genes. A paired sample design was used in edgeR, which employs a negative binomial generalized linear model (NB-GLM) for statistical calculations. The edgeR package implements advanced empirical Bayes methods to

estimate gene-specific biological variation under minimal levels of biological replication. The RNA composition in each sample was normalized in edgeR using the trimmed mean of M-values (TMM) method. The significance p-values were adjusted for multiple hypotheses testing by the Benjamini and Hochberg method (Benjamini & Hochberg, 1995) as modified by Storey (Storey & Tibshirani, 2003), providing a false discovery rate q value for each differentially expressed gene. Genes with an absolute fold difference ≥ 2 and q ≤ 0.05 were considered statistically significant.

Analysis

RNAseq data were organized in a Microsoft Excel table for subsequent analyses. The fold changes between Stage 1 and Stage 2 were converted to log base 2 for down-stream analysis. The normalized read counts (counts per million- CPM) were used when calculating correlations between samples and generating heat-maps.

Correlation matrices and heat maps, as well as volcano plots, were generated in Excel. Functional overrepresentation analysis on the Gene Ontology set of pathway annotations (Harris et al., 2004) was performed using DAVID pathway analysis. (Huang da, Sherman, & Lempicki, 2009)

AmiGO Gene Ontology annotations (http://www.geneontology.org/) for pathways of interest were cross-referenced with significantly differentially expressed genes. The gene ontologies visualized in heat maps were selected based on their relevance to peripheral nerve repair. Statistical criteria of q <= 0.05 and |FC|>= 2 were selected, yielding 3,641 differentially expressed genes in this analysis. Heat maps of the

qualifying genes were generated using JMP Pro 13 (SAS). Hierarchical clustering was performed using Ward's method in JMP.

Results

Tissue Samples

The mass and freezing delay time (time from when the nerve was removed from participant to when it was snap-frozen in dry ice) were calculated for tissue collected for RNAseq from Stage I (Table 2.1) and Stage II (Table 2.2). Freezing Time Delay was nearly three times longer at Stage II (59 ± 25 min; Mean ± SD) compared to Stage I (18 ± 5 min). The peripheral nerve tissue in Stage II was successfully grafted into the participant before the companion, excess tissue was frozen.

Histology

The morphology of the surgical tissue samples was assessed using standard H&E histology and MCOLL staining, which differentiates myelin, collagen fibers, and cells in peripheral nerve (see Figure 2.1). The tissue morphology was consistent with peripheral nerve, confirming that the tissue harvested in the surgery and used in grafts was peripheral nerve fascicles. Myelinated fibers, extracellular matrix, and cells were all visible in both Stage I and Stage II tissue. Of note, Stage II samples showed increased numbers of cell nuclei compared to Stage I, suggesting infiltration and proliferation of immune cells as described in the literature (Cattin & Lloyd, 2016). These findings are supported by increased cytokines and immune cell markers as described below. Tissue analyzed by

histology was collected from study participants using the procedure described in the methods section, but tissue from these participants was not assessed with RNAseq.

Correlation Matrix and Volcano Plot

When we correlated the gene profiles of individual participants' samples with respect to nerve injury status (Stage I or Stage II), we found high correlation between participant's gene profiles within the acute injured status samples (Stage I) and within the regenerative status samples (Stage II), but not across Stage I and Stage II status (Figure 2A). The analysis of the samples showed consistent and robust changes in RNA levels among all 6 participants in response to the nerve cut injury. These results provide evidence that the nerve regeneration was successful in inducing phenotypic changes in the peripheral nerve tissue used in the grafting procedure. A consistent gene profile in Stage II tissue also allows for greater confidence in interpreting the individual participant responses to the grafting procedure.

The Volcano plot (Figure 2B) estimates the most meaningful changes in the gene transcriptome, between Stage I and Stage II that exceeded a cutoff of $q \leq 0.0003$; and fold change $|FC| >= 4$. Of those significantly differentially expressed genes, 693 gene transcripts (orange) were increased and 576 gene transcripts were decreased (blue). This analysis demonstrates that even with a very conservative criteria, there are hundreds of differentially expressed genes between the Stage I and Stage II tissue samples

DAVID Pathway Analysis

DAVID is an online software tool that groups genes based on their functional similarity. Given a list of significantly differentially expressed genes, DAVID uses a fuzzy clustering algorithm, that uses information in its knowledge base on genes and their functional associations, to group genes that are together, statistically significant in their association to a set of common functional categories. (Huang da et al., 2009)

DAVID analysis revealed that the majority of the most significantly increased pathways in the injury-conditioned samples (Table 3) were related to cell cycle, cell proliferation, and immune cell function. The majority of the most significantly down-regulated pathways (Table 4) were related to synaptic structure and neuron function. These transcriptome trends match the phenotype of peripheral nerve responding to injury: immune cell infiltration plus cell proliferation with Wallerian degeneration of axons. (Arthur-Farraj et al., 2012; Arthur-Farraj et al., 2017; Cattin & Lloyd, 2016)

Heat Maps

Heat maps were generated from gene ontology terms of interest to this study drawing from the peripheral nerve regeneration literature (Arthur-Farraj et al., 2012; Arthur-Farraj et al., 2017; Cattin et al., 2015; Cattin & Lloyd, 2016) and pathways of interest to the research teams.

Growth Factors

Figure 2.3 shows all significantly differentially expressed ($q < 0.05$, $|FC| > 2$) gene transcripts annotated with the Gene Ontology (GO) term “Growth factor Activity”

(GO:0008083), indicating that there is literature evidence of these genes being involved in “The function that stimulates a cell to grow or proliferate.” Out of 166 unique genes with this ontology, 43 (25.9%) of these genes were significantly differentially expressed between Stage I and Stage II. 26 genes were more abundant and 17 genes were less abundant in Stage II samples than Stage I. Of particular note were the neurotropic factor genes for GDNF, FGF, and others. These results showed significant increases after injury in transcripts of growth factor genes known to promote neuroprotection in the CNS, including GDNF.

Myelination

Out of 110 unique gene transcripts identified with the GO term “Myelination” (GO:0042552), “the process in which myelin sheaths are formed and maintained around neurons,” 48 (43.6%) were significantly differentially expressed (7 genes were more abundant and 41 genes less abundant in Stage II samples than Stage I samples, Figure 2.4). These results show significant decreases in myelination-related gene transcripts consistent with loss of myelinating-phenotype Schwann Cells.

Schwann Cell De-Differentiation

Using the GO term “Epithelial-Mesenchymal Transition” (GO:0001837), “a transition where an epithelial cell loses apical/basolateral polarity, severs intercellular adhesive junctions, degrades basement membrane components and becomes a migratory mesenchymal cell,” yielded 130 unique genes. Of these, 29 (22.3%) were significantly differentially expressed (24 genes were more abundant and 5 genes less

abundant in Stage II samples than Stage I samples, Figure 2.5). Likewise, the GO term "Schwann Cell Differentiation" (GO:0014037), "the process in which a relatively unspecialized cell acquires the specialized features of a Schwann cell" identified, 36 unique genes. Of these, 15 (41.7%) were significantly differentially expressed, with only 1 gene being more abundant while the other 14 genes were less abundant in Stage II samples than Stage I samples (Figure 2.5). These results indicate a loss of markers of mature (myelinating) Schwann cell phenotype and increase in markers of trans-differentiation of cells. Taken together, these gene changes are consistent with the trans-differentiation of Schwann cells following injury from a mature (myelinating) form to a repair phenotype.

Neuroprotection

Figure 2.6 shows all significantly differentially expressed gene transcripts annotated with the GO term "Negative Regulation of Apoptotic Processes" (GO:0043066) or "Negative Regulation of Neuron Death" (GO:1901215). "Negative Regulation of Apoptotic Processes" is defined by "the process in which myelin sheaths are formed and maintained around neurons. This ontology yielded 916 unique genes, of which, 128 (14.0%) were significantly differentially expressed (all 128 genes were more abundant in Stage II samples than in Stage I samples). Meanwhile, for Negative Regulation of Neuron Death, "any process that stops, prevents or reduces the frequency, rate or extent of neuron death," we found 67 genes out of 206 (32.5%) were significantly differentially expressed (45 genes more abundant and 22 genes less abundant in Stage II samples than

in Stage I samples). These gene changes indicate significant increases in transcripts of genes related to the reduction of neuron death.

Discussion

A major finding from the current study is that the RNAseq data support the feasibility and reproducibility of the time-delayed approach for producing trans-differentiation of the sural nerve tissue into a repair cell phenotype. Histology and transcriptome profiles were consistent with peripheral nerve that has undergone injury response (Arthur-Farraj et al., 2017; Weiss et al., 2016; Yi et al., 2015). Furthermore, the correlation matrix visualizes the consistent RNA levels between participants as a result of the pre-lesioning approach we call Stage I (van Horne et al., 2017; van Horne et al., 2018). The participants who contributed tissue for this study had all been diagnosed with idiopathic PD for over 5 years and ranged in age from 53 to 70, with Movement Disorders Society- Unified Parkinson's Disease Rating Scale (MDS-UPDRS) Part III motor function scores, while having stopped PD medication, that ranged from 22-75 points (the scale ranges from 0 [normal] to 132 points [most affected]). It is possible that the disease processes of this neurodegenerative disorder had some effect on peripheral nerve gene expression and its injury response. For example, recent evidence has identified that phosphorylated forms of alpha-synuclein are found in some peripheral nerves of Parkinson's patients. (Donadio et al., 2014; Doppler et al., 2014; Zhang et al., 2019) In other participants of the clinical trial (not included here), who have had a history of neuropathy, we have used clinical nerve conduction velocity tests to assess the sural

nerve before grafting. None of those participants showed remarkable decrement in nerve conduction velocity (data not shown). A future study comparing baseline levels of RNA in patients with Parkinson's versus age-matched healthy controls could help determine potential baseline differences. These results are also valuable from a clinical trial perspective as they provide evidence for homogeneity of the treatment being applied in the PD population. The transcriptome of the nerve tissue is similar between all participants even though they range in age and PD severity and also in other co-morbidities that might affect the trans-differentiation process. In addition, based on the high degree of correlation within the samples from Stage I and from Stage II, shown in the correlation matrix results, the results support that the surgical procedure and sample collection protocols, in spite of the variability involved in collecting the tissue, e.g. the Freezing Time Delay, led to consistent results from the tissue samples. These benchmarks could serve as potential quality-assurance metric if this technique were to be adopted as part of a multicenter trial.

Another major finding of the study is that it provides RNAseq quantification of gene transcripts in peripheral nerve tissue being grafted into the brain. These data answer pressing questions from the clinical trial as to the phenotype of the nerve tissue used in the grafting procedure (van Horne et al., 2018). There is now evidence for the expression of specific growth factors such as GDNF, which is known to be neuroprotective, especially to dopaminergic neurons of the substantia nigra (Gash et al., 1996; Grondin et al., 2002; Kirik, Georgievska, & Bjorklund, 2004). Given that the target of this grafting trial is the substantia nigra, this finding supports the neuroprotective potential of this graft tissue.

Along these lines, the freezing time delay differences between Stage I and Stage II is one of the variabilities within the design of our study. This was inherent to the trial design where the focus was to optimize the grafting procedure and the availability of tissue to be grafted. So while in Stage I the tissue was processed to isolate the fascicles immediately after extraction, in Stage II, the tissue was extracted, placed on saline ice, the sural nerve incision was closed, the target areas were prepared, and the tissue was grafted. We recognize this delay represents a difference in the freezing time delay between the stages; however, because the fascicles that are snap-frozen at Stage II are parts of the tissue that was actually implanted, by content and exposure time, we think the analysis of this tissue, after the delay, best reflects our "study intervention."

This study also highlights gene changes that play a role in the injury response of the human peripheral nerve. This analysis of peripheral nerve repair is in the context of a full transection injury. Other injury modalities, for example crush injuries, have been shown to induce different repair processes than transection injury (Lago & Navarro, 2006). This should be considered when applying these findings to other injury models. Comparing differentially expressed genes between Stage I and Stage II reveals many functional pathways involved in the tissue response to injury.

Growth factor activity showed multiple differentially expressed genes. One increased gene of note is GDNF, which is neuroprotective of dopaminergic neurons and has been tried as a therapeutic for Parkinson's disease (Gash et al., 1996; S. S. Gill et al., 2003; A. Whone et al., 2019; A. L. Whone et al., 2019). Multiple interleukins were also upregulated, which in addition to being cytokines, also play a role in neurogenesis (for

review see (Borsini, Zunszain, Thuret, & Pariante, 2015)). The myelination genes showed that the most significantly differentially expressed genes of this ontology were decreased two weeks after injury. This is consistent with myelin degradation after peripheral nerve injury as described in the literature (Gomez-Sanchez et al., 2015). In general the Schwann cells showed overall decreased transcripts related to differentiation. This could be interpreted as de-differentiation of Schwann cells described in the literature (Arthur-Farraj et al., 2012). However, this comparison was not straightforward. We expected to see that the JUN gene, which codes for the c-Jun transcription factor that regulates Schwann cell trans-differentiation, would be increased (Arthur-Farraj et al., 2012). However, this gene was not significantly differentially expressed in these samples. We currently hypothesize that the time course of JUN transcription did not match with the two samples taken two weeks apart, or that post-translational processing of c-Jun has more influence on its activity. In fact, the present study is limited in that it only addresses the levels of gene transcripts in the fascicles two weeks after the transection. Ideally, future studies should strive to investigate both shorter (3 to 7 days) and longer 3-6 weeks post injury to understand more about the trans-differentiation process.

Negative regulation of apoptotic processes describes genes that decrease apoptosis, and includes anti-apoptotic properties that are of interest to us for therapeutic benefit. The visualization of this ontology were striking: over one hundred genes were significantly differentially expressed and all were up-regulated. This suggests that marked suppression of apoptosis is occurring in this tissue. Whether these anti-apoptotic processes confer neuroprotection after tissue grafting merits study.

Human studies of the peripheral nerve transcriptome are few. To our knowledge only Weiss and colleagues (Weiss et al., 2016) have performed RNAseq on human peripheral nerve fascicles. They studied human peripheral nerve fascicles collected during surgeries, ex vivo degenerated nerves (8 days post-lesion), cultured Schwann cells, and cultured fibroblasts. Their method of obtaining degenerated nerves differed from ours in that our model was degenerated in vivo and collected 14 days post-injury. Studies in animal models are more common. Arthur-Farraj (Arthur-Farraj et al., 2017) and colleagues reported transcriptome and DNA methylome findings from mouse models. They found changes in epithelial-mesenchymal transition, which we also observed. They also analyzed non-coding RNA. Yi (Yi et al., 2015) and colleagues studied a rat model of sciatic nerve crush injury. Of note: both Arthur-Farraj et al. and Yi et al. performed sequencing at multiple time points post-injury. This approach allowed them to show the change in differentially expressed pathways over time.

Our RNAseq analysis limits the genes visualized to those exceeding a fold change threshold of $|FC|>2$ or 4. This is an arbitrary convention meant to refine the results of RNAseq to a more "biologically relevant" dataset. However, this convention may exclude genes that are in actuality biologically relevant. For example, transcripts of NF2, a marker of Schwann Cell proliferation, were statistically significantly increased ($p=0.0192$). However, the average magnitude of increase was less than 2, so it was not included in the visualized data. Furthermore, we focused in this paper only on transcripts that were differentially expressed between Stage II and Stage I while recognizing that some genes could be highly expressed in both stages, but not differentially so. This is one limitation of

this broad analysis approach, and in the specific case of NF2 levels in this tissue merits further study.

The sural nerve fascicles analyzed included all cells and tissue components within the peripheral nerve. It should be noted that the tissue analyzed does not include the epineurium of the sural nerve. This was intentional, as it reflects the graft tissue composition (fascicles only), which is intended to contain mostly Schwann cells, macrophages, and extracellular matrix. This yielded an aggregate of all RNA in the tissue from multiple cell types. A future study using single-cell RNA-seq would be able to investigate the responses of individual peripheral nerve cells and cell types, which would likely be of great value to the field of neural regeneration and repair. Thus, it is possible that while we have emphasized the importance of the Schwann cells in the RNAseq data and the "repair cell" properties of the tissue that the results cannot at this time be solely attributed to changes in Schwann cells.

Taken together, the results from this study present whole-tissue transcriptome-scale data of human peripheral nerve shortly following transection injury versus two weeks post-injury from the distal portion of the same injured nerve. The findings of this study reveal significant changes in the transcriptome of an injured human peripheral nerve after two weeks of repair processes in situ. These results provide data for future researchers to analyze and incorporate into their bioinformatics models information regarding normal peripheral nerve and its injury response. Such models may provide insight into the peripheral nerve repair process and its relevance to basic science research into peripheral nerve and Schwann cells as well as clinical and translational research

looking to adapt peripheral nerve tissue for use in promoting neuroprotection, neural repair, axonal regeneration, or other therapeutic strategies for nervous system disorders.

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Chapter 2 Tables

Participant	Mass (g)	Freezing Time Delay* (min)
1	0.0205	16
2	0.0158	28
3	0.0254	20
4	0.0256	14
5	0.0294	14
6	0.0197	17
Mean ± SD	0.0227± 0.0049	18 ± 5

* Freezing Time Delay includes the gross dissection time during which fascicles were separated from the whole nerve.

Table 2.1: Stage I Mass and Freezing Time Delay

Mass and storage information for nerve samples collected during Stage I DBS surgeries.

Participant	Mass (g)	Freezing Time Delay* (min)
1	0.0354	41
2	0.0301	108
3	0.0613	64
4	0.0363	52
5	0.0757	39
6	0.0256	50
Mean ± SD	0.0441 ± 0.0198	59 ± 25

* Freezing Time Delay includes the gross dissection time during which fascicles were separated from the whole nerve and the time required for fascicle segment grafting into participants during Stage II.

Table 2.2: Stage II Mass and Freezing Time Delay

Mass and storage information for nerve samples collected during Stage I DBS surgeries.

Term	Upregulated	Number of gene transcripts	P Value
GO:0000278	mitotic cell cycle	83	2.42E-32
GO:0007067	mitotic nuclear division	57	3.97E-31
GO:0000819	sister chromatid segregation	41	1.20E-27
GO:0044770	cell cycle phase transition	49	4.75E-19
GO:0071103	DNA conformation change	32	1.21E-16
GO:0002682	regulation of immune system process	62	4.67E-14
GO:0000228	nuclear chromosome	41	3.38E-13
GO:0006952	defense response	58	1.11E-11
GO:1901987	regulation of cell cycle phase transition	29	5.00E-11
GO:0051303	establishment of chromosome localization	14	2.42E-10
GO:0006259	DNA metabolic process	46	4.49E-09
GO:0002366	leukocyte activation involved in immune response	19	6.52E-09
GO:0042129	regulation of T cell proliferation	16	1.64E-08
GO:0051321	meiotic cell cycle	17	2.41E-07
GO:0006342	chromatin silencing	13	5.18E-07
GO:0033043	regulation of organelle organization	46	5.84E-07
GO:0032640	tumor necrosis factor production	12	7.16E-07
GO:0000910	cytokinesis	13	1.42E-06
GO:0046631	alpha-beta T cell activation	12	2.69E-06
GO:0001932	regulation of protein phosphorylation	45	3.85E-06
GO:0006302	double-strand break repair	16	6.15E-06
GO:0007051	spindle organization	12	1.01E-05
GO:0050663	cytokine secretion	13	1.54E-05
GO:0032101	regulation of response to external stimulus	28	2.78E-05
GO:0032675	regulation of interleukin-6 production	10	2.86E-05
GO:0019899	enzyme binding	54	3.65E-05
GO:0060089	molecular transducer activity	35	3.80E-05
GO:0044774	mitotic DNA integrity checkpoint	11	5.42E-05
GO:0006270	DNA replication initiation	7	5.53E-05
GO:0002704	negative regulation of leukocyte mediated immunity	7	6.60E-05
GO:0032760	positive regulation of tumor necrosis factor production	8	7.62E-05
GO:0034501	protein localization to kinetochore	5	8.20E-05
GO:0009897	external side of plasma membrane	14	8.27E-05

Table 2.3: Top Significantly Increased Pathways During Regeneration

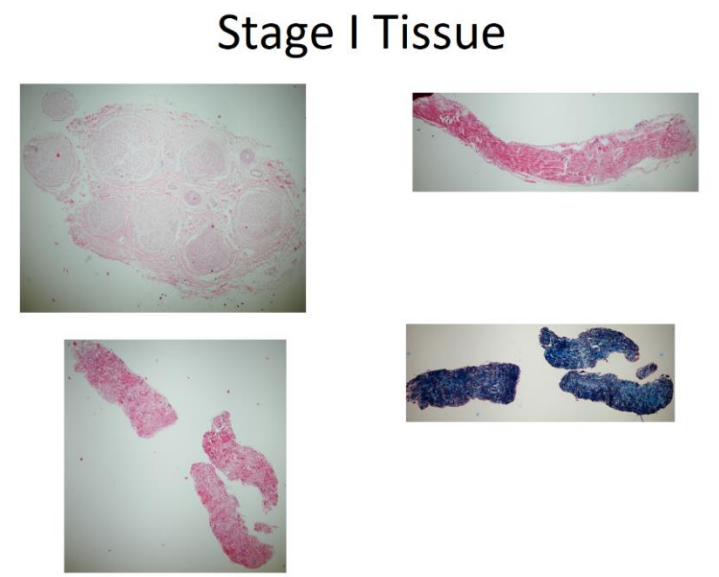
DAVID pathway analysis of RNAseq data showing the pathways of most significantly increased gene transcripts.

Term	Downregulated	Number of gene transcripts	P Value
GO:0042391	regulation of membrane potential	17	2.69E-08
GO:0098590	plasma membrane region	27	4.06E-08
GO:0050803	regulation of synapse structure or activity	14	8.01E-08
GO:0050877	neurological system process	22	7.00E-07
GO:0050808	synapse organization	13	8.20E-07
GO:0048812	neuron projection morphogenesis	18	4.23E-06
GO:0030426	growth cone	10	1.42E-05
GO:0050807	regulation of synapse organization	8	4.21E-05
GO:0034765	regulation of ion transmembrane transport	13	5.56E-05
GO:0005578	proteinaceous extracellular matrix	13	6.25E-05
GO:0035725	sodium ion transmembrane transport	8	7.87E-05
GO:0098794	postsynapse	13	9.39E-05

Table 2.4 Legend: Top Significantly Decreased Pathways During Regeneration

DAVID pathway analysis of RNAseq data showing the pathways of most significantly increased gene transcripts.

Chapter 2 Figures



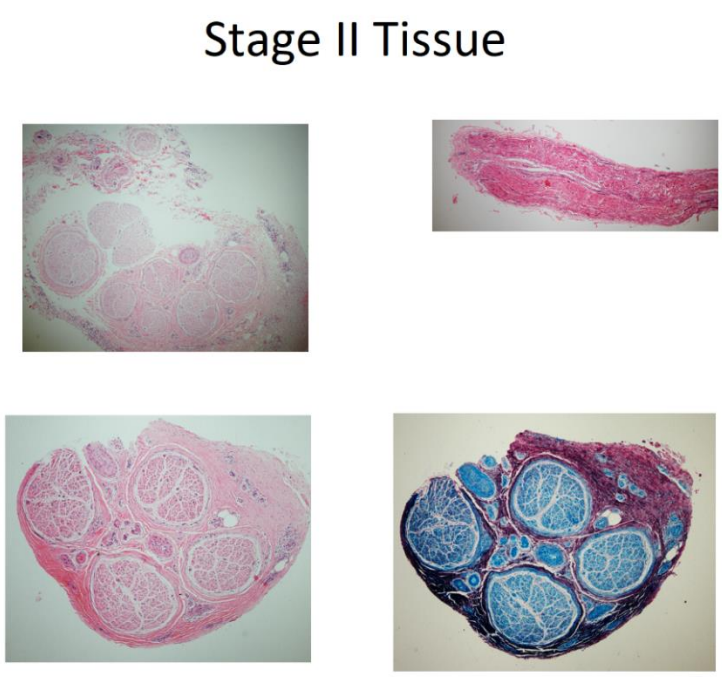


Figure 2.1: Sural Nerve Histology

H&E and MCOLL (LBF, picrosirius, haematoxylin) staining of representative peripheral nerve fascicles. MCOLL stains myelin in blue and collagen in red.

Figure 2.2A: Correlation Matrix

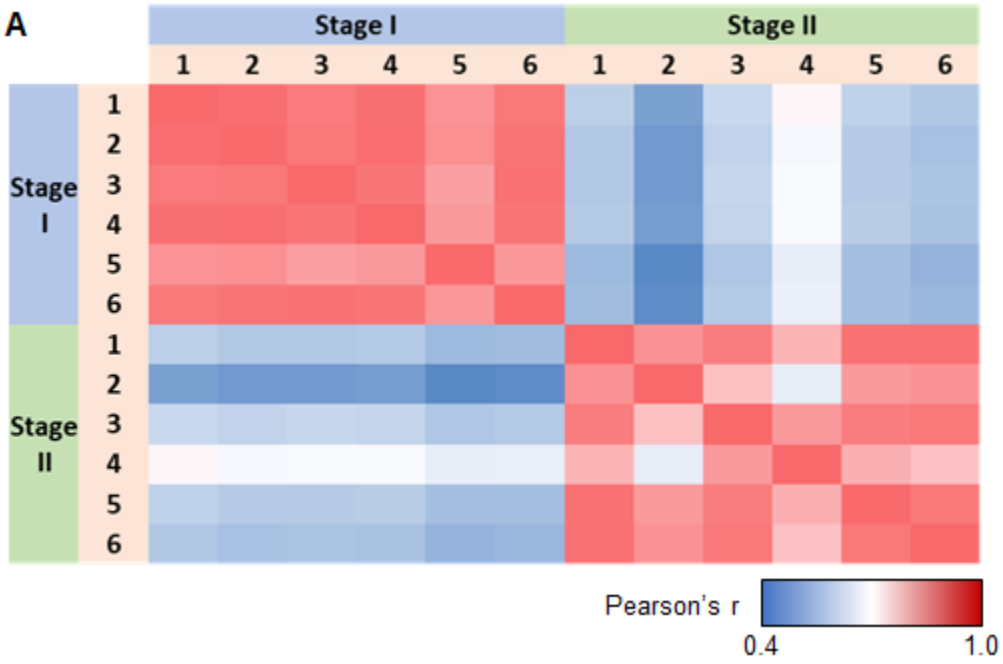


Figure 2.2B: Volcano Plot

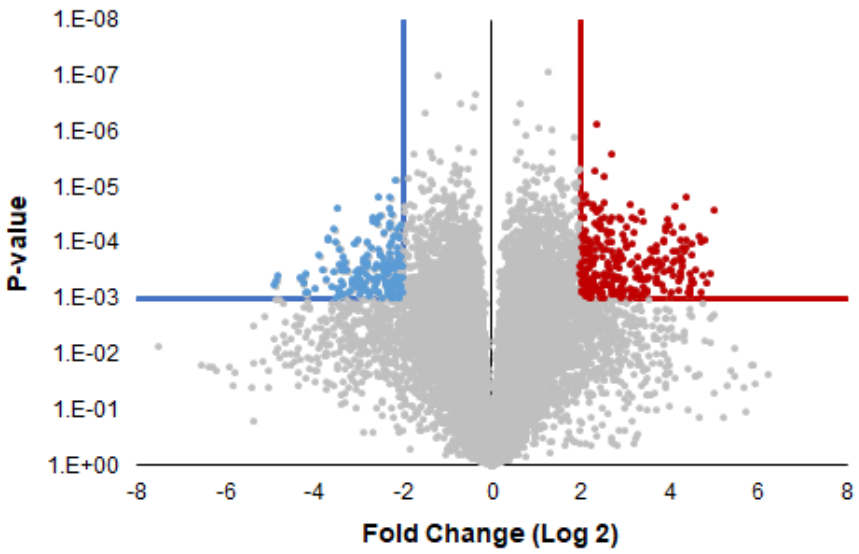


Figure 2.2A: Correlation matrix

Pearson's r for the transcriptional profile of every subject vs every other subject. Scale bar: Correlation values range from 0.4 (blue- less similar) to 1 (red- more similar). This visualization shows strong agreement among different profiles within each Stage, and a sharp distinction between Stages.

Figure 2.2B: Volcano Plot

For differences between Stage 1 and Stage 2, log 2 scale fold changes (x axis) are plotted as a function of p-value (inverted log 10 scale- volcano plot). Results that exceed conservative q-value ($q \leq 0.0003$) and fold change ($|FC| >= 4$) cutoffs are highlighted (blue- downregulated in Stage 2, red- upregulated in Stage 2).

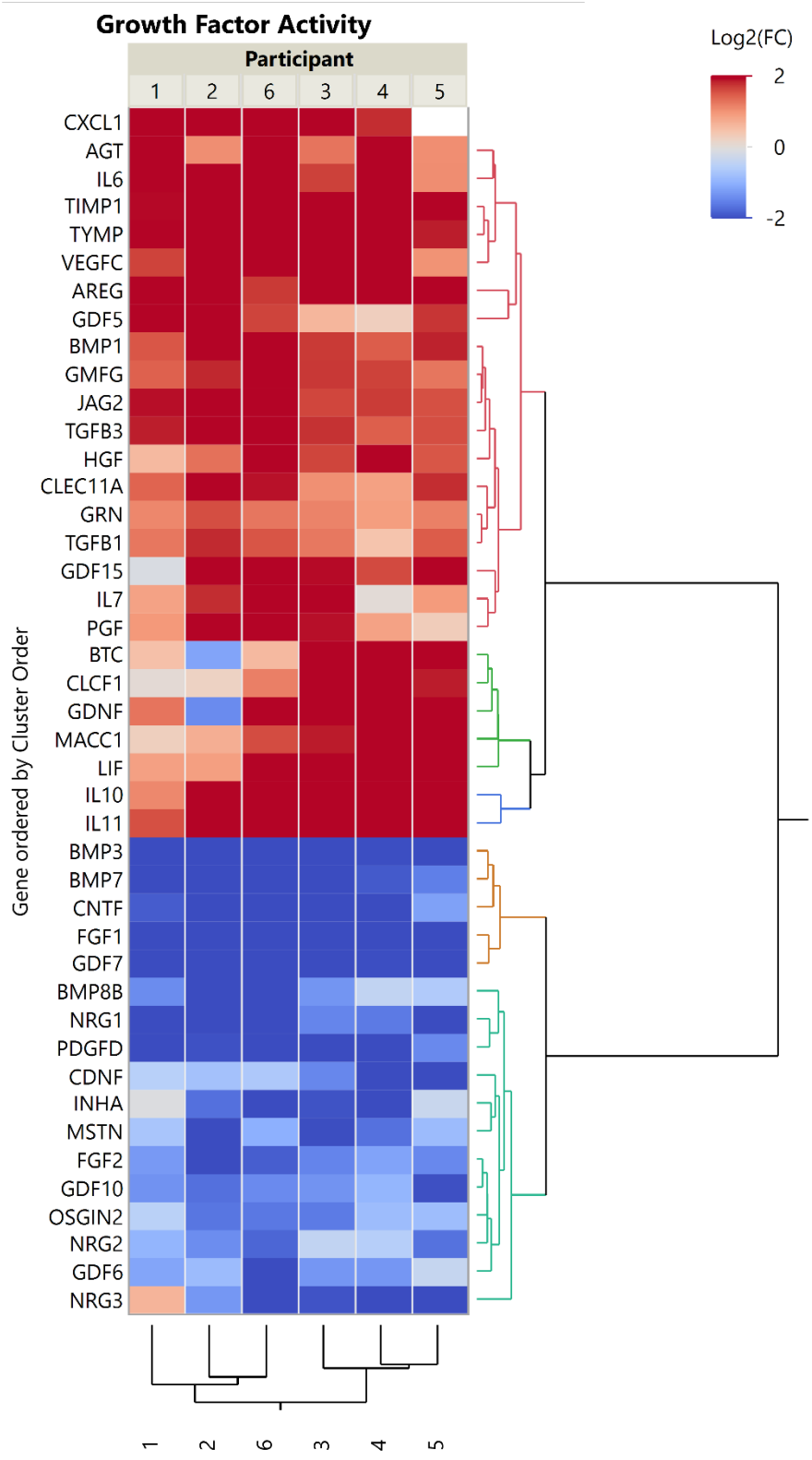

Growth Factor Activity
Participant
1
2
6
3
4
5
Log2(FC)
2
0
-2
Gene ordered by Cluster Order
CXCL1
AGT
IL6
TIMP1
TYMP
VEGFC
AREG
GDF5
BMP1
GMFG
JAG2
TGFB3
HGF
CLEC11A
GRN
TGFB1
GDF15
IL7
PGF
BTC
CLCF1
GDNF
MACC1
LIF
IL10
IL11
BMP3
BMP7
CNTF
FGF1
GDF7
BMP8B
NRG1
PDGFD
CDNF
INHA
MSTN
FGF2
GDF10
OSGIN2
NRG2
GDF6
NRG3
1
2
6
3
4
5

Figure 2.3: Growth Factor Activity

Heat map showing all significant differentially expressed ($q < 0.05$, $|FC| > 2$) gene transcripts annotated with the GO term “Growth factor Activity” (GO:0008083). Genes are organized by Ward hierarchical clustering. Dendrograms are scaled to hierarchical clustering distance; longer branches represent more distant clusters.

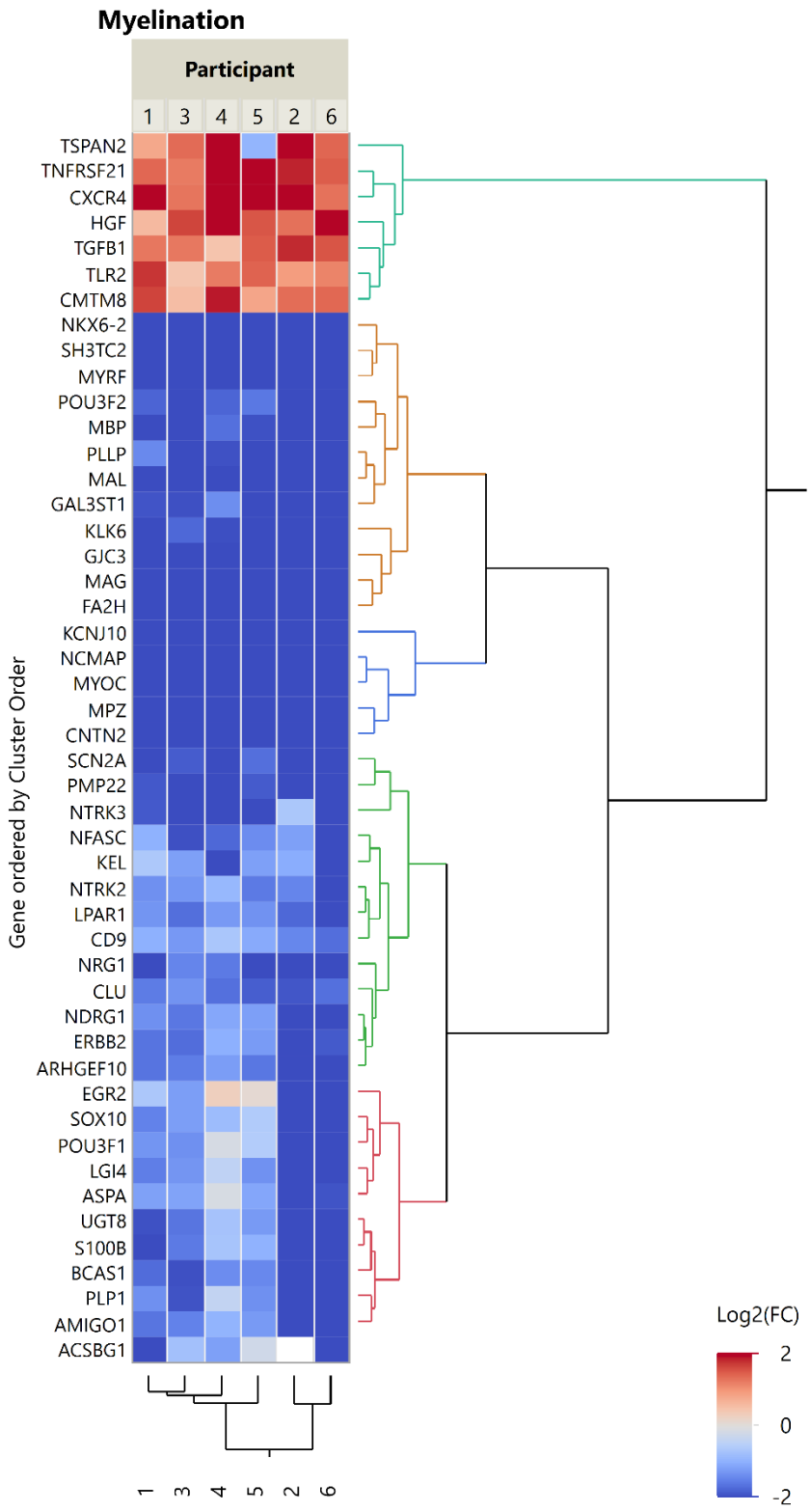

Myelination
Participant
1
3
4
5
2
6
TSPAN2
TNFRSF21
CXCR4
HGF
TGFB1
TLR2
CMTM8
NKX6-2
SH3TC2
MYRF
POU3F2
MBP
PLLP
MAL
GAL3ST1
KLK6
GJC3
MAG
FA2H
KCNJ10
NCMAP
MYOC
MPZ
CNTN2
SCN2A
PMP22
NTRK3
NFASC
KEL
NTRK2
LPAR1
CD9
NRG1
CLU
NDRG1
ERBB2
ARHGEF10
EGR2
SOX10
POU3F1
LGI4
ASPA
UGT8
S100B
BCAS1
PLP1
AMIGO1
ACSBG1
Gene ordered by Cluster Order
1
3
4
5
2
6
Log2(FC)
2
0
-2

Figure 2.4: Myelination

Heat map showing all significantly differentially expressed ($q < 0.05$, $|FC| > 2$) gene transcripts annotated with the GO term “Myelination” (GO:0042552). Genes are organized by Ward hierarchical clustering. Dendrograms are scaled to hierarchical clustering distance; longer branches represent more distant clusters.

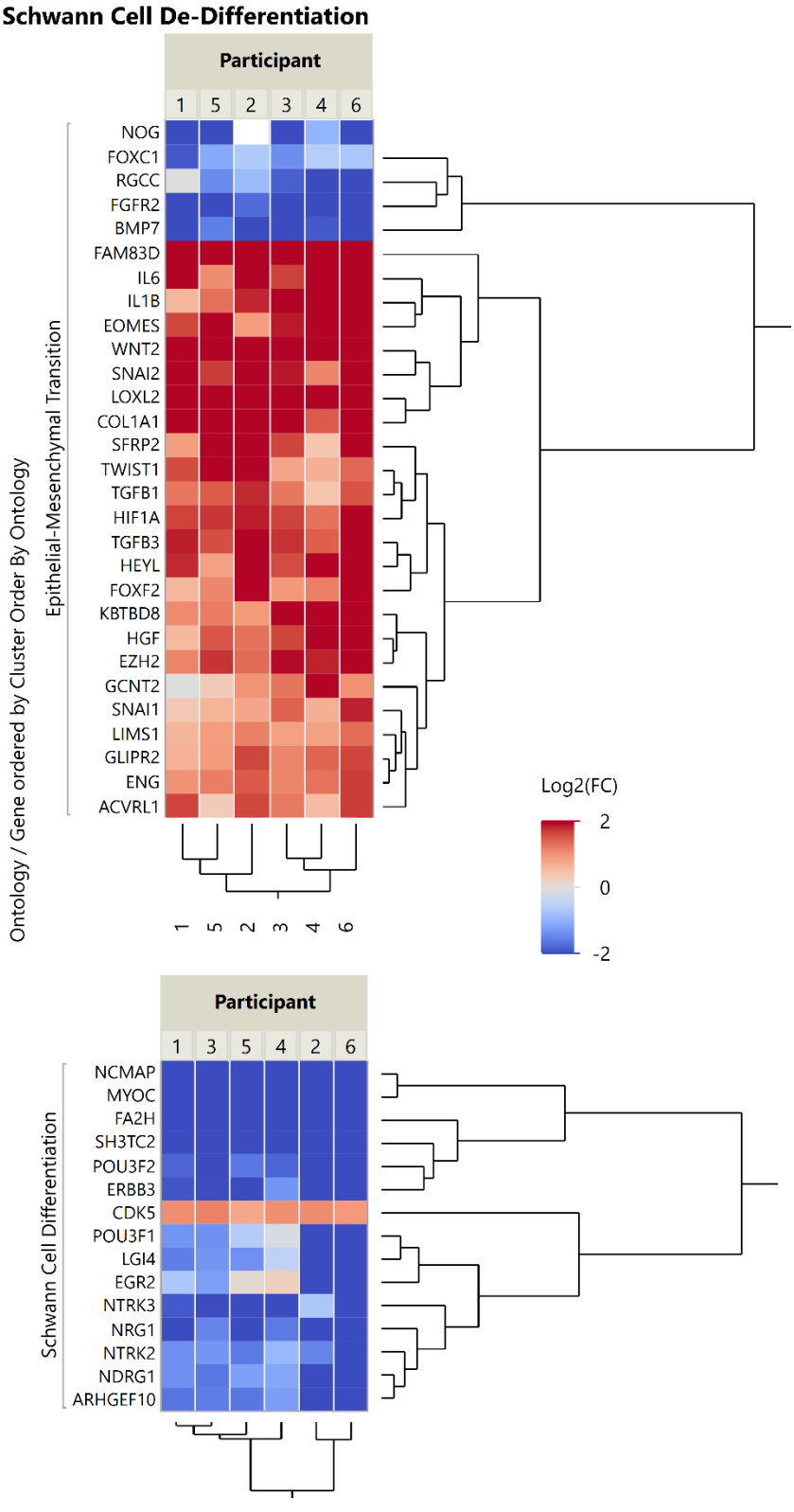

Schwann Cell De-Differentiation
Participant
1 5 2 3 4 6
NOG
FOXC1
RGCC
FGFR2
BMP7
FAM83D
IL6
IL1B
EOMES
WNT2
SNAI2
LOXL2
COL1A1
SFRP2
TWIST1
TGFB1
HIF1A
TGFB3
HEYL
FOXF2
KBTBD8
HGF
EZH2
GCNT2
SNAI1
LIMS1
GLIPR2
ENG
ACVRL1
Epithelial-Mesenchymal Transition
Ontology / Gene ordered by Cluster Order By Ontology
1 5 2 3 4 6
Log2(FC)
2
0
-2
Participant
1 3 5 4 2 6
NCMAP
MYOC
FA2H
SH3TC2
POU3F2
ERBB3
CDK5
POU3F1
LGI4
EGR2
NTRK3
NRG1
NTRK2
NDRG1
ARHGEF10
Schwann Cell Differentiation

Figure 2.5: Schwann Cell Transdifferentiation

Heat map showing all significantly differentially expressed ($q < 0.05$, $|FC| > 2$) gene transcripts annotated with the GO term "Epithelial-Mesenchymal Transition" (GO:0001837) or "Schwann Cell Differentiation" (GO:0014037). Genes are organized by Ward hierarchical clustering. Dendrograms are scaled to hierarchical clustering distance; longer branches represent more distant clusters.

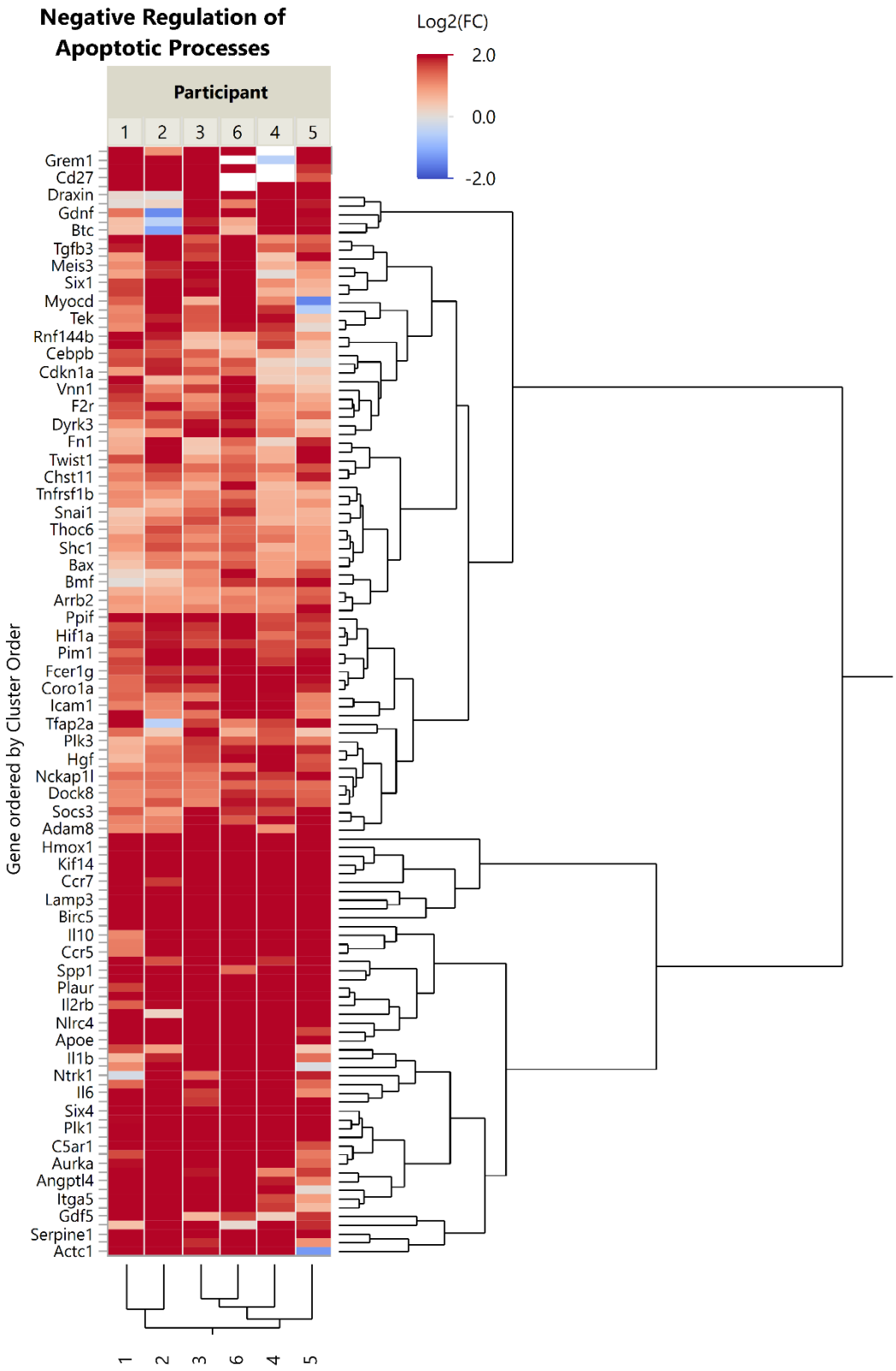

Negative Regulation of Apoptotic Processes
Log2(FC)
2.0
0.0
-2.0
Participant
1
2
3
6
4
5
Gene ordered by Cluster Order
Grem1
Cd27
Draxin
Gdnf
Btc
Tgfb3
Meis3
Six1
Myocd
Tek
Rnf144b
Cebpb
Cdkn1a
Vnn1
F2r
Dyrk3
Fn1
Twist1
Chst11
Tnfrsf1b
Snai1
Thoc6
Shc1
Bax
Bmf
Arrb2
Ppif
Hif1a
Pim1
Fcer1g
Coro1a
Icam1
Tfap2a
Plk3
Hgf
Nckap1l
Dock8
Socs3
Adam8
Hmox1
Kif14
Ccr7
Lamp3
Birc5
Il10
Ccr5
Spp1
Plaur
Il2rb
Nlrc4
Apoe
Il1b
Ntrk1
Il6
Six4
Plk1
C5ar1
Aurka
Angptl4
Itga5
Gdf5
Serpine1
Actc1
1
2
3
6
4
5

Figure 2.6: Anti-Apoptosis

Heat map showing all significantly differentially expressed ($q < 0.05$, $|FC| > 2$) gene transcripts annotated with the GO term “Negative Regulation of Apoptotic Processes” (GO:0043066) or “Negative Regulation of Neuron Death (GO:1901215). Genes are organized by Ward hierarchical clustering. Dendrograms are scaled to hierarchical clustering distance; longer branches represent more distant clusters.

Chapter Three: The Neuro-Avatar Project

Neuro-Avatar: A Reverse Translational Model of an Ongoing Cell Therapy Trial for the Treatment of Parkinson's Disease[3]

Introduction

With the pro-regenerative phenotype of the sural nerve graft supported by transcriptomics, the next scientific questions posed by the clinical trial related to the neurobiology of the graft after implantation. How many graft cells survive the transplantation? Do the graft cells integrate with the surrounding tissue? How long do these cells survive? Furthermore, while the use of pre-lesioned tissue for its pro-regenerative properties was supported by the scientific literature and now laboratory evidence, the investigators wanted to evaluate whether there were measurable differences between injury-naive versus post-injury nerve tissue used in grafts. Specifically, differences between graft cell count, integration with the host brain, and morphology of the graft cells between stage I and stage II graft tissue would illustrate differences in the graft's effectiveness.

In order to address these questions, the second aim of my project was to develop an animal model of the grafting procedure. This animal model would allow us to study the post-graft brain with histology and immunohistochemistry at multiple time points

[3] Andrew S. Welleford, Nader El Seblani, Francois Pomerleau, Victoria Thompson, George E. Quintero, Craig van Horne, and Greg A. Gerhardt
University of Kentucky College of Medicine

after grafting. The challenge posed by this project was to use the actual human nerve tissue collected from the grafting procedure; to best replicate the neurobiology of the human procedure. Considering the largest challenge to this approach to be immune rejection of human graft tissue, this led us to evaluate athymic nude rats as our model organism.

Athymic nude rats are an immunodeficient strain of Sprague Dawley rats with reduced immune rejection of foreign tissue. The most common strain, the rnu strain, was first discovered in 1953 by Rowett et al. (Festing, Lovell, Sparrow, May, & Connors, 1978) These rats were observed to lack normal fur development, hence the nude moniker, and were also observed to have reduced immune system activity. This immune deficiency was found to be due to lack of thymus development and consequently impaired T cell maturation. This trait was linked to homozygous mutations of the rnu gene on chromosome 10.

Due to their reduced rejection of foreign tissue, athymic nude rats, along with athymic nude mice, have been used as an animal model for the grafting of tissue from other organisms (xenografting), including human tissue. In oncology research, athymic nude rats have been used for both anatomical and toxicology studies following grafting with human cancer cell and tissue lines. Kristjansen (Kristjansen, Roberge, Lee, & Jain, 1994) describes the implantation of human tumor lines in athymic nude rats. Ozawa (Ozawa et al., 2002) describes growth of human glioblastoma xenografts. Chen (L. Chen, Tai, Brar, Leung, & Hsiao, 2015) describes changes in gut microbiome as a consequence of tumor grafting. Maruo (Maruo et al., 1982) describes grafting with human tumor lines

and an age-dependence of the rat host, with increased immune rejection observed in older rats.

Other athymic nude rat xenografting experiments include Beretta's (Beretta et al., 2017) grafting of human neural stem cells after TBI. Chesmel (Chesmel, Branger, Wertheim, & Scarborough, 1998) showed that human demineralized bone grafts elicit bone repair in a cranial defect in athymic nude rats compared to autografts. Keating (Keating, Isaacs, Mallu, & Baxi, 2015) demonstrated that human acellular nerve xenografts could be used to promote functional nerve repair in an athymic nude rat model of peripheral nerve injury.

These studies demonstrate the success of using athymic nude rats for human tissue xenograft experiments. However, there is no history of human peripheral nerve tissue xenografts within the brain of athymic nude rats. Thus, the aim of this project was to assess the feasibility of an athymic nude rat model of the human sural nerve grafting procedure. This model was dubbed the "Neuro-Avatar" for its use of human nerve tissue xenografted into an interspecies host. First, the logistics of coordinating human and animal surgeries are described. Next, a rodent stereotactic surgical grafting procedure was evaluated for feasibility, accuracy, and precision. Lastly, preliminary results of human graft tissue survival at two weeks and six months were assessed.

Methods

Experimental Design

Table 3.1 summarizes the experimental design. 16 total animals were planned: 8 to be collected at 2 weeks post-surgery and 8 to be collected 6 months post-surgery. Within each group of 8 animals 4 were to receive tissue from Stage I surgerys (injury-naive tissue) and 4 were to receive tissue from Stage II surgeries (injury-conditioned tissue). Because each human patient underwent a Stage I and Stage II surgery, a total of 4 human patients would provide the tissue.

Animal Surgery

Athymic nude (Hsd:RH-Foxn1rnu) male rats (which have decreased immune rejection of human graft tissue) aged 2 months were ordered from [Vendors]. Animals were kept in University of Kentucky Division of Laboratory Animal Resources (DLAR) facilities in an infection control room except when in the surgical suite.

Human sural nerve samples were collected during DBS surgeries. DBS at our institution is carried out in two stages: Stage I is followed by Stage II two weeks later (van Horne et al., 2017). For the Stage I tissue collection, the surgeon made a transverse incision through the skin of the lateral ankle, 2 cm superior to the lateral malleolus. The surgeon then identified the neurovascular bundle containing the sural nerve and associated artery and vein. The sural nerve was then separated from the bundle using blunt dissection. Next two black silk sutures were tied around the nerve roughly 1 cm

apart to mark the nerve. The section of the sural nerve between these sutures was then cut, a piece removed, and the surgical area sutured closed.

The removed section of nerve was then stripped of its epineurium using microsurgical dissection in sterile saline. Individual fascicles of nerve fibers were separated using jeweler's forceps and the perineurium was discarded. These fascicles were placed in conical microcentrifuge tubes and snap-frozen on dry ice before long-term storage at -80°C until assayed. For Stage II tissue collection, the surgical area from Stage I was reopened at the original incision. The surgeon located the suture markers on the sural nerve and removed a new piece of tissue from the distal stump of the nerve. This tissue was kept on sterile saline ice before implantation. Tissue was prepared for study intervention as in (van Horne et al., 2017) Sural nerve fascicles was transported from DBS surgery on ice. The fascicles were sectioned into 1 mm^3 pieces, covered, and left on ice until insertion.

The surgical field was prepared by laying out a sterile surgical drape over a preheated surgical warming pad set to 40 °C. The sterile empty surgical cannula was mounted to the arm of the stereotactic frame. This cannula was a 9-gauge needle with the tip blunted and smoothed with a dremel tool. A metal 0.1 mL syringe plunger was used as an inner stylet.

Anaesthesia was induced using 5% isoflurane with supplemental oxygen in an anesthesia induction box. After anaesthesia induction, animals were secured to the stereotactic frame using a tooth bar and anaesthesia nose cone with 2.5% isoflurane with supplemental oxygen. The tooth bar was adjusted so that the dorsal surface of the

animal's skull was level. Ear rods were placed securely in both auditory canals and adjusted until the animal was symmetrically secured to the stereotactic frame. Animals were kept on surgical warming pads during and after surgery.

The dorsal surface of the animal's skull was shaved using an electric razor from brows to ears. Next the skin was swabbed two times with cotton swabs soaked in betadine solution followed by wipe-down with two alcohol pads.

After anaesthesia induction the animal was treated with the following: Rymadyl (10 mg/kg, SQ), Buprenorphine SR (1 mg/kg SQ), and Baytril (5 mg/kg IM). Dosing was individually calculated for each animal based on the animal's mass, and the sustained release formulation of Buprenorphine provided analgesia for the entire post-surgery period.

The animal's body posterior to the surgical site was then covered with a second sterile drape.

The surgeon donned sterile surgical gloves and made an incision down to bone with a scalpel along the midline from mid-brow through to the posterior crown ridge of the skull. The skin flaps were separated, blood was cleaned using sterile cotton swabs, and the superficial fascia was clipped using seraph clips and retracted, letting the weight of the seraph clips hold the incision open. Any actively bleeding blood vessels on the skull surface were cauterized using a cauterization pen. The skull surface was flushed with saline and dabbed dry with surgical gauze. The bregma was located at the convergence of the anterior suture and lateral sutures and marked using a permanent marker. The

cannula plus inner stylet tip was positioned directly on bregma and the Anterior-Posterior (AP) and Lateral-Medial (LM) stereotactic coordinate readout was zeroed. The coordinates used for the graft to the dorsal striatum were 1 AP, ±1 LM, and 4.5 DV.

The cannula plus inner stylet was then repositioned to the coordinates of 1 (anterior) AP and -1 (Right) LM and lowered to just above the skull surface. This location on the skull was marked with a permanent marker. The cannula plus inner stylet was returned to bregma and the same procedure was repeated to mark the left side.

Burr holes were drilled into the marked skull locations using a ball-tip drill bit. Care was taken to drill completely through the skull without applying pressure to the dura mater. After drilling, the burr hole was flushed with saline and cleared using sharp-tipped forceps. The dura was then pierced using the sharp-tipped forceps. The cannula plus inner stylet was reset to the 1 AP and -1 LM coordinates and lowered to the surface of the brain, watching for a reflection change of the dural surface indicating that the cannula had contacted the brain surface. The Dorsal-Ventral (DV) coordinate readout was set to zero, and the cannula plus inner stylet was inserted to a depth of 4.5 at a rate of 1 mm/minute to create a tract through the brain. This tract emulates the human surgery, which involves an insertion of a bullet-tipped rod prior to graft cannula insertion to create a tract through the brain. This insertion speed was achieved by lowering the cannula plus inner stylet 0.2 mm at a time and waiting 10 seconds. After reaching a depth of 4.5, the cannula plus inner stylet was removed at the rate of 1 mm/minute. This process was repeated on the left side.

The cannula plus inner stylet, still mounted to the arm, was returned to the right side at 1 AP and -1 LM coordinates, the inner stylet was raised, and one 1 mm^3 piece of nerve fascicle was loaded into the open end of the cannula. The cannula was lowered to the surface of the brain again, the DV coordinates were set to zero, and the cannula was inserted to a depth of 3.5 at a rate of 1 mm/minute to create a tract through the brain. This left a 1 mm space for the graft tissue to be inserted. This insertion speed was achieved by lowering the cannula plus inner stylet 0.2 mm at a time and waiting 10 seconds. After reaching a depth of 3.5, the inner stylet was depressed, pushing the graft tissue into the brain. The cannula plus inner stylet was removed at rate of 1 mm/minute. If the graft tissue was seen to be adhered to the cannula then it was removed, as was the case in approximately half of the cases, a new piece was loaded into the cannula, and the insertion was repeated 1-2 times until successful graft delivery was achieved. After grafting, a sham insertion was performed on the control side using the cannula using the same procedure but without the graft tissue.

After sham insertion, the burr hole was sealed using bone wax. The bone and tissue were wet thoroughly with saline and the skin flaps were pinched shut using curved blunt-end forceps. The surgical incision was stapled closed using 3-4 staples, taking care to align the cut ends of the incision. The animal was transferred to a cage on a preheated surgical warming mat until recovered from anaesthesia. Animal activity indicating anaesthesia was generally observed within 15 minutes of ending the surgery, and more vigorous activity was observed within one hour. Animals were kept for observation overnight, then returned to the animal care facility the day after surgery. No motor or

behavioral deficits were observed in the animals in the days or weeks following the surgery.

Brain Collection

At the indicated date, animals were euthanized with 1 mL sodium pentobarbital solution (Lethal Plus) intraperitoneally followed by decapitation. Brains were dissected out and stored in 4% paraformaldehyde solution overnight. Brains were then transferred to a 40% sucrose solution with 0.1% sodium azide until tissue sectioning.

Brains were sectioned using a cryostat set to 45 um section thickness. Sections were collected sequentially in a 24 well plate in phosphate buffer with 0.1% sodium azide.

Immunohistochemistry

Tissue sections were washed 3 times with 1x TBS for 6 minutes each. Sections were then etched with 7:2:1 etching solution (7mL TBS, 2 mL MeOH, and 1 mL freshly added H2O2) for 10 minutes, then washed with 1x TBS. Sections were then etched in sodium metaperiodate solution for 20 minute, then washed 3 times with 1x TBS-T for 10 minutes.

Sections were blocked in 10% neutral goat serum (NGS) in TBS-T for 60 minutes.

Sections were then incubated in primary antibody (mouse anti-Human Nuclear Antigen, Abcam, Lot:GR183983-5) solution (1:500 in 3% NGS TBS-T) overnight.

The following day the sections were washed in ~1 mL 3% NGS for 10 minutes, then incubated in 5 mL secondary antibody (biotinylated goat anti-mouse, Vector) solution (1:200 in 3% NGS TBS-T) for 60 minutes.

ABC development solution was mixed according to manufacturer instructions (Vectastain ABC HRP Kit, Vector).

Sections were washed in 1 mL 3% NGS TBS for 10 minutes 3 times, then incubated in 500 uL of ABC solution for 60 minutes. Sections were washed in TBS 1 mL for 10 minutes three times.

VIP solution was added for 3 minutes, until the tissue visibly began to turn dark. Sections were washed in TBS for 10 minutes 3 times, then transferred to PB overnight. Sections were floated in PB onto slides, then cover slipped using Cytoseal.

Remaining avatar brain regions were preserved in cryoprotectant (300 mL ethylene glycol, 250 mL glycerol, PB added to 1 L volume) and kept at -20° C.

Results

Feasibility of tissue grafting procedure

The first concern in developing this animal model of the sural nerve grafting procedure was the feasibility of implanting fresh human nerve tissue collected from surgery into rodents. The coordination of human tissue collection with animal implantation was successful. All 16 animals indicated in the experimental design received a graft of human tissue from four human participants. Following surgical dissection of

fascicles the human peripheral nerve fascicles were delivered to the animal surgery suite on ice and kept on ice until grafted into the animals. This was achieved by beginning the animal surgery while the human peripheral nerve tissue was being collected and surgically dissected, so that the insertion of graft tissue occurred shortly after the human tissue arrived to the surgical suite. Communication between the animal surgical team and the human tissue collector ensured that these events were coordinated.

Each rodent surgery session involved two animals being implanted with tissue from the same human subject's surgery: one animal to be evaluated two weeks later, and one animal to be evaluated six months later. These two rodent surgeries were performed sequentially, with the first insertion occurring as soon as the human tissue arrived, and the second insertion occurring after closing the first animal and preparing the second animal. The animal to be collected at two weeks was not indicated at the time of surgery, and was assigned by the experimenters at two weeks post-surgery. One or two human surgeries occurred per day, meaning that up to four total rodents were implanted each surgery day. On days with two human surgeries the tissue collection was separated by several hours, allowing for the completion of the first set of rodent surgeries before the second tissue sample arrived. Each animal's surgery took approximately two hours from anaesthesia induction to surgical incision closing.

Each human subject underwent two surgeries: a Stage I and Stage II DBS surgery spaced two weeks apart. With two animals grafted from each human surgery, a total of four human participants provided tissue for 16 rodent surgeries.

Athymic Nude Rat Tolerance of Stereotactic Surgical Grafting Procedure

The surgical procedure was well-tolerated overall by athymic nude rats. All 16 animals indicated in the experimental design successfully underwent the grafting procedure. One uncounted animal died of cerebral hemorrhage during the surgery as a result of the bone drill slipping approximately one millimeter deep into the brain while drilling a burr hole. This occurred before grafting the human tissue, and a backup animal was used to carry out the grafting procedure for that tissue sample. A different animal recovered from the grafting procedure anaesthesia and was observed to resume activity that day, but died the following night from suspected respiratory arrest. This animal was counted in the total of 16 grafted animals but excluded from analyses as it did not survive to the two week time point.

Accuracy and Precision of Stereotactic Surgical Grafting Procedure

Histology of the brains post-implantation shows that not all cannula insertions were successful in reaching the dorsal striatum. The depth of the graft or control tracts did not reach the targeted depth. Evidence of brain compression and resulting contusion were visible. Taken together, these suggest that the graft cannula in its current design creates drag when it is inserted into the brain tissue, which compresses brain tissue and causes contusions while not reaching the desired depth.

Preliminary Analysis of Human Graft Cell Survival

In the brains of animals successfully grafted, HNA staining was positive on the graft side along the tract of tissue insertion. (Figure 3.1 and 3.2) One control tract out of the

eight animals was also positive for HNA. Peripheral nerve tissue was visible two weeks following the grafting procedure. (Figure 3.3) This tissue is striated and darkly-staining against HNA.

HNA positive nuclei were observed throughout the brain of grafted animals. Figure 3.4 shows HNA-positive nuclei within the corpus callosum. Two main HNA-positive cell types were observed: HNA-positive nuclei and HNA-positive cell surfaces. (Figure 3.5)

As Figure 3.7 summarizes, at two weeks post-surgery there was no difference in diameter of HNA-positive staining from the graft tract between animals grafted with Stage I (injury-naive) tissue and animals grafted with Stage II (injury-conditioned) tissue.

In addition, from preliminary staining of the six months post-implantation group, immunofluorescence showed presence of HNA-positive cell nuclei within the target region of the dorsal striatum in some animals, HNA-positive cell nuclei in the cortex but not dorsal striatum of other animals, and no HNA staining in some animals. These results support the survival of human graft cells in the animal model up to and exceeding six months. (El Seblani et al. *In preparation.*)

Discussion

Surgical procedure

Successful delivery of human peripheral nerve tissue to the rat dorsal striatum presented several challenges that needed to be overcome. First, a custom cannula was designed for the Neuro-Avatars. The cannula used in human surgeries was designed to

hold five 1 mm^3 graft pieces, whereas the rodents were to receive only one 1 mm^3 piece. The relative size of even one 1 mm^3 nerve segment is much larger than the human counterpart, necessitating a rodent cannula to be designed to minimize brain volume displacement. This limited the size of the graft cannula. We improvised using a 9-gauge needle blunted and rounded using a Dremel tool. The inner stylet used to deploy the tissue was a small metal syringe plunger from a 0.1 mL syringe, which fit securely within the cannula.

Even with the customized cannula, the grafting cannula did not reach the targeted brain depth in all insertions as evidenced by histology of the track. This is possibly due to blunt end of the cannula not effectively pushing into the brain tissue, even following piercing of the dura mater with sharp-tipped forceps. The empty cannula with inner stylet inserted was used to both create a track and insert the nerve tissue. The human surgery uses a bullet-tipped rod to create a tract for the graft cannula. A similar tool with an appropriate diameter will be needed to reliably create a tract to the desired depth in the Avatars.

A delivery cannula of a different design would improve the Neuro-Avatar animal model. There was a small gap between the cannula and the inner stylet and peripheral nerve tissue would often stick to the cannula and be drawn back out along with the cannula, necessitating re-insertion of the graft in several animals. Using the human cannula is not feasible given the proportionately large diameter compared to the rat brain. In the human surgery there are instances of the same problem occurring: nerve tissue getting caught between the cannula and inner stylet and not successfully being

deployed in the brain. A surgically appropriate material that can create a complete seal between the cannula and inner stylet, such as an inert silicone tip, may improve the reliability of tissue delivery. The sticky nature of peripheral nerve tissue itself is another contributing factor. An inert coating of the nerve tissue might improve the reliability of tissue delivery; however such an addition would not match the human trial, and the human trial is limited by FDA approval for additional substances to be inserted into the brain. Because the nerve tissue is autologous and not exposed to any treatment except surgical dissection it is considered permissible by the FDA for use in this clinical trial. If the Avatars are to model the human surgery as closely as possible then inserting additional substances must be avoided.

An athymic nude rat atlas should be used for precise surgical planning. Athymic nude rats are smaller than their wild-type (WT) counterparts and have smaller skulls and brains. Stereotactic coordinates may differ between athymic nude and WT rats, especially for more precise targets such as the Substantia Nigra. This led to the amendment of stereotactic coordinates from the first surgeries to the last.

As this project was the first step in developing the Neuro-Avatar animal model, the researchers decided to use an unlesioned animal rather than a dopamine-depletion model using 6-OH-dopamine lesion. This allowed for the evaluation of the grafting procedure independent of other experimental challenges of consistent animal dopamine depletion. Future studies could use a 6-OH-dopamine lesion model or another model of Parkinsonism to study effects of the graft tissue in a Parkinsonian brain.

Contamination of the control side with HNA-positive staining was observed in one animal. This was likely due to human cells remaining on the graft cannula after the insertion of the graft. In the future two identical cannulas should be used, and only one allowed to come in contact with the graft tissue.

Human graft tissue

HNA-positive cell staining supports the survival of human cells at two weeks, and preliminary analysis suggests graft cell survival up to and beyond six months. (El Seblani et al. *In preparation.*) The presence of HNA-positive cells in the area of the graft indicates that the graft survives in the brain of immunodeficient athymic nude rats. This finding suggests that in an autologous human graft recipient we would also expect graft cell survival at six months.

Conclusions

These results support the development of xenograft animal model of the human sural nerve graft procedure. Optimizations of the surgical protocol, in particular the development of a tract-forming rod analogous to the human surgery, are described. Survival of human tissue for at least six months in the athymic nude rats is evidenced by HNA staining, suggesting that within humans the nerve graft tissue implanted in the DBS Plus clinical trial survives within the host brain.

Chapter 3 Tables

Target	Total n	Treatment	Tissue Source	Group total n	Time Post-Graft of Collection	
					2 weeks	6 months
Dorsal Striatum	16	Nerve graft (R) Sham insertion (L)	Stage I DBS	8	4	4
			Stage II DBS	8	4	4

Table 3.1: Avatar Project Experimental Design

A total of 16 animals were grafted with the target of dorsal striatum. Each animal received a nerve graft inserted in the right hemisphere and a sham insertion of an empty cannula on the left hemisphere. A total of eight animals received tissue from Stage I of human DBS surgery and eight animals from Stage II of human DBS surgery. Within each group of eight, brains from four animals were collected at 2 weeks post-surgery and brains from the remaining four animals were collected at 6 month post-surgery.

Chapter 3 Figures

Figure 3.1: Coronal Histology of Avatar brain with HNA Staining

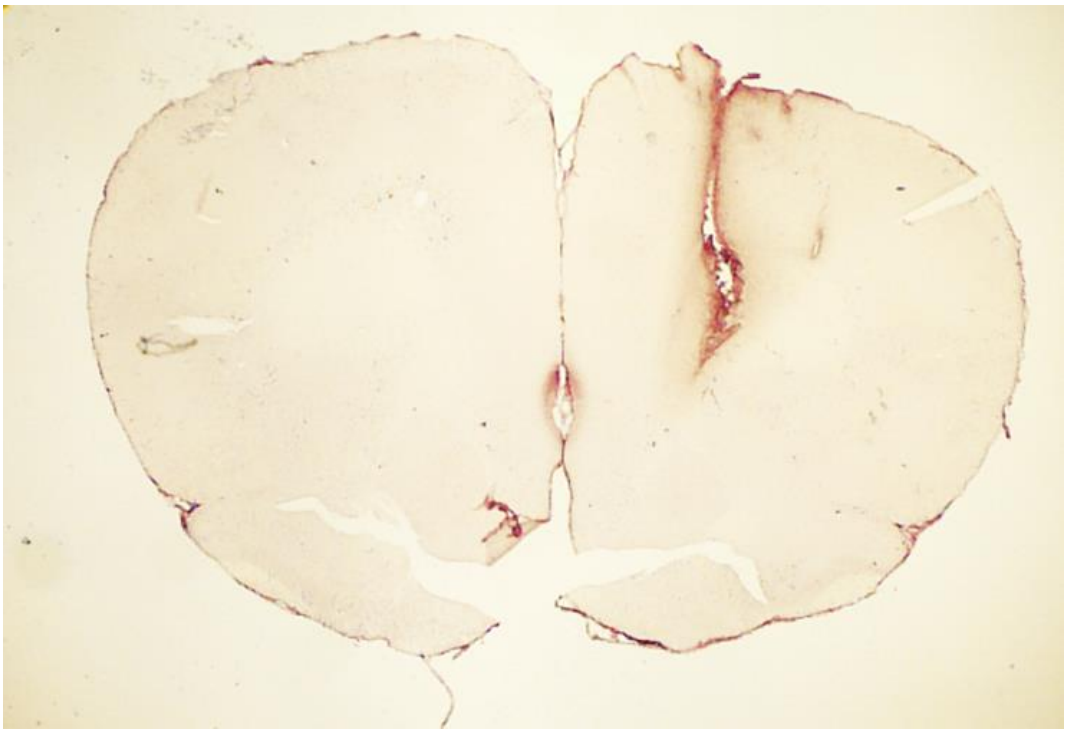


Figure 3.2: Coronal Histology of Avatar brain with Nissl Staining

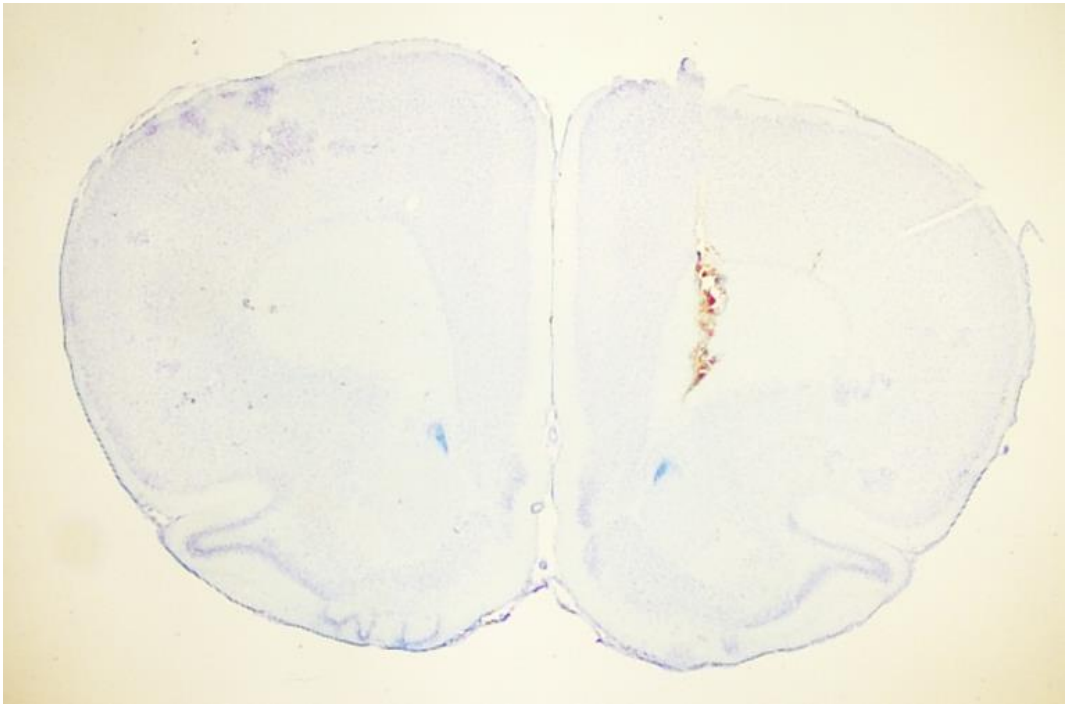


Figure 3.1 and Figure 3.2: Coronal Histology of Avatar brain

Anti-Human Nuclear Antigen (HNA) (Fig 1) and Nissl (Fig 2) staining of animal R1, two weeks post-graft. The right hemisphere is marked with a cut visible on the right. The graft tract is visible in the right hemisphere. A control tract was not visible in this animal. Evidence of brain contusion from blunt pressure of the control cannula is visible in the left hemisphere.

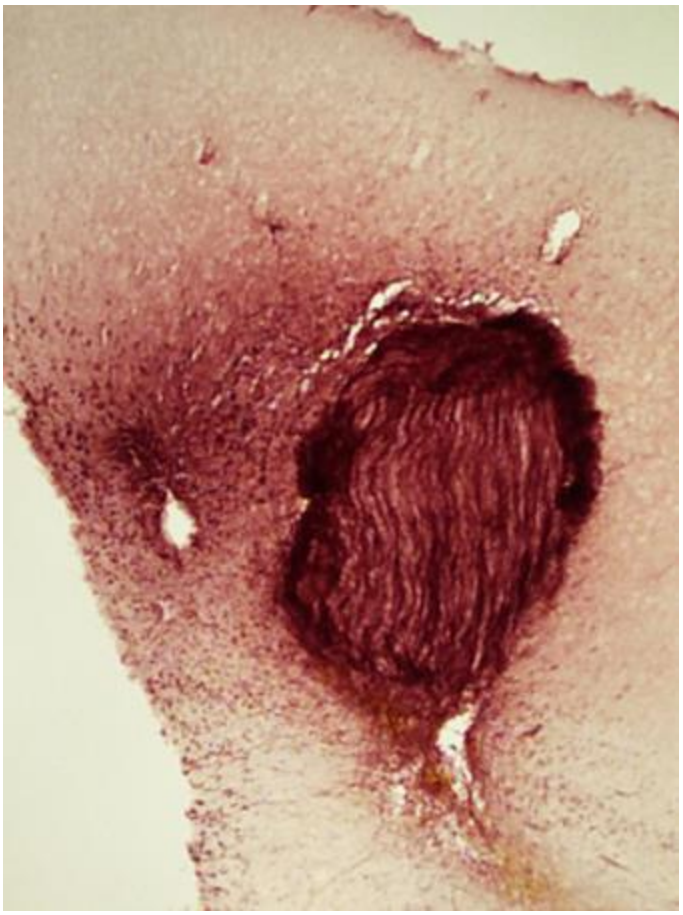


Figure 3.3: Graft tissue with HNA staining

HNA staining showing putative graft tissue. Longitudinal fibers of the peripheral nerve tissue are visible with robust anti-HNA staining. Multiple HNA-positive cells are visible surrounding the graft tissue. Diffuse anti-HNA staining is also seen surrounding the graft and HNA-positive cells.

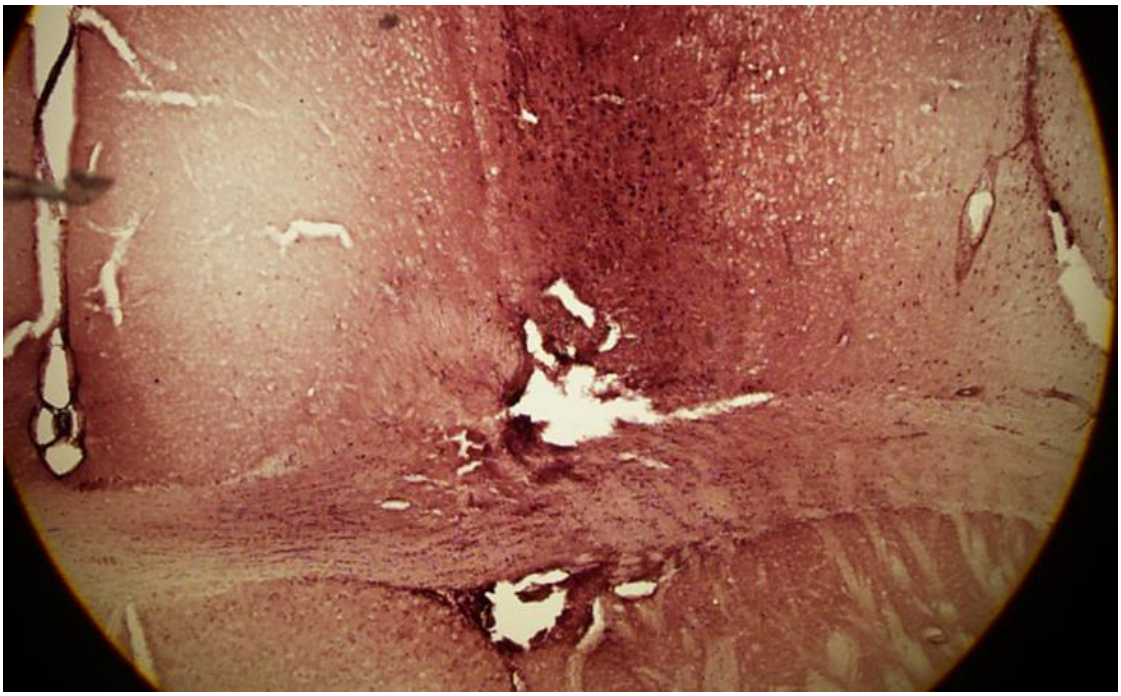


Figure 3.4: Graft Cell Infiltration with HNA Staining

HNA-positive cells are visible throughout the right hemisphere. Note HNA-positive cell nuclei within the corpus callosum. This suggests migration of HNA-positive cells through the brain of the grafted animal beyond the borders of the graft.

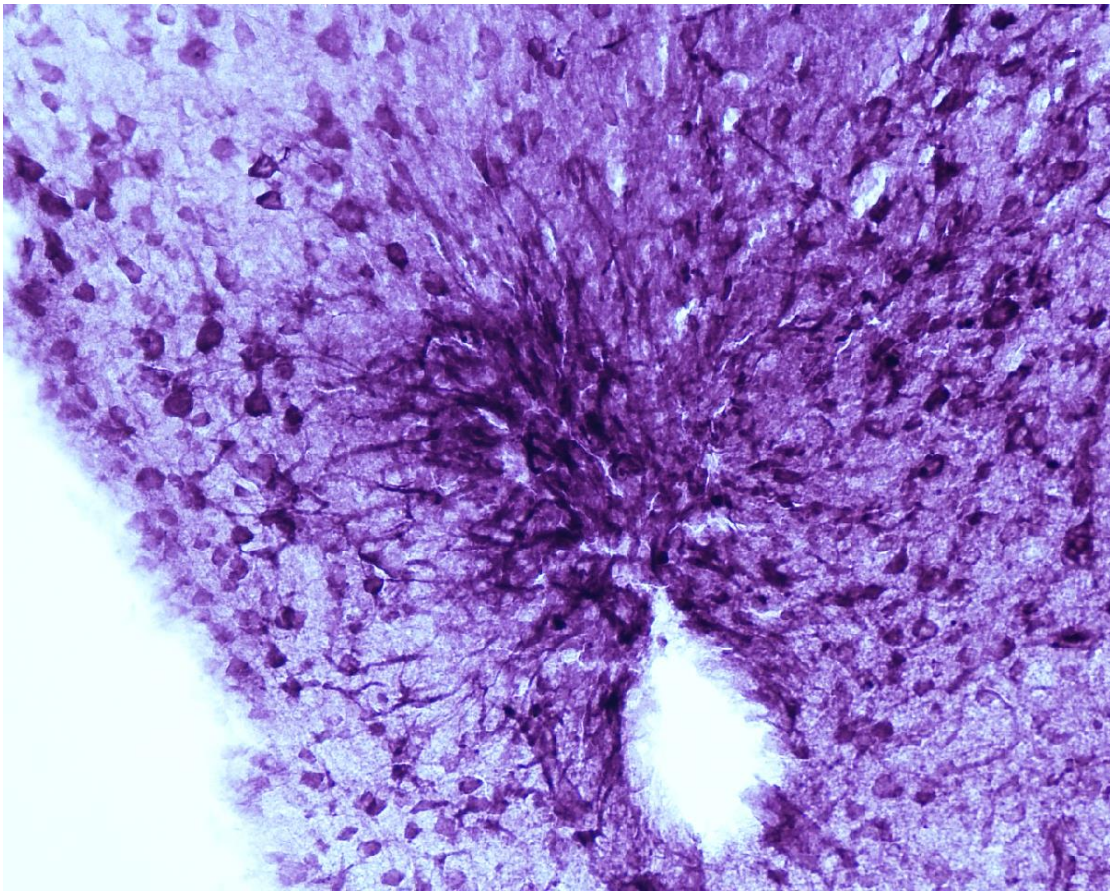


Figure 3.5: Cell Morphology with HNA Staining

20x magnification showing morphology of HNA-positive cells. These cells appear to be part of the rat brain, and their shape suggests that they are neurons. The nuclei of these cells are not positive for HNA. It is possible that these are MHC-expressing cells that are presenting HNA after graft cell death.

Figure 3.5: Radius of HNA-positive Staining, Graft vs. Sham

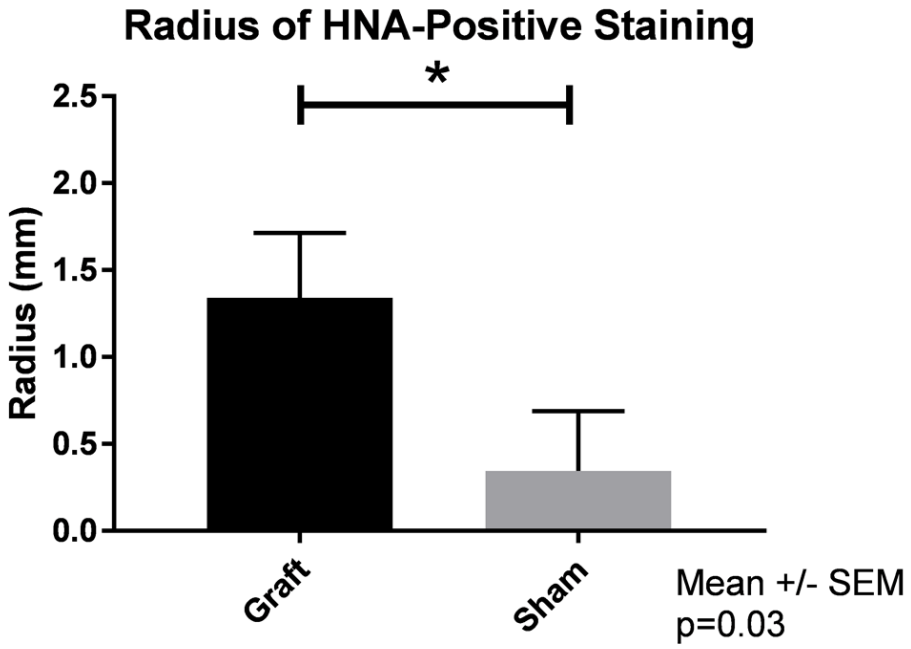


Figure 3.7: Radius of HNA-positive Staining, Stage I vs. Stage II Tissue

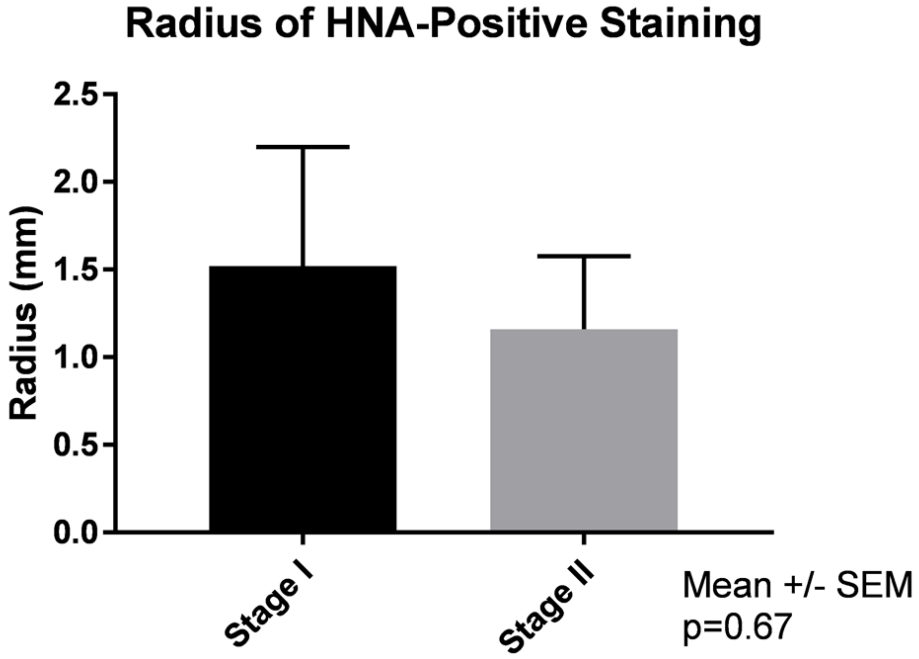


Figure 3.6 and Figure 3.7: Radius of HNA-positive Staining

Quantification of HNA-positive staining radius in each brain. There was significantly higher staining radius in graft vs control tracts. Note that only one rat brain showed HNA staining on the control tract; a suspected contamination of the control cannula with human tissue. There was no statistically significant different in the radius of HNA staining from Stage I vs Stage II graft tracts.

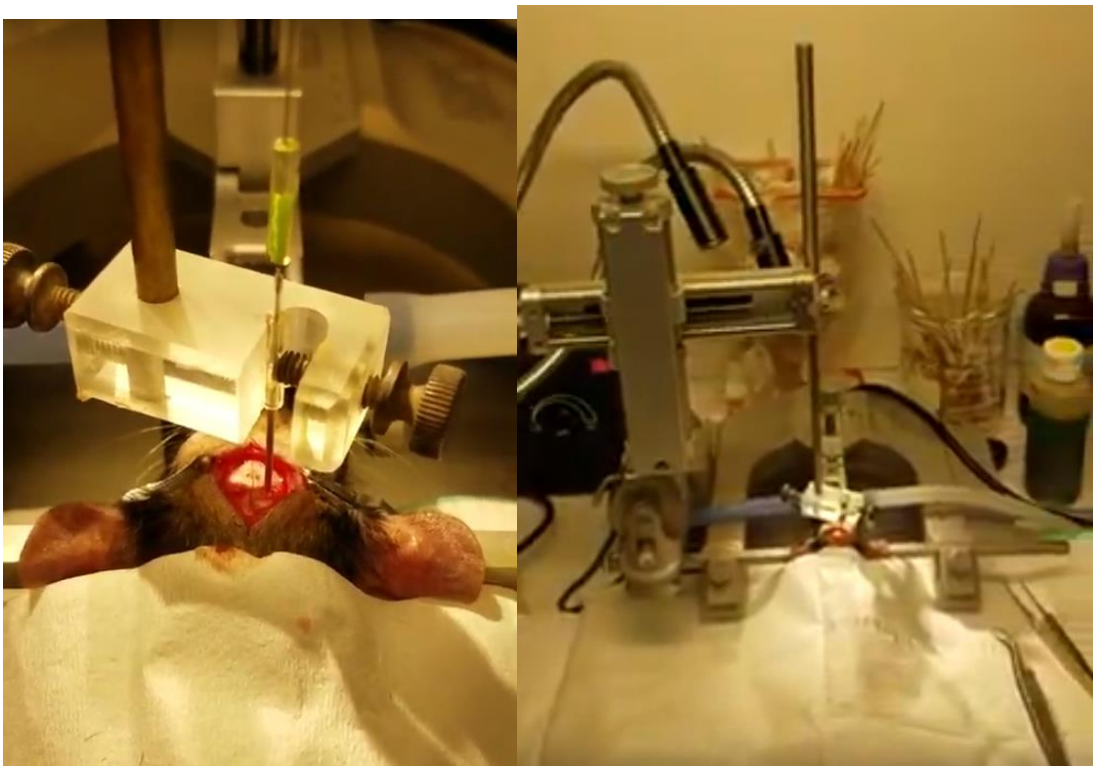
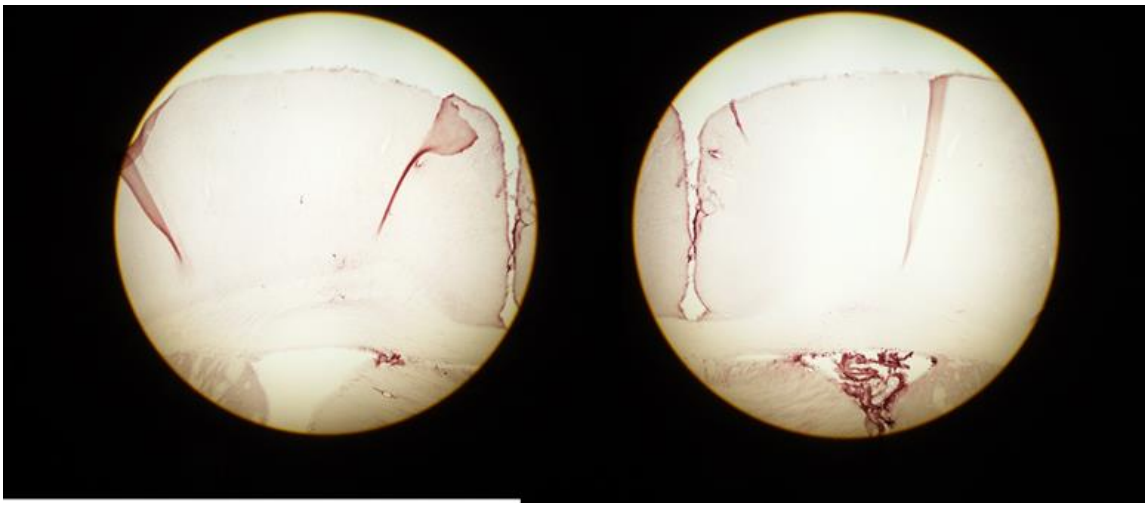


Figure 3.8: Avatar Surgery Setup

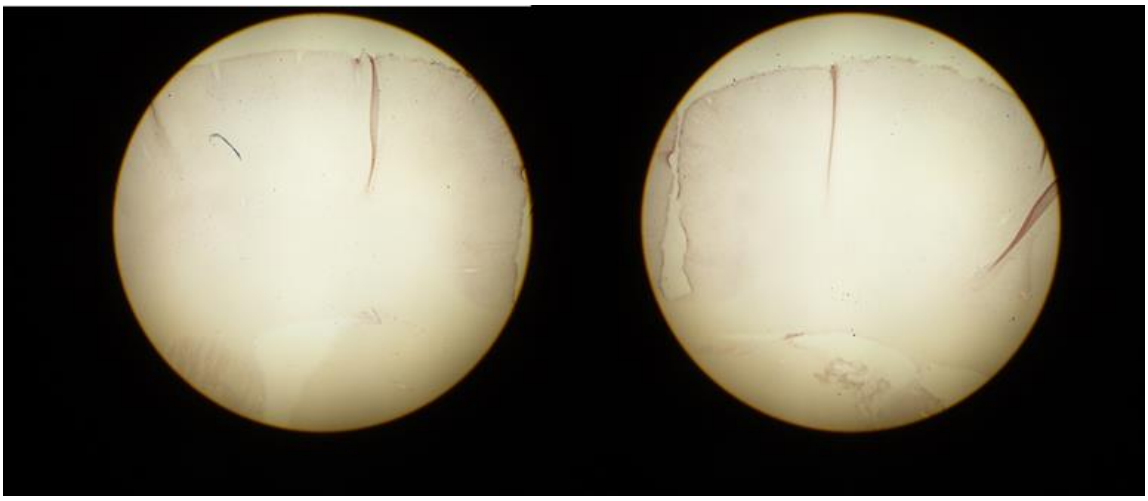
Picture of rat within stereotactic frame.

Figure 3.9: Avatar graft and Control Tracts

No Primary Antibody (Negative Control)



No Secondary Antibody (Negative Control)



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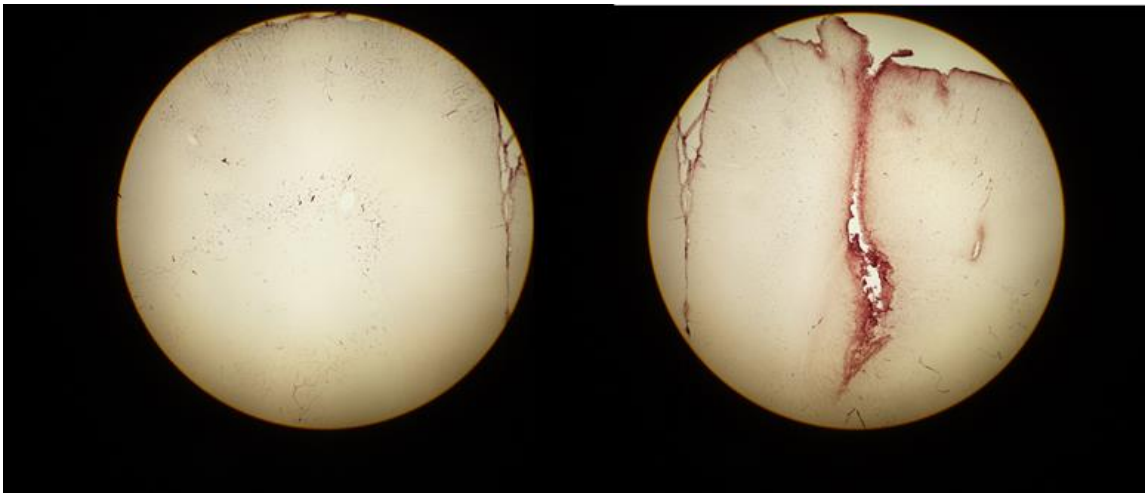

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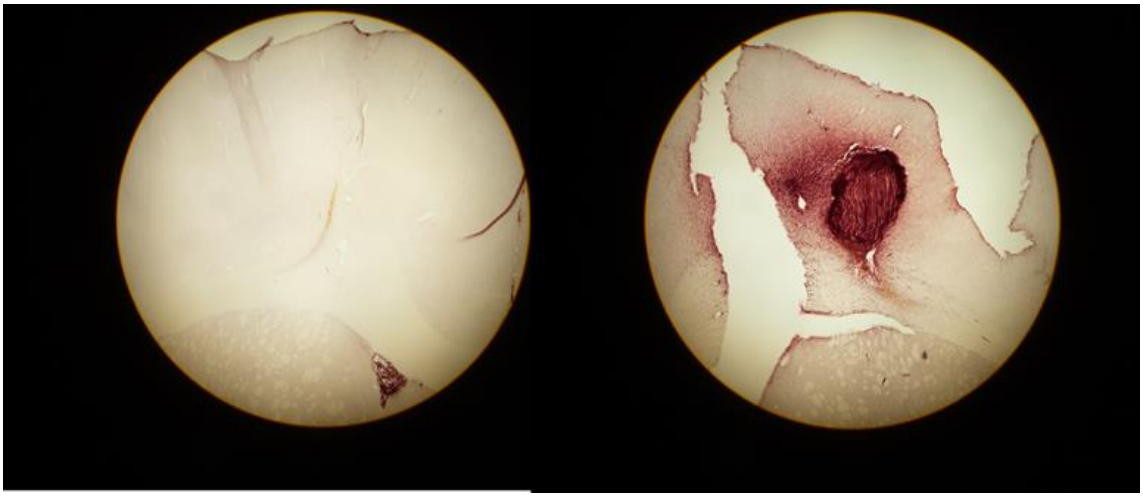

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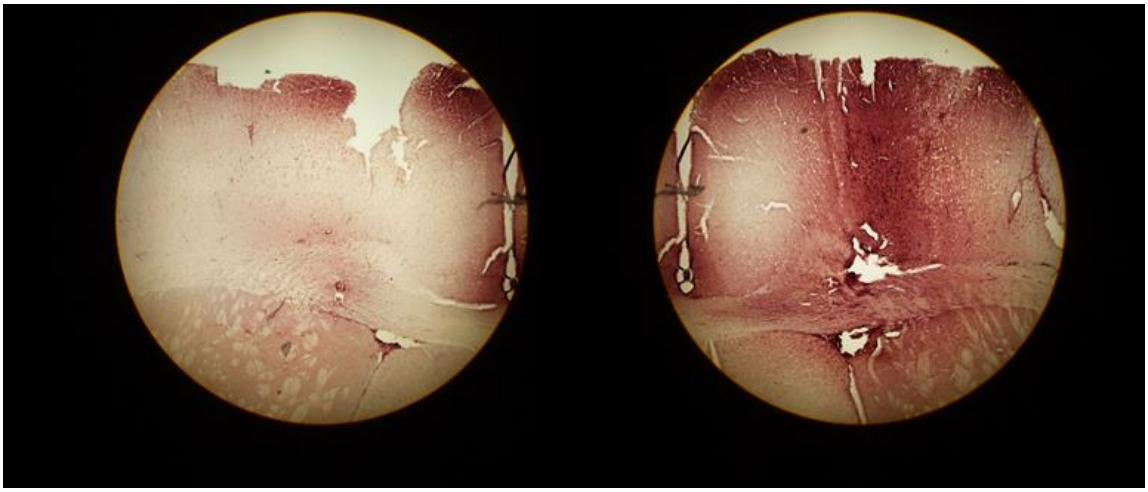

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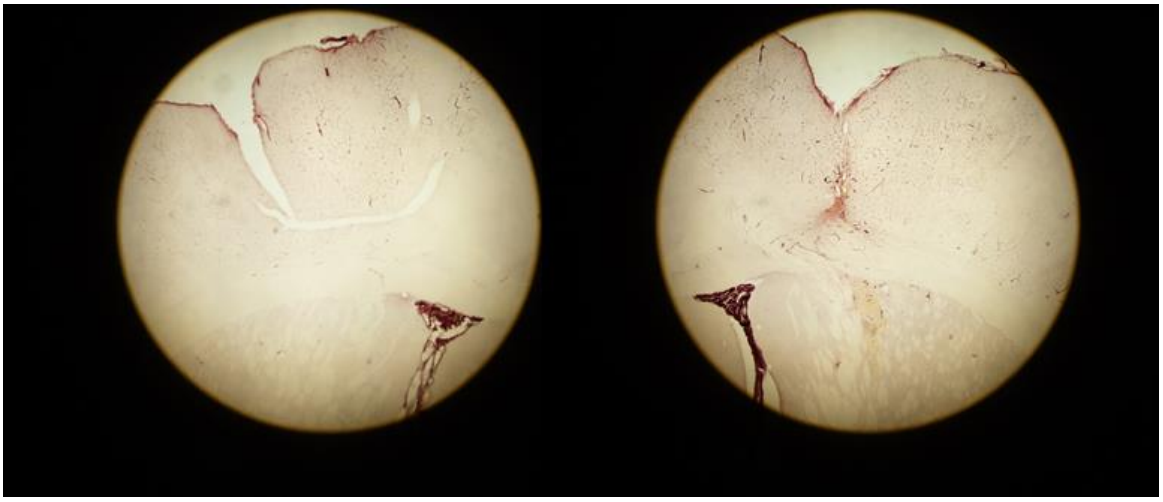

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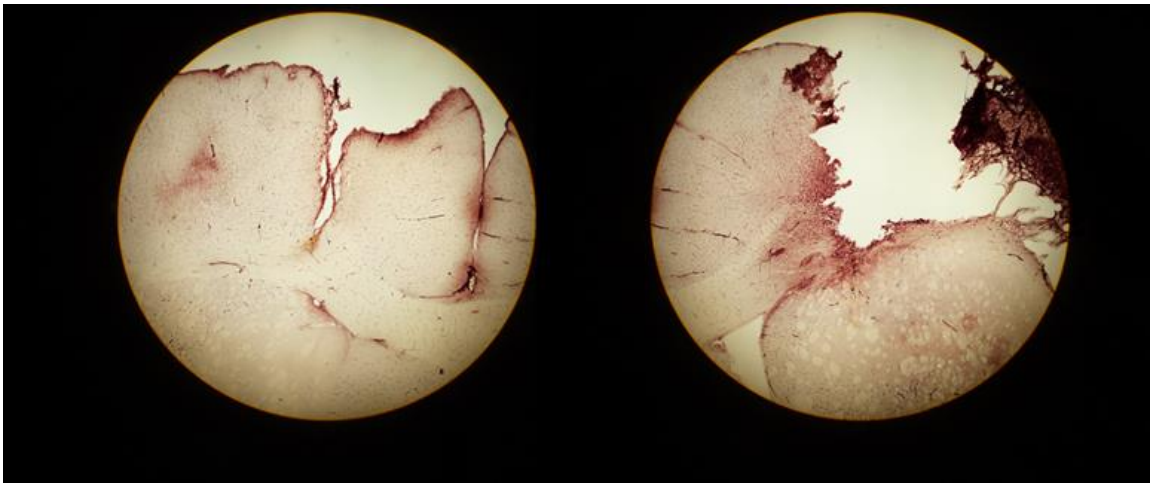

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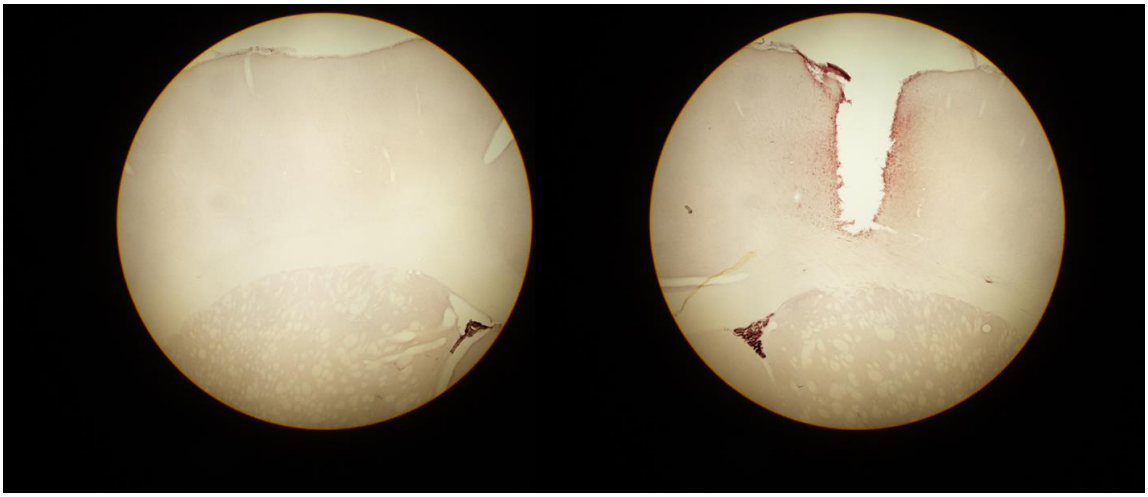

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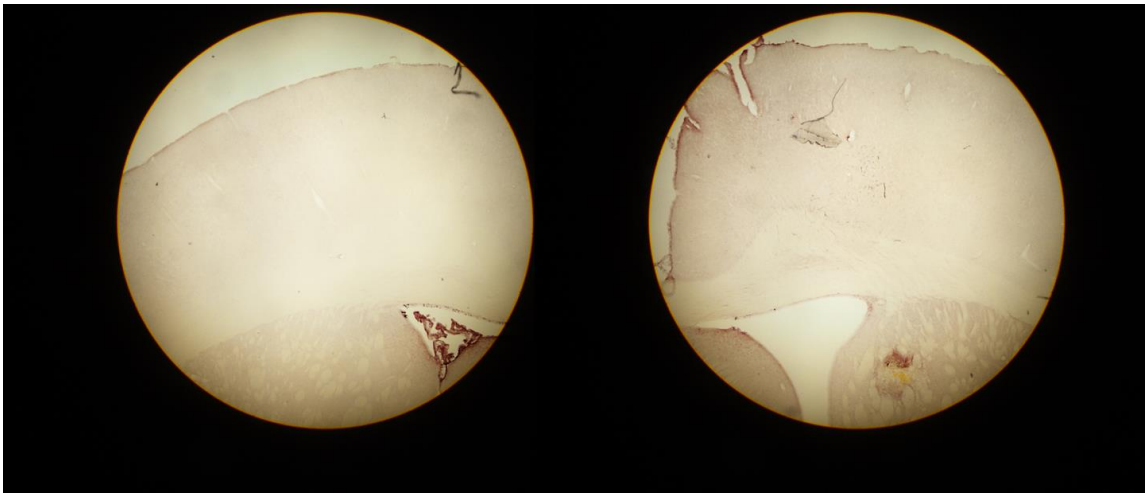

Randomized 8

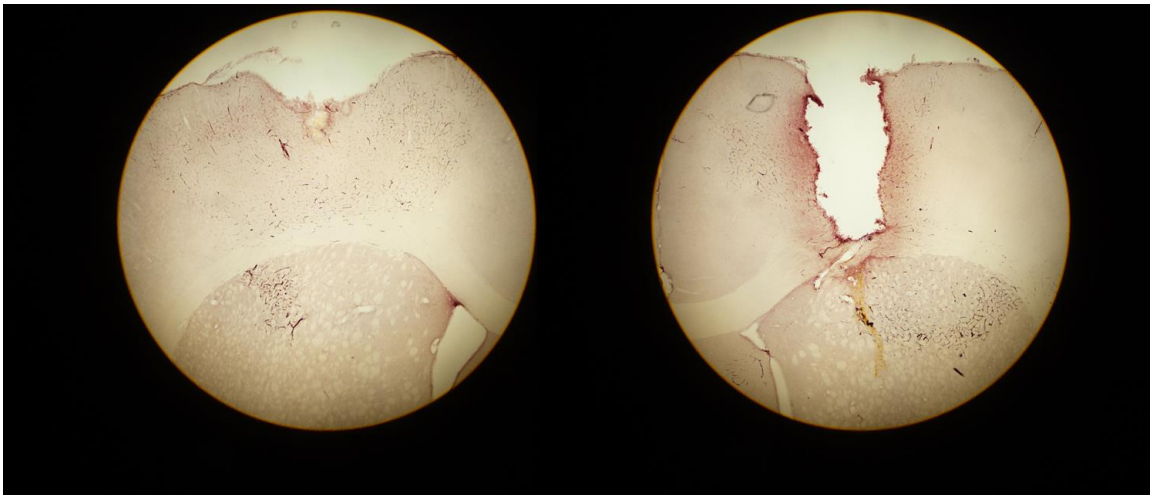

Figure 3.9: Avatar graft and Control Tracts

Images of graft and control tracts for all grafted animals.

Chapter Four: Clinical Data

Peripheral nerve grafts implanted into the substantia nigra in patients with Parkinson's disease during deep brain stimulation surgery: 1-year follow-up study of safety, feasibility, and clinical outcome[4]

Introduction

The motor symptoms of Parkinson's disease (PD) are primarily related to the degeneration of dopaminergic neurons within the midbrain. One of the hallmarks of the disease is the continual progression of symptoms associated with ongoing cell loss. Although medical and surgical therapies can ameliorate some of these symptoms, disease progression leads to treatment-resistant symptoms that are often accompanied by troublesome fluctuations and medication side effects. Unfortunately, no treatment slows or halts disease progression. Thus, altering the course of clinical decline in PD is perhaps the largest unmet need facing patients, researchers, and clinicians. (Kalia & Lang, 2015)

The majority of surgical research efforts to alter disease progression have focused on either cell replacement strategies or the delivery of growth factors that target the restoration of dopaminergic neurons. Transplantation of fetal dopaminergic tissue has not demonstrated significant clinical improvement in randomized double-blinded control studies. (Kidd et al., 2013) However, pronounced long-term

[4] Published as: Craig G. van Horne, Jorge E. Quintero, John T. Slevin, Amelia Anderson-Mooney, Julie A. Gurwell, Andrew S. Welleford, John R. Lamm, Renee P. Wagner, and Greg A. Gerhardt. "Peripheral nerve grafts implanted into the substantia nigra in patients with Parkinson's disease during deep brain stimulation surgery: 1-year follow-up study of safety, feasibility, and clinical outcome." *Journal of neurosurgery* 129, no. 6 (2018): 1550-1561.

improvements in several patients (Barker, Barrett, Mason, & Björklund, 2013) have prompted a new round of investigations in Europe (TRANSEURO; clinicaltrials.gov database registration no.: NCT01898390), despite ongoing moral and ethical issues surrounding the use of human fetal tissue as donor material. Meanwhile, strategies for growth factor delivery have focused on the use of a single factor and have included direct infusion of the factor, such as glial cell–derived neurotrophic factor (GDNF) (Steven S Gill et al., 2003), (John T. Slevin et al., 2005) or the infusion of a viral vector, such as AAV-neurturin, capable of initiating the production of the factor. (Bartus et al., 2013) Intraparenchymal GDNF delivery has shown promise in preclinical studies that failed to show significance in a phase II trial. (Lang et al., 2006) Despite promising early results, 2 randomized controlled studies with AAV-2 neurturin (CERE-120) did not meet their primary end points. (Marks et al., 2010; Marks et al., 2008)

These clinical studies have clearly identified some of the hurdles in developing neurorestorative therapies. Identifying the right factor(s), target(s), and methodology for optimum delivery poses major challenges. An additional consideration is that patients who are appropriate for enrollment in surgical implantation therapy trials are often at a stage in their disease where deep brain stimulation (DBS) is now the standard of care. This is especially relevant for studies aimed at changing disease progression. From a patient-centric view, choosing to participate in a long-term trial to assess alteration of disease progression may mean foregoing the benefits of DBS. For a study that may last 1, 2, or more years, having patients with PD forego the standard of care for so long for unproven therapies creates a set of ethical dilemmas.

Considering these challenges, we have identified a new approach to investigate the ability to alter disease progression. We use a cell-based graft that consists of peripheral nerve tissue as a source of growth factors and growth-promoting proteins for delivery into the parenchymal region with dopaminergic cell loss. We deploy the tissue grafts in the substantia nigra immediately following stimulating electrode placement into the subthalamic nucleus (STN) in patients with PD undergoing DBS surgery.

Peripheral nerve tissue is an abundant source of Schwann cells, which support and maintain axonal function and metabolism. (Kidd et al., 2013) Following injury to the nerve, Schwann cells transdifferentiate into repair cells and support axonal regeneration in the peripheral nervous system. (Jessen & Mirsky, 2016) The specific mechanism involves the activation of the transcription factor, c-Jun, which enables transdifferentiation through the upregulation of the production and release of growth factors, (Taylor & Bampton, 2004) the downregulation of regulatory maintenance programs, the reentry of the cell into the cell cycle, and the initiation of chemical signaling in order to attract the migration of macrophages to assist in the process of regeneration. (Arthur-Farraj et al., 2012) The growth factors produced include those that have been shown to promote and maintain dopaminergic neurons in experimental conditions: GDNF, (Henderson et al., 1994) brain-derived neurotrophic factor, (Meyer, Matsuoka, Wetmore, Olson, & Thoenen, 1992) and neurotrophin 3. (Funakoshi, 1993)

We previously reported the safety and feasibility of grafting autologous peripheral nerve tissue into the substantia nigra. (van Horne et al., 2017) This study

presents the full experience of the clinical trial that enrolled 8 participants and the clinical outcome data at 1-year postimplantation.

Methods

This study was designed as a 12-month, investigator-initiated, open-label, single-center, phase I trial to assess safety and feasibility as the primary end points. This study was registered with (registration no. NCT01833364, clinicaltrials.gov). Clinical data were collected to evaluate the potential of the graft to affect the clinical course of PD symptoms. This study was approved by the University of Kentucky institutional review board, and informed consent was obtained from all study participants.

Patient Selection

Eight patients with a diagnosis of idiopathic PD, who had been selected and provided consent to receive bilateral DBS of the STN, were informed about the study and gave both assent and formal consent to participate. Inclusion criteria were age 40–75 years; greater than 5-year history of symptomatic, idiopathic PD; and an ability to participate in all follow-up appointments. As part of our standard preoperative DBS protocol, participants were evaluated using the Unified Parkinson's Disease Rating Scale (UPDRS), both on and off medication, and underwent a formal neuropsychological evaluation. The Parkinson's Disease Questionnaire–8 was used to assess overall quality of life.

Safety and Feasibility

Patients were monitored for both perioperative and long-term postoperative adverse events. Clinical monitoring included the perioperative time points through the conclusion of the study at the 12-month time point. During this time period, adverse events were recorded from clinical reports, chart reviews, and specific queries during the clinical visits. All adverse events were submitted to and reviewed by the Data and Safety Monitoring Board (DSMB) at the University of Kentucky. Adverse events were graded as mild, moderate, or severe, as defined by the Medical Dictionary for Regulatory Activities (version 11.0). Serious adverse events were designated as any event that resulted in death, disability, prolonged or new hospitalization, or were life threatening or required medical or surgical intervention. A 1.5-T MRI study was performed within 48 hours postoperatively and at 12 months. Adverse events were categorized as either related to the graft, graft procedure, DBS surgery, DBS therapy, or not related to either surgical or therapeutic component.

Surgical Implantation

The surgical components of stimulator placement and graft placement have been described in detail (van Horne et al., 2017) and are summarized in brief below. As is typical at our institution, DBS surgery takes place over two stages. (van Horne et al., 2015) Stage I involves the implantation of the pulse generator and extensions. Stage II is when the DBS electrodes are implanted. The trajectory of the stimulating electrode to the STN was planned based on visualized targeting. Preoperative susceptibility-weighted imaging (SWI) MR sequences were used for target localization, and T2-weighted and

magnetization-prepared rapid acquisition gradient-echo sequences with contrast were used to establish trajectories that avoided the sulci, ventricles, and vasculature. Entry through the cortical gyri in the vicinity of the coronal suture allowed for lead trajectories that passed through the long axis of the STN. Plans were created using Brainlab software (iPlan Stereotaxy 3.0) for use with the Cosman-Roberts-Wells frame (Integra). DBS surgeries were performed while the patient was awake with microelectrode recording and test stimulation. (van Horne et al., 2015)

Once the stimulating electrodes were successfully placed, the graft was implanted unilaterally into the substantia nigra contralateral to the most affected side based on the UPDRS part III scores. The 5-mm-long target zone within the substantia nigra was located between 1 mm and 6 mm deep to the inferior border of the STN. The inferior border was identified intraoperatively by the electrophysiological activity that was recorded during stimulating electrode implantation. A guide cannula (1.8-mm-wide outer diameter; FHC, Inc.) was then placed within the graft target zone. The graft implantation trajectory was parallel to that of the stimulating electrode, at approximately 3 mm posterolateral, and did not require the creation of an additional burr hole.

The sural nerve that had been prepared and identified in stage I (van Horne et al., 2017; van Horne et al., 2015) was then re-identified, and a 2-cm-long segment of the nerve was harvested distal to the previous transection site. The nerve segment was rinsed in sterile saline, the epineurium was removed, and the fascicles were then cut to produce smaller, approximately 1-mm-long segments. Five to 6 segments were then

loaded into the custom-made graft cannula, and the graft cannula was placed to the target through the same burr hole used for the stimulating electrode. After graft placement, the incisions were closed with subcutaneous sutures.

Postoperative Evaluation

After DBS and graft implantation, participant were admitted overnight. Each participant underwent postoperative MRI to confirm electrode placement and to evaluate the region of the graft target zone. Stimulator programming was initiated in the immediate postoperative period before discharge. Patients underwent clinical follow-up through routine postoperative visits, programming visits, and postoperative study visits. Medication changes were documented and reported as the levodopa equivalent dose (LED). (Pahwa, Wilkinson, Overman, & Lyons, 2003) For UPDRS testing, participants stopped taking medication at least 12 hours before undergoing UPDRS testing. After surgery, participants (if on medication) stopped medication 12 hours before UPDRS testing and turned off DBS 12 hours before UPDRS testing, except for the one participant (participant 2) who stopped taking medication and turned off the DBS stimulator 3 hours before UPDRS testing.

Neurocognitive Testing and Analysis

The neurocognitive assessment battery was designed to meet the Movement Disorders Society's Task Force Guidelines for determining mild cognitive impairment and PD dementia. (Emre et al., 2007; Litvan et al., 2012) Level II criteria for comprehensive assessment of cognition in patients with PD include multiple measures of executive

functioning, attention, working memory, language, memory, and visuospatial functions. Some subtests of these measures were used to assess both verbal and psychomotor speed. In addition, an estimate of premorbid function and a brief measure of general intelligence were included to aid clinical judgments of changes in individual cases and provide a comprehensive neurocognitive assessment. Affective and emotional symptoms were measured using a self-reported measure to complement clinical assessment during the patient interview. Postoperative evaluations were completed approximately 1 year after the DBS procedure (mean ± SD 14.98 ± 4.73 months) using alternate forms of measures, where available, to minimize practice effects between assessments. The full assessment protocol for both the preoperative and postoperative evaluations involves a clinical interview with a neuropsychologist, with participation by both the participant and an informant, and battery administration by a clinically licensed, master's level psychometrician. All performances on the neurocognitive measures were quantified using appropriate normative sources, and all neuropsychological data were interpreted by a neuropsychologist. Analysis focused on individual changes between the preoperative and postoperative scores.

Data Analysis

Results were analyzed using the t-statistic (or Wilcoxon matched-pairs test for nonparametric values) for paired group means between the baseline and 12-month assessments (Prism 6.0, GraphPad Software Inc.). Mean values are presented with standard deviation. The minimal level of significance was $p \leq 0.05$ (2-tail criterion). Evaluation of the neurocognitive data were completed using IBM SPSS (version 23) with

the majority of scores in standard form (mean 100, SD 15). Raw scores were compared as the affective measure. Due to the small sample size, the normality of the score distribution was examined, and the results suggested discordant normality for some scores (e.g., $p = 0.03$ for the presurgical riddles subtest of the Kaufman Brief Intelligence Test-2 vs $p = 0.10$ for the postsurgical results, Kolmogorov-Smirnov test). Given this finding, nonparametric analysis with the Wilcoxon signed-rank test was employed to examine the neurocognitive data. One participant was included in only some analyses because of differences in the presurgical battery construction.

Results

Patient Summary

Eight participants—6 males and 2 females—met the criteria for participation and successfully completed the study protocol. The mean participant age was 62.9 ± 9.2 years, and the mean disease duration was 9.8 ± 3.9 years. Because of family issues, 1 participant was not able to participant in the intermediate therapeutic evaluation visits (6 and 9 months) but was able to complete the 12-month evaluations. With respect to safety and adverse event reporting, the participant was evaluated by the local movement disorders neurologist and was in close communication with our study team throughout the study.

Safety

All adverse events were submitted to and reviewed by the DSMB at the University of Kentucky and are summarized in Table 4.1. The events listed include all

events that presented from the first stage of the surgery through study closeout at 12 months postimplantation. One adverse event, superficial cellulitis surrounding the ankle incision, was graded as mild and categorized as serious and related to the graft procedure. The participant was treated with a 10-day course of antibiotics, and the cellulitis resolved. No other adverse events were related to graft harvesting or graft implantation. Two adverse events, urinary retention and headache, were mild, not serious, and related to the DBS surgery and resolved without further intervention. Three events were deemed to be mild, not serious, and not related and included falls and headache. One event, hypomania, was mild, not serious, and related to DBS stimulation and resolved promptly after changing the stimulation of the more proximal set of contacts of the stimulating electrodes. With respect to DBS surgery, there were no observed infections, hardware malfunctions, or skin erosions. With respect to feasibility, all participants underwent successful graft harvesting and implantation without complication or significant delay.

MRI Data

MR images (1.5 T) were obtained within 48 hours and at 12 months to evaluate the midbrain region for evidence of abnormalities, including swelling, edema, or contrast enhancement. The MR images from the 48-hour time point have been reported previously.38 There was no evidence of swelling or edema on the T2-weighted images and no evidence of enhancement on the T1-weighted contrast images within 48 hours or at 12 months. The MRI data also confirmed the appropriate placement of the DBS

electrodes within the STN (Figure 4.1). There was no evidence of complications, such as hemorrhage or stroke, along the trajectories of the electrodes.

Clinical Measures

A summary of the clinical data is included in Table 4.2, with individualized scores listed in Table 4.3 and 4.4. Analysis of each of the 4 parts of UPDRS revealed that all parts showed a decrease (improvement) from baseline. Because UPDRS part III in the off state is the most appropriate score to evaluate any potential influence from the graft, we focused our subgroup analysis on the UPDRS part III scores. Collectively, the part III off scores at 12 months (25.1 ± 15.9 points) decreased from baseline (32.5 ± 9.7 points), but the difference was not statistically different (Table 4.2). We also individually scored each participant (Figure 4.2) with respect to changes in baseline using values based on clinically important differences (CIDs). According to Shulman et al., (Shulman et al., 2010) minimal, moderate, and large CIDs are 2.5, 5.2, and 10.8 points from baseline, respectively.

Individual changes in motor scores (UPDRS part III) while off DBS and off medication therapy from baseline to 12 months after surgery. Minimal (2.5 points), moderate (5.2 points), and large (10.8 points) CIDs are indicated. Each symbol represents a different participant.

We further analyzed the UPDRS part III data by performing subgroup evaluations of the collective data of all participants to investigate potential differences among symptom subsets, including tremor, rigidity, bradykinesia, and axial symptoms (speech,

facial expression, gait, posture, postural instability, and body bradykinesia), and between lateralized scores relative to graft placement (ipsilateral vs contralateral). Subgroup analysis demonstrated larger improvements in tremor and rigidity and minor improvements in bradykinesia and axial symptoms (Figure 4.3. The analysis of the lateralized scores showed a greater overall reduction in scores on the side contralateral to the graft than the ipsilateral side (Figure 4.4).

Neurocognitive Data

Analysis revealed that neurocognitive performance remained largely stable between the preoperative and postoperative time points, with some notable exceptions (Table 4.5). Some degree of improvement was postoperatively seen on the constructional praxis task (block design test of the Wechsler Adult Intelligence Scale–Fourth Edition; $T = 0$, $p = 0.04$) with a large effect size ($r = -0.55$). A subtle change was observed in the approach to the problem-solving task, on which responses became less nonsystematic and random at the postoperative time point (nonperseverative errors on the Wisconsin Card Sorting Test–64 Card Version; $T = 3$, $p = 0.6$). This change approached statistical significance, and the effect size was large ($r = -0.50$). The clinical significance of these changes is unknown because both may be influenced by repeated exposures to the stimuli and/or improvements in fine motor function. A significant decline was observed in phonemic fluency only (Controlled Oral Word Association Test; $T = 0$, $p = 0.02$) with a large effect size ($r = -0.62$). Levels of affective distress remained stable between the presurgical and postsurgical evaluations ($T = 10$, $p = 0.92$).

Discussion

As the first-ever reported attempt at combining a surgically delivered therapy with DBS, we designed this pilot study to test the safety and feasibility of this approach. Although we did not have a formal control group, we compared our results to similar patients in our database and to results in the literature where applicable. Specific risks for the procedure itself have been covered in detail in our previous publication, (van Horne et al., 2017) and as such we focus on the inclusive, longer term adverse events of the graft itself.

The adverse events collected throughout the study and monitored by our institutional DSMB showed only 1 serious adverse event that that could be attributed to the grafting procedure itself. This was superficial cellulitis at the graft harvest site that responded to oral antibiotics. The other adverse events reported were not related to the graft procedure or the graft and were similar to the adverse events reported by the DBS patients without a graft through 12 months (University of Kentucky DBS database; data not shown). Our total adverse events were also similar to those previously reported by other centers. (Fenoy & Simpson, 2014; Patel et al., 2015)

MR images obtained at 12 months postoperatively showed no evidence of signal abnormality or contrast enhancement in the region of interest (ROI) based on preoperative targeting. These images were compared with the baseline and 24-hour postoperative MR images. Used in this way, MRI is able to provide information regarding potential adverse interactions of the graft with the surrounding parenchyma, such as inflammation, edema, or breakdown of the blood-brain barrier. MRI does have its

limitations in relation to our protocol. Our ROI is very small, measuring only 1.5 mm in diameter, and the presence of a DBS electrode limits our scan potential to 1.5 T. Additionally, the artifact from the electrode can be substantial, especially on SWI sequences. Functional MRI is not practical due to poor resolution with 1.5 T and the need for the patient to remain still for a prolonged period of time, which is especially difficult for many patients because the stimulator must be turned off during the scanning process. Although DaTscan (123I-ioflupane) analysis (GE Healthcare) does hold some promise for evaluating the potential effects on the dopaminergic terminal field in the striatum, the lack of dopamine transporters on the cell bodies in the substantia nigra precludes its use for directly evaluating the region. MRI remains a useful tool for evaluating tissue integrity within the ROI of the graft but lacks the utility for assessing graft survival or function.

Clinical Data

In this study, we report all components of the UPDRS examination. All components of our clinical evaluation, which are noted in Table 4.2, showed improvements, although none of the changes were statistically significant. Taken together, these results provide evidence that the grafting procedure in addition to DBS therapy does not negatively influence the therapeutic effect of DBS.

As noted previously, UPDRS part III is the most relevant clinical measure for evaluating potential influence from the graft; the scores from the other components of the examination are heavily influenced by the continual presence of DBS and medication

therapy. Thus, the UPDRS part III off-therapy scores give a reasonable and practical way to measure a participant's baseline progression of motor symptoms.

Using each individual's preoperative scores as baseline, the composite scores of all participants showed an overall decline by 7.4 points. This compares to an expected annual increase of 3–5 points over time in patients with PD. (Guimaraes et al., 2005; Kieburtz, 2003; Venuto, Potter, Dorsey, & Kieburtz, 2016)

In considering potential outcome measures for this and future studies that employ this paradigm, we chose to evaluate participants according to clinically important differences, as described previously. (Caplan, 2007; Shulman et al., 2010) The observed changes in the scores of the individual participants demonstrated that 6 of the participants scored lower (better) than baseline while 2 scored higher (worse). Using the UPDRS part III categorization for clinically important differences to assess the 6 participants who improved, 2 participants had a large CID, 3 had a moderate CID, and 1 had minimal CID.

Further analysis of the data subset showed that the biggest improvement in scores was in the domain of tremor. We do not think that this could be accounted for as a holdover from DBS therapy because tremor is one of the first symptoms to return after DBS is turned off. (Temperli et al., 2003) In addition, we chose a more conservative DBS-off time interval of 12 hours for motor testing. Although 3 hours off DBS is adequate for off testing, (Temperli et al., 2003) we thought that an extended time would provide a more accurate assessment for scoring.

A comparison of unilateral versus contralateral scores showed improvements on both sides, but a larger improvement was noted on the side of the body contralateral to graft placement. Improvements unilateral to surgical therapies for PD have been reported for DBS (Chung, Jeon, Kim, Sung, & Lee, 2006; Walker, Watts, Guthrie, Wang, & Guthrie, 2009) and for the infusion of GDNF. (John T. Slevin et al., 2005)

We documented the change in PD medications in LDEs and report a large decrease in the total daily doses. The large variability is accounted for by the 2 participants who continued on their regimen with lower doses, whereas the other 6 participants remained off their medications for the 12-month study period. We cannot determine whether the grafts had any impact on this finding. DBS of the STN allows for a substantial reduction in PD medication (Molinuevo et al., 2000) compared with DBS of the globus pallidus internus. (Williams et al., 2014) We also note that with our protocol we implemented an intention-to-treat approach and therefore did not manipulate medications as indicated by the UPDRS part III results while DBS was on. As such, in future studies that evaluate potential disease-altering therapies in the setting of DBS therapy, we recognize that medication changes are important to monitor but are not a critical end point for measuring graft function.

Neurocognitive Outcome

Our analysis suggests that, despite some changes, postoperative neurocognitive functioning remained largely stable in this group and the levels of affective distress remained stable as well. Significant decline was limited to phonemic verbal fluency only, and this is commonly observed after DBS, in particular with STN stimulation. (Combs et

al., 2015) Potential improvements were observed in constructional praxis and abstract problem solving. However, these changes are of unknown clinical significance, as noted above, because these outcomes may have been affected by repeated exposure to these stimuli and improvements in fine motor functions. Furthermore, potential practice effects may have masked the underlying decline to some degree, although the true extent of that potential influence cannot be estimated accurately. However, because the time between evaluations was an average of more than 14 months, practice effects may have been less of a concern. In addition, the salient literature suggests that individuals with cognitive vulnerabilities such as mild cognitive impairment may not benefit from practice effects like control individuals without cognitive impairment.7 Thus, practice effects may not have significantly influenced the current data, because some degree of mild cognitive impairment is prevalent in individuals with sufficient and severe PD who present for DBS. However, because this effect is difficult to quantify, the potential influence of repeated exposure to stimuli in the present data remains unknown. In any case, potential improvements in some skills, as expected, circumscribed the decline in specific neurocognitive functions, and stable neuropsychiatric status indicates encouraging safety and feasibility outcomes for this procedure.

DBS Plus

The positive safety and feasibility results presented in this project provide the first prospective, proof-of-concept evidence for the strategy of combining a cellular or biological therapy that is delivered in conjunction with DBS, although several investigators have previously described the theoretical concept of combined therapy.

(During, Kaplitt, Stern, & Eidelberg, 2001; Rowland et al., 2016; Rowland et al., 2015) Meanwhile, in several cases, some patients received DBS therapy after receiving cell therapy (fetal transplant after the development of runaway dyskinesias). (Graff-Radford et al., 2006)

We did not observe the development of off- or on-therapy dyskinesias in our patients through the course of our study or at any routine follow-up visits beyond the study timeline. Although it could be argued that the grafted tissue possibly caused the dyskinesias and the STN stimulation masked them, we did not observe any dyskinesias even during the 12 hours off stimulation at the testing time points. Thus, we report that tissue grafts can be implanted into the substantia nigra directly following the implantation of stimulating electrodes into the STN, and this adds minimal clinical risk for up to 1 year. Additionally, when performed in this manner, clinical evaluations for assessing graft function are straightforward and can be carried out easily and in accordance with current off-therapy protocols. Thus, we fully support the platform—which we have termed DBS Plus—for studying cellular or biological disease-modifying therapies in patients who are candidates for DBS therapy. The main advantage to the patients is that they are able to receive the full benefits of DBS therapy while participating in a clinical trial.

While it is possible that DBS therapy may influence graft function, as seen with the regional metabolic effects of DBS lead implantation, (Pourfar et al., 2009) we think that testing this cellular therapeutic approach in patients receiving DBS is important. The overall clinical goal of any PD therapy is to improve patient outcomes. Given the

complexity and clinical variability of PD, it is unlikely that a new therapeutic approach will directly result in a frontline, monotherapeutic treatment. Rather, a more likely process is that a new therapeutic approach will at first become an adjunctive therapy to current treatment strategies, much in the same way that DBS has become integrated into accepted adjunctive therapies for symptom control. If future studies show that an invasive, cellular, or biological approach could produce a disease-altering effect, then it could be appropriately tested in patients who are in earlier stages of illness.

Although we are proponents of the DBS Plus platform, there are limitations. For our study, the main limitation is our inability to track the survival and biological influence of the graft on the host environment. At this stage, we can only draw on the results of Kordower et al., (Kordower, Fiandaca, Notter, Hansen, & Gash, 1990) who showed that sural nerve grafts to the nonhuman primate caudate and putamen contain surviving, nerve growth factor–rich Schwann cells at 3 months after grafting. As noted above, the ROI represented by the graft is very small and restricts the type of imaging, such as PET or SPECT scans, that could be beneficial. Because of the presence of the DBS system, MRI is limited to 1.5 T, and with some sequences, such as SWI series, the artifact produced by the stimulating electrode may obscure the graft ROI. Our ongoing studies (www.clinicaltrials.gov, registration no. NCT02369003) have addressed some of these issues by including participants who are receiving DBS to the globus pallidus internus, thereby freeing the ROI of the graft from lead artifacts. We have also added DaTscan imaging to the protocol to investigate the possibility of the graft's influence on the nigral projections to the striatum. (Marek et al., 2001) Another overall limitation of our current

study is the inability to draw conclusions from the clinical efficacy data because of the small sample size. This is an expected limitation inherent in the design of this pilot study that focused on safety and feasibility. Other study design limitations include the lack of a control group and nonblinding, which does not take into account the possibility of the placebo effect on clinical measures. Furthermore, long-term follow-up will be required to fully assess the disease-altering capabilities, if any.

Conclusions

We present the results of our study in which we investigated the safety and feasibility of placing single, autologous, peripheral nerve grafts into the substantia nigra of patients with PD undergoing DBS therapy and report our clinical outcome measures. This study is unique in that it is the first to provide prospective data on using the DBS Plus platform that combines cellular therapy with DBS therapy, and this is the first study to evaluate the potential effects of peripheral nerve grafts on the progression of PD. We demonstrated that the procedure can be performed in a straightforward manner with minimal additional risk to patients for up to 1 year. In addition, we present preliminary evidence of the possible influence of the graft on motor measures (UPDRS part III) in the off state at 1 year. In the context of the inherent limitations of this pilot study design, we think that the overall positive findings presented here warrant further clinical investigation.

Chapter 4 Tables

Case No.	Event	Significant Adverse Event	Status	Relatedness
1	Urinary retention	No	Resolved	DBS surgery
2	Hypomania	No	Resolved	Stimulation
3	None	NA	NA	NA
4	Superficial cellulitis of ankle incision	Yes	Resolved	Graft harvesting
5	Cough	No	Resolved	Not related
	Headache	No	Resolved	DBS surgery
	Falls	No	Resolved	Not related
6	Fall & head laceration	No	Resolved	Not related
7	None	NA	NA	NA
8	None	NA	NA	NA

NA = not applicable.

Table 4.1: Cumulative 1-year adverse events

Summary of all clinical adverse events for study participants.

	Mean ± SD	
Variable	**Baseline**	**12 Mos**
UPDRS		
Part I*	2.6 ± 1.8	2.1 ± 1.7
Part II†	15.6 ± 3.7	14.5 ± 5.8
Part III‡	32.5 ± 9.7	25.1 ± 15.9
Part III§	13.4 ± 9.7	10.9 ± 9.7
Part IV¶	5.5 ± 1.9	1.6 ± 2.3
Other scales		
Modified Hoehn & Yahr Scale**	2.8 ± 0.5	2.8 ± 0.7
Schwab & England Activities of Daily Living Scale††	75.0 ± 9.3	81.3 ± 13.6
Parkinson's Disease Questionnaire-8‡‡	9.4 ± 4.4	7.5 ± 4.5

* Mentation, behavior, and mood (range 0–16).
† Activities of daily living while on therapy (range 0–52).
‡ Motor examination while off therapy (range 0–108).
§ Motor examination while on therapy (range 0–108).
¶ Complications of therapy (range 0–23).
** Measured while off therapy (range 0–5).
†† Range 0–100.
‡‡ Range 0–32.

Table 4.2: Participant scores

The mean +/- SD UPDRS and other scale scores across all participants at baseline

Table 4.3: Individualized participant characteristics

Participant No.	Age (yrs)	Duration w/ PD (yrs)	UPDRS Score						LED (mg)	Modified Hoehn & Yahr Scale	Activities of Daily Living Scale	Parkinson's Disease Questionnaire-8
			Part I	Part II	Part III	Part III	Part IV	Part IV				
1												
Baseline	71	14	2	19	10	30	4	0	300	3	80	6
12 mos			5	18	13	32	0	0	0	4	60	8
2												
Baseline	64	13	3	20	18	31	7	2	1800	3	70	11
12 mos			3	19	10	23 §	2	0	0	3	90	6
3												
Baseline	55	6	6	9	30	48	8	3	900	3	70	17
12 mos			4	16	18	43	1	1	0	3	70	14
4												
Baseline	48	12	4	16	5	34	3	1	375	3	80	11
12 mos			1	5	0	8	0	0	0	2	100	0

Table 4.3 cont.

5												
Baseline	56	5	2	12	11	28	3	0	500	2	90	4
12 mos			1	21	7	17	0	0	0	2	90	6
6												
Baseline	75	14	3	18	6	28	6	3	1300	3	80	8
12 mos			2	11	6	17	1	1	225	3	80	5
7												
Baseline	65	6	1	16	3	17	7	1	1280	2	60	13
12 mos			1	18	3	9	7	2	600	2	90	13
8												
Baseline	69	8	0	15	24	44	6	1	380	3	70	5
12 mos			0	8	30	52	2	0	0	3	70	8

* Baseline measurements were performed during the rising phase of the levodopa challenge. Measurements at 12 months were performed after the DBS had been on for at least 45 minutes and during the rising phase of the levodopa challenge (if applicable).

† Baseline measurements were performed after the PD medications had been stopped for > 12 hours. Measurements at 12 months were performed after the DBS had been off for > 12 hours and PD medications had been stopped for > 12 hours (if applicable).

‡ Measures dyskinesia on a scale of 0–13. § DBS had been off for only 3 hours at measurement.

Table 4.3: Individualized participant characteristics

All UPDRS and other scale scores for each participant at baseline and 12 months later.

Table 4.4: Individual lateralized scores while off medication and/or off stimulation

	Total Lateralized Scores*		Tremor†		Rigidity‡		Bradykinesia§	
Participant No.	**Ipsilateral**	**Contralateral**	**Ipsilateral**	**Contralateral**	**Ipsilateral**	**Contralateral**	**Ipsilateral**	**Contralateral**
1								
Baseline	8	10	3	0	2	2	3	8
12 mos	8	6	1¶	0**	0¶	0¶	7††	6¶
2								
Baseline	7	11	3	5	0	0	4	6
12 mos	6	7	1¶	0¶	0**	2††	5††	5¶
3								
Baseline	12	23	2	6	3	6	7	11
12 mos	12	19	2**	4¶	2¶	3¶	8††	12††
4								
Baseline	10	12	4	7	0	2	6	3
12 mos	2	5	0¶	2¶	0**	1¶	2¶	2¶
5								
Baseline	8	15	1	7	3	4	4	4
12 mos	5	7	1**	2¶	2¶	2¶	2¶	3¶
6								
Baseline	7	11	0	2	1	1	6	8
12 mos	4	8	0**	2**	1**	2††	3¶	4¶

Table 4.4 cont.

7								
Baseline	5	7	0	1	1	1	4	5
12 mos	3	4	0**	0¶	1**	2††	2¶	2¶
8								
Baseline	14	18	3	4	4	4	7	10
12 mos	20	14	4††	0¶	5††	5††	11††	9¶

* Range 0–36. Includes tremor at rest, action or postural tremor of hands, rigidity, finger taps, hand movements, rapid alternating movements of the hands, and leg agility.

† Range 0–12. Includes tremor at rest and action or postural tremor of the hands.

‡ Range 0–8.

§ Range 0–6. Includes finger taps, hand movements, rapid alternating movements of the hands, and leg agility.

¶ Improved scores from baseline.

** No change from baseline.

†† Worse score from baseline.

Table 4.4: Individual lateralized scores while off medication and/or off stimulation

All UPDRS and other scale scores for the left and right of each participant at baseline and 12 months later.

Table 4.5: Neurocognitive measures of various neurocognitive domains and skills

Neurocognitive Measures*	**Preop Mean ± SD**	**Postop Mean ± SD**
Premorbid functioning & intelligence estimates		
Premorbid status		
Wide Range Achievement Test 4, word-reading subtest	94.63 ± 16.60	95.05 ± 18.23
General intelligence		
KBIT-2, intelligence quotient composite	89.14 ± 16.94	88.43 ± 14.91
Verbal intelligence		
KBIT-2, verbal	88.57 ± 16.32	86.86 ± 14.92
Nonverbal & perceptual intelligence		
KBIT-2, nonverbal	90.71 ± 20.06	90.71 ± 16.76
Verbal learning & memory		
List-based learning		
HVLT-R, total recall	81.37 ± 15.01	78.38 ± 15.57
List-based recall		
HVLT-R, delayed recall	85.63 ± 18.70	79.25 ± 20.19
List-based recognition memory		
HVLT-R, recognition discrimination index	97.12 ± 10.37	84.50 ± 33.02

Table 4.5 cont.

Story-based learning		
WMS-IV, logical memory I	89.29 ± 16.94	85.71 ± 14.27
Story-based recall		
WMS-IV, logical memory II	81.14 ± 16.29	89.29 ± 13.97
Expressive & receptive language		
Confrontation naming		
Boston Naming Test–2nd Edition	87.43 ± 25.08	84.71 ± 25.34
Semantic fluency		
Animal fluency	92.38 ± 17.24	79.63 ± 13.55
Phonemic fluency		
Controlled Oral Word Association Test	91.29 ± 15.81	75.43 ± 12.56†
Expressive vocabulary		
WAIS-IV, vocabulary subtest	113.33 ± 7.64	102.50 ± 8.66
Auditory comprehension		
Multilingual Aphasia Examination, token test	98.00 ± 17.94	93.14 ± 19.80
Visuospatial skills		
Spatial perception		
Benton Judgment of Line Orientation	89.63 ± 17.25	94.88 ± 21.38

Table 4.5 cont.

Constructional praxis		
WAIS-IV, block design subtest	90.00 ± 18.03	95.00 ± 18.03†
Attention & executive functions		
Attention & working memory		
WAIS-IV, digit span subtest	95.00 ± 8.45	93.75 ± 12.17
Mental flexibility & divided attention		
TMT-B	82.57 ± 33.22	85.17 ± 20.60
Response inhibition		
Stroop, color-word	91.29 ± 12.54	82.29 ± 18.25
Stroop, interference	106.29 ± 10.31	100.29 ± 16.61
Abstract problem solving		
WCST-64, total error rate	77.71 ± 15.53	85.43 ± 17.82
WCST-64, perseverative error rate	86.43 ± 14.07	83.14 ± 19.83
WCST-64, nonperseverative error rate	76.86 ± 15.55	95.86 ± 13.23
WCST-64, categories completed*	1.57 ± 1.27	2.14 ± 1.77
Information processing speed		
Verbal speed		

Table 4.5 cont.

Stroop, word reading	82.14 ± 14.65	79.71 ± 15.01
Stroop, color naming	79.57 ± 8.92	75.57 ± 15.24
Psychomotor speed		
TMT-A	83.86 ± 21.08	85.29 ± 15.73
Emotional functioning		
Psychiatric symptoms		
Psychiatric Diagnostic Screening Questionnaire, total score*	21.29 ± 25.02	26.50 ± 27.36

HVLT-R = Hopkins Verbal Learning Test–Revised; KBIT = Kaufman Brief Intelligence Test; TMT = Trail Making Test; WAIS-IV = Wechsler Adult Intelligence Scale–Fourth Edition; WCST-64 = Wisconsin Card Sorting Test–64 Card Version; WMS-IV = Wechsler Memory Scale–Fourth Edition.

* Values are provided as standard index scores with a mean ± SD of 100 ± 15 unless otherwise noted with an asterisk.

† Significant at $p < 0.05$.

Figure 4.5: Medication Reduction Following Surgery

Mean and SD (*error bars*) LEDs were significantly lower at 12 months postoperatively than before surgery. L-DOPA = levodopa.

Chapter 4 Figures

Figure 4.1: Graft Locations in All Participants

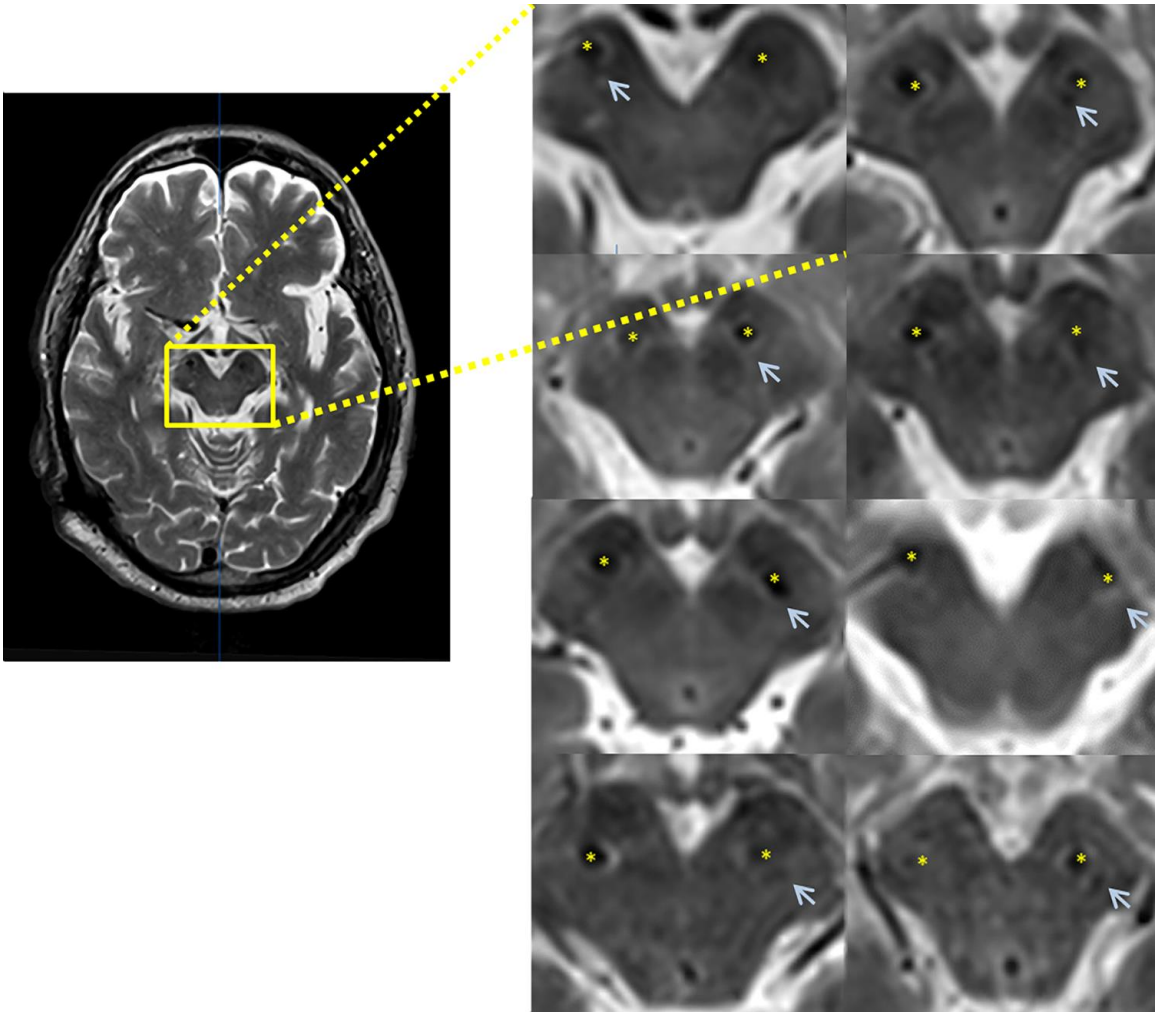

Figure 4.1: Graft Locations in All Participants

Axial T2-weighted MR images obtained at 12 months in all participants, showing the area targeted for graft implantation (*arrows*). *Asterisks* indicate DBS electrode locations.

Figure 4.2: Motor Score Changes in All Participants

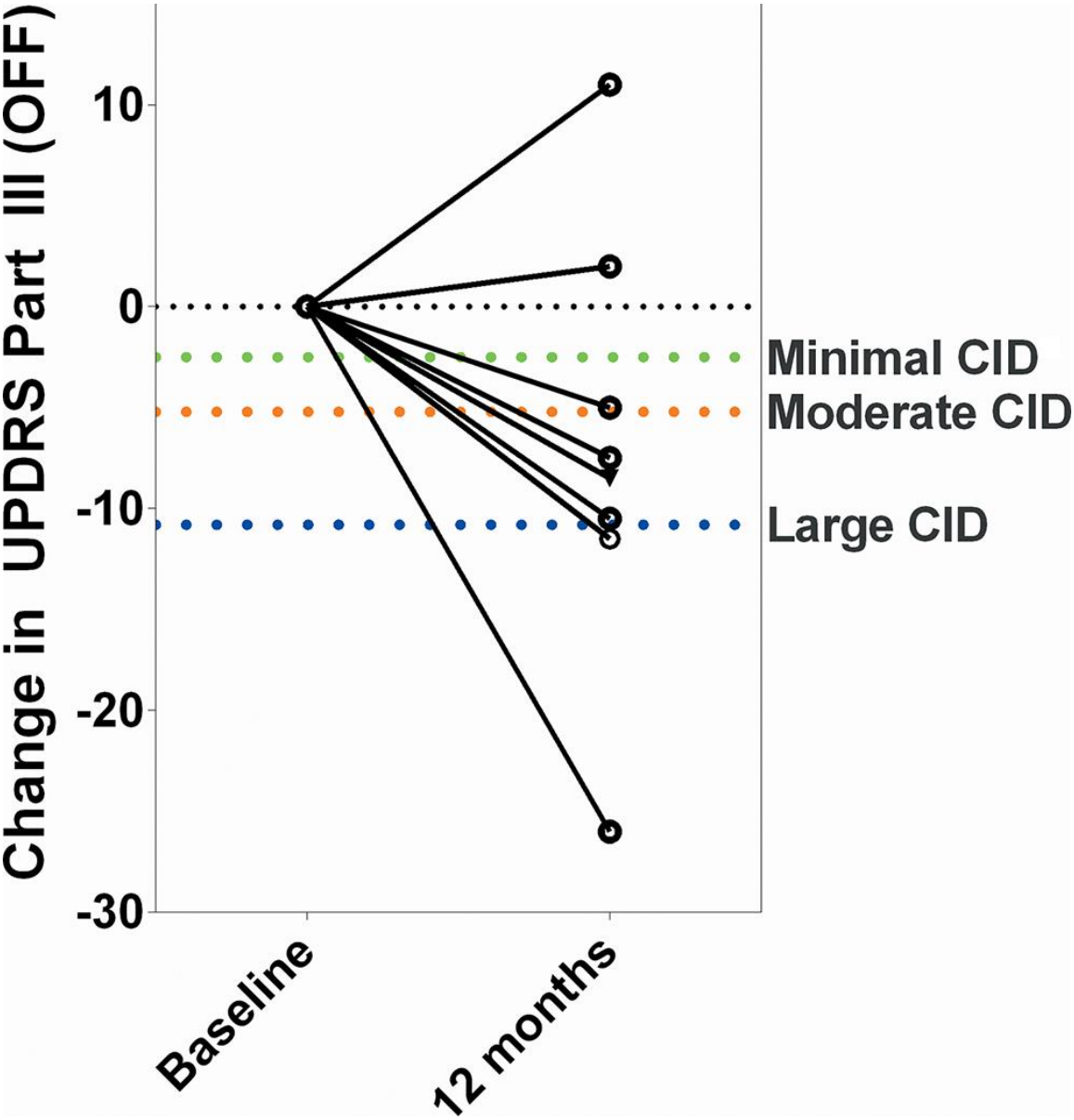

Figure 4.2: Motor Score Changes in All Participants

Individual changes in motor scores (UPDRS part III) while off DBS and off medication therapy from baseline to 12 months after surgery. Minimal (2.5 points), moderate (5.2 points), and large (10.8 points) CIDs are indicated. Each symbol represents a different participant. Figure is available in color online only.

Figure 4.3: Average Improvement or Worsening in Motor Deficit Scores

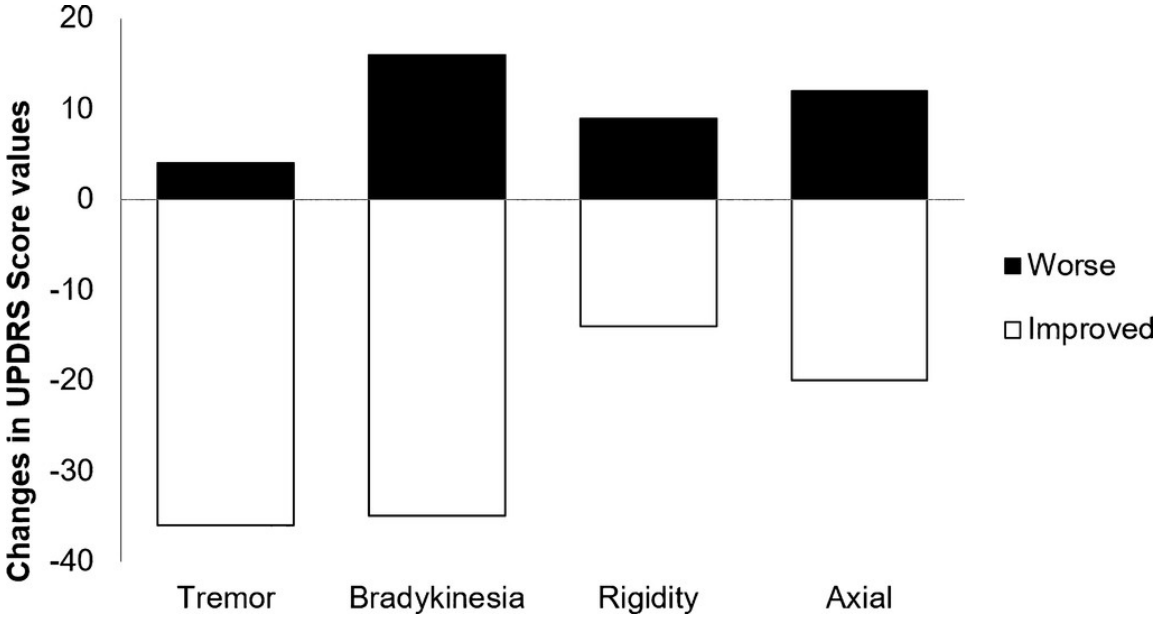

Figure 4.3: Average Improvement or Worsening in Motor Deficit Scores

For each of the domains of the UPDRS part III test, the total change in points for all participants was combined. The changes in the scores for all participants who performed worse in each domain were combined, and the changes in scores for all participants who showed improvements in each domain were combined.

Figure 4.4: Average Improvement or Worsening in Lateralized Motor Score

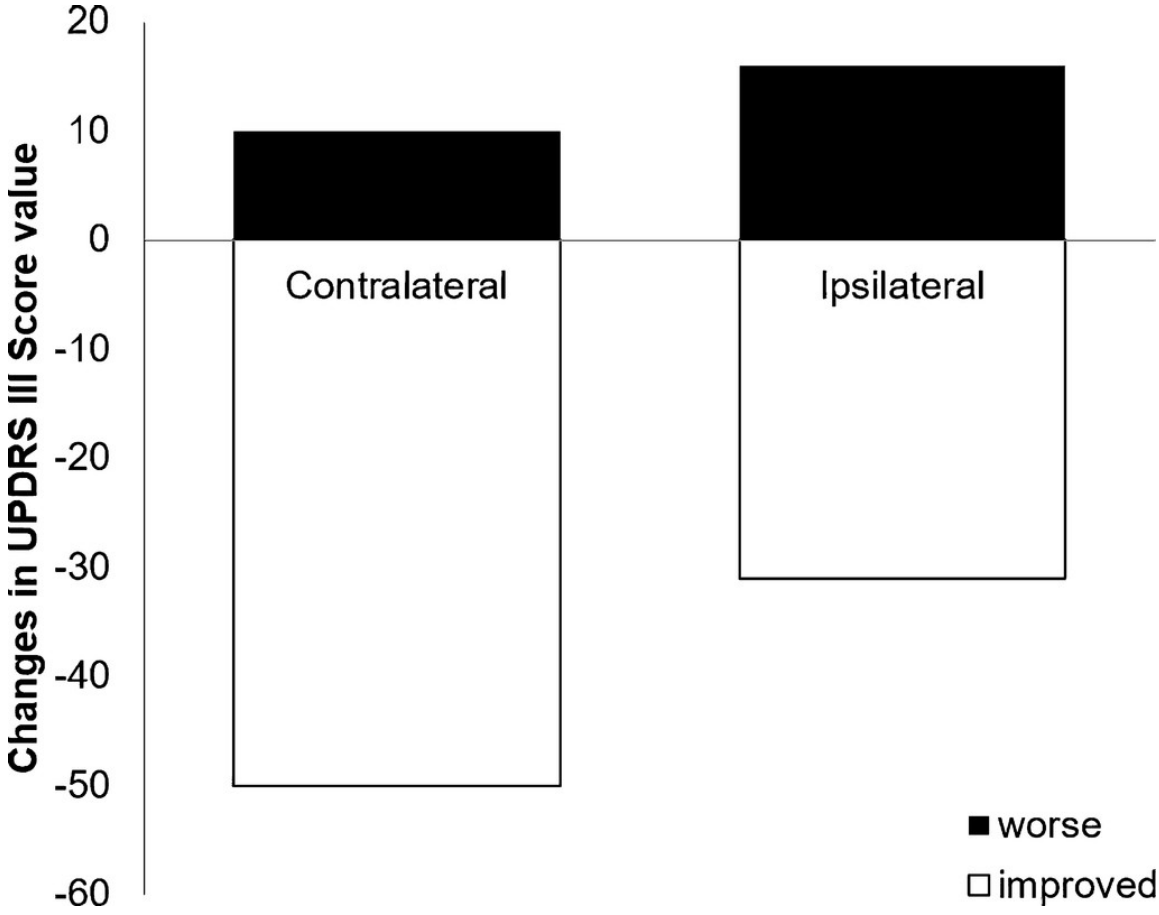

Figure 4.4: Average Improvement or Worsening in Lateralized Motor Score

Lateralized scores on the UPDRS part III test were used to identify differences in motor performance on the side contralateral or ipsilateral to graft placement. The changes in the scores of all participants who performed worse on the lateralized test were combined, and the changes in scores of all participants who showed improvements on the lateralized test were combined. Lateralized scores include tremor at rest, action or postural tremor of hands, rigidity, finger taps, hand movements, rapid alternating movements of the hand, and leg agility.

Figure 4.5: Medication Reduction Following Surgery

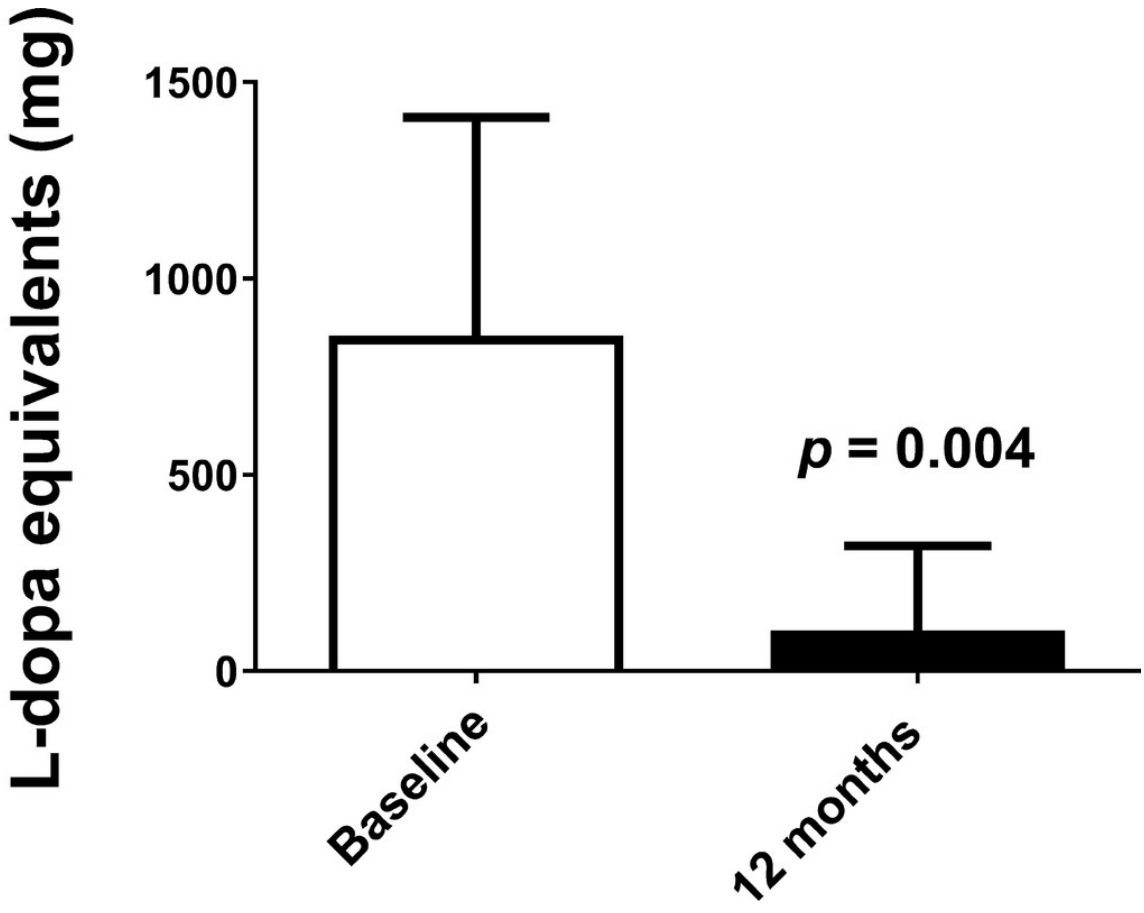

Figure 4.5: Medication Reduction Following Surgery

Mean and SD (*error bars*) LEDs were significantly lower at 12 months postoperatively than before surgery. L-DOPA = levodopa.

Chapter Five: Conclusions

Research Summary and Implications for the DBS Plus Clinical Trial

Summary of Findings

Histology of the human sural nerve tissue supports the nerve fascicle composition of the nerve graft used in the DBS Plus clinical trial. RNAseq analysis showed transcriptome changes between the injury naive (Stage I) and the injury conditioned sural nerve (Stage II) samples in all six patients analyzed. Consistent changes in all six patients were seen in gene ontologies of myelin production, growth factor activity, Schwann cell dedifferentiation, negative regulation of neuron death, and negative regulation of apoptosis. All of these changes were consistent with literature descriptions of the peripheral nerve repair process, including Schwann cell transdifferentiation, immune cell infiltration, and myelin clearance.

The Avatar animal model development has demonstrated the feasibility of using athymic nude rats as a host for human peripheral nerve tissue grafts in the brain. Preliminary results show that the human graft cells survive in the brain for at least six months. The animal model has potential applications for a dopamine lesion or other PD model paradigms such as alpha synuclein overexpression viral vectors, further histology, and/or *in vivo* transcriptome analysis.

The clinical trial data supports the safety and feasibility of the grafting procedure. Participants who received the graft have showed an overall decrease in the severity of their PD motor symptoms when off DBS stimulation and off medication. With the

expected increase in severity of motor symptoms over time in PD, these results suggest that the sural nerve grafting procedure may have benefits in reducing the severity and progression of PD motor symptoms.

Discussion

These results support the clinical trial's goal of delivering reliably pro-regenerative peripheral nerve tissue to the substantia nigra of patients with Parkinson's disease. In addition to elucidating transcriptome changes in human peripheral nerve injury response, the RNAseq analysis identified the consistency of transcriptome changes between multiple patients, which supports the reliability of the pre-lesioning approach to nerve conditioning used in the clinical trial. The Neuro-Avatar model demonstrates that both the injury-naive and injury-conditioned nerve tissue survives within the CNS. The clinical data supports the safety and feasibility of this procedure and provides preliminary evidence for efficacy in reducing the severity of the motor symptoms of PD.

This work also revealed unexpected findings. Before performing histology on the nerve grafts it was assumed that the surgical dissection of the nerve separated fascicles completely. Histology revealed that the many of the dissected nerve pieces contained multiple fascicles connected by perineurium. With the RNAseq transcriptome analysis the researchers had expected several transcription factors (namely NRF2, c-Jun, and NFkB) to be upregulated. This was not the case, as each of these genes was not significantly differentially expressed between samples. However, the magnitude of some gene ontology changes, for example 100% of differentially expressed "Negative Regulation of

Apoptotic Processes" (anti-apoptosis) genes being upregulated was an surprising but intriguing results of the pre-lesioning method of conditioning nerve tissue.

With respect to the advantages of the pre-lesioning approach, the RNAseq analysis demonstrates pro-regenerative changes within the injury-conditioned nerve tissue. It is still an open question as to whether the nerve tissue would adopt pro-regenerative phenotype if the injury-naive tissue were to be implanted in the CNS. The survival of injury-naive tissue for six months in the Neuro-Avatars suggests that the injury-naive tissue adapts to the environment of the CNS. It is possible that grafting injury-naive nerve tissue would also have therapeutic benefit in this clinical trial.

Thus, this dissertation work has identified an open question: would injury-naive tissue be an equally effective alternative to the injury-conditioned tissue currently grafted in the clinical trial?

The case for the pre-lesioning approach

The pre-lesioning approach used in this clinical trial aims to make use of the most neuro-regenerative tissue found in the human body. Many factors interact in peripheral nerve to facilitate the regeneration of peripheral axons and functional recovery after injury. In addition to Schwann cells, peripheral nerve tissue contains a matrix of connective tissue, fibroblasts, and macrophages. These elements respond to peripheral nerve injury in a coordinated fashion to promote functional regeneration. Removing peripheral nerve from this environment, as would be the case when grafting injury-naive tissue, may interrupt these process. For example, macrophages may not successfully

infiltrate the nerve tissue if removed and implanted into the brain, where it would be disconnected from vasculature.

Secondly, and more importantly, the clinical trial has already demonstrated the safety and feasibility of this approach. Preliminary results from the clinical trial also support the potential efficacy of the injury-conditioned graft tissue in reducing the severity of motor symptoms as assessed by UPDRS Part III Motor Score. (Figure 4.2)

The case for the injury naive approach

Currently DBS surgeries at other institutions are staged differently than at the University of Kentucky. Most groups implant the stimulating electrodes during the first stage of the surgery, then connect the electrodes to a stimulator in the second stage. The nerve grafts must be implanted at the same time as DBS electrodes in order to take advantage of surgical access to deep brain structures. Thus, applying a pre-lesioning approach at other institutions is less feasible, because the pre-lesioning cannot occur during DBS surgeries.

In addition, there is some additional medical risk of accessing the site of peripheral nerve collection multiple times. One case of superficial cellulitis at of the lateral ankle following a second nerve collection was observed in the clinical study. Though it was quickly resolved without incident, it constitutes an adverse event

When considering efficacy, figures 5-1 and 5-2 highlight that patients 4 and 2 experienced the most significant changes in patient motor symptoms. Of note, patient 4 exhibited a different pattern of sural nerve transcriptome change between Stage I and Stage II as visualized in the correlation matrix (Figure 2-2A). Patient 4 exhibited a

transcriptome more similar to Stage I tissue. While only a single instance, this finding suggests that peripheral tissue with a more injury-naive transcriptome is potentially effective in reducing the severity of motor symptoms when grafted into the SN of patients with PD.

Implications for the clinical trial

These observations taken together support the development of an arm of the clinical trial testing the use of injury-naive nerve grafts. The more challenging option (pre-lesioning the graft tissue by two weeks) has already been done in over 60 patients. Phase I clinical trials demonstrate safety and feasibility. It would be appropriate to evaluate the feasibility of using injury-naive tissue grafts before proceeding to a Phase II clinical trial evaluating efficacy. When considering efficacy, a demonstration of non-inferiority would make using injury-naive tissue more feasible than injury-conditioned nerve tissue. A randomized cohort of participants who would receive DBS alone would be an ideal comparison for efficacy measures related to disease progression.

A lesson from this clinical trial is that translational research aiming to be applied in humans is best conducted in humans. Properly designed in vitro experiments and animal models can answer some questions, but ultimately questions of human safety, feasibility, and efficacy can only be answered in humans. Finite resources for clinical trial research and development must balance groundwork with application. With this in mind, a comparison group within the clinical trial would be best answer the question of the relative safety, feasibility, and efficacy of injury-naive nerve graft tissue versus injury-conditioned nerve graft tissue.

Future Research Directions

PN tissue as a source of cell-based therapy for neurodegenerative diseases shows great promise. PN tissue may have the ability slow down the progression of neurodegenerative diseases and contribute in the future to a disease modifying therapy. Modification of peripheral nerve tissue to improve this therapeutic potential merits further research. Further work is needed to test how manipulating PN tissue could deliver vital therapeutic agents to CNS neurons to stop or reverse the degenerative processes.

In the future we hope to continue the animal model work with Parkinsonian models such as dopamine depletion using 6-OH dopamine or alpha synuclein overexpression using viral vectors. In addition, evaluating biological processes such as axon growth, myelination by grafted Schwann cells, and growth factor activity may reveal more about the biological behavior of the graft tissue in the CNS.

With analysis of more patient tissue we could further correlate clinical disease progression with the graft tissue transcriptome. This approach could identify undescribed neuroprotective genes in a data-driven manner. Future study of human graft recipients after autopsy can also answer questions about the biological activity of the graft. If possible, RNA sequencing of the graft tissue *in situ* would reveal much as to the behavior of the peripheral nerve tissue after grafting into the CNS.

Conclusion

From this work we have validated the consistent response of human patients to a conditioning injury of peripheral nerve used as grafts to the CNS. This work has also

described the pro-regenerative phenotype of pre and post-injury peripheral nerve tissue at a transcriptome scale. We have also developed an animal model of the grafting procedure that demonstrates survival of human nerve tissue in the brains of immunodeficient rats for at least six months. The ongoing clinical trial can provide future insights into the safety, feasibility, and efficacy of peripheral nerve grafts for the treatment of PD and other neurodegenerative disorders.

Chapter 5 Figures

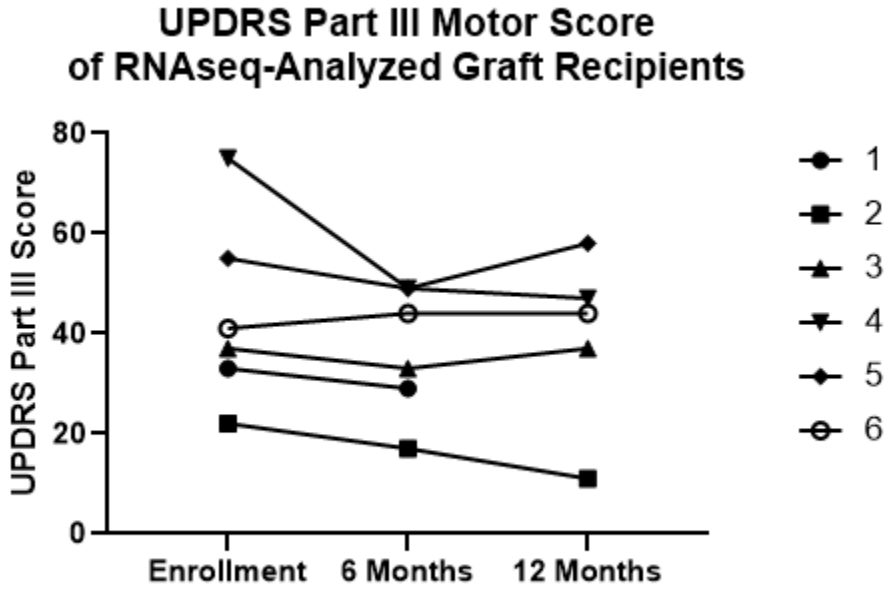

Figure 5.1: UPDRS Part III Motor Score of RNAseq-Analyzed Graft Recipients

UPDRS scores of participants who received graft tissue analyzed by RNAseq. A higher score more severe motor symptoms.

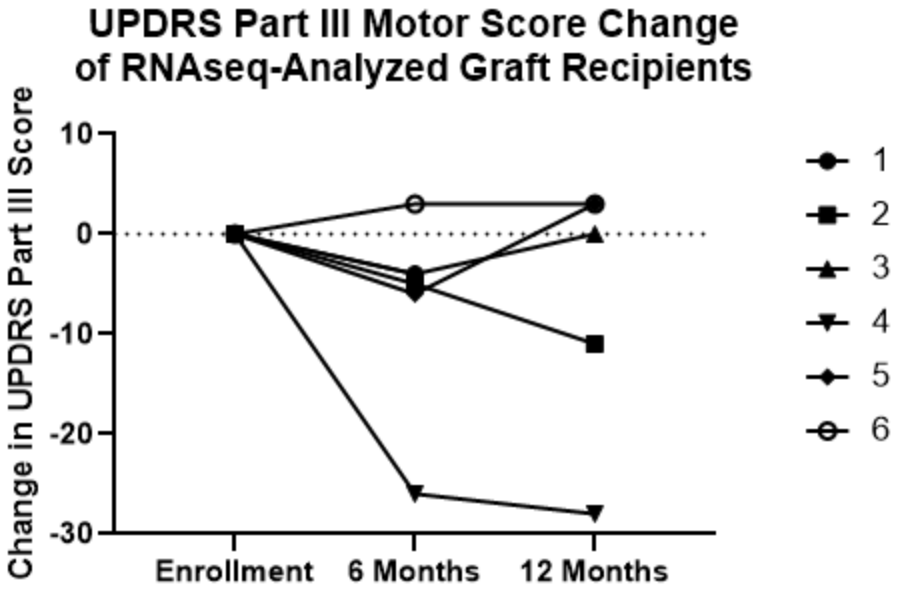

Figure 5.2: UPDRS Part III Motor Score Change of RNAseq-Analyzed Graft Recipients

Changes of UPDRS scores from baseline of participants who received graft tissue analyzed by RNAseq. A reduction of score indicates an improvement in motor symptoms.

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Vita

Andrew S. Welleford

Education

University of Kentucky College of Medicine, MD-PhD Program

Graduate Certificate in Anatomical Sciences Instruction - 2018

Graduate Certificate in Neuroscience Instruction - 2018

University of Kentucky

BS in Chemistry, BS in Biology, Minor in Neuroscience, Graduate of the UK Honors Program with Departmental Honors in Chemistry and Biology, *magna cum laude* - 2013

Awards and Honors

Travel Award, American Society for Neural Therapy and Repair - 2018

"Neuroscience 2017 *Hot Topic*", Society for Neuroscience Annual Meeting - 2017

2nd Place, University of Kentucky 3-Minute Thesis Competition - 2016

University of Kentucky College of Medicine Scholarship – 2013, 2014

Dr. Hume and Ellen Towle Bedford Scholarship – 2012

Thomas B. Nantz Memorial Tuition Scholarship – 2011

Department of Chemistry Scholarship – 2010

Freshman Chemistry Award – 2009

University of Kentucky Academic Excellence Scholarship – 2009

Dean's List – various semesters, 2008-2013

Professional Experience

Graduate Research Assistant

Supervisor: Greg Gerhardt, PhD

University of Kentucky Department of Neuroscience, Aug 2015-Present

Graduate Teaching Assistant for Anatomy and Physiology I & II Courses

Supervisor: Kristen Platt, PhD

University of Kentucky Department of Neuroscience, Aug 2015-Present

Graduate Teaching Assistant for Graduate Neuroscience Course

Supervisors: Sam Franklin, PhD and Richard Grondin PhD

University of Kentucky Department of Neuroscience, Aug 2017-Dec 2017

Undergraduate Research Assistant

Supervisor: D. Allan Butterfield, PhD

University of Kentucky Department of Chemistry, May 2012 – July 2013

Research Experience and Collaborations

Sural Nerve Autograft in Combination with Deep Brain Stimulation Surgery for the Treatment of Parkinson's Disease

PI: Greg Gerhardt, PhD

A Modular, Adaptable, and Self-Contained Neuroscience Curriculum for Elementary School Students

PI: Amy Hessler, DO

Alpha-Synuclein Seeding Activity from Parkinson's and Multi-System Atrophy Brain Extracts

PI: Tritia Yamasaki, MD, PhD

Multimodal Review Sessions for Undergraduate Anatomy Education

PI: Kristen Platt, PhD

Lifestyle Factors in Alzheimer's Disease Progression

PI: Gregory Jicha, MD, PhD

Chemical Markers for Oxidative Stress in Alzheimer's Disease

PI: D. Allan Butterfield, PhD

Publications

Andrew S. Welleford, Craig G. van Horne, Jorge E. Quintero, Eric Blalock, Sumedha Gunewardena, John A. Stanford, Steven M. Shapiro, Sean M. Riordan, Greg A. Gerhardt. "RNAseq Analysis of Human Peripheral Nerve Fascicles Pre- and Post-Injury." In preparation.

Nader El Seblani, **Andrew S. Welleford**, George Quintero, Craig van Horne, and Greg A. Gerhardt. "Peripheral Nerve Tissue Grafting for Repair of the CNS in Trauma and Degenerative Disease." In preparation.

Andrew S. Welleford, Lauren M. Weaver, Kristen Platt. “Multimodal Review Sessions for Undergraduate Muscle Anatomy.” In submission.

Craig G. van Horne, MD, PhD, Jorge E. Quintero, PhD, John T. Slevin, MD, MBA, Amelia Anderson-Mooney, PhD, Julie A. Gurwell, PA-C, PhD, **Andrew S. Welleford**, BS, John R. Lamm, MD, Renee P. Wagner, RN, and Greg A. Gerhardt, PhD. "Peripheral nerve grafts implanted into the substantia nigra in patients with Parkinson's disease during DBS surgery: one year follow-up of safety, feasibility, and clinical outcome." Journal of Neurosurgery. 2018 Feb 16:1-12

Platt, K.M., **Welleford, A.S**., Naze, G.S. and Hatcher, A.R. “Embracing your Eureka Moments: Perspectives on Fostering Impactful and Innovative Teaching Styles.” Journal of Faculty Development. 2017 Sep;31(3):87-94.

Susan A. Farr; Michael L. Niehoff, Michael A. Ceddia, Kelli A. Herrlinger, Brandon J. Lewis, Shulin Feng, **Andrew Welleford**, D. Allan Butterfield, and John E. Morley. "Effect of botanical extracts containing carnosic acid or rosmarinic acid on learning and memory in SAMP8 mice." *Physiology & Behavior* 165 (2016): 328-338. doi: 10.1016/j.physbeh.2016.08.013

Sarah Förster; **Andrew S Welleford**; Judy C Triplett; Rukhsana Sultana; Brigitte Schmitz; David Allan Butterfield. “Increased O-GlcNAc levels correlate with decreased O-GlcNAcase levels in Alzheimer disease Brain.” Biochim Biophys Acta. 2014 Sep;1842(9):1333-9. doi: 10.1016/j.bbadis.2014.05.014. Epub 2014 May 23.

Abstracts:

Oral Presentations

A. S. Welleford, C. G. van Horne, J. E. Quintero, E. M. Blalock, J. A. Stanford, S. M. Shapiro, S. M. Riordan, G. A. Gerhardt. “RNA-seq and Histological Characterization of Human Peripheral Nerve Tissue for Use in Brain Grafts for the Treatment of Parkinson’s Disease.” American Society for Neural Therapy and Repair. April 28, 2018.

Andrew S. Welleford, Lauren M. Weaver, Kristen Platt. “Multimodal Review Sessions for Undergraduate Anatomy Education.” American Association of Clinical Anatomists Annual Meeting. July 19, 2017.

Platt, K.M., **Welleford, A.S**., Naze, G.S. and Hatcher, A.R. “Embracing your Eureka Moments: Perspectives on Fostering Impactful and Innovative Teaching Styles.” Pedagogicon. May 19th, 2017.

Andrew S. Welleford, Craig G. van Horne, Jorge E. Quintero, Yi Ai, Greg A. Gerhardt. “Peripheral nerve grafts to the brain of patients with Parkinson’s disease: Microscopic, biochemical, and immunohistochemical characterization.” Neuroscience Departmental Seminar. March 2, 2017.

Andrew S. Welleford, Craig G. van Horne, Jorge E. Quintero, Yi Ai, Greg A. Gerhardt. “Peripheral Nerve Autografts to the Substantia Nigra in Combination with Deep Brain Stimulation Surgery for the Treatment of Parkinson’s Disease.” Anatomy and Neurobiology Departmental Seminar. April 28, 2016.

Andrew S. Welleford, Craig G. van Horne, Jorge E. Quintero, Yi Ai, Greg A. Gerhardt. "Peripheral Nerve Autografts to the Substantia Nigra in Combination with Deep Brain Stimulation Surgery for the Treatment of Parkinson's Disease." Center for Clinical and Translational Research Fall Seminar, University of Kentucky. December 9, 2015.

Andrew S. Welleford, D. Allan Butterfield. "Chemical Markers for Oxidative Stress in Alzheimer's Disease." National Conference for Undergraduate Research. April 12, 2013.

Poster Presentations

El Seblani, N., **Welleford, A.**, Quintero, G., Qian, A., Pomerleau, F., Huettl, P., van Horne, C., & Gerhardt, G. (2019). Peripheral Nerve Grafting as a Disease Modifying Cell Therapy for Parkinson's Disease American Academy of Neurology Annual Meeting. October 2017.(N3. 001).

A. S. Welleford, C. G. van Horne, J. E. Quintero, E. M. Blalock, J. A. Stanford, S. M. Shapiro, S. M. Riordan, G. A. Gerhardt. "Neuro-Avatar: A reverse translational model of an ongoing cell therapy clinical trial for Parkinson's disease." University of Kentucky Center for Clinical and Translational Science Spring Conference. April 13, 2018.

A. S. Welleford, C. G. van Horne, J. E. Quintero, E. M. Blalock, J. A. Stanford, S. M. Shapiro, S. M. Riordan, N. El Seblani , G. A. Gerhardt. "Neuro-Avatar: A reverse translational model of an ongoing cell therapy clinical trial for Parkinson's disease." Society for Neuroscience Annual Meeting, San Diego, CA. November 7, 2018

A. S. Welleford, C. G. van Horne, J. E. Quintero, E. M. Blalock, J. A. Stanford, S. M. Shapiro, S. M. Riordan, G. A. Gerhardt. "RNA-seq and Histological Characterization of Human Peripheral Nerve Tissue Used in Brain Grafts for the Treatment of Parkinson's Disease" American Society for Stereotactic and Functional Neurosurgery, Denver, CO. June 22, 2018.

A. S. Welleford, C. G. van Horne, J. E. Quintero, E. M. Blalock, J. A. Stanford, S. M. Shapiro, S. M. Riordan, G. A. Gerhardt. "RNA-seq and Histological Characterization of Human Peripheral Nerve Tissue Used in Brain Grafts for the Treatment of Parkinson's Disease" University of Kentucky Center for Clinical and Translational Science Spring Conference. April 13, 2018.

Yamasaki, T. R., **Welleford, A. S.**, & Gerhardt, G. A. "Biochemical Differences in Pathologic alpha-Synuclein in Synucleinopathies." American Academy of Neurology Annual Meeting. October 2017. In *ANNALS OF NEUROLOGY* (Vol. 82, pp. S180-S180).

Andrew S. Welleford, Craig G. van Horne, Jorge E. Quintero, Yi Ai, Greg A. Gerhardt. "Microscopic, biochemical, and immunohistochemical characterization of peripheral nerve tissue used in brain autografts for the treatment of Parkinson's disease." American Physician-Scientist Annual Meeting, Chicago, IL. April 22, 2017

Andrew S. Welleford, Craig G. van Horne, Jorge E. Quintero, Yi Ai, Greg A. Gerhardt. "Peripheral Nerve Grafts to the Brain of Patients With Parkinson's Disease: Microscopic, Biochemical, and Immunohistochemical Characterization" University of Kentucky Center for Clinical and Translational Science Spring Conference. March 30, 2017.

Andrew S. Welleford, Craig G. van Horne, Jorge E. Quintero, Yi Ai, Greg A. Gerhardt. "Microscopic, biochemical, and immunohistochemical characterization of peripheral nerve tissue used in brain autografts for the treatment of Parkinson's disease." Society for Neuroscience Annual Meeting, San Diego, CA. November 16, 2016

Andrew S. Welleford, Craig G. van Horne, Jorge E. Quintero, Yi Ai, Greg A. Gerhardt. "Peripheral Nerve Grafts to the Brain of Patients With Parkinson's Disease: Microscopic, Biochemical, and Immunohistochemical Characterization" University of Kentucky Center for Clinical and Translational Science Spring Conference. April 21, 2016.

Andrew S. Welleford, Craig G. van Horne, Jorge E. Quintero, John T. Slevin, Julie A. Gurwell, Greg A. Gerhardt. "Peripheral Nerve Autografts to the Substantia Nigra in Combination with Deep Brain Stimulation Surgery for the Treatment of Parkinson's Disease." AOA Student Research Day Symposium, Student Poster Session, University of Kentucky. February 4, 2016.

Keith Albrektson, Sarah Tarrant, Amanda Pursell, Dasha Kenlan, **Andrew Welleford** and Gregory Jicha. "An inverted U-shaped dose-response curve for aerobic exercise intensity associated with improved cognitive function in an elderly, community-dwelling cohort." American Academy of Neurology Annual Meeting. April 23, 2015.

Dasha Kenlan, Sarah Tarrant, Amanda Pursell, **Andrew Welleford**, Keith Albrektson and Gregory Jicha. "Refined sugar intake is associated with lower cognitive performance across the cognitive continuum but is not associated with preclinical cognitive decline in intact elderly subjects." American Academy of Neurology Annual Meeting. April 23, 2015.

Andrew S. Welleford, Sarah D. Tarrant, Erin L. Abner, Keith R. Albrektson, Dasha E. Kenlan, Amanda B. Pursell, Andrew S. Welleford, Gregory A. Jicha. "Self-Report of Bio-Psycho-Social Function in Elders as a Predictor for Mild Cognitive Impairment." AOA/MD/PhD Student Research Symposium, University of Kentucky. February 3, 2015.