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COMBINATION OF INVESTIGATIONAL CELL-BASED THERAPY AND DEEP BRAIN STIMULATION TO ALTER THE PROGRESSION OF PARKINSON'S DISEASE

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COMBINATION OF INVESTIGATIONAL CELL-BASED THERAPY AND DEEP BRAIN STIMULATION TO ALTER THE PROGRESSION OF PARKINSON'S DISEASE

DISSERTATION

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

By

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2020

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ABSTRACT OF DISSERTATION

COMBINATION OF INVESTIGATIONAL CELL-BASED THERAPY AND DEEP BRAIN STIMULATION TO ALTER THE PROGRESSION OF PARKINSON'S DISEASE

Parkinson's disease (PD) is the second most common neurodegenerative disorder and the motor symptoms are caused by progressive loss of midbrain dopamine neurons. There is no current treatment that can slow or reverse PD. Our current "DBS-Plus" clinical trial (NCT02369003) features the implantation *in vivo* of autologous Schwann cells (SCs) derived from a patient's sural nerve into the substantia nigra pars compacta (SNpc) in combination with Deep Brain Stimulation (DBS) therapy for treating patients with advanced PD.

The central hypothesis is that transdifferentiated SCs within conditioned nerve tissue will deliver pro-regenerative factors to enhance the survival of the degenerating dopaminergic cells in the SNpc. The main goal of our studies is to determine if implantation of peripheral nerve tissue into SNpc in combination with DBS surgeries is safe, feasible and can possibly slow the loss of the midbrain dopamine neurons. First, RNA sequencing was used to validate the repair phenotype of human sural nerve tissue two weeks after transaction injury. The transcriptomic analysis showed that 3641 genes were differentially expressed in conjunction with the upregulation of multiple neurotrophic factors and the enhancement of axonogenesis. Secondly, to study the neurobiology of the implant, we grafted human nerve implant into the dorsal striatum of athymic nude rats (called Neuro-Avatars). Immunostaining studies showed a remarkable survival of the implanted human SCs up to 6 months post-implantation in Neuro-Avatar animals. In addition, there were significant increases in the numbers of surviving human-derived cells in the Neuro-Avatar's using pre-degenerated human sural nerve tissue as compared to the same sural nerve tissue that was harvested in its normal state.

Finally, we studied data from 27 human subjects with PD that had received DBS plus autologous nerve-implants. The safety of the combined intervention and the progression of the motor symptoms were evaluated at baseline, 12, and 24 months using the Unified Parkinson's Disease Rating Scale part III (UPDRS). The safety of the studies

at 2 years post implantation showed adverse events (AE's) that were similar to those seen with standard DBS therapy. In addition, there was a significant motor improvement on the side contralateral to the tissue implantation in comparison to the ipsilateral one. Taken together, our data support that combining DBS with *in vivo* pre-degenerated peripheral nerve tissue containing SCs can serve as a safe and promising disease-modifying therapy to alter the progression of PD.

KEYWORDS: Schwann cell, Peripheral Nerve, Neurotrophic factor, Deep Brain Stimulation, Parkinson's disease

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06/09/2020

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Dedicated to my parents, Fouad and Nada, who have always
inspired me to work hard and never give up,
And to Dalia, my beautiful wife who introduced me to the worlds of Love and Science

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CHAPTER ONE: INTRODUCTION_UTILIZING PERIPHERAL NERVE REGENERATIVE ELEMENTS TO REPAIR DAMAGE IN THE CNS

Abstract

An ongoing question in neuroscience is how the peripheral nervous system can repair itself following an injury or insult whereas the central nervous system has a profoundly limited ability for repair. The recent and rapid advancement of our understanding of the gene expression and corresponding biochemical profiles of Schwann cells, within the distal segments of injured peripheral nerves, has helped elucidate the potential mechanisms underlying the unique ability for these cells to enable regeneration of peripheral nerve tissue. Meanwhile, with a new understanding and appreciation for the capabilities of the peripheral nervous system, we are beginning to unlock the potential for neural regeneration and repair within the central nervous system. The aim of this introduction is to briefly outline the historical advancements and the scientific background that lead to the concept of utilizing peripheral nerve tissue implants or Schwann cell culture implants to serve as repair mechanisms for the central nervous system in the clinical setting of spinal cord injury, multiple sclerosis, and neurodegenerative disorders such as Parkinson's disease.

El Seblani, N., Welleford, A., Quintero, J. E., van Horne, C., & Gerhardt, G. A. (2020). Invited Review: Utilizing Peripheral Nerve Regenerative Elements to Repair Damage in the CNS. *Journal of Neuroscience Methods*, 108623 (El Seblani, Welleford, Quintero, van Horne, & Gerhardt, 2020)

Historical Perspective of Peripheral Nerves and Their Regenerative Capacity in the Central Nervous System

The interesting and unpredicted finding that peripheral nerves (PN) had the ability to regenerate was demonstrated in the 1500's (1596) when a surgeon, Gabriele Ferrara, published his meticulous technique for suturing together the ends of a transected nerve following injury (Artico, Cervoni, Nucci, & Giuffre, 1996). Schwann and Schleiden (1847) later recognized the complexity and dynamic nature of the PN cells (Schwann & Schleiden, 1847). Schwann, in his treatise on cell theory, attempted to clarify the origins and differences between nerve fibers and their ensheathing components. Schwann offered several possibilities, including that the nerve fibers formed from the membranous coalescence of the ensheathing cells. Waller studied and characterized the changes taking place in the distal PN segment following disruption or transection, but he did not yet have an electron microscope to truly define the reorganization (Waller, 1850). In 1891, Von Büngner noted that in the distal segment, Schwann cells (SCs) proliferated and formed bands along the remaining collagen components (Koeppen, 2004). These bands are now known as "Bands of Büngner". Büngner noted that this construct was the key component allowing the impressive ability of PN to regenerate following injury. It is now understood that the Bands of Büngner are formed when transdifferentiated SCs change their profile from myelinating into a phagocytic phenotype first and recruit neutrophils and macrophages through chemotactic signaling (Lindborg, Mack, & Zigmond, 2017). This initiates the Wallerian degeneration, which is important to clear the distal stump of axonal and myelin debris. Transdifferentiated SCs disperse along Bands of Büngner to support and guide regenerating growth cones.

The concept that Wallerian degeneration and Bands of Büngner were important for nerve repair was understood by some of the early researchers asking questions regarding neuronal plasticity. One of Cajal's protégés, Francisco Tello, designed a set of experiments in 1911 through which he utilized segments of a sciatic nerve implanted into the cortex of rabbits (TELLO, 1913). The nerve was transected 8-14 days before implanting thus allowing Wallerian degeneration to occur. Tello found that predegenerated PN grafts could promote CNS regeneration and postulated that the release of neurotrophic substances from PN cells played a key role. Cajal later used a microscope and was able to better define not only the degenerative changes but also the regenerative potential of PN. While commenting on his findings in 1928, Cajal noted that the CNS fibers acted as if "they were attracted by an irresistible force" (Cajal, 1928).

Technical signs of progress revitalize the concept of using peripheral nerve implants

In the early eighties, Aguayo and his colleagues' pioneering work with PNS and CNS regeneration has encouraged other investigators to design different grafting techniques to test how the PN environment augments the capacity of CNS neurons to regenerate and grow new fibers (Aguayo, Björklund, Stenevi, & Carlstedt, 1984; Bray, David, Carlstedt, & Aguayo, 1983; Kao, Chang, & Bloodworth Jr, 1977). In addition to validating the ability of central axons to regenerate along the PN graft, they observed that such regeneration is dependent on the distance between the central neuron cell body and the graft location (Richardson & Issa, 1984).

Axonal regeneration and elongation in response to grafted PN tissue can occur in cortical as well as in subcortical areas. Grafting a segment of the sciatic nerve into the basal

ganglia and the cortex of rats showed that the largest number of neurons grew from the striatum (Benfey & Aguayo, 1982). Such an observation indicated that the striatal cells are able to regenerate and extend new axons when they are in a close proximity to the PN grafts.

The remarkable collaboration between Aguayo, Björklund, Stenevi, and Carlstedt resulted in a finding that highlighted the potential of PN grafts in supporting the viability and regeneration of the central neurons in disease states (Aguayo et al., 1984; Gage et al., 1985). Their animal experiments were undertaken in two stages. In stage I: The dopaminergic cells of the striatum were depleted by 6-hydroxydopamine (6-OHDA), a state analogous to the striatum pathology in Parkinson's disease (PD), and grafted with mesencephalic rat fetal tissue over the superior colliculus. Then, an approximate 2 cm segment of heterologous sciatic nerve was placed in the skull. One end of the nerve was connected to the mesencephalic graft, while the other nerve end was left freely hanging over the frontal bone. In stage II, two months after stage I, the nerve end over the frontal bone was transected and the freshly cut free end was inserted into the already depleted dorsal striatum. Five months after implantation, the immunohistochemical staining for tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine synthesis, showed that the PN grafts were able to support the survival and extension of the axons from the remotely implanted fetal mesencephalic neurons into the adult rat striatum. This experiment strongly validated that PN grafts have the capacity to promote fetal neuron survival, axonal regeneration, and guidance, noted that non-neuronal cells that were known to be supportive of PNS repair were also essential for the growth of axons from the transplanted cells into the CNS.

The critical role of these non-neuronal cells was highlighted in Anderson's work which demonstrated how nerve axonal growth into freeze-dried PN grafts was suspended until SCs invaded the grafts (Anderson et al., 1983). SCs secrete neurotrophic factors in the first few days following the nerve injury and have been shown to induce cholinergic neuron sprouting *in vivo* (Carey & Bunge, 1981; Manthorpe et al., 1983). Furthermore, examination of the PN grafts at different time intervals up to 12 months post-transplantation of long nerves into the CNS confirmed that the PN tissue survives and myelinates CNS axons (Munz, Rasminsky, Aguayo, Vidal-Sanz, & Devor, 1985).

Meanwhile, Ebner, Erzurumlu, and Lee recognized the importance of PNS injury signals in augmenting the viability and the regenerative capacity of implanted embryonic cells within adult brain environment (Ebner, Erzurumlu, & Lee, 1989). In their experiments, they grafted embryonic neocortical cells into the rat cortex of topographically injured sensory nerve and measured the extent of the specific thalamic fiber ingrowth and activation in the implanted cells. Interestingly, they found that introducing what they called a "conditioning lesion" of the PN resulted in an extensive growth and later a functional innervation of the implant. Basically, their research raised the question of how damage to a PN enhances the regenerative capacity of the central neurons several synapses away. One of their main explanations for that observation was that lesioned PN tissue releases growth signals and molecules which were transported retrogradely to the distant brain neurons to enhance their survival and regeneration. They were able to physiologically record, at 35 days after grafting, spontaneous and organized discharges of action potentials within the implant. Additionally, they demonstrated the growth of thalamic fibers and their terminals into the grafts using anterograde horseradish peroxidase staining. Hence, their work

supported that the CNS has, in fact, a regenerative capacity that could be unleashed in response to a specific stimulus and the PNS milieu can be a source for such a stimulus in the CNS.

The regenerative interaction between PN and the CNS was also described in a set of elegant experiments by Chi and Dahl who grafted an autologous sciatic nerve into the rat CNS (Chi and Dahl, 1983). They performed the transplantation using two techniques. The first technique was called the "through-and-through" model during which a segment of the sciatic nerve was passed through two craniotomy holes. The second technique was a "nerve-within-tube" model during which a small tube of polyethylene containing a nerve piece was implanted in the rat CNS. Axonal sprouting from brain tissue to the nerve in the "nerve-within-tube" model started as early as 2 weeks post-grafting and was characterized by a slow-moving and organized pattern of axonal regeneration. In comparison, the axons in the "through-and-through" model followed a disorganized and tortuous type of regeneration. That could be a result of different CNS axons exiting the graft and entering the brain tissue from the sides. Chi and Dahl also noticed that the axonal fibers in the "nerve-within-tube" model were able to grow from the brain into the graft with less damage to the brain. Remarkably, they identified "reacting" SCs secreting a unique type of Glial Fibrillary Acidic Protein (GFAP) throughout the graft. On average, the nerve grafts survived longer with the "through-and-through" model (up to 6 months) in comparison to the nerve-within-tube model (2 months). Regeneration of the non-myelinated axons of the brain tissue was observed around 1 month while that of the myelinated fibers was not observed before 3 months. Central gliosis did not prevent the axonal regeneration but affected the orientation of the new axonal fibers. The grafts that completely faced the brain

tissue were well innervated by new axons, which supported the idea that PN tissue has the potential to induce formation and regeneration of axons in the CNS.

Interestingly, implantation of a PN in rat brain induces the formation of fenestrated capillaries within the graft after 5 weeks and myelinated and non-myelinated axons in the implant 8 weeks after implantation (Mitchell, Stauber, Anderson, & Mayor, 1985). In their attempt to trace the source of the regenerating axons, Mitchell and his colleagues used a fluorochrome true blue label, which labeled the cortical areas adjacent to the graft in addition to the ipsilateral trigeminal and superior cervical ganglia. The authors could not trace the dye to distant CNS areas and concluded that the bulk of the regenerating axons originated from pial nerves. Nevertheless, the ability of the central neurons to regenerate their axons for a significant distance in an injured CNS environment had been thoroughly demonstrated by other research groups (Katzman, Björklund, Owman, Stenevi, & West, 1971). Björklund and his team, observed that catecholamine fibers were able to sprout after iris tissue was grafted into injured rat brain.

In response to an axonal damage, CNS neuronal cell bodies are at a great risk for chromatolysis and retrograde death. Villegas-Perez demonstrated the potential of tibial nerve graft to significantly preserve axotomized retinal ganglionic cells (Villegas-Perez, Vidal-Sanz, Bray, & Aguayo, 1988) . In addition, they showed that the PN graft guided the regenerating axons to synapse with the appropriate CNS targets instead of randomly synapsing in the injured CNS. Yet, one of the main limitations of their work was that the PN graft had to be anastomosed to the severed optic nerve thus indicating how important the proximity of the graft is to the damaged CNS region of interest. Meanwhile, there was

little axonal growth into the nerve graft when the axons were injured a long distance away from the perikarya.

Later, it was proposed that the gliosis at the interface between the nerve graft and the CNS tissue can limit the ability of the CNS originated axons to expand beyond of the graft (Bovolenta et al., 1993; Bovolenta and Fernaud-Espinosa, 2000). Yet, recent research showed that the axonal regeneration could be further enhanced by modulating the extracellular matrix in the CNS. For instance, applying chondroitinase to CNS lesions before/after transplantation of SCs or PN tissue facilitated the regeneration of injured axons and restored their functioning (Bradbury et al., 2002; Bradbury and Carter, 2011; Caggiano et al., 2005, 2003; DePaul et al., 2015; Fouad et al., 2005; Houle et al., 2006; K R Jessen and Mirsky, 2016; Liu et al., 2006).

Overall, observations from the use of different PN grafting techniques, which were mainly pursued in the twentieth century (see Tables 1.1 and 1.2), concluded that: a) certain CNS neurons can grow their axons at distances equivalent to the long tracts in rodents that join widely separated areas of neuraxis (So and Aguayo, 1985); b) axons of some neurons can potentially grow into a longer length after injury (Bray et al., 1987; Gage et al., 1985; Villegas-Perez et al., 1988); c) many of the regenerating axons in the CNS arise from injured neurons rather than sprouting from dormant ones (Friedman and Aguayo, 1985); d) PN grafts stimulate central axons' regeneration and significantly enhance the functional recovery and early survival of axotomized ganglion cells such as retinal ganglion cells (Aguayo et al., 1984; van Horne et al., 1991; Villegas-Perez et al., 1988) and e) grafting PN tissue near neuronal cell bodies upregulates the expression of regeneration-associated genes (RAGs) such as Tα1-tubulin, GAP-43 and c-Jun (Kobayashi et al., 1997; Merzenich

and Jenkins, 1993; Schaden et al., 1994; Tetzlaff et al., 1991), which support the extension of regenerating axons into the CNS. The question then becomes, where does the capacity of the PN to regenerate come from? Findings from a wide variety of studies support that the unique repair cell properties of SCs are the key.

The Role of Schwann Cells in Neuronal Survival and Axonal Regeneration

SCs derive from the neural crest cells which initially differentiate into Schwann cell precursor. Eventually, immature SCs mature into myelin-forming and non-myelin (Remak) SCs (Frostick et al., 1998; Jessen et al., 2015; Jessen and Mirsky, 2005). In addition to maintenance of the axonal sheath and myelin production, the myelin-forming and Remak SCs play a crucial role in the Wallerian degeneration of PN (Figure 1.1). About a week after an axonal injury, those SCs start to divide and form Bungner's bands, which accept newly regenerating sprouts from the proximal axonal stumps (Gomez-Sanchez et al., 2017). In fact, these dividing SCs acquire a novel phenotype with new characteristics in addition to regaining some traits from their undifferentiated precursor state (Figure 1.1).

i. SCs re-express the molecules that characterize immature SCs in developing nerves, including L1 adhesion molecule, p75 neurotrophin receptor (p75NTR), and glial fibrillary acidic protein (GFAP) their precursors before being turned into myelinating cells (Chen and Rajewsky, 2007; Jessen et al., 2015; Jessen and Mirsky, 2008).

ii. Acutely after a nerve injury, reprogrammed SCs downregulate myelinating genes and their related-transcription factors like Egr2 (Krox20), myelin-associated glycoprotein (MAG), and periaxin (Arthur-Farraj et al., 2012; Fazal et al., 2015).

iii. SCs secrete neurotrophic factors like nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/5, ciliary neurotrophic factors (CNTF), and glial-cell line derived neurotrophic factor (GDNF) (Chan et al., 2004; Höke et al., 2003; Ma et al., 2011; Michailov et al., 2004; Taveggia et al., 2005). However, it is worth noting that changes in these factors differ in timing and site of nerve injury. The upregulation of the neurotrophic factors tends to be higher in the distal portion of an injured nerve during the acute phase of the injury (Boyd and Gordon, 2003; Chen et al., 2007). SCs also secrete cytokines to recruit macrophages and neutrophils (Lindborg et al., 2017). Some of these cytokines, like LIF and IL-6, act directly on neurons to promote survival. Later, the recruited macrophages act as a maintaining source of vital cytokines as the macrophages are polarized by the SCs toward an alternative, the M2 phenotype. This phenotype promotes axonal outgrowth by releasing essential anti-inflammatory mediators like IL10, arginase-1, chitinase-like 3/YM1, and mannose receptor C type 1/CD206 (Al-Darraji et al., 2018; Haydar et al., 2019; Kroner et al., 2014).

iv. SCs release VEGF and other factors to promote vascularization (Barrette et al., 2008).

v. Together with macrophages, SCs clear the myelin that inhibits new axon regeneration (Hirata and Kawabuchi, 2002).

vi. SCs form regeneration columns which are known as bands of Büngner. These bands are important in guiding the newly regenerating axons to their correct targets (Jessen et al., 2015).

Signaling pathways and mechanisms involved in reprogramming SCs

The emergence of the SCs' repair phenotype is controlled mainly by the transcriptional factor c-Jun (K R Jessen and Mirsky, 2016). Activation of other transcription factors like Sox2, Id4 and Pax3 is also essential in initiating the reprogramming process, but what differentiates c-Jun from other transcriptional factors is that it simultaneously downregulates myelinating genes and upregulates regeneration-associated genes (Parkinson et al., 2008).

How c-Jun is activated remains unclear but inhibiting the activity of SCs' c-Jun greatly reduces the regenerative capacity of injured PN. Normally, c-Jun is suppressed by Krox-20 in myelinating SCs. However, upon nerve injury, c-Jun becomes activated in the distal stump (Arthur-Farraj et al., 2012; Chen et al., 2002; Parkinson et al., 2008). Jessen and Mirsky's group in 2012 used a c-Jun knock out mouse model to study the role of c-Jun in the trans-differentiation of SCs after PN injury. The nerves of c-Jun mutant mice appeared like those of wild type mice before injury. However, after an injury, a comparison of the distal nerve stumps of wild type and c-Jun knock out mice showed that a remarkable number of genes involved in regeneration and trophic support, like *BDNF, GDNF, Artn, Shh,* and *GAP-43*, failed to upregulate. On the other hand, myelin-related genes such as *Mpz, Mbp,* and *E-cadherin* failed to downregulate after an injury in c-Jun mutant mice.

Importantly, reactivation of c-Jun by adenovirus transfer upregulated *BDNF* and *GDNF* expression and fully restored the number of axons after injury(Arthur-Farraj et al., 2012).

The c-Jun signaling pathway also affects neuronal cell adhesion processes. Injured nerves, from c-Jun knockout mice, expressed reduced N-cadherin and p75NTR but elevated levels of NCAM (Arthur-Farraj et al., 2012). In addition to N-cadherin and NCAM, Neurofascin-155, TAG-1, and Gliomedin are other major cell adhesion proteins that are expressed during the regeneration process. Additionally, other studies reported that SCs produce extracellular matrix molecules (like Fibronectin, Collagen I and IV, and Laminin) that facilitate and guide axonal growth in PN environment (Eshed et al., 2005; Spiegel and Peles, 2006; Tait et al., 2000; Traka et al., 2002).

Despite the major role of c-Jun in inducing the repair cell phenotype of SCs, other transcriptional factors can act independently of c-Jun. For instance, the function of Sox-2, a regeneration associated protein, is not affected by knocking down c-Jun after a nerve injury (Arthur-Farraj et al., 2017, 2012). Nevertheless, the key function of the repair SCs phenotype in preventing the neuronal death relies heavily on the activation of c-Jun. Selectively mutating c-Jun in SCs reduces the number of DRG neurons by about 50 % after an injury to PN. Interestingly, the number of proliferating SCs in the c-Jun knockout mice was comparable to that of the wild type animals thus demonstrating that the lack of axon regeneration and the increased neuronal death in c-Jun knock outs was dependent on the new SCs' phenotype rather than simply their proliferation.

The complete chain of events leading to c-Jun activation after PN injury has yet to be fully elucidated. The events below describe the set of signaling pathways that are involved in c-Jun activation after injury. Signaling molecules in the MAPK pathway are

activated intracellularly in response to an injury including Erk1/2, JNK, and p38. These are important regulators of the AP-1 transcriptional complex of which c-Jun is a key component (Harrisingh et al., 2004; Napoli et al., 2012, 2009; Yang et al., 2012). Activation of the Raf / ERK pathway with and without a nerve injury initiates the repair phenotype of the SCs and induces a robust inflammatory response. This response is reversible when the Raf/ERK pathway is switched-off (K. R. Jessen and Mirsky, 2016).

Furthermore, switching into the migratory repair phenotype is crucial for SCs to form the regenerative tracks that cross-bridge the wound site. Formation of a new extracellular matrix is crucial for SCs to travel along the regenerative tracks. Ephrin B expressed by the fibroblasts activates EphB2 receptor on SCs which then induces the relocalization of N-cadherin to the SC surface through the Sox2 transcription factor (Jessen et al., 2015; Parrinello et al., 2010). While the extracellular tissue that is bridging the wound sites is initially not vascularized, it becomes hypoxic. The macrophages sense the ongoing hypoxia and secrete VEGFA, that is vital to induce the vascularization of the bridge and subsequent SC migration (for review see Cattin et al, 2016, Stierli S, Glia 2019). Formation and maintenance of the regeneration tracks by repair SCs, bands of Büngner, is decisive for guiding the regenerating axons back to the terminal SCs at the original targets while providing the essential trophic factors to support the survival of the proximally injured neurons.

The repair capacity of SCs is increasingly motivating the curiosity of researchers. A better understanding of this repair phenotype and how it is activated may, in the future, change our ability to treat injury or damage in the CNS for conditions such as neurodegenerative diseases. A recent study published by Parkinson and his team

investigated the role of the Hippo/YAP (Yes-associated protein) pathway in controlling the repair capacity of SCs (Mindos et al., 2017). Using a sciatic nerve crush injury model in mice, Merlin-null nerves had a severely impaired axonal regeneration and remyelinating capacity. The failure of Merlin-null nerves to repair was mediated by activating YAP, which is a major Hippo pathway effector. To better test the relationship between YAP and c-Jun expression, rat SCs were infected with adenovirus expressing the YAP protein. YAP was localized to the nucleus and was associated with a significant downregulation of c-Jun (Mindos et al., 2017). On the other hand, removing YAP restores c-Jun levels, neurotrophin expression, and the functional recovery of the nerve after an injury. Taken together, these findings indicate that Merlin expression and YAP inhibition help in switching SCs into their repair phenotype.

Finally, the complexity of the different layers involved in driving the repair phenotype of SCs has yet to be fully investigated. The recent work published by Arthur-Faraj et al. raised several questions regarding the role of the appropriate regulation of epigenetics for a proper repair of injured nerves. For instance, c-Jun-null nerves expressed significantly lower levels of miR-21a-5p and miR-34b than controls. In contrast, other miRNAs (miR96-5p, miR-124-3p, miR-183-5p, and miR-204-5p) were significantly overexpressed (Arthur-Farraj et al., 2017). What is the physiological importance of such micro RNAs and others in the repair process? Only the recent advances in the epigentic research and its methodologies will help answering such key questions.

Pre-clinical and clinical studies

Next, we review the preclinical and clinical literature exploring the hypothesis that grafting PN tissue into the CNS might be utilized as a therapeutic approach to helping repair the CNS and, in part, slow down, halt, or reverse the progression of PD and other neurodegenerative diseases by employing the "repair cell' properties of SCs. Ultimately, we ask: can we use the signaling properties of the SC to explore how to repair the CNS?

Use of SC implants in *spinal cord repair research*

Several groups have tried to implement the regenerative properties of SCs to repair the spinal cord after an injury. In different animal models of spinal cord injury, transplantation of SCs has demonstrated tissue preservation, axonal regeneration and myelination in addition to functional recovery (Kanno et al., 2014; Pearse et al., 2004; Takami et al., 2002). To enhance the viability of transplanted SCs and reduce the astrogliosis in an injured spinal cord, different matrices have been used to suspend SCs pre-transplantation like Matrigel, Puramatrix, and Alginic acid sodium hydrogel (Kanno et al., 2015; Moradi et al., 2012). These matrices were composed of a variety of extracellular matrix components including collagen type IV, heparin sulfate proteoglycans, and entactins (Kanno et al., 2014; Pearse et al., 2007). Additionally, several transplantation methods were investigated to augment SCs survival and axonal regeneration including co-transplanting bone marrow stem cells (BMSCs) with genetically engineering SCs that produce different neurotrophic factors (Table 1.1). The migration of SCs within the host spinal cord tissue and their integration with the propriospinal axons were further facilitated

by adding chondroitinase ABC to SCs or PN grafts with or without acidic fibroblast growth factors (Bradbury and Carter, 2011; DePaul et al., 2015; Kanno et al., 2014; Zhao and Fawcett, 2013). Chondroitinase degrades the chondroitin sulfate proteoglycans, which is secreted by the astrocytes and leads to the formation of the glial scar. Also, grafting SCs with olfactory ensheathing cells after spinal cord contusion unduces axonal growth and the functional outcome (Fouad et al., 2005; Pearse et al., 2007; Ramón-Cueto and Avila, 1998). Furthermore, co-transplantation of SCs with neural stem cells and bone marrow mesenchymal stem cells overexpressing trophic factors promotes neuronal differentiation, neuroprotection and outgrowth of serotonergic fibers, and enhances locomotor recovery (Oraee-Yazdani et al., 2016; Pourheydar et al., 2012; Yazdani et al., 2013).

The preclinical work by Bastidas et al. (2017) investigated the use of cultured human SCs to repair an injured spinal cord. Xenografts of human SCs were transplanted into the spinal cord of nude rats and demonstrated both safety and ability of the cells to survive and support histological regeneration and functional recovery of the CNS (Bastidas et al., 2017). To study the role of human SCs in treating demyelinating diseases like multiple sclerosis, Kohama and colleagues implanted human SCs in a demyelinating rat spinal cord. Five weeks after transplantation, the electrophysiological recordings and immunohistochemistry analysis showed a prominent survival of the transplanted human SCs and extensive myelination with an increase in average conduction velocity (Kohama et al., 2001). Thus, SCs which are normally contained in PN, can survive transplantation and promote neural repair in a model of demyelinating diseases.

Meanwhile, Saberi group has tested autologous SCs transplantation on 33 human participants who had chronic spinal cord injury. In the six months before the

transplantation, study participants did not show any clinical improvement. Three to four million cultured SC's were injected into the injury site of the spinal cord (Saberi et al., 2008). After two years, MRI results showed no evidence of abnormal tissue or tumor growth at the injection sites. Clinical assessments showed significant improvement in the sensory light touch test but no improvement in the pinprick sensation test. Only subtle motor improvements were reported in the cases where the spinal cord injury had occurred within three years; however, there were no significant improvements in sexual, sphincteric, or functional assessments (Saberi et al., 2011). The lack of a significant improvement after the transplantation could be due to many reasons including the chronic changes that might have occurred between the injury onset and the transplantation timing. A larger enrollment of participants along with a longer follow-up period could help clarify the potential clinical significance of the effects this cell-based intervention. On the other hand, additional elements of PN tissue, namely the macrophages, neutrophils, fibroblasts and disintegrated axonal cytoskeleton, are needed to support the function of SCs and allow appropriate repair of injured or degenerating CNS cells.

The first clinical trial under FDA approval was an open-label, unblinded, nonrandomized and non-placebo-controlled Phase I study that involved six subjects with subacute spinal cord injury. The primary endpoints of the trial were to evaluate the safety and feasibility of implanting cultured SCs intro an injured spinal cord. Autologous SCs were cultured *in vitro* from sural nerve and injected into the lesioned spinal cord. One year after grafting, there were no major surgical, medical, or neurological adverse events related to the transplanted SCs (Anderson et al., 2017).

Human experience with implantation of SCs in neurodegenerative diseases

Neurodegenerative diseases, such as Alzheimer's and PD are complex, chronic disorders with cognitive and motor symptoms that result from the progressive neuronal loss within cortical and subcortical areas of the human brain. The current pharmacological treatments act on symptoms without slowing or reversing the ongoing deterioration in neurons and their function. Nevertheless, preclinical research shows that at earlier pathological stages, the neurons retain some characteristics to recover if they get enriched with optimal neurotrophic media, such as NGF, BDNF or GDNF (Quintino et al., 2019). Different "repair" strategies have been proposed to treat or replace degenerating neurons in the CNS, especially in PD. These therapeutic strategies include transplantation of fetal ventral mesencephalic tissue (Bakay, 1993; Freed et al., 2011, 2001; Hallett et al., 2014; J.H. et al., 2017; Kordower et al., 2000; Li et al., 2008; Olanow and Fahn, 2006), delivering neurotrophic factors to degenerating areas of the CNS (Kordower et al., 2000; Lindahl et al., 2017; Slevin et al., 2007; Sullivan and O'Keeffe, 2016; Whone et al., 2019), and autologous PN grafting (van Horne et al., 2018, 2016). Previous and current clinical attempts to restore neuronal loss by transplanting fetal ventral mesencephalic tissue have faced serious ethical and technical challenges although a few subjects did show some improvement. We would argue that because of the ability of SCs to support neuronal recovery in PNS, we may gain further insight into neuroregeneration through examining these experimental models of grafting SCs in the CNS. Unfortunately, great difficulties have been encountered in standardizing different isolation and culture protocols of SCs.

Neurotrophic Factors as Putative Therapies

Pilot studies that have investigated the delivery of neurotrophic factors like GDNF (Hoffer et al. 1994, Gash et al. 1996, Gash et al. 2005, Grondin et al. 2002, Grondin et al. 2019), Neurturin (Reosenblad et al. 1999; Oiwa et al. 2002, Gasmi et al., 2007, Grondin et al. 2008), cerebral dopaminergic neurotrophic factor (CDNF; Voutilainen et al. 2011, Airavaara et al. 2012), or BDNF (Tsukahara et al. 1995, Yurek et al. 1996), have shown considerable promise in promoting, restoring or protecting dopamine containing fibers and neurons. These are the primary type of neuronal cells affected in PD and are affected in rodent and nonhuman primate models of dopamine depletion. Furthermore, co-administration of GDNF with NT-4/5, has a synergistic effect on increasing dopaminergic neuron survival and stimulating dopamine release in rat organotypic explants (Di Santo and Widmer, 2018). Nevertheless, the use of neurotrophic factors in humans, either by direct delivery of GDNF (Nutt et al. 2003, Gill et al. 2003, Patel et al. 2013, Slevin et al. 2005, Lang et al. 2006), platelet-derived growth factor (PDGF-BB, Paul et al. 2015) or AAV delivery of Neurturin (Marks et al. 2008, Marks et al. 2010, Bartus et al. 2013, Olanow et al. 2015), have shown limited clinical benefits while identifying practical dilemmas concerning the specificity of the therapeutic target. In addition, manufacturing and purification of biologics, such as growth factors, is expensive, complicated, and can complicate their use. Penetration of the neurotrophic proteins into brain parenchyma was so problematic that a need evolved for direct versus gene therapy-based approaches to CNS delivery (Marks et al., 2015, 2010; Salvatore et al., 2006). Recently, to improve striatal GDNF exposure and optimize the pharmacodynamics and pharmacokinetics of GDNF, a research group conducted a randomized, placebo-controlled study of convection-enhanced

delivery of GDNF (Whone et al. 2019). The drug was administered intermittently every 4 weeks for 40 weeks using an intra-putamenal and bilateral skull-mounted, multi-cannula transcutaneous port device (Whone et al., 2019). The study failed to meet its primary endpoints, but a subsequent analysis of the extended treatment (80 weeks) showed significant differences in motor activity and quality of daily living. But if the trophic factor hypothesis remains valid, one question becomes: is one growth factor enough to slow or stop the degeneration and/or restore injured neurons in the nigrostriatal system?

SCs within the autologous nerve grafts may serve as an alternative and natural delivery source of many "repair molecules" including neurotrophic factors After experimental results (Watts et al., 1997) demonstrated no major functional improvement, possibly in part because of sample size limitations (only 5 participants), this area of research had been suspended for more than two decades. Nevertheless, that trial and a recent, single-case transplantation (Tabakow et al., 2014) of repair SCs, in the form of PN grafts in combination with olfactory ensheathing cells, were able to show safety of the procedure and some indications of possible efficacy. This work has helped revitalize the concept of potentially using PN grafts in repairing the CNS.

PN grafting in nonhuman primate models of neurodegenerative diseases

Preclinical and clinical attempts to restore the nigrostriatal dopaminergic pathway using dopamine secreting cells located within adrenal medulla grafts and human fetal mesencephalic tissue were carried out in the 1980's to possibly treat PD. Autologous adrenal medullary transplantation alone resulted in poor graft survival while the human fetal grafts appeared to initially produce little clinical improvement (Bakay, 1993; Brundin

and Björklund, 1987). However, co-grafting adrenal medullary tissue with an autologous PN tissue was tested by Watts and his team in n-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP)-treated parkinsonian monkeys (Date et al., 1990; Watts et al., 1997, 1995). Two small cavities were created in the caudate nucleus of two monkeys. Each cavity was implanted with an adrenal medulla-sural nerve co-graft. Dexamethasone and nonsteroidal anti-inflammatory drugs were administered postoperatively. The grafted monkeys demonstrated significant improvements in their parkinsonian motor scores 6 months after the surgery. These results were also supported by EMG traces with increased peak velocity of movements after grafting. Their study was unblinded and lacked surgical sham controls thereby limiting the scientific judgment of the effect of the graft versus the surgery itself on the study outcomes. However, this research group was able to histologically demonstrate prolonged survival of the adrenal medulla cells along with the peripheral SCs for months after the grafting procedure. These results may reflect the potential of the PN grafts in biodelivering adequate trophic support to enhance the survival of adrenal medullary cells and possibly repair the damaged host neurons at earlier stages of a neurodegenerative disease.

Clinical trials investigating the potential of PN cell / tissue-based grafts to repair the CNS

The concept of tissue transplantation into the CNS has been explored as a therapy for the treatment of multiple conditions, including PD (Freed et al., 2011), multiple sclerosis (Stangel, 2004), Huntington's disease (Bachoud-Lévi et al., 2006; Gaura et al., 2004), and spinal cord injury (Levi et al., 2002; Saberi et al., 2011). The safety of

implanting autologous PN grafts in combination with other tissues into the CNS is supported by the results of investigations in both non-human primate models (Levi et al., 2002) and in patients with PD (Watts et al., 1997). Following positive results in a study examining the efficacy of intrastriatal co-grafts of autologous adrenal medulla and sural nerve in a macaque model of PD (Watts et al., 1995), the research team placed similar co-grafts in the basal ganglia of five human subjects with advanced PD (Watts et al., 1997). Segments of the 11th intercostal nerve were harvested and stereotactically placed, together with autologous adrenal medullary tissue, into the caudate nucleus and, putamen. The concept was that SCs from transected PN segments could produce NGF to enhance the survivability of transplanted adrenal chromaffin cells and the recovery of the host nigrostriatal system (Date et al., 1990). Overall, the human surgery was reported to result in no major complications and was ultimately deemed both safe and successful. The two-year follow-up showed a mild to moderate improvement in Unified Parkinson's Disease Rating Scale (UPDRS) motor scores; yet, the improvement was not statistically significant. This could be explained by the small sample size and the chosen locations of the grafts.

The safety of transplantation into the basal ganglia has been demonstrated by Mendez et al. by placing fetal cell suspensions bilaterally into the substantia nigra, as well as the putamen, without any perioperative or long-term complications (Mendez et al., 2002). Meanwhile in 2001, Timothy Vollmer and his team at Yale implanted autologous SCs into lesions in the frontal lobe of the brains of patients with multiple sclerosis. While, to our knowledge, the results were never published, reports from the funding agency state the surgical procedure was safe, with no adverse effects from the transplantation (The Myelin Project). Other studies in both humans and animals have also shown the safety of

transplanting purified SCs into the brain or spinal cord (Anderson et al., 2017; Kohama et al., 2001; Pizzorusso et al., 1994; Saberi et al., 2011, 2008). One of the key benefits of implanting autologous SCs is that each patient could serve as his/her own donor, thus minimizing the need for immunosuppression (Stangel, 2004).

Could it be that perhaps because of these unpromising clinical results, and the rising trend in the use of single growth factors such as GDNF or Neurturin to restore dopaminergic function in PD (Funakoshi et al., 1993; Gash et al., 1996; Kordower et al., 2000; Slevin et al., 2007) the therapeutic benefits of PN grafts for treating CNS disorders have been undervalued over the past two decades? In addition, an emerging understanding of the PN repair process helped further incorporate this cell-based therapeutic into clinical trials (see Tables 1.1 and 1.2).

Using regenerative PN implants in PD to alter disease progression

In 2013 our research group initiated the first clinical trial to investigate the hypothesis of using autologous PN grafts as a source of SCs to deliver crucial repair molecules to the degenerating dopaminergic neurons in the SN of patients with PD undergoing deep brain stimulation (DBS) surgery (Figure 1.2) (van Horne et al., 2018, 2016). The goal of the trial was to determine the safety and feasibility of implanting PN grafts at the time of DBS surgery. This was the first clinical study that investigated the use of autologous PN grafting in conjunction with DBS, an FDA-approved adjunctive therapy to treat the symptoms of PD. The participants were tested with the DBS system turned off and medications removed to washout their effects. Eight participants (6 males and 2 females) were included in the study with the mean (± Standard Deviation) age of 63 ± 9

years, and mean disease-duration of 10 ± 4 years. All the participants consented for DBS surgery targeting the subthalamic nucleus (STN) and for sural nerve grafting to the substantia nigra (a procedure we have termed DBS Plus). The surgeries were done in two stages on two different days (van Horne et al., 2015) (Figure 1.2).

During the first stage, the sural nerve was exposed and transected. The initial injury to the nerve was expected to stimulate the SCs to reprogram themselves into the "repair cell" phenotype as described previously in this review. The second stage was done 3-5 days later, during which DBS electrodes were inserted bilaterally followed by unilateral grafting of distal sural nerve fascicles, approximately 5 segments each about 1 mm in length into the SN, contralateral to the most affected side. The targeting of the DBS electrodes and the graft target location were confirmed to be accurate by postoperative 1.5 T MRI.

Adverse events of the grafting were comparable to that of DBS surgery alone. The postoperative follow-up at 12 months reflected safety and tolerability of the grafting procedure (van Horne et al., 2018). Only one participant suffered from superficial cellulitis at the sural nerve incision site and 3 participants reported numbness along the sural nerve dermatome. MRI of the midbrain showed no abnormalities acutely or at 12 months after the surgery. Motoric impairment was measured using the UPDRS Part III while participants were off therapy at 12 months. The mean scores were, 25 ± 16 points at 12 months vs 33 ± 10 points at baseline. The UPDRS test is scaled from 0-108 points, 0 is not affected and 108 being most affected). This pilot study was able to reflect the safety and feasibility of transplanting autologous SCs within conditioned PN tissue into the human brain. To further validate the safety and explore the efficacy of this therapeutic approach,

we have optimized the sample size in our ongoing studies (clinicaltrials.gov, registration no. NCT02369003).

Our current ongoing studies (NCT02369003) have included patients who receive DBS electrodes to the internal segment of the Globus Pallidus (GPi), another target for DBS. One benefit of this approach is that the location is remote enough from the SN that the grafts can be readily visualized with MR imaging. In addition to MRIs, SPECT imaging using the radioligand, Iofluopane I_{123} injection (DaTscan™) has been implemented to evaluate dopamine neuronal function in participants pre- and post-implantation of the sural nerve grafts

In our studies, transplanting repair SCs within PN autografts is being investigated as a conceivable disease-modifying therapy in PD. With no known cures, disease modification remains one of the greatest unmet medical needs in the treatment of neurodegenerative diseases like PD (Kalia et al., 2015). We continue to investigate questions regarding dosing and optimization of bilateral placements of the PN grafts to help lay the foundation for a blinded Phase II trial to better determine the efficacy of the combined DBS and cell-based intervention.

Table 1. 1 Critical studies that investigated the use of peripheral nerve tissue components in treating trauma and spinal cord injury.

Trauma & Spinal Cord Injury

Research Group	**Experimental Design**	**Model**	**Results**
(David & Aguayo, 1981)	PN graft alone	Bridging model in transected model of SCI in rats	Regenerating axons were guided by the PN graft from the medulla oblongata to the lower cervical and upper thoracic spinal cord
(Xu, Guénard, Kleitman, Aebischer, & Bunge, 1995)	Graft of SCs + BDNF + NT-3	Transection model of SCI in rats	Neurotrophins infused with the graft enhanced propriospinal axonal regeneration and promoted axonal regeneration of specific distant populations of brain stem neurons into the grafts
(Ramón-Cueto & Avila, 1998)	Combined SC-filled guidance channels + OECs transplants	Transection model of SCI in rats	Robust ingrowth of 5HT-positive axons across SC graft–cord interfaces and promotion of long-distance regeneration of descending supraspinal and ascending propriospinal axons
(Levi et al., 2002)	Intercostal nerve autografts + fibrin glue containing aFGF	Transection model of SCI in Cynomolgus monkeys	Regeneration of theproximal spinal axons into the PN grafts; the grafts significantly enhanced the regeneration of myelinated axons into the region of the hemisected spinal cord

Table 1.1(Continued)

(Takami et al., 2002)	Co-graft of OE glia and cultured SCs	Contusion model of SCI in rats	At 12 weeks after injury, SC-containing grafts expressed more intense staining for glial fibrillary acidic protein and chondroitin sulfate proteoglycan compared with OEC-only grafts. Propriospinal and brainstem axons reaching 5–6 mm beyond the grafted area. SC graft is more effective in promoting axonal sparing/regeneration than an SC/OEC or OEC graft in the moderately contused adult rat thoracic spinal cord
(Pearse et al., 2004)	Schwann cell grafts with phosphodiesterase IV inhibitor (Rolipram)	Contusion model of SCI in rats	Inhibition of cAMP hydrolysis by the phosphodiesterase IV inhibitor rolipram promoted significant supraspinal and proprioceptive axon sparing and myelination
(Fouad et al., 2005)	Graft of SCs/OECs + Chondroitinase ABC	Transection model of SCI in rats	Increased numbers of both myelinated axons in the SC bridge and serotonergic fibers that grew through the bridge and into the caudal spinal cord
(Pearse et al., 2007)	Co-grafts of SCs cultured from sciatic nerves + OEC	Contusion model of SCI in rats	At 9 weeks post-implantation, OEC grafts showed improved survival when transplanted with SCs; modest improvements in open-field locomotion and hind paw positioning

Table 1.1(Continued)

(Golden et al., 2007)	Grafts of SCs transduced with D15A neurotrophic factor	Contusion model of SCI in rats	At 6 weeks post-transplantation, SCs survived, myelinated 5-HT, DβH, and CGRP axons and their lengths were up to 5-times longer within the transduced grafts
(Vavrek, Pearse, & Fouad, 2007)	Grafting of SCs with prolonged ChABC infusion	Contusion model of SCI in rats	Regrowth of raphe-spinal fibers from the brain stem areas (vestibular nuclei and reticular formation) into the SC bridge
(Ma et al., 2010)	Sural nerve transplants + aFGF	Transection model of SCI in monkeys	Improvement in walking performance at 16 weeks after surgery
(Moradi et al., 2012)	Xenografts of human fetal SCs encapsulated in PuraMatrix scaffold	Contusion model of SCI in rats	At 8 weeks post-transplantation, grafted SCs infiltrated the injury site, suggesting that PuraMatrix may play an important role in the repair after human fetal SC is transplanted with SAPNS
(Pourheydar et al., 2012)	BMSCs + SCs	Contusion model of SCI in rats	At 8 weeks post-transplantation, SCs survived around the injury site, co-transplanted animals showed an improvement of functioning but greater allodynia

Table 1.1(Continued)

(Kanno, Pearse, Ozawa, Itoi, & Bunge, 2015; Kanno et al., 2014)	Graft of SCs transduced with D15A neurotrophic factor + ChABC	Contusion model of SCI in rats	Increase of axonal regeneration caudal to the graft; increase of locomotor and sensory function
(DePaul et al., 2015)	Graft of PN + aFGF + ChABC	Transection model of SCI in mice	Regeneration of serotonergic and tyrosine hydroxylase-positive axons across the lesion; cytometry analysis and external urethral sphincter electromyograms showed improved urinary bladder function; reduction in collagen scaring
(Bastidas et al., 2017)	Xenografts of human SCs	Contusion model of SCI in rats	At 6 months post-transplantation, there was no evidence of tumorigenicity with preservation of the white matter as well as axon growth and myelination
Clinical Trials			
(Tadie et al., 2002)	Surgical bypass using segments of autologous sural nerve from thoracic cord levels into the lumbar ventral roots	Humans with chronic SCI (N=1)	At 8 months after transplantation, partial return of motor function in the paralyzed legs after nerve autografts
(Saberi et al., 2011; Saberi et al., 2008)	Intramedullary graft of autologous SCs cultured from sural nerve	Humans with SCI for ≥6 months (N=33)	At two years follow up, no major adverse events were reported, but statistical improvement in sensory scores. The duration of SCI had no significant influence on sensory results. Significant motor changes were observed in those with duration of SCI < 3years

Table 1.1(Continued)

(Zhou et al., 2012)	Autologous SCs cultured from sural nerve + Neurorehabilitation	Humans with SCI 1wk-20 months after injury (N=6)	At 5 years of follow-up, grafting procedure was feasible, safe, with mild to moderate clinical improvement on ASIA motor and sensory index; Smaller volume of myelomalacia and cystic degeneration after SCs transplantation.
(Amr et al., 2014)	Combined sural nerve grafts + chitosan-laminin scaffold + BMSCs	Humans with chronic SCI (N=14)	Mild to moderate motor and sensory improvements in the lower limbs; regaining sensation of bladder fullness in all but 3 patients
(Tabakow et al., 2014)	Co-grafts of autologous sural nerve + OEC	Humans with SCI (N=1)	At 19 months post-transplantation, no adverse effects observed; Functional regeneration of both efferent and afferent long-distance fibers; partial bridging of the spinal cord at the nerve grafts; Neurophysiological restitution of the integrity of the corticospinal tracts
(Yazdani et al., 2013)	Combined intrathecal injection of autologous SCs + MSCs	Humans with chronic SCI (N=6)	No evidence of tumor overgrowth observed in MR imaging for a mean of three years after cell transplantation; no serious adverse effects were reported

Table 1.1(Continued)

(Anderson et al., 2017)	Intra-spinal transplantation of highly purified autologous SCs	Humans with subacute SCI (N=6)	At 1-year post-transplantation, no serious adverse events related to the cell therapy were reported. There was no evidence of additional spinal cord damage, mass lesion, or syrinx formation. In one patient, there was an atrophy and focal tethering of the spinal cord

PN: Peripheral nerve, SCI: Spinal cord injury; BDNF: Brain derived neurotrophic factor; NT-3: Neurotrophin-3; 5-HT: 5-hydroxytryptamine, aFGF: acidic fibroblasts growth factor; OEC: Olfactory ensheathing cells; DβH: dopamine-β-hydroxylase; CGRP: Calcitonin gene-related peptide; BMSCs; Bone marrow stromal cells; MSCs: Mesenchymal stem cells; SAPNS: self-assembling nanofiber scaffold; ChABC: Chondroitinase ABC; ASIA: American Spinal Injury Association score

Table 1. 2 Critical studies that investigated the use of peripheral nerve tissue components in treating demyelinating and neurodegenerative diseases.

Demyelinating and Neurodegenerative Pathologies

Research Group	**Graft Design**	**Model**	**Results**
(Aguayo et al., 1984)	Combined PN grafts + mesencephalic fetal transplants	6-OHDA lesioned rat model for Parkinson's disease	Fetal mesencephalic neurons survived and extended long axons for about 2 cm along PN grafts inserted into the striatum
(Gage et al., 1985)	Combined PN grafts + mesencephalic fetal transplants	6-OHDA lesioned rat model for Parkinson's disease	7/26 implanted rats showed 70% recovery of motor deficits with amphetamine-induced rotational behavior. DA neuron grafts survived in both behaviorally compensated and uncompensated rats; Neurites extended the entire length of the PN bridge
(Kordower, Fiandaca, Notter, Hansen, & Gash, 1990)	Intrastriatal implantation of minced adrenal medulla combined + minced sural nerve or sural nerve alone	MPTP-treated rhesus monkeys	Monkeys receiving an implant of nerve only, exhibited survival of SCs and enhanced survival of adrenal cell 3 months after implant surgery
(Date et al., 1996; Date et al., 1994)	Intrastriatal co-graft of adrenal medulla + mouse sciatic nerve	MPTP-treated mouse model of dopamine system depletion	Immunocytochemistry indicated that co-grafts into the striatum significantly enhanced adrenal cell survival and tyrosine hydroxylase-positive host cells with a large number of cells extending neurites

Table 1.2 (Continued)

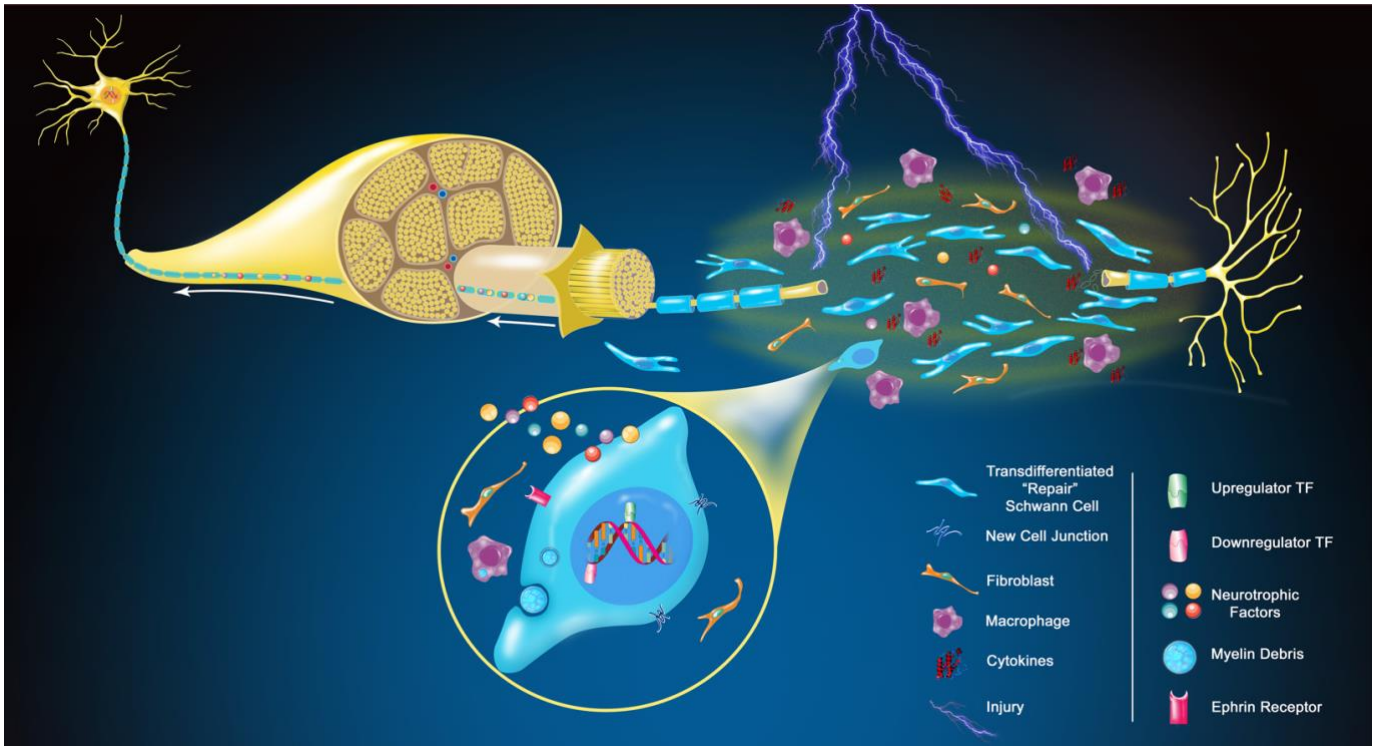
(Collier and Springer, 1991)	Mesencephalic neurons + PN co-grafted into the adjacent lateral ventricle	6-OHDA lesioned rat model for Parkinson's disease	Increase in density and area of reinnervation of the host striatum, with co-grafts clearly providing more extensive reinnervation
(van Horne et al., 1991)	Combined DA neurons grafts and minced sciatic nerve tissue	6-OHDA rat model of Parkinson's disease	Co-grafted animals exhibited significantly better functional recovery than animals receiving only DA neuron grafts
(Watts et al., 1997, 1995)	Combined autologous sural nerve + adrenal medullary tissue intrastriatal	MPTP-induced Parkinsonian Monkeys	Adrenal cell survival was good to excellent in the grafted striatum
(Kohama et al., 2001)	Grafts of SCs cultured from human sural nerves	X-irradiation /ethidium bromide lesioned demyelination in rats	Remyelination throughout the lesion with improved conduction velocity and action potentials conducted over a greater distance into the lesioned dorsal columns
(Date et al., 1995)	Co-graft of adrenal medulla and PN into the bilateral caudate nuclei	Humans with Parkinson's disease (N=1)	No postoperative complications with gradual and significant amelioration of the parkinsonian symptoms starting 2 weeks and continued for 2 year after transplantation

Table 1.2 (Continued)

Clinical Trials			
(Date et al., 1995, 1996)	Cograft of adrenal medulla and PN into the bilateral caudate nuclei	Humans with Parkinson's disease (N = 1)	No postoperative complications with gradual and significant amelioration of the parkinsonian symptoms starting 2 weeks and continued for 2 year after transplantation
(Watts et al., 1997)	Combined unilateral intrastriatal adrenal medulla plus intercostal nerve cografts into the right striatum	Humans with Parkinson's disease (N=5)	At two-year follow up, no morbidity was encountered. Motor test scores during the "off" state improved by 35% compared to baseline mainly in the side contralateral to the graft.
(Timothy Vollmer et al, 2011)	Grafts of cultured autologous SCs in frontal lobe lesions	Humans with Multiple Sclerosis (N=3)	Reports from the funding agency state the surgical procedure was safe, with no adverse effects from the transplantation
(van Horne et al., 2017; van Horne et al., 2018)	Combined DBS with autologous sural nerve graft implanted into the unilateral substantia nigra	Humans with Parkinson's disease (N=8)	Adverse event profiles were comparable to those of standard DBS surgery; UPDRS III motor scores suggest improvement at one year compared to baseline mainly on the side contralateral to the graft

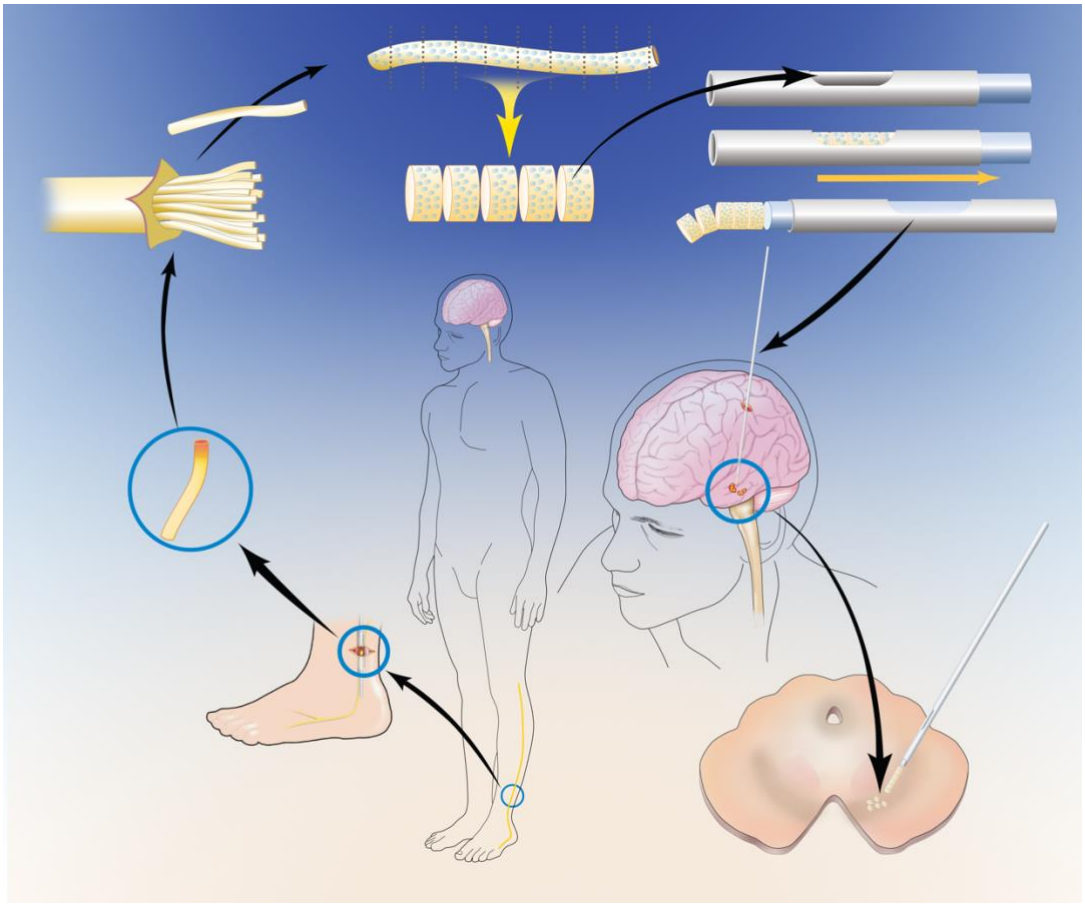
PN: Peripheral nerve graft; 6-OHDA: 6-hydroxydopamine; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; DA: dopaminergic neurons; DBS: Deep Brain Stimulation; UPDRS III: Unified Parkinson Disease Rating Scale Part III.

Figure 1. 1 Reprogramming of Schwann cells into Repair Cells.



After a nerve injury, distal SCs dedifferentiate, re-express precursor markers, alter their morphology, and migrate within regenerating tracks (Büngner bands). These tracks guide the regenerating axons back to their correct targets. Ephrin B expressed by the fibroblasts activates Eph receptor B2 on SCs to promote expression of cell junctions and extracellular matrix molecules. SCs crosstalk with the macrophages and 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 new axons. Pro-myelinating genes are downregulated through the cross-inhibition of Krox-20, POU domain class 3 transcription factor 1 (Pou3f1 or Oct-6), myelin protein zero (MPZ) and myelin basic protein (MBP) by c-Jun, Sox-2, Pax-3, Id2, and Egr1/3 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 are retrogradely transported towards the perikaryon to upregulate anti-apoptotic pathways and to enhance the function of injured neurons.

Figure 1. 2 Overview of DBS Plus Trial.



Grafting of autologous peripheral nerve implants in the substantia nigra of a human subject with Parkinson's disease. During Stage I 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 Schwann cells, is harvested after two weeks during the stage II surgery. The epineurium is removed, nerve fascicles are stripped and sectioned into petite segments and loaded into a custom-made graft cannula. The grafts are stereotactically deployed into the degenerating substantia nigra.

CHAPTER TWO: MATERIALS AND METHODS

Clinical Trial Design

DBS Plus trial was designed as an open-label, single-center, Phase I clinical trial that aims to assess the safety and feasibility of grafting autologous peripheral nerve implants into the substantia nigra of patients with PD who were undergoing DBS surgery. The secondary outcome measure of this trial is the motor signs assessment after surgery in comparison to the baseline. The time frame is 24 months for participants who received single-unilateral implants, and 12 months for the group of participants who received double dose of the implants to the unilateral substantia nigra. The trial was registered with the ClinicalTrials. gov database (http://clinicaltrials.gov), and its registration number is NCT02369003. The study was reviewed and approved by the University of Kentucky IRB committee, and the informed consent was obtained from all participants.

Participants Selection and Eligibility Criteria

Participants who have met the criteria for DBS surgery had been selected, informed about the study, and consented for the DBS Plus the cell implantation procedure. Male and female participants who are 40 to 75 years show a positive response to Sinemet (Carbidopa/Levodopa), capable of tolerating the surgical procedure, and can follow up with their appointments were included in the study. Those who showed mild to moderate neurocognitive scores were excluded from entering the trial. UPDRS III assessments, both

OFF and ON-medications, were done before the surgery, i.e., baselines scores. Participants who have any medical condition that would not make them a candidate for DBS of the STN or GPi or unable to give informed consent were excluded from the trial enrollment. Females who are pregnant, lactating, or of child-bearing potential and unwilling to use an adequate birth control method during the trial were also excluded from the study.

PPMI Database for PD Control

The data used for the PD Control group were obtained from the Parkinson's Progression Markers Initiative (PPMI) database (www.ppmi-info.org/data). PPMI is funded by the Michael J. Fox Foundation for Parkinson's Research and other funding partners (www.ppmi-info.org/fundingpartners). PPMI is a result of the collaboration of research centers of multiple sites around the word and biotechnology companies to find a biomarker of PD progression. Those centers have obtained approval from the local IRB committees and acquired consent from the participants. All observational data were then de-identified and shared on the PPMI database. The PPMI website details description of the PPMI methods in addition to the acquisition and dispensation of the data. The PPMI Data were downloaded in November 2019. The database included 2117 PD participants with a total of 14861 visits and an average of 7 visits per participant over a period of 6 years. Our analysis was focused on three-time points: Baseline, 12 months from baseline, and 24 months from baseline. We aimed at refining the PPMI-control PD group to match the inclusion and exclusion criteria of the DBS-Plus trial with the baseline set at the time point when the participants score for MDS-UPDRS III of 30 and above on his/her visit.

Subsequently, 80 participants from the PPMI Control PD database have matched the inclusion criteria and completed a 24-month visit after.

Deep Brain Stimulation Plus PN Implantation Surgery

Deep Brain Stimulation (DBS) is a standard treatment for movement disorders such as Parkinson's disease, Essential Tremors, and Dystonia. Also, DBS was demonstrated to be effective in treating Epilepsy, obsessive-compulsive disorder, depression, and other neurocognitive disorders. By sending out electrical stimuli, DBS acts on several mechanisms to excite or inhibit different neuronal networks and modulate their synaptic transmission (Herrington, Cheng, & Eskandar, 2016). The downstream effects of electrical stimulation mainly depend on the chosen brain targets. The most common deep brain targets for treating PD are the subthalamic nucleus (STN), Globus Pallidus Internal (GPi), and caudal zona incerta nucleus(cZi). Those targets are carefully chosen with a multidisciplinary team of neurologists, neurophysiologists, psychologists, and a neurosurgeon to guarantee delivery of the best out symptomatic relief with a minimum side effect. Participants were evaluated before surgery using MRI. The trajectories and entry points of the DBS electrodes were mapped using Brainlab software (IPlan 3.0 Stereotaxy). The grafting trajectory was also mapped in parallel to that of the leads. The DBS surgery was done in two stages (van Horne et al., 2018).

At stage I, the pulse generator was implanted, and lead extensions were projected from the chest to the skull. Next, the patient ankle was repositioned for a standard sural nerve biopsy incision. The sural nerve was exposed and transected about 10 cm between the lateral malleolus and the posterior border of the ankle. A 1 cm nerve sample was taken,

and two silk ties were secured distal to the transection for better identification of the nerve during stage II surgery. The incision was then closed in layers. The second stage of the DBS Plus was completed two weeks later. Coordinates of the targets and trajectories were calculated based on a fusion of the participants' CT scan and susceptibility-weighted images sequences of MRI for better and precise targeting. After implantation of the DBS electrodes, electrodes contacts were tested for efficacy and side effects. Once testing is completed, a second burr hole was drilled and a guide cannula (FHC Inc., 1.8-mm outer diameter) was then placed to target the Substantia Nigra contralateral to the most affected parkinsonian side of the patient. The previously transected sural nerve was prepared, and the distal stump of the nerve was identified. A 2-cm segment was harvested and rinsed with normal saline. The epineurium was delicately removed, and the fascicles were removed and dissected into five small segments of approximately 1 mm length. The segments were then loaded in the graft cannula, which was placed to target the SN (Figure 1.2). The graft cannula was then removed, and the dura was covered with the Durepair matrix from Medtronic. The bur hole was filled with HydroSet. This sequence of events was planned carefully not to intervene with the regular DBS procedure.

The participants received MRI 24-48 hours post-operatively and a complete MDS-UPDRS III assessment during their follow-ups. A total of 18 participants with idiopathic PD have consented for a bilateral DBS surgery targeting the GPi plus a single unilateral nerve implant targeted to the substantia nigra pars compacta. A total of 9 participants with idiopathic PD have consented for a bilateral DBS surgery targeting the GPi plus a unilateral yet double dose of the PN implant targeted to the substantia nigra pars compacta.

Safety and Adverse Events Assessment

Human participants were evaluated for long-term postoperative adverse events (AE). Clinical monitoring timepoints for this dissertation report includes perioperative time points, the 2-year evaluation visits for single and unilateral implanted participants, and 1-year evaluation visits for participants who received double-unilateral implants. Through those time periods, AE was recorded from the clinical reporting, chart review, and specific queries during the clinical visits (van Horne et al., 2018). Postoperative 1.5-T MRI was used for evaluation 24-48 hours after the implantation surgery. AEs were classified into those which are related to DBS insertion or activation, graft-related procedure, or not related to both. AEs were also categorized in severity from mild to severe according to the Medical Dictionary for Regulatory Activities, version 11.0.

Motor Assessment: MDS-Unified Parkinson's Disease Rating Scale Part III

Movement Disorder Society-Unified Parkinson's Disease Rating Scale (UPDRS) Part III is a standard clinical scale used to evaluate and assess the PD motor signs. The PD participants were examined and scaled by one of 3 neurologists who are specialized in movement disorders. Assessments were done in two states. During the OFF-medication State, participants were assessed and scored after being off Levodopa-Carbidopa or any other PD medications for about 12 hours. Then, participants were allowed to take their prescribed Levodopa-Carbidopa dose, and they were re-assessed 1-2 hours afterward (State 2: ON-medication score). All post-op assessments are done with DBS stimulation being turned off in order to tease out the effect of the electrical stimulation on the PD signs. MDS-

UPDRS Part III is made of 18 items. Each subscale has 0-4 ratings, where 0 = normal, 1 = slight, 2 = mild, 3 = moderate, and 4 = severe.

Tissue Collection for translational studies

Experimental procedures, including handling and implanting human peripheral tissue, were reviewed and approved by the IRB of the University of Kentucky. Sural nerve fascicles were obtained from living adult participants with PD (aged 45-70 years). The donors had no known diagnosis of peripheral nerve pathologies or neurological illness other than PD. The sural nerve samples were collected in two stages: Stage I was followed by Stage II two weeks later. The sural nerves were dissected, and the nerve fascicles were collected on average 30 and 60 minutes after harvesting from stage I and stage II surgeries, respectively. Human peripheral nerve fascicles were obtained from 6 human subjects undergoing DBS-Plus surgery for Parkinson's disease for RNA sequencing studies. For the Neuro-Avatar experiments, human peripheral nerve fascicles, collected from both stage I and stage II surgeries, were obtained from 4 human subjects undergoing DBS-Plus. The removed section of the nerve was then stripped of its epineurium using microsurgical dissection in cold, 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, snap-frozen on dry ice, and stored at -80°C until they were assayed using RNA sequencing technique. The nerve fascicles that were collected for the in vivo experiments, Neuro-Avatar, the fascicles were collected in sterile conditions, placed on ice, and transported immediately from the operation room to the laboratory for implantation.

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. 200µl of chloroform was added, and the tube was shaken vigorously by hand for 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. The 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 25µl 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 sequencing

RNA-Seq 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 the six individual samples were sequenced pre and post

lesioning; thus, resulting in a total of 12 samples. The 12 samples were multiplexed in two lanes of a flow-cell, resulting in between 25 and 34 million reads per sample. The read quality was assessed using the FastQC software (Andrew S. 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 S et al. 2015). Expression normalization and differential gene expression calculations were performed in edgeR (release 2.14) (Robinson & Oshlack, 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 methods and as modified by Storey (Benjamini & Yekutieli, 2005) providing a false discovery rate "q-value" for each differentially-expressed gene. Genes with an absolute fold difference ≥ 2 and $q \leq 0.05$ were considered statistically significant.

RNA-seq Analysis

RNA-seq data were organized in a Microsoft Excel table for subsequent analyses. These normalized read counts (counts per million- CPM) were used to calculate fold-change between the pre-lesion and post-lesion samples, and the Log base 2 of fold change was used for further analysis. Correlation matrices between different differentially expressed transcription factors, and the respective genes were generated in JMP Pro 14 software. AmiGO Gene Ontology (GO) annotations (http://www.geneontology.org/) for pathways of interest were cross-referenced with significantly differentially expressed genes, yielding a list of differentially expressed genes related to each GO annotation's respective biological function. The GO annotations chosen to be visualized in heat maps were selected based on their relevance to peripheral nerve repair.16 Statistical criteria of q <= 0.05 and |FC|>= 2 were selected, yielding a total of 3,641 differentially expressed genes included in this analysis. Heat maps of the qualifying genes were generated using JMP Pro 14 (SAS). When applicable, hierarchical clustering was performed using Ward's method in JMP.

Animal Care

All animal procedures were approved by the IACUC of the University of Kentucky and were performed in accordance with the NIH guidelines for the care and use of laboratory animals. Twenty-three adult male athymic nude rats (weigh 100-450 g; Charles River Laboratories, Chicago, IL) were housed in micro-isolated cages and kept in a sterile room with a 12-hr light/dark cycle. Autoclaved food and water were available ad libitum.

Cages and bedding were changed every three days. Clinical observations, as well as health and mortality records, were noted.

Implantation of Human Peripheral Neve in Neuro-Avatar

Young (4-8 weeks old) athymic nude rats (NIH-Foxn1rnu) were anesthetized under isoflurane and received implants of human nerve fascicles into the dorsal striatum. A total of 16 animals received nerve grafts (8 of stage I and 8 of stage II nerve fascicles). Half of each animal group was randomly selected for brain collection and histological analysis at two weeks, while the other half were euthanized at 6-month post-implantation. One mm3 of human nerve fascicle was delivered over 4 minutes using a 23-gauge adjusted needle with a personal style and directed at the following stereotaxic coordinate within the striatum: AP: +1.0mm, ML: -3.0 mm, DV: -5.0 mm. After implant deployment, a 4-minute wait period was maintained to allow attachment of the implant to the host brain tissue, the skin wound was stapled, and the animal was allowed to recover from anesthesia.

Histological Procedures

At the endpoints (two weeks and six months) post-transplantation, animals were deeply anesthetized using a single dose of pentobarbital (100 mg/kg IP) and transcardially perfused with 250 mL of normal cold saline followed by 180 mL of 4% Paraformaldehyde (PFA). The brain was extracted and postfixed with 4% PFA for at least 24 hours. Following post-fixation, the implantation site was identified and cut into a 1 cm section in brain mold and cryoprotected in 30% sucrose for three days. Cryopreserved brains were cut coronally into 40 µm thick sections for staining.

Immunostaining

To identify the transplanted human peripheral nerve cells and examine their interaction with the host CNS, immunostaining with antibodies against Human Nuclear Antigen (HNA) was employed. Tissue series from the grafted brain sections were subjected to the Anti-HNA antibody together with DAPI (4′,6-diamidino-2-phenylindole). The primary antibody used was Mouse Anti-human HNA (1:500; Catalog#: ab191181, Abcam, Cambridge, MA). The fluorescent secondary antibody was Alexa conjugated goat Anti-mouse IgG (1:500; Catalog#: ab150113, Abcam, Cambridge, MA). To ensure the specificity of observed immunostaining, both positive and negative controls were included during staining. Negative controls consisted of the incubation of the primary or secondary antibodies alone, in addition to a non-grafted and intact rodent brain collected after six months of the experiment. Positive Controls consisted of incubation of sections of human sural nerve collected during both stage I and stage II surgeries. The brain sections were transferred from their wells and washed once with PBS. The tissue was permeabilized using 0.2% Triton X-100 for 5 minutes and then washed with PBS once. The slides were blocked with 5% goat serum, 1%Triton X-100 for 1 hour. Afterward, the slides were incubated with the primary antibody in PBS, 0.1% goat serum overnight at 4'C. The next day, the slides were washed with PBS, 3x5 minutes, and incubated with fluorescence-conjugated secondary antibody in PBS, 0.1% normal serum for 1-2 hours at RT in the dark. Finally, the slides were rinsed in PBS, mounted on coverslips, and examined under a fluorescent microscope.

Magnetic Resonance Spectroscopy

Magnetic Resonance Spectroscopy is possible on any nucleus producing a magnetic moment. The metabolically active proton nuclei (1H) is the most sensitive for NMR, and it allows us to detect a large number of important amino acids and metabolic pathways' end products like N-acetyl aspartate (NAA) and Creatine (Cr) /phosphocreatine. In healthy brain tissue, the most prevalent resonance originates from the methyl group of NAA at 2.02 ppm (Moffett et al., 2007). The chemical shifts are referenced in regards to that of 3-(trimethylsilyl)1-propane sulfonic acid at 0.00 ppm. NAA is exclusively localized in the CNS as well as the PNS. NAA has been used as a marker for viable neurons as it is only found in neurons, and its concentration decreases in the neurodegenerative diseases which affect the CNS (Schuff et al., 2006). The NAA concentration is yet not uniform and varies between gray matter (8-11 mM) and white matter (6-9 mM) (Moffett, Arun, Ariyannur, & Namboodiri, 2013; Moffett & Namboodiri, 1995). The methyl and methylene protons of creatine and phosphocreatine resonate at 3.03 ppm and 3.93 ppm. Since the sum of both forms, total creatine (tCr), is constant in adulthood, the later can be used as a suitable in vivo concentration reference. Glutamate and Glutamine peaks were determined at 2.1-2.5 ppm and 3.7 ppm, respectively. Brain morphology and metabolism were assessed using T2WI magnetic resonance and imaging MRI and MRS (7-tesla Horizontal bore magnet; Bruker/Siemens scanner) at three months post-implantation in Neuro-Avatar animals. Two animals were used for MRS studies at three months post-implantation. The first one was a neuro-avatar athymic rat that has received a stage II human nerve implants into the right dorsal striatum. The second animal was a sham animal where a cannula with PBS, and no nerve implant was inserted into its striatum. Rats were deeply anesthetized by 2%

isoflurane. The heads of the rats were fixed in a body retainer, the respiratory rate was monitored, and the body temperature was maintained at 37 oC with a body heating pad. The spectra were acquired at TR/TE/NS=2500/3/200 for ipsilateral as well as the contralateral striatum with the voxel (volume = 3×2×3 mm3) placed just next to the graft trajectory. Ipsilateral metabolic concentrations were normalized to the contralateral (intact) ones before comparing the two treatment groups.

Thesis Outline

The following chapters investigate the use of peripheral nerve implants in combination with DBS to alter the disease progression of PD. Peripheral nerves have a remarkable potential for repair and regeneration in response to an injury compared to the CNS (Gu et al. 2014, Sheng Yi et al., 2018). Schwann cells, within the distal stump of the injured nerve, play a major role in promoting and maintaining axons regeneration from the proximal stump. Those cells release neurotrophic factors and cross-talk with the basal lamina, fibroblasts, and immune cells to ensure adequate regeneration. In our first study, we analyzed, using high throughput RNAseq analysis, the impact of the conditioning transectional injury on human sural nerve's molecular pathways at 14 days following the injury. Our main goal was to validate if the nerve implants collected from participants with PD will contain the repair molecules that are required to induce neuroprotection and axons regeneration of the degenerating nigrostriatal system.

In Chapter Four, we studied the viability and trophic effect of the nerve implants after being implanted in the CNS. Pre-lesion and post-lesion sural nerve implants were collected from participants undergoing DBS Plus and implanted into the dorsal striatum of

athymic and immunodeficient rats. Immunostaining for human Schwann cells at six months post-implantation (longest period examined) demonstrated the ability of those cells to tolerate the grafting procedure and to survive the central nervous environment. Furthermore, stage II (14 days post-lesioning) nerve implants contained a higher load of cells and were able to survive better up to 6 months post-implantation when compared to Stage I (pre-lesion) nerve implants. Additionally, the brain tissue reaction to the human nerve implants was evaluated using in vivo magnetic resonance spectroscopy

In Chapter Five, we examined the safety and efficacy of combining DBS surgery with autologous nerve implant stereotactically targeted to the substantia nigra of human subjects with PD. In this study, two treatment groups of participants were studied. The first is a group of 16 human subjects who received a single and unilateral dose of nerve implant while in the second group, nine human subjects with PD have received a double-unilateral dose of nerve implants. The participants were followed up to 24 months after the surgery. The adverse events reported by both study groups were similar to those of DBS surgery. On the other hand, the clinical motor assessments indicated an improvement in UPDRS III scores up to two-years after-grafting. The post-hoc analysis demonstrated that the improvement in the parkinsonian signs was mostly lateralized to the side contralateral to the implanted substantia nigra. Finally, we discuss how the combination of Deep Brain Stimulation Surgery and autologous peripheral nerve implantation could be a safe and promising intervention to alter the disease progression in Parkinson's disease.

CHAPTER THREE: TRANSCRIPTOME ANALYSIS OF IN VIVO PREDEGENERATED PERIPHERAL NERVE

Abstract

The development of regenerative therapies for central nervous system diseases can likely benefit from an understanding of the peripheral nervous system repair process, particularly in identifying potential gene pathways involved in human nerve repair. This study employed RNA sequencing technology (RNA-seq) to analyze the whole transcriptome profile of the human peripheral nerve in response to an injury. The distal sural nerve was exposed, wholly transected, and a 1-2 cm section of nerve fascicles were collected for RNA-seq from six participants with Parkinson's disease, ranging in age between 53 and 70 years old. Two weeks after the initial injury, another section of the nerve fascicles of the distal and pre-degenerated stump of the nerve were dissected and processed for RNA-seq studies. An initial analysis between the pre-lesion status and the post-injury gene expression revealed 3,641 genes that were significantly differentially expressed. Our results provide evidence for the trans-differentiation process that occurred by the end of the 2-weeks post-injury. Gene ontology and hierarchical clustering were used to identify the major signaling pathways affected by the injury. In contrast to previous non-clinical studies, substantial changes were observed in molecular pathways related to glial cell proliferation, neurotrophic factors release, Axonogenesis, neural synaptic plasticity, and PD-causative genes. The results of our current study provide new insights regarding

the essential interactions of different molecular pathways that drive neuronal repair and axonal regeneration in humans.

Introduction

Peripheral nerves have a spectacular capacity for regeneration in comparison to the CNS (Scheib & Höke, 2013). Regardless of the type of the injury, the distal stump of the peripheral nerve initiates an orchestrated pattern of complex cellular and molecular events leading to proximal neuron survival and axon regeneration towards the original target. This series of events, which have been known as Wallerian degeneration, take place over mainly two stages (K. Jessen & Mirsky, 2016). The early-stage happens within the first five days, and the later stage occurs 1-2 weeks after injury. During the early stage, the axonal debris and myelin are cleared by SCs and the invading immune cells followed by morphological changes of the SCs to facilitate their migration and formation of the regenerative tracks (Bands of Büngner). Promotion of the Axonogenesis and neuronal repair occurs mainly during the later stage. This regenerative capacity of the peripheral nerve highly depends on genetic changes driving adequate SCs-axon interaction, appropriate immune cells' response, and suitable release of neurokines, chemokines, and growth factors. Trophic factors such as GDNF, NGF, the FGFs, neurotrophin-3 (NT-3), BDNF, PDGF, interleukin-1, and apolipoprotein E, facilitate SC and neuronal survival, axonal elongation as well as an anti-inflammatory response for (review see (Li et al., 2020)). Moreover, the adequate delivery of cytokines, including tumor necrosis factor α (TNFα), tumor growth factor β (TGFβ) and leukemia inhibitory factor (LIF) is essential to avert exaggerated inflammatory

cascades and production of chondroitin sulfate proteoglycans (CSPGs) which can impede neuronal survival and axonal outgrowth (K. Jessen & Mirsky, 2016).

Identification of the genes involved in peripheral nerve regeneration and how they interact is crucial to exploring strategies that enhance the neural protection, regeneration, and repair processes. On the other hand, promoting successful regeneration in CNS has been difficult in neurodegenerative diseases, traumatic brain injury, stroke, epilepsy, and PD. Yet, understanding the gene expression changes that drive effective neural repair within the PNS may also help in identifying new therapeutic targets or methods that could enhance CNS neural regeneration.

Whole-transcriptome profiling of gene expression in response to peripheral nerve injury can now be feasibly studied using RNA sequencing (RNA-seq) technology. RNA-seq combines molecular biology approaches of RNA amplification with bioinformatics tools 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 reviewing the applications of RNA-seq see (Han, Vickers, Samuels, & Guo, 2015)). The study reported herein presents the analysis of the transcriptome profile of *in vivo* predegenerated sural nerve tissue in patients with PD and to help evaluate how a conditioning injury of peripheral nerve tissue can induce pro-regenerative changes.

Our study was conducted in conjunction with an ongoing clinical trial (clinicaltrials.gov:NCT#02369003;https://clinicaltrials.gov/ct2/show/NCT02369003?term=NCT02369003&cond=Parkinson+Disease&draw=2&rank=1), the “DBS Plus” trial, which involves grafting of autologous peripheral nerve fascicles in patients with

Parkinson's disease (PD) at the time of deep brain stimulation (DBS) surgery. To our knowledge, this study is the first of its type studying and analyzing the human peripheral nerve genetics in response to a transection injury within the same human subjects. The trial involved a collection of two samples of the sural nerve from the same participants at two distinct time points. The first sample, referred to as the "pre-lesion" sample, was collected from the participant's sural nerve during Stage I of the surgery, which involved DBS hardware implantation. The second sample, referred to as the "post-lesion" sample, was taken two weeks later from the distal end of the same nerve during Stage II of the surgery, which is the stage when the DBS leads are positioned into the STN or GPi nuclei. This two-stage approach corresponded with the two stages of DBS surgery and was designed to induce pro-regenerative changes in the peripheral nerve following an injury (van Horne et al., 2017).

In our current study, we hypothesize that pre-conditioned implants harvested from the distal stump of the sural nerve in participants undergoing the stage II of DBS Plus, 14 days post-lesioning, will hold a transcriptional profile that promotes nerve regeneration during the later stage of Wallerian degeneration.

Research Subjects

The nerve samples were collected from human participants with PD who electively participated in the clinical trial testing the safety and feasibility of peripheral nerve implants to the CNS for the treatment of PD. The nerves samples of 6 participants, (two females, four males) aged 63.8 ± 6.9 SD years (range 53-70 years), were used for RNA-seq analysis.

The study was approved by the University of Kentucky institutional review board, and informed consent was obtained from all study participants.

RNA Collection and Sequencing

RNA collection and sequencing were done as described in the Methods section of Chapter Two. In a repeated measure design, mRNA from the six individual samples were sequenced pre and post lesioning; thus, resulting in a total of 12 samples. These normalized read counts (counts per million- CPM) were used to calculate fold-change between the pre-lesion and post-lesion samples, and the log base 2 of fold change (log2FC) was used for further analysis. Genes with an absolute fold difference ≥ 2 and $q \leq 0.05$ were considered statistically significant, yielding a total of 3,641 differentially expressed genes included in this analysis. AmiGO Gene Ontology (GO) annotations (http://www.geneontology.org/) for terms of interest were cross-referenced with significantly differentially expressed genes, yielding a list of differentially expressed genes related to each GO annotation's respective biological function. Heat maps of the qualifying genes were generated using JMP Pro 14. Hierarchical clustering was performed using Ward's method in JMP.

Results

Nerve Tissue Collection

The mass and freezing delay time (time from when the nerve was harvested from the participant to when it was snap-frozen in dry ice) were calculated for the nerve tissue collected for RNAseq from Stage I and Stage II (Table 3.1). Freezing Time Delay was

significantly longer (p-value= 0.0022, Figure 3) at Stage II (two weeks after injury) (59 ± 25 min; Mean ± SD) compared to Stage I (Intact state) (18 ± 5 min). This difference was a result of the longer technical surgical procedures needed for the clinical trial during Stage II of the DBS surgery. The mitigation of Mass Effect during the RNA Access library prep is accomplished in multiple ways through normalizations incorporated at multiple steps through the entire process of library prep and sequencing.

Glial Cell Proliferation

Figure 3.2 shows all significantly differentially expressed gene transcripts annotated with the GO term "Positive Regulation of Glial Cell Proliferation" (GO:0060252) in Homosapiens. Out of 19 unique genes with this GO annotation, 6 of those genes (*IL1B, LYN, E2F1, PRKCH, MYB, IL6*) were significantly differentially expressed while only one gene (*PLAG1*) appeared to be less expressed 14 days after the initial nerve transection injury. On the other hand, 7 genes (I*DH2, CERS2, SOX10, DICER1, PTN, HES1, ADCYAP1R1*) out of 16 genes associated with "Negative regulation of glial cell proliferation" (GO:0060253) were differentially significantly expressed.

Growth Factor Activity

Figure 3.3 shows all significantly differentially expressed ($q < 0.05$, $|FC| > 2$) gene transcripts annotated with the Gene Ontology (GO) term "Growth Factor Activity" (GO:0008083). Out of 166 unique genes with this GO annotation, 43 (25.9%) were significantly differentially expressed two weeks after the initial injury (collected during

Stage II). Twenty-six of those genes were enriched in the predegenerated nerve tissue in comparison to normal levels while 17 genes were less abundant

Regulation of Axons Regeneration and Extension

Out of 96 genes associated with gene ontologies: "Regulation of Axon Extension"-(GO: 0050772) and "Axon Regeneration" (GO: 0045773), 12 genes were significantly upregulated in the distal nerve tissue after injury in comparison to their initial transcript levels which were sequenced and measured in the sural neve samples collected during stage I surgery (Figure 3.4). The expressed genes included *PLXNC1, SCARF1, HGF, BDNF, NRP1, FKBP1A, GRN, LIMK1, FN1, HCLS1, TRPV2*, and *TNFRSF12A*. On the other hand, 12 genes were transcribed at significantly lower levels two weeks after the initial conditioning injury to the sural nerve during stage I surgery.

Regulation of Neural Synaptic Plasticity

All five human genes associated with the gene ontology term "Positive Regulation of Neural Synaptic Plasticity" (GO:0048170) demonstrated higher transcript levels in the distal nerve tissue harvested during stage II surgery in comparison to that from stage I surgery (Figure 3.5). Of particular interest, *SHANK3* and *KIT* genes were significantly expressed with FCs levels of 2.44 (*p-value* = 2.56183E-12) and 2.14 (*p-value* = 7.16588E-07) respectively.

Associated Transcription Regulatory Factors

624 unique transcription factors (TFs) with regulatory functions related to the 26 growth activity-associated genes, which were significantly upregulated (figure 3.2), were identified based on the Genecards database (a repository of a database for human gene and proteins with links to other databases https://www.genecards.org/). 267 TFs were differentially and significantly between stage I and Stage II with p or q values ≤ 0.05 and $|FC| \geq 2$. Yet, only 38 TFs (figure 3.3) were differentially expressed and with an expression profile that is significantly correlated ($|\text{Pearson correlation}| \geq 0.8$) to the expression trends of the growth factors. The correlation matrixes were calculated and plotted by JMP Pro14 software. Out of the total 38 TFs, 31 TFs were significantly expressed and had a strong positive correlation of expression with at least one of 21 Growth factors. On the other hand, 22 TFs were significantly downregulated after injury, while their expression profile had a significant and strong negative correlation with at least one out of 18 Growth factors (figure 3.6). Of particular interest was the downregulation of AHR transcription factor (FC= -2.023) while its downregulation was significantly correlated to the upregulation of GDNF (FC=3.54, Correlation = -0.98, and p-value = 0.0006).

Transcriptome Profile of PD-Related Genes

Mutations in more than 20 genes have been identified and linked to Parkinson's disease. The hallmark pathology of PD is the deposition of Lewy bodies within the dopaminergic neurons. Lewy bodies are protein aggregates consisting of different proteins, including α-synuclein (encoded by the *SNCA* gene). PD is one of the most common

synucleinopathies; other synucleinopathies include multiple system atrophy and Lewy body dementia, which clinically overlap with PD (Cornelis Blauwendraat et al. , 2020). We were interested in studying the expression of *SNCA* and other PD-related genes in the peripheral nervous system of patients with PD and how their transcriptomic profile would change in response to an injury. Differential expression analysis of RNA transcripts-count per million (CPM) values using $p < 0.05$, FDR < 0.05, and $|Log2FC| \geq 1$ as cutoff criteria produced a total of 8 transcripts differentially expressed between intact nerve tissue (stage I) and post-injury profile (stage II). Among these differentially expressed genes, seven transcripts (*SNCA, LRRK2, PLA2G6, DNAJC6, MAPT, SIPA1L2,* and *PDZRN4*) were downregulated and only one (*GCH1*) was upregulated two weeks after nerve injury (figure 3.7a, 3.7b). Of particular note, *SNCA* gene transcript was significantly downregulated (Log2FC = -2.5, p-value=1.5E-18, FDR=2.49E-17).

To further study the molecular signaling that might have played a role in the downregulation of those PD-related genes, we identified through the Reactome database a total of 103 candidate transcription factors (TFs). Those TFs were either directly or indirectly correlated to the signaling pathways involved in the expression of PD-related genes. Only 25% of those TFs were differentially expressed after injury, and their transcriptomic profiles were significantly correlated to the expression of the PD-related genes with a cutoff threshold of $|\text{Pearson's correlation}| \geq 0.8$, and p-value < 0.05 (figure 3.8). 7 TFs (SMARCA4, TFDP1, XRCC5, ZNF792, MAZ, SOX13, and DRAP1) were identified as potential repressors of 5 PD-related genes (*SNCA, MCC1, LRRK2, PARK2,* and *DNAJC6*). The expressions of those TFs were upregulated and significantly correlated to the downregulation of PD genes' expression. Two noteworthy potential

repressors for SNCA gene expression were identified: ZNF792 and DRAP1. On the other hand, 17 potential enhancers were identified and positively correlated to the expression of 7 PD-related genes (*SNCA, LRRK2, PDZRN4, DNAJC6, MCCC1, PARK2,* and *PINK1*).

Discussion

In this study, we present the relevant transcriptome of human sural nerve tissue and characterize transcriptional changes at 14 days following a transectional injury. We provide evidence that the transection lesion paradigm used in the DBS Plus surgeries induces phenotypic changes in the peripheral nerve tissue consistent with the peripheral nerve repair response: immune cell infiltration plus cell proliferation, Wallerian degeneration of axons, and up-regulation of growth factors production (Arthur-Farraj et al., 2012; Arthur-Farraj et al., 2017; Cattin & Lloyd, 2016; K. R. Jessen & Arthur-Farraj, 2019). Our experimental design included harvesting sural nerve tissue from 6 human subjects who were undergoing DBS surgery for PD. The mRNA from a total of 12 individual samples were sequenced and measured pre and post lesioning using RNA Sequencing technique. Normalized read counts (CPM) were used to calculate fold-change between the pre-lesion and post-lesion samples, and Log2FC was used for further analysis. Genes with an absolute fold difference ≥ 2, $p \leq 0.05$, and FDR ≤ 0.05 were considered statistically significant, yielding a total of 3,641 differentially expressed genes. AmiGO Gene Ontology (GO) annotations for specific signaling pathways were cross-referenced with significantly differentially expressed genes, yielding a list of differentially expressed genes related to each GO annotation's respective biological function.

A large number of differentially expressed genes were identified, most of which were involved in growth factor activity, glial cell proliferation, axon regeneration and extension, and positive regulation of neural synaptic plasticity, based on Gene Ontology. To better refine the results of our RNA-seq analysis to a more "biologically relevant" dataset, we decided to limit our observations to the genes whose transcript levels exceeded a fold change threshold of |FC|>2. However, this convention might have excluded genes that were biologically relevant at lower fold changes. For example, the transcription of *NF2* gene, a marker of Schwann Cell proliferation, was statistically significantly increased (p-value =0.0192). However, the fold change of transcript levels was less than 2, so it was not included in the visualized data. Likewise, Mesencephalic Astrocyte Derived Neurotrophic Factor (MANF) transcripts level was significantly higher post-injury (p-value= 3.03E-07), yet its FC was 1.754 (data not shown). MANF plays a vital role in different reparative phases during the neuronal regeneration processes, and MANF therapeutics are expected to enter clinical trials (Sousa-Victor, Jasper, & Neves, 2018). Furthermore, we focused in this paper only on transcripts that were differentially expressed between pre- and post-lesion samples while recognizing that some genes could be highly expressed in both stages, but not necessarily differentially. That could be one limitation of this broad analysis approach, and in the specific case of NF2 levels in this tissue merits further study.

The gene cluster of the growth factor terms showed multiple differentially expressed genes, with the majority being increased. One increased gene of note is GDNF, which is neuroprotective and neurorestorative of dopaminergic neurons and has been tried as a therapeutic intervention for PD in preclinical and clinical studies (Quintino et al., 2019; Slevin et al., 2007; Whone et al., 2019). All post-lesion nerve samples, except one,

demonstrated an upregulation of GDNF transcription. Only the nerve sample collected from participant number 2 showed a lower number of GDNF transcripts after injury (Figure 3.3). The significant longer freezing time delay for this sample (Table 1) might have negatively affected GDNF-mRNA stability and its relevant count during RNAseq processing. Multiple interleukins were also upregulated, which, in addition to being cytokines, play a role in neurogenesis (Borsini, Zunszain, Thuret, & Pariante, 2015).For example, the levels of gene transcript for IL-6, which has been described as neuroprotective against focal brain injury, were increased in response to the nerve injury (Penkowa et al., 2003). The growth factor activity genes, which were decreased (for example, CDNF) at two weeks post-injury, are also of interest and may indicate the complexity of the neuronal repair process in regard to the changes of individual growth factors over time in response to nerve injury. That was evident in the work of Lin et al., 2019 as PPAR, PI3K-Akt, and chemokine signaling pathways were dominant in early Wallerian degeneration (Lin, Xie, Zhou, Yin, & Lin, 2019). Whereas at the later stage, the main signaling pathways were ErbB, tumor necrosis factor, AMPK, MAPK, PPAR, and Wnt.

To our knowledge, this is the first report to publish data about the transcriptional regulators and the enriched growth factors after human peripheral nerve injury. Over 600 transcription factors potentially related to the expression of upregulated growth factors were generated through Reactome libraries (http://www.reactome.org/). Only 38 transcription regulators were differentially expressed and significantly correlated in the upregulation of 24 growth factors, which were upregulated (figure 3.6). These findings substantially add to our understanding of the signaling pathways which intervene in enhancing or repressing the synthesis of several essential growth factors. For example, the

aryl hydrocarbon receptor (AHR) may act as a potential repressor of GDNF expression. Previous studies have shown that knocking out AHR upregulated vascular endothelial growth factor (VEGF) expression and markedly enhanced the ischemia-induced angiogenesis (Ichihara et al., 2019). Hence, inhibiting AHR may enhance GDNF synthesis after injury.

We have previously demonstrated the upregulation of several genetic pathways involved in trans-differentiation and reprogramming of Schwann cells into "repair" cells in response to nerve injury to humans (Welleford et al., 2019). Nonetheless, the current transcriptome analysis adds to our understanding of the reprogramming process of those cells through mapping the expression of essential regulatory genes that are clustered to the proliferation of those glial cells (figure 3.2). The transcript levels of 8 of those genes were enriched (*E2F1, IL1B, IL6, LYN*, *MYB, PRKCH, IDH2*, and *ADCYAP1R1*) while the expression of only 2 genes (*SOX10, PLAG1*) was significantly downregulated at 2 weeks post-injury. The later genes were clustered as negative regulators of Schwann cells proliferation (Fujiwara et al., 2014); thus, repressing their expression may have helped to induce the proliferation of Schwann cells. Furthermore, several genes were significantly expressed and involved in axonogenesis, axons regeneration and extensions, and enhancing neural synaptic plasticity such as *PLXNC1 (Chabrat et al., 2017), GRN* (Rosen et al., 2011), and *SHANK3* (Huang et al., 2019) (figure 3.4, figure 3.5).

PD is a progressive neurodegenerative disease accompanied by the degeneration of dopaminergic neurons within the substantia nigra of the midbrain. Approximately 10% of the PD cases are familial with a spectrum of PD-causative genes that have been identified (Ferreira & Massano, 2017). Through this study, we analyzed the transcriptional regulation

of the major PD-related mendelian genes and the PD-risk loci (Hernandez, Reed, & Singleton, 2016). A total of 8 PD-related genes (*SNCA, DNAJC6, PLA2G6, LRRK2, PDZRN4, SIPA1L2, MAPT,* and *GCH1*) were differentially expressed. Most importantly was the consistent downregulation of alpha-synuclein (*SNCA*) gene among the 6 subjects (FC = -5.56, p =1.5 E-18, FDR = 2.5E-17). At least 30 mutations in the *SNCA* gene have been found to cause PD. Two main types of alterations of the *SNCA* gene are related to PD (Petrucci, Ginevrino, & Valente, 2016). In one mutation, the amino acid alanine is replaced with threonine at protein position 53 or with the amino acid proline at position 30. These mutations cause the alpha-synuclein protein to be misfolded and hazardously aggregates in the cells. In the other type of alteration, *SNCA* gene is inappropriately duplicated or triplicated, leading to an excess of alpha-synuclein deposition in patients with PD (Zafar et al., 2018). It is unclear how SCNA gene causes PD, but alpha-synuclein proteins cluster together to form Lewy bodies leading to selective death or impairment of neurons that produce dopamine. There is evidence that inhibition of α-synuclein aggregation can improve the survival of neurons after injury (Teil et al., 2020). To our knowledge, eight transcription factors have been demonstrated to be involved in regulating *SNCA* transcription. GATA2 and p53 promote SNCA transcription; PARP1, EMX, C/EBPβ, and NKX6/1 repress it while ZSCAN21 and ZFN210 can have both effects; for review see (Piper, Sastre, & Schüle, 2018). To further explore the signaling pathways involved in the expression *SNCA* and the other PD-related gene in Stage II nerve samples, hundreds of unique regulatory transcription factors were functionally mapped to be Parkinson's related genes through Reactome pathway database. 20 transcription factors were differentially expressed and significantly correlated to the PD-related genes (figure 3.8). 8 transcription

factors (EGR2, NBN, ZXDB, NRF1, ZNF792, ZBTB20, ZFHX2, and DRAP1) were significantly correlated to the downregulation of *SNCA* expression. In particular, the upregulation of ZNF792 (FC = 2.0, p =4.6E-6, FDR =1.8E-5) was significantly correlated (r = -0.837, p = 0.037) to the repression of *SNCA* gene in stage II. This finding strongly points to ZNF792 as a potential repressor of *SNCA* gene. Future studies using gene therapy to target such repressors of SNCA may help reduce alpha-synuclein aggregation in the neurons (Valente et al., 2020).

Conclusion

In summary, this study provides evidence that the sural nerve autologous implants, which are harvested from PD participants two weeks after a conditioning injury, embrace a repair phenotype consistent with a release of growth factors, a proliferation of Schwann cells, axons regeneration, and enhancement of neural synaptic plasticity. We have also demonstrated significant changes in several major PD-associated genes. We have identified novel transcription regulators of several PD-associated genes, like SNCA, which will expand our knowledge about the different molecular pathways involved in the regulation of PD-causative genes. We believe that this transcriptomic analysis will be of great benefit for future therapeutic strategies to reduce the levels of α-synuclein in PD and other synucleinopathies.

Table 3. 1 Mass and Freezing Time Delay of the nerve samples collected during Stage I vs. Stage II.

Participants	STAGE I SAMPLES		STAGE II SAMPLES	
	Mass (g)	**Freezing Time Delay (min)**	**Mass (g)**	**Freezing Time Delay (min)**
1	0.0205	16	0.0354	41
2	0.0158	28	0.0301	108
3	0.0254	20	0.0613	64
4	0.0256	14	0.0363	52
5	0.0294	14	0.0757	39
6	0.0197	17	0.0256	50
Mean ± SD	0.0227± 0.0049	18 ± 5	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 implantation into participants during Stage II.

Figure 3. 1 Lesion-Freezing delay of the nerve samples collected during Stage I and Stage II .

Freezing time delay was significantly longer (Paired two-tailed t-test: $t(5)$=4.863, p = 0.0046) for post-lesion nerve samples (59 ± 25 min; Mean ± SD) compared to pre-lesion ones (18 ± 5 min).

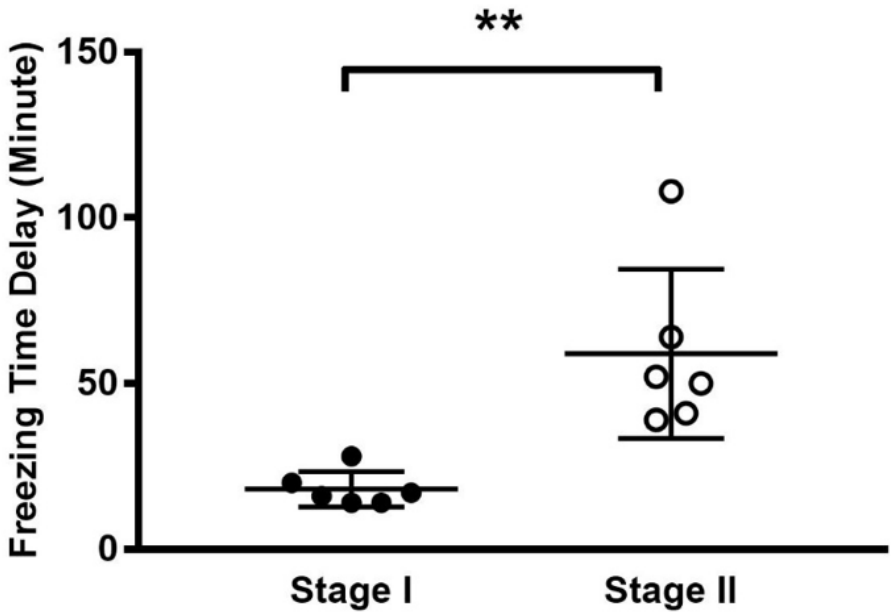


Figure 3. 2 Differentially expressed genes involved in regulating glial cell proliferation. Abundance of the transcripts levels of 8 genes annotated with the positive regulation of glial cell proliferation (GO:0060252). The two genes annotated (*SOX10*, *PLAG1*) with negative regulation of glial cell proliferation (GO:0060253) were downregulated transcript levels in stage II nerve tissue. Differential expression cutoff criteria were |log FC| >1, $p < 0.05$ and FDR <0.05).

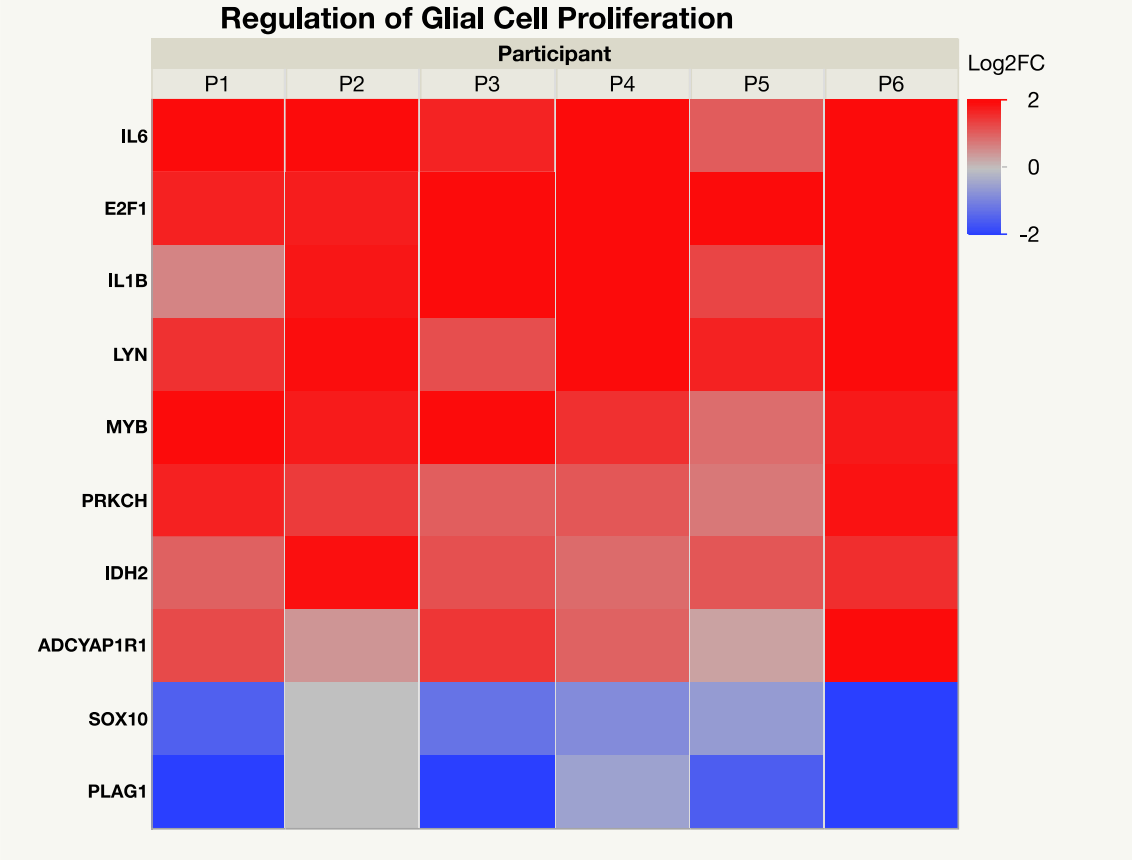


Figure 3. 3 Analysis of growth factors activity.

Differentially and significantly expressed growth factors defined under Growth Factor Activity according to gene ontology (GO:0008083). Differential expression cutoff criteria were |log FC| >1, p < 0.05 and FDR <0.05).

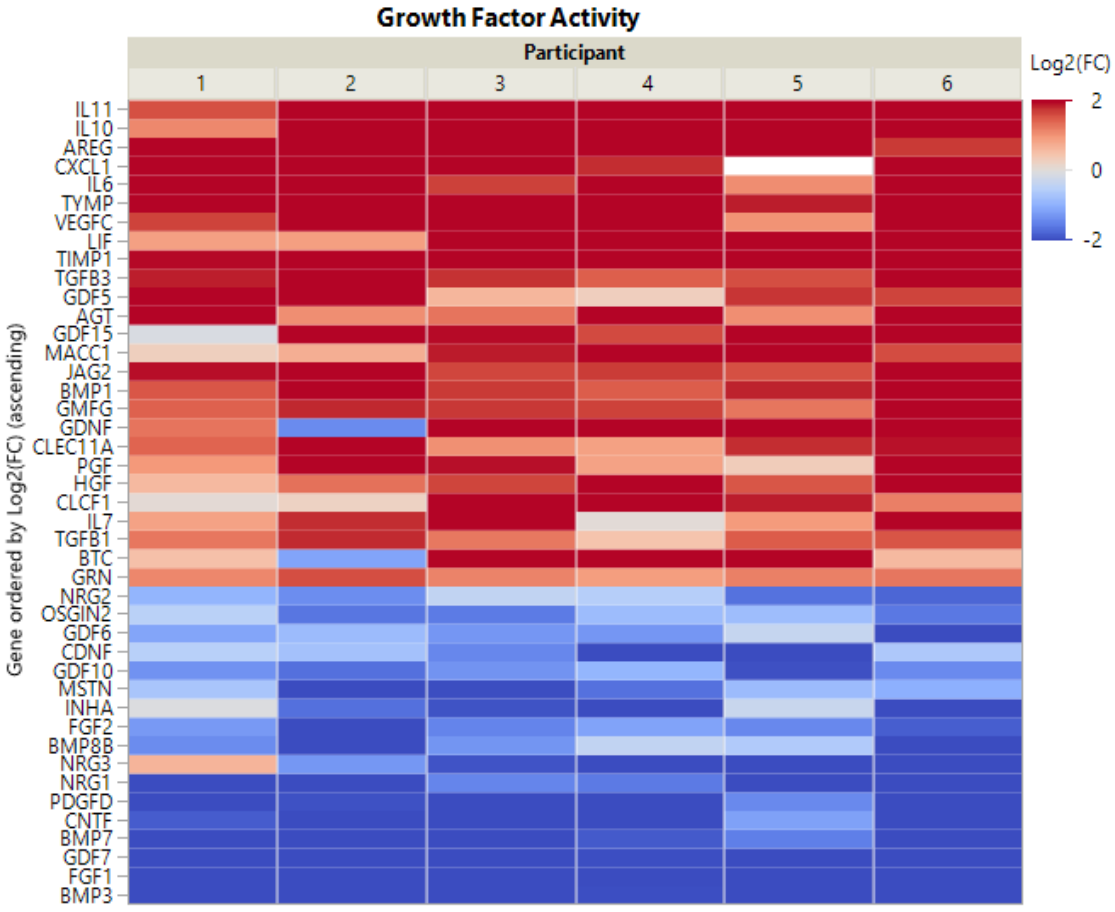


Figure 3. 4 Analysis of the key genes involved in the axonal repair.

Heat map obtained for the differentially-expressed genes that are annotated with the ontology terms: Regulation of Axons Regeneration (GO: 0045773) and Axons Extension (GO: 0050772). The transcript levels were obtained from the sural nerve tissue of 6 participants during stage II surgery and compared to that of stage I. The expression of main positive regulators of Axonogenesis. such as *PLXNC1* gene, was upregulated at two weeks after the initial nerve injury. Significant levels were determined at |Log FC| >1, $p < 0.05$ and FDR <0.05.

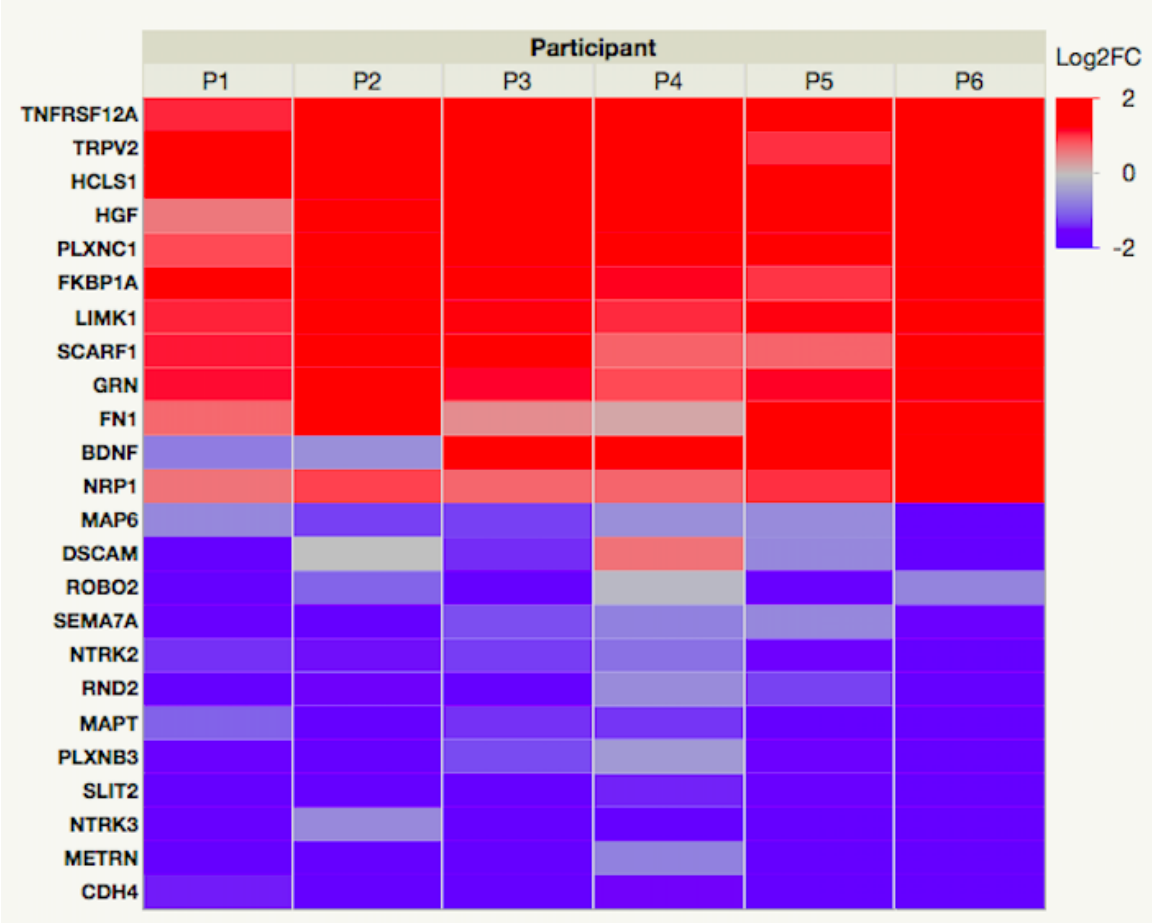


Figure 3. 5 Analysis of neural synaptic plasticity two weeks after sural nerve injury.

There was a significant abundance of the transcript levels of the genes (*SHANK3* and *KIT*) that are positive modulators of the long-term neural synaptic plasticity in nerve tissue obtained from all participants during stage II surgery. |Log FC| >1, $p < 0.05$ and FDR <0.05).

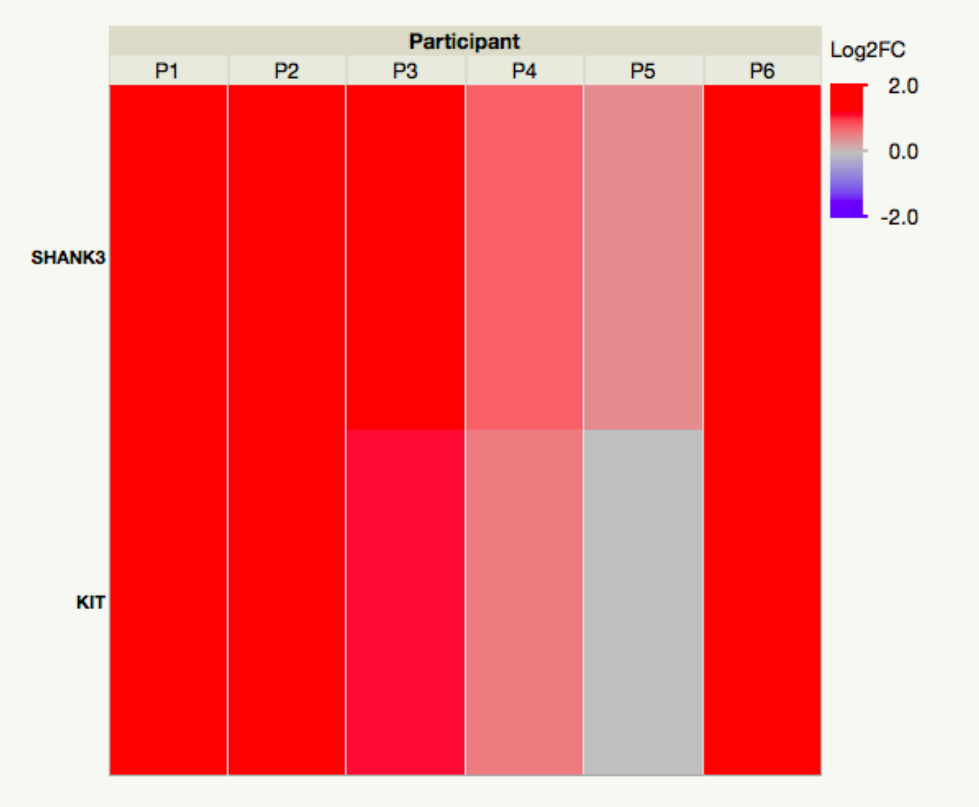


Figure 3. 6 Correlation matrix between significantly expressed TFs and growth factors in response to nerve injury

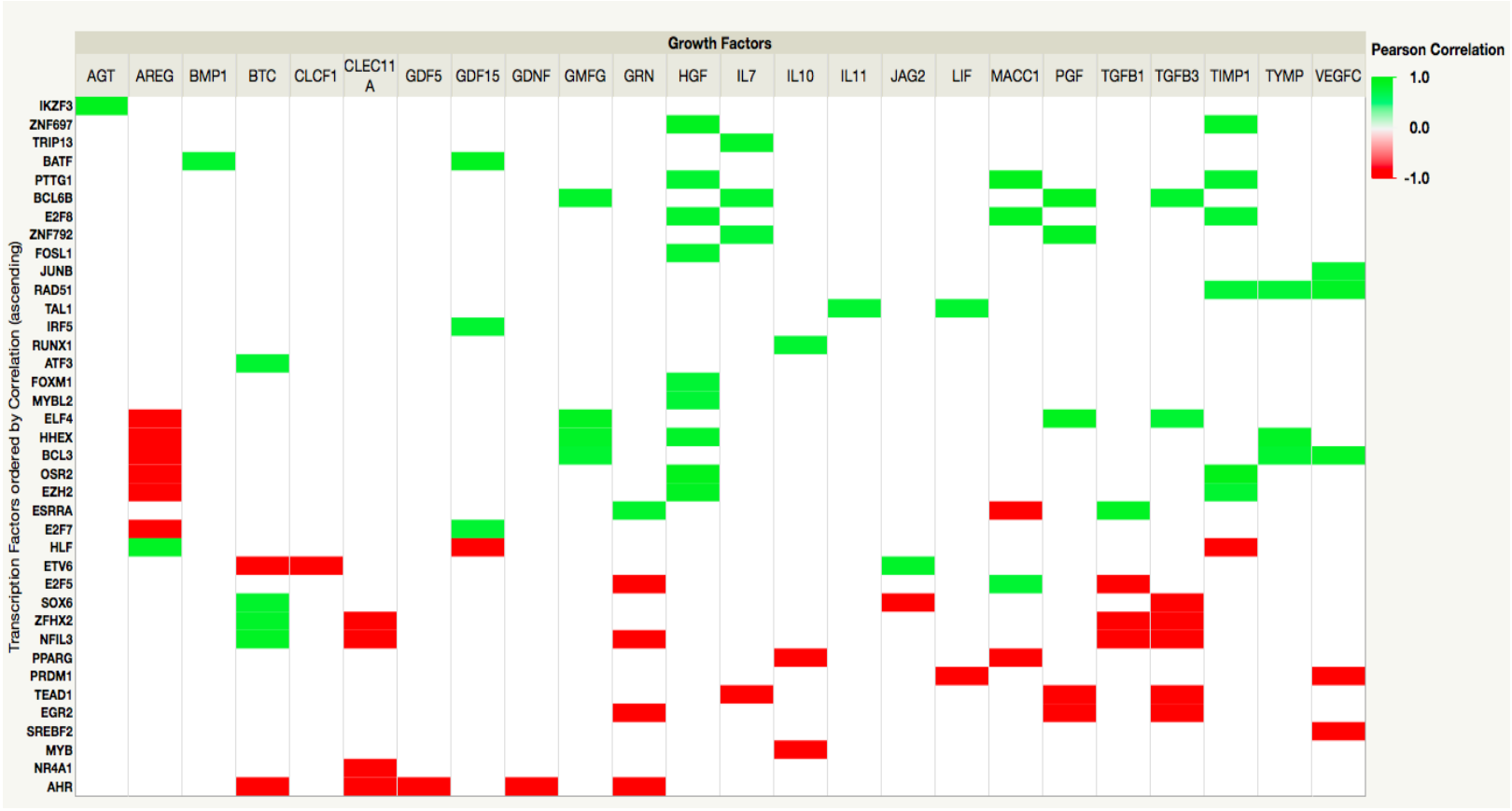
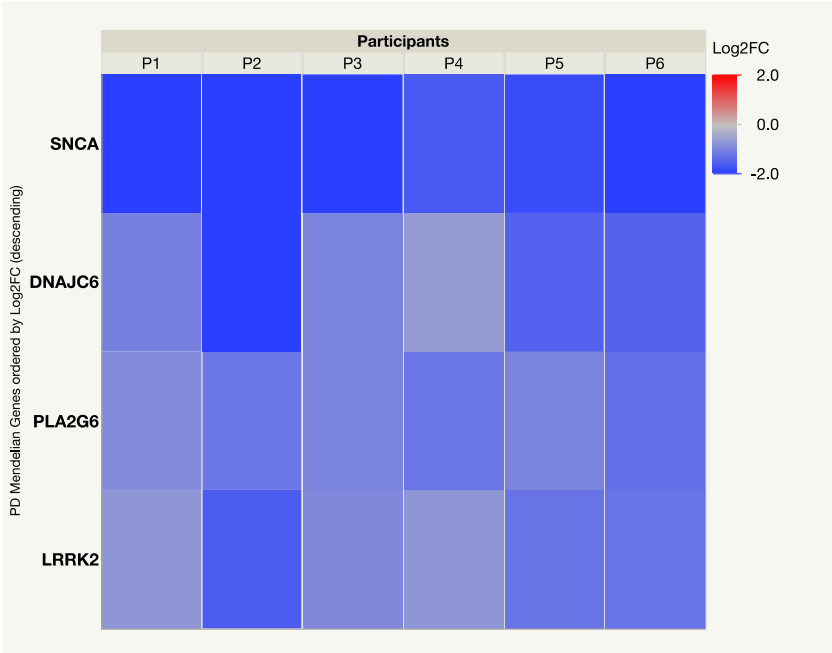


Figure 3. 7 Comparison of the transcripts' levels of differentially-expressed PD-related genes.

Heat maps obtained from RNA-Seq data of stage II sural nerve tissue and compared to that of stage I. The abscissa indicates the participants and ordinate indicates **A)** PD mendelian genes and **B)** PD-Risk loci that were significantly and differentially expressed with $|\log2FC| \geq 1$ and p-value < 0.05.

A)



B)

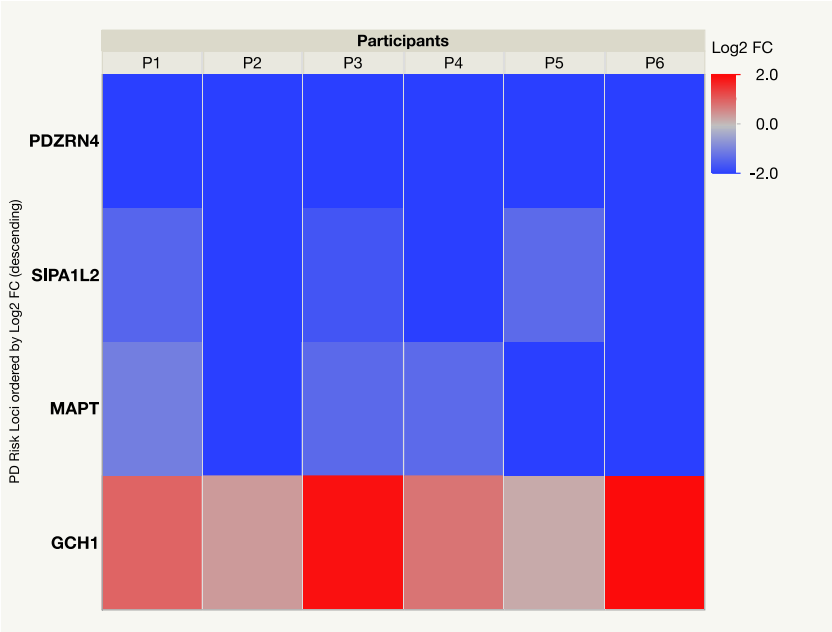


Figure 3. 8 Identification of transcriptional regulators of PD-related genes.

The expression pattern of the differentially-expressed transcription factors (TFs) during stage II was compared to that of PD-related genes. The Correlation matrix was mapped in JMP Pro14 with |Pearson's correlation| ≥ 0.8 and p-value < 0.05. Seven potential repressors were significantly upregulated in coordination with the subsequent downregulation of 5 PD-related genes, while 13 TFs were significantly downregulated in a strong correlation with the suppression of the PD-related genes.

CHAPTER FOUR: THE NEURO-AVATAR PROJECT

Abstract

The implantation of peripheral nerve tissue and cells provides anatomical and functional restoration in a variety of CNS injury models. To date, the reported results of implanting peripheral nerve cells within the brain environment have been limited to the usage of rodent peripheral nerve tissue. Transplanting human peripheral nerve fascicles in the dorsal striatum of rodents have never been examined experimentally. Herein, we describe the survival, biodistribution, and host responses to human peripheral nerve tissue implants, harvested from human donors according to the protocol developed for the Phase I DBS-Plus clinical trial. Repair human Schwann cells (huSCs) persisted within the injured rat dorsal striatum through 6 months after implantation and displayed restricted biodistribution within the implantation site. Besides, histological comparison between injury-naive and conditioned implants (14 days after injury) was also reported. Further, studying the bio-response of the brain tissue, using proton magnetic resonance spectroscopy, displayed signs of a positive influence of the PN implants on the integrity of the central neurons.

Introduction

Cell therapeutics have been one of the frontline approaches that aimed at promoting neuron replacements or repair in the central nervous system (CNS). The introduction of a

diversity of cell types, including transplantation of SCs, in the form of a peripheral nerve (PN) implant or as an injected-purified cell suspension, has been examined as a therapeutic strategy in various CNS injury animal models over the last three decades (Wiliams & Bunge, 2012). This experimental approach was recently implemented clinically in Poland, where human SCs transplanted as PN implants showed indications for safety and efficacy in subjects with spinal cord injury (Tabakow et al., 2014).

Our transcriptomic results reported in Chapter 3 add to our understanding of the repair potential adopted by the human PN two weeks after an injury. Yet, we still needed to assess the neurobiology of the conditioned PN implants following implantation in the midbrain. To our knowledge, all preclinical data supporting the safe and effective use of SCs upon transplantation, in the form of PN implants, into the CNS were almost exclusively employed in rodent-derived cells (Bastidas et al., 2017). To date, no research study has examined the effects of human SCs when transplanted into the brain. Thus, to better learn about the survivability, distribution, and interaction of human SCs, when transplanted in the form of PN implants, we designed the Neuro-Avatar project. This xenotransplantation project aimed to examine cellular function and host response to human SCs when transplanted into the dorsal striatum of the athymic nude rat. Athymic nude rats are an immunodeficient strain of Sprague-Dawley rats with a reduced immune rejection of foreign tissue (Hanes, 2006). To better characterize the transplantation paradigm used in DBS Plus clinical trial, we utilized human PN fascicles harvested during Stage I and Stage II surgeries and implanted them in the brain of athymic nude rats. First, we studied the bioavailability and distribution of human SCs at two weeks and six months post-transplantation. Then, we

assessed the host bio-response to the xenograft using the modern applications of *in vivo* ^{1}H Magnetic resonance spectroscopy (MRS) (Ligneul et al., 2019).

Experimental Design and Procedures

Four human participants undergoing the DBS Plus trial have donated sural nerve fascicles. Grafts from the donated nerve fascicles were implanted in a total of 16 animals: 8 brains were collected at two weeks post-surgery and 8 were collected six months post-surgery. Within each group of 8 animals, 4 were implanted with human nerve tissue from Stage I surgery (injury-naive tissue), and 4 received nerve tissue from Stage II surgeries (*in vivo*- conditioned tissue).

Harvesting of Human Peripheral Nerve Implants

Experimental procedures, including handling and transplantation of human peripheral tissue, were approved by the IRB (B18-3211-M) of the University of Kentucky. Human peripheral nerve fascicles were obtained from 4 human subjects (aged 45-70 years) who are undergoing DBS-Plus surgery for Parkinson's disease. The sural nerves were dissected, and the nerve fascicles were collected from stage I and stage II. The nerve fascicles were collected in sterile conditions, placed on ice, and transported from the operating room to the laboratory for grafting. Up to 9 nerve fascicles were harvested from 1 cm section of the sural nerve during stage I surgery and two weeks later during stage II surgery.

Animal Care

All animal procedures were approved by IACUC of the University of Kentucky and performed per NIH guide for the care and use of Laboratory animals. 23 adults male athymic nude male rats (Hsd:RH-Foxn1rnu) were obtained from Charles River Laboratories, Chicago, IL. On average, the animals weighed 225g ± 20, were at eight weeks of age, and were housed in micro-isolated cages and kept in a room designated for immunodeficient rodents with a 12-hr light/dark cycle. Autoclaved food and water were available ad libitum. Cages and bedding were changed every three days. Clinical observations, as well as health and mortality records, were noted.

Animal Surgery

The surgical field was prepared by laying out a sterile surgical drape over a preheated surgical warming pad set to 38-40 °C. The sterile, empty surgical cannula was mounted to the arm of the stereotactic frame. This cannula was a 20 gauge needle with the tip blunted and smoothed with a Dremel tool and an inner stylet of 0.1 mm diameter. Anesthesia was induced using 5% isoflurane with supplemental oxygen in an anesthesia induction box. After anesthesia induction, animals were secured to the stereotactic frame using a tooth bar and anesthesia nose cone with 2.5% isoflurane with supplemental oxygen. Ear bars (45° Non-Rupture tip) 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 a wipe-down with two alcohol pads. After anesthesia induction, the animal was treated with the following: Rimadyl (10 mg/kg, SQ), Buprenorphine SR (1 mg/kg SQ), and Baytril (5 mg/kg IM). After making the skin incision, Bregma was located on the skull surface and a burr hole was made over the right stratum (1 AP, -2.5 LM) and the dura carefully broken. The cannula containing the PN implants, was lowered slowly at a rate of ~1 mm/minute. Once the target was reached (-4.5 DV), a 1 mm^3 of human PN implant was implanted in the right striatum. The cannula was removed slowly and the burr hole was sealed using bone wax. The surgical incision was stapled. The animals were transferred for recovery on a surgical warming mat.

Histological Processing

At the endpoints (2 weeks and six months post-transplantation), animals were deeply anesthetized using a single dose of pentobarbital (100 mg/kg IP) and then transcardially perfused with at least 250 mL of normal cold saline followed by 180 mL of 4% Paraformaldehyde (PFA). The brain was extracted and postfixed with 4% PFA for at least 24 hours. Following post-fixation, the implantation site was identified and cut into a 1 cm section in brain mold and cryoprotected in 30% sucrose for three days. Cryopreserved brains were cut coronally into 40 µm thick sections and stored in 30% sucrose solution with 0.1% sodium azide until being stained.

Immunostaining

To identify the transplanted human peripheral nerve cells and examine their interaction with the host CNS, immunostaining with antibodies against Human Nuclear Antigen (HNA) was employed. Tissue series from the transplanted brain sections were subjected to anti-HNA antibody together with DAPI (4′,6-diamidino-2-phenylindole). The primary antibody used was Mouse Anti-human HNA (1:500; Catalog#: ab191181, Abcam, Cambridge, MA). The fluorescent secondary antibody was Alexa conjugated goat Anti-mouse IgG (1:500; Catalog#: ab150113, Abcam, Cambridge, MA). To ensure the specificity of observed immunostaining, both positive and negative controls were included during staining. Negative controls consisted of the incubation of the primary or secondary antibodies alone, in addition to a non-grafted and intact rodent brain collected after 6 months of the experiment. Positive Controls consisted of incubation of sections of human sural nerve collected during both stage I and stage II surgeries. The brain sections were transferred from their wells and washed once with PBS. The tissue was permeabilized using 0.2% Triton X-100 for 5 minutes and then washed with PBS once. The slides were blocked with 5% goat serum, 1%Triton X-100 for 1 hour. Afterward the slides were incubated with the primary antibody in PBS, 0.1% goat serum overnight at 4°C. The next day, the slides were washed with PBS, 3x5 minutes, and incubated with fluorescence-conjugated secondary antibody in PBS, 0.1% normal serum for 1-2 hours at RT in the dark. Finally, the slides were rinsed in PBS, mounted on coverslips, and examined under a fluorescent microscope.

Magnetic Resonance Spectroscopy

Magnetic Resonance Spectroscopy is possible on any nucleus producing a magnetic moment. The metabolically active proton nuclei (^{1}H) is the most sensitive for NMR, and it allows us to detect a large number of important amino acids and metabolic pathways' end products like N-acetyl aspartate (NAA) and Creatine (Cr) /phosphocreatine. In healthy brain tissue, the most prevalent resonance originates from the methyl group of NAA at 2.02 ppm (Moffett et al., 2007). The chemical shifts are referenced in regard to that of 3-(trimethylsilyl)1-propanesulfonic acid at 0.00 ppm. NAA is exclusively localized in the central as well as the peripheral nervous systems. NAA has been used as a marker for viable neurons as it is only found in neurons, and its concentration decreases in the neurodegenerative diseases which affect the CNS (Schuff et al., 2006). The NAA concentration is not uniform and varying between gray matter (8-11 mM) and white matter (6-9 mM) (Moffett et al., 2013). The methyl and methylene protons of creatine and phosphocreatine resonate at 3.03 ppm and 3.93 ppm. Since the sum of both forms, total creatine (tCr), is constant in adulthood, the later can be used as a suitable *in vivo* concentration reference. Glutamate and Glutamine peaks were determined at 2.1-2.5 ppm and 3.7 ppm, respectively. Brain morphology and metabolism were assessed using T2WI magnetic resonance and imaging MRI and MRS (7-tesla Horizontal bore magnet; Bruker/Siemens scanner) at three months post-grafting in Neuro-Avatar animals. Two animals were used for MRS studies at three months post-grafting. The first one was a neuro-avatar athymic rat that has received a stage II human nerve tissue implanted into the right dorsal striatum. The second animal was a sham animal where a cannula with PBS, and no nerve implant was inserted into its striatum. Rats were anesthetized using 2%

isoflurane. The heads of the rats were fixed in a body retainer, the respiratory rate was monitored, and the body temperature was maintained at 37 ^{o}C with a body heating pad. Spectra were acquired at TR/TE/NS = 2500/3/200 for ipsilateral as well as the contralateral striatum with the voxel (volume = 3×2×3 mm^3) placed just next to the implantation trajectory. Ipsilateral metabolic concentrations were normalized to that of the contralateral (intact) hemisphere before comparing the two treatment groups.

Results

Accuracy and Precision of Stereotactic Surgical Implantation Procedure

Gross histology of 5 transplanted brains showed that some of the transplantation trajectories did not reach the deep targets in the dorsal striatum. Some of the trajectories were deflected when passing through the corpus callosum. This could partially be caused by the tract made by the blunted cannula in the brain tissue leading to lateral deviation of the trajectory in fibers-dense brain areas like in corpus callosum. However, the grafted brains with deviated trajectories were not excluded from the final analysis since the main objective of the experiment was to assess the viability of the human nerve cells post-implantation in the central nervous system environment.

Long-Term Survival Of Human Sural Nerve Implant After Implantation

Human Schwann cell transplantation, in the form of PN implants, in the brain of injury rats was followed by analysis for evidence of the long-term survival *in vivo* (figure

4.1). At two weeks post-implantation, animal brains implanted with pre-degenerated PN implants (Stage II) contained a higher count of +HNA cells than the animals implanted with PN tissue that were harvested from stage I (figure. 4.2). Second, transplanted +HNA human cells derived from stage I PN tissue significantly are significantly reduced 6 months after transplantation. However, the viability of human cells was significantly higher in animals receiving implants from stage II rather than from Stage I samples (figure 4.3). In addition to that, the cystic cavity and the surrounding edema were smaller at 6 months post-implantation from stage II implants.

Brain Reaction To Human PN Implants

Brain morphology and metabolism were assessed using an *in vivo* proton magnetic resonance spectroscopy (MRS). Proton spectra (Figure 4.4) were acquired in a cubic volume of 18 mm^3 in the right striatum of sham and avatar groups, and the concentration ratios of N-acetylaspartate/choline (NAA/Cr) and other metabolites (Myo-inositol (Ins), Taurine (Tau)) were determined after identifying their respective peaks on the spectra (Figure 4.5). One animal was randomly chosen from each group to undergo the MRS study at three months of the surgery. Brain T2WI showed a similar insertion trajectory with mild hyperintensity at the implantation socket of the avatar brain. The N-acetylaspartate/choline (NAA/Cr) ratio, a neuronal marker, measured by MRS inside the striatum of the avatar brain showed a mild increase around the implant tip. The astrogliosis marker, Ins, was slightly increased in comparison to the sham animal. Tau, a marker of inhibitory neurons, was decreased in the striatum of the avatar animal.

Discussion

In response to peripheral nerve injury, Schwann cells transdifferentiate and gain new potential to proliferate, survive, and migrate in a hypoxic PNS environment (Chang et al., 2011; Yao et al., 2016; Yu et al., 2012). Our goal is to identify the cellular viability and molecular changes after transplanting conditioned and pre-injured human peripheral nerve implant in the deep brain areas. To our knowledge, this is the first study that utilizes fresh and *in vivo* conditioned human peripheral nerve implants to the rat's striatum. To avoid rejection of the nerve xenografts, this Neuro-Avatar project used athymic nude rats which are immunocompromised animals and have been successfully utilized in xenotransplantation research (Bastidas et al., 2017; Hanes, 2006). Human sural nerve tissue, donated by patients with PD participating in our DBS Plus clinical trial, were implanted in the dorsal striatum of the animals. We have shown that huSCs remained viable in the dorsal striatum up to six months post-transplantation (longest period examined). The implanted human derived cells demonstrated limited migration outside the grafting socket, a low proliferation rate, and no tumorgenicity features. When compared to Stage I implants, the Stage II implants displayed a higher cell count and viability *in vivo*. This is consistent with previous studies that indicated following a nerve injury, the distal stump undergoes Wallerian degeneration which optimizes cells' long-term viability and endurance (Gordon, Wood, & Sulaiman, 2019; Yang et al., 2008).

In this study, we report the implementation of T2WI MRI and proton MR spectroscopy to measure the biochemical response of the host striatum to conditioned-human (Stage II) PN implants. Brain MRS is a non-invasive method to analyze the concentration of metabolites in the cerebral and deep brain areas (VAN ZIJL & Barker, 1997). This technique

allows us to acquire noninvasive metabolic data and help to monitor subtle changes in health and disease states in humans as well as in animal models (Bozza et al., 2010; Cho, Choi, Lee, & Kim, 2003; Manganas et al., 2007). In comparison to the sham group, we observed that the relative concentration of NAA was increased around the implant and at the borders of the substantia nigra. N-acetylaspartate has been used as an *in vivo* biochemical marker for neurons integrity and neurogenesis (Moffett & Namboodiri, 1995; Moffett, Ross, Arun, Madhavarao, & Namboodiri, 2007). The observed increase in NAA levels indicates enhancement of neuronal integrity that is consistent with the RNA-Seq analysis findings reported in Chapter Three, which demonstrated that pre-degenerated (Stage II) PN implants embrace a transcriptome profile that drives neurogenesis and axonal regeneration. We also observed a slight increase in Ins surrounding the implant, which is consistent with predicted gliosis (Haïk et al., 2008) produced by host astrocytes in response to the implantation procedure (Reier, 2012). However, this gliotic reaction was not noticeable on T2WI images, in contrary to other studies which reported significant CNS gliosis in response to fetal nigral grafts (Barker, Dunnett, Faissner, & Fawcett, 1996; Chi & Dahl, 1983; Lee et al., 2008). These results support incorporating MRS analysis in the DBS Plus clinical trial to improve our evaluation of brain response to PN implants in human subjects.

Conclusion

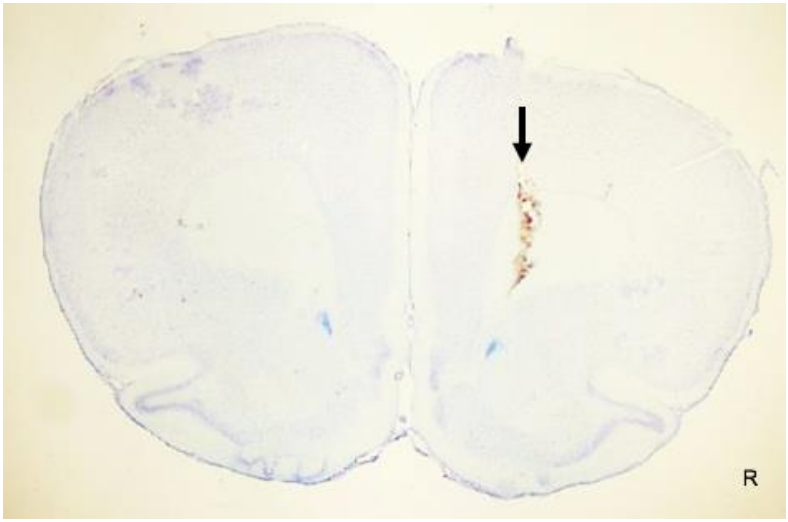
In conclusion, throughout this study, we investigated the viability and function of human Schwann cells following implantation of human nerve implants into the dorsal striatum. We have demonstrated that sural nerve implants collected from PD patients can persist long-term after transplantation (up to 6 months). The conditioning injury to the sural

nerve, induced during stage I of DBS surgery, incites the Wallerian degeneration processes *in vivo*; thus, prompting the proliferation of huSCs and enhancing their survival potential when collected at two weeks post-injury during the Stage II surgery of DBS procedure. We also demonstrated that nerve implants lead to striatal cell protection and central axons growth support. The histological and *in vivo* spectroscopy results do not display changes indicative of toxicity while showing signs of host striatal environment reacting positively to the nerve implants following implantation. Future studies employing transplantation of proregenerative nerve tissue in the transgenic animal model of PD will help us understand how such cell-based therapy can support the nigrostriatal cells in a disease state.

Figure 4. 1 Gross histology of Avatar brain.

A) Nissl stained-coronal section of 40 μm thickness with human PN implant located in the right striatum (black arrow indicates the transplantation trajectory). B) MRI coronal section of the implantation trajectory within the striatum.

A)



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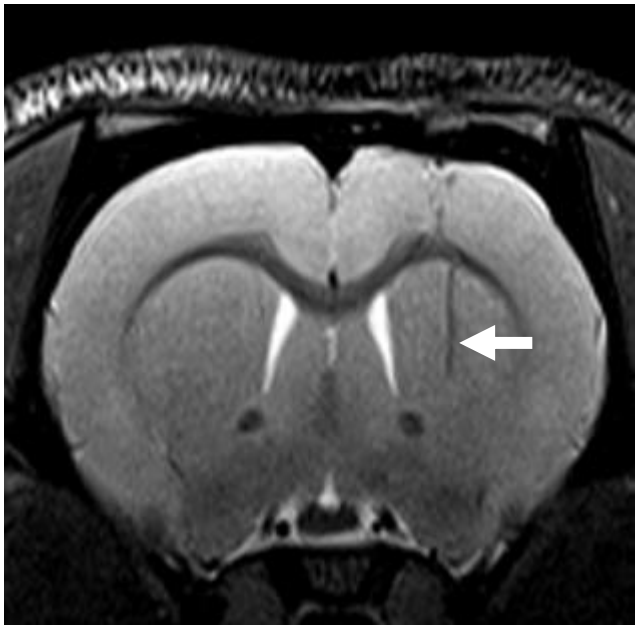


Figure 4. 2 Human PN derived cells remain viable in rat brain.

The immunofluorescence staining results showed that the HNA labeled cells remain viable up to 6 months post-transplantation (longest period examined). HNA(green), DAPI (blue).

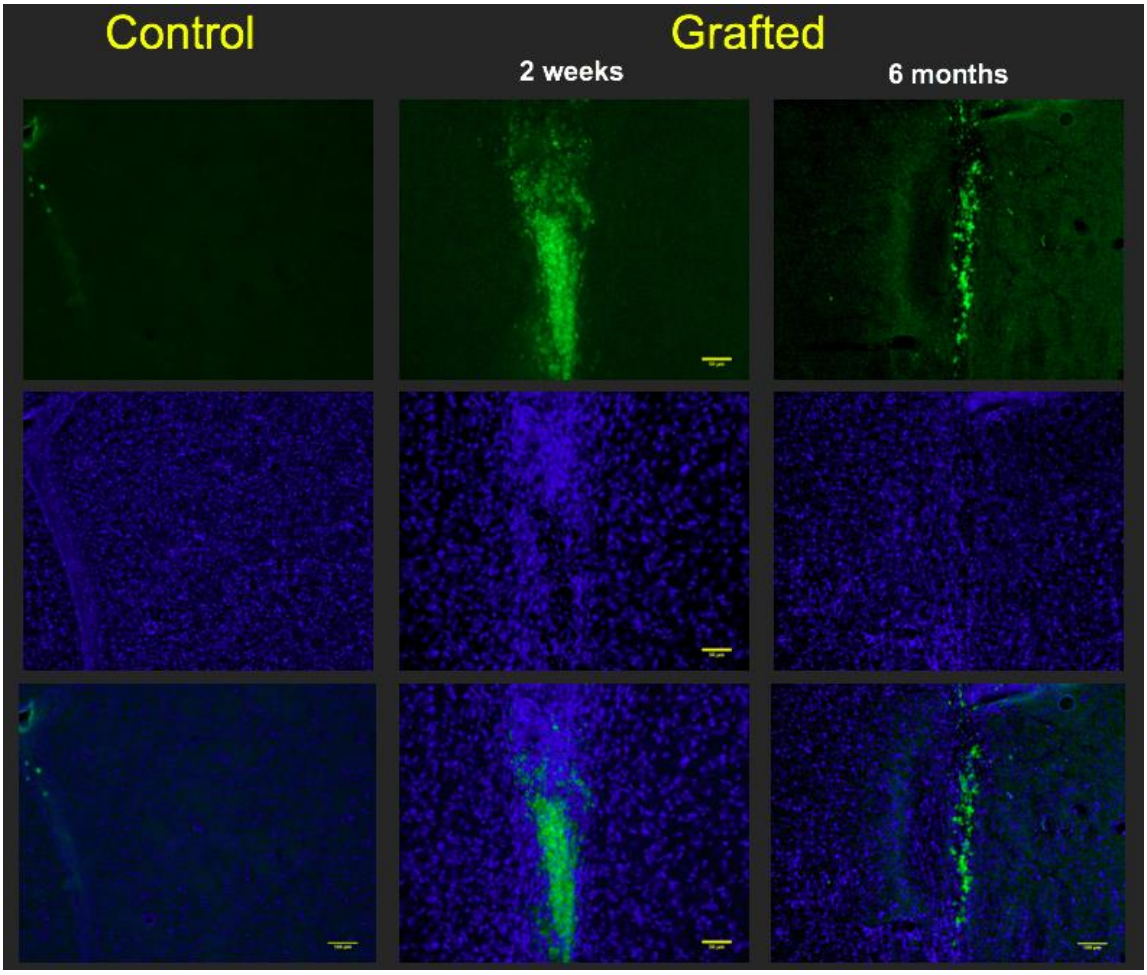


Figure 4. 3 Survivability of the implanted autologous PN-derived cells.

Implanted PN cells survived for 6 months post-implantation in the rat brain. There is greater viability when the cells were transplanted from Stage II (*in vivo* pro-regenerative or conditioned tissue) in comparison to Stage I cells. At two weeks following implantation into the striatum, nerve implants collected from Stage II DBS Plus displayed a significantly higher count of HNA positive SCs, compared to nerve implants harvested from Stage I surgery. Stage I implanted cell count was significantly decreased six months post-implantation. At six months, human SC viability was significantly higher in animals receiving implants from stage II compared to Stage I. Statistical significance indicated a $*p<0.05$ or $**p<0.01$.

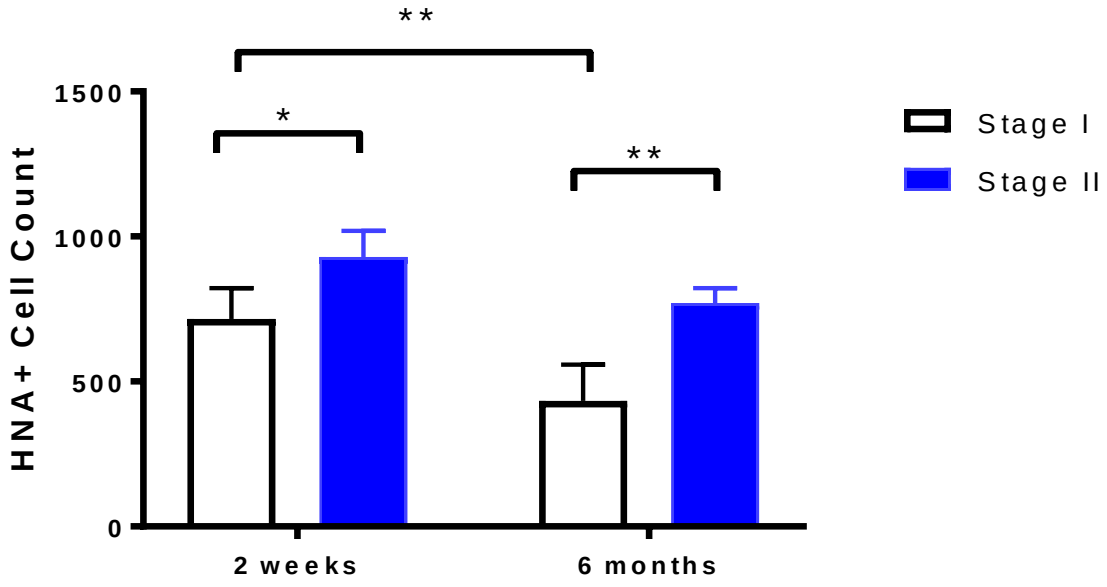


Figure 4. 4 In vivo 1H MR spectroscopy analysis of brain metabolites.

(A) Spectra obtained and brain MRI images of the sham and human PN transplanted groups, and the relative amounts of N-acetylaspartate (NAA), Myo-inositol (Ins), Glutamate (Glu), Taurine (Tau), and creatine (Cr).

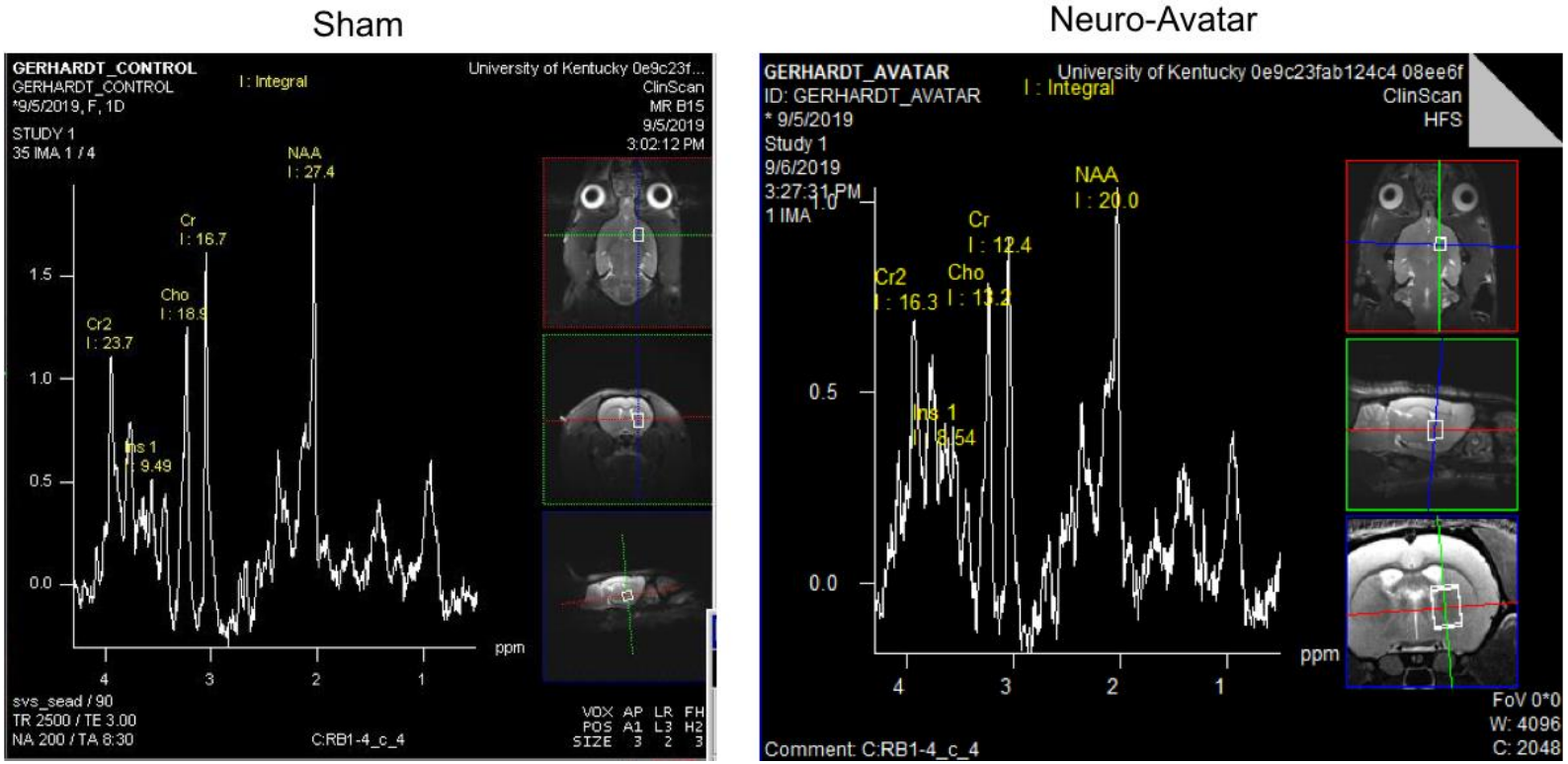
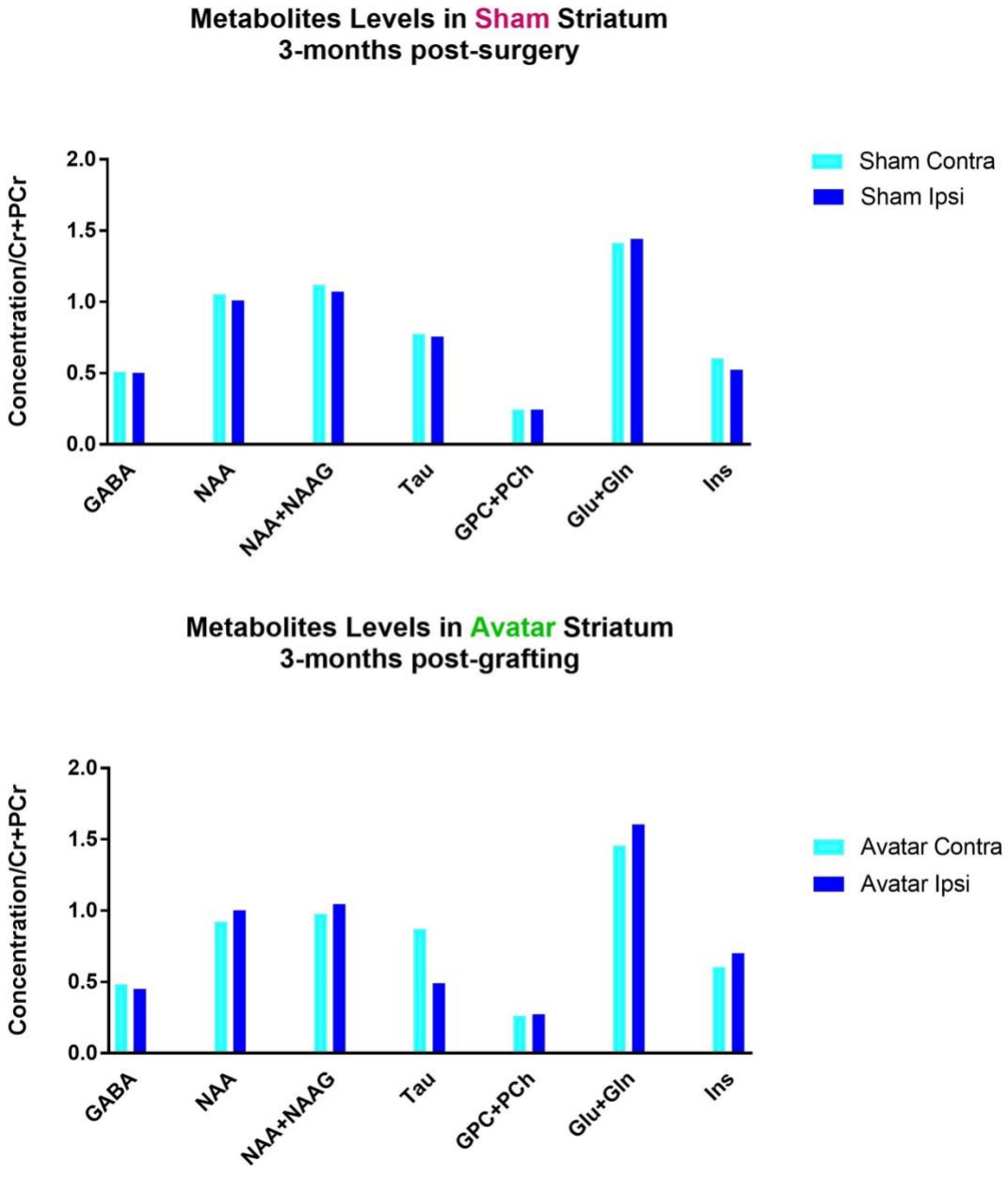


Figure 4. 5 Concentration ratios of brain metabolites as identified by ^{1}H MRS.

The relative concentrations in the grafted striatum were compared to the intact contralateral side within each group (N=1). An increase of the neuronal marker (NAA) as well as a mild increase in the gliosis metabolite (Inositol) was observed surrounding the PN implant in the avatar group.



CHAPTER FIVE: DBS PLUS CLINICAL TRIAL

Introduction

With no known cure, disease modifying therapies are one of the greatest unmet medical needs in the treatment of neurodegenerative diseases like Parkinson's disease (PD) (Kalia, Kalia, & Lang, 2015). Prior cell therapies investigated for replacing degenerating dopaminergic cells in the substantia nigra pars compacta (SNc), including fetal nigral cells, autologous carotid body cells, and bone marrow-derived mesenchymal stem cells, have failed to alter the progression of the disease (Yasuhara, Kameda, Sasaki, Tajiri, & Date, 2017). On the other hand, direct administration of non-neuronal cells that can deliver trophic factors has demonstrated therapeutic potential in protecting the sick neurons and possibly ameliorating the toxic environment in the brain (C. Chen et al., 2020). In 2013, our research group initiated a clinical trial to investigate the hypothesis of using reprogrammed Schwann cells (SCs), in a form of autologous peripheral nerve (PN) implants, to deliver crucial repair molecules to the degenerating dopaminergic neurons in PD patients undergoing DBS surgery (a procedure we have termed DBS Plus) (van Horne et al., 2017). This clinical study was the first to investigate the use of autologous PN tissue implants in conjunction with DBS, an FDA-approved adjunctive therapy to treat the symptoms of PD. The main goal of the initial pilot study was to determine the safety, feasibility, and tolerability of implanting PN implants at the time of DBS surgery. Eight participants (6 males and two females) were included in the first pilot study, which

demonstrated safety and feasibility of implanting sural nerve tissue containing autologous SCs into the SNc from conditioned PN tissue.

To further validate the safety and to explore the potential "open label" efficacy of this therapeutic approach, we have optimized the sample size in our ongoing studies (clinicaltrials.gov, registration no. NCT02369003). The study reported here includes participants with PD who elected to receive DBS plus pre-injured sural nerve tissue into the SNc with targeting of DBS leads to the internal segment of the Globus Pallidus (GPi). One benefit of this approach is that the location of the DBS leads is remote enough from the SNc tissue implants so that they can be readily visualized with MR imaging. We continue to investigate questions regarding dosing and optimization of bilateral placements of the PN tissue implants to help lay the foundation for a blinded Phase IIa trial to optimize the efficacy of the combined DBS and cell-based intervention.

Patients Selection

This clinical study is a part of an open-labeled Phase I clinical trial (clinicaltrials.gov, registration no. NCT02369003) conducted by the Brain Restoration Center at the University of Kentucky Medical Center. The study aims to investigate the safety, feasibility and motor outcome of the DBS Plus intervention in two groups of PD participants. Group A includes participants (N=18) who received Five, 1 mm^3 sections of pre-injured sural nerve tissue into the SNc. Group B participants (N=9) received a double dose (2 sets of Five, 1 mm^3 segments of nerve fascicle) of the sural nerve tissue. Both groups of participants met the inclusion and exclusion criteria described in Chapter Two and consented to receive DBS targeting bilateral GPi plus the designated cell therapy. For

the control group, we collected UPDRS III OFF-medication scores of patients with PD (w/o any history of surgical intervention) from the PPMI Database (Parkinson's Progression Markers Initiative). We collected UPDRS III data for 80 control subjects, matched by age (45-75 years) and disease severity (Baseline OFF UPDRS III ≥30, Hoehn & Yahr II-IV), for whom at least two years of follow-up was available. Since no OFF-Stimulation scores were reported in the database for PD subjects who elected to receive DBS, such a group of subjects (+DBS, w/o implant) was not included in our comparison analyses. We compared UPDRS III total OFF Medication/ OFF Stimulation scores, parkinsonian motor subcomponents, change from baseline, striatal reserve measured by Levodopa Response (OFF-ON difference), and adverse events at the end of one and two years of clinical follow up. The study was approved by the institutional IRB and Ethics committee.

Statistical Analyses

The statistical analyses and data plotting were performed using GraphPad Prism 8. The quantitative data were plotted using mean ± SEM. Analysis of the motor scores between the groups at different time points was done using a Two-way ANOVA followed by Tukey's multiple comparison test. A comparison between the lateralized scores at the Two-year time point was made using Wilcoxon Signed-Rank test. A multiple comparison test was used to compare the motor subcomponents of the UPDRS III scale. A Spearman correlation was used to test the relationship between the Levodopa Response at baseline and the Change of the OFF Score at the post-op follow up examination.

Results

Patient Summary

Twenty-seven participants (20 males and 7 females) met the criteria for participation in the study (Table 5.1). 18 participants of group A and 9 of group B were randomly assigned to receive unilateral implantation of single and double dose of PN implants respectively. The mean age (SD) of group A participants was 62.9 ± 8.1 years, and the mean disease duration was 9.3 ± 4.4 years. The mean age of group B participants was 61.88 ± 6.66 years, and the mean disease duration was 9.3 ± 4.18 years. The baseline UPDRS III OFF scores of group A participants ranged from 20 to 56, while those of group B participants ranged from 22 to 75. One participant of group A has failed to follow up while another participant has sadly passed away due to non-study related causes 20 months post-surgery. Hence, a total of 16 participants of group A have completed their follow ups. Only 8 participants of group B were able to complete their follow up assessments at the 12-month time point. For monitoring safety and adverse event reporting, each participant was evaluated by a movement disorder specialist at the University of Kentucky Medical Center and was in close communication with our study team throughout the study.

Safety of DBS Plus

All adverse events were submitted to and reviewed by the Data and Safety Monitoring Board (DSMB) at the University of Kentucky. One adverse event, superficial cellulitis around the ankle incision, was related to the transplantation procedure and was graded as

mild. The infection was treated with oral antibiotics. Most of the participants complained of ankle paresthesia and numbness, which was expected and consented as a result of sectioning the sural nerve. Three adverse events (urinary retention, headache, and dyspnea) were mild, related to the DBS surgery and the general anesthesia (but not to the implantation procedure), and resolved without further intervention. Three events, included falls, were considered to be mild, not serious, and not related to the implantation procedure as well. One event, hypomania, was mild, not serious, and was related to DBS stimulation and resolved promptly after changing the stimulation of the more proximal set of contacts of the stimulating electrodes. With regards to DBS surgery, there were no observed infections, hardware malfunctions, or skin erosions. Concerning feasibility, all participants underwent successful sural nerve resection and tissue implantation without complications or significant delay.

MRI Imaging

Post-op imaging was performed using a 1.5T MRI to verify targeting of the DBS electrodes and the tissue implant location. The MRI images were performed within 48 hours post-surgery. There was no evidence of edema on the T2 weighted images (T2WI) and no enhancement on the T1WI contrast images. Post-op MRI images were fused to pre-op CT scan data and the implantation trajectories were mapped using Lead-DBS software (Hanes, 2006) to verify the placement of the electrodes and the tissue implants in all participants (figure 5.1). There was no evidence of stroke, hemorrhage, or brain deviation resulting from the procedures.

Motor Outcomes

Analysis of the UPDRS III motor scores at a two-year follow up of participants who received DBS plus a single dose of PN implant (Group A) showed a remarkable motor improvement in comparison to the control PD group (Table 5.2). We observed a substantial improvement in motor score at the 6 month time point (UPDRS reduction of 7.82). This improvement in motor score persisted for 24 months post-surgery. This change from the baseline was significant ($p<0.0001$) when compared to Control PD subjects who continued to progress clinically and scored on average 6.53 ± 10.6 more points (figure 5.2).

Also, we report here UPDRS III scores of the participants who received DBS plus the double dose of tissue implants (Group B). Out of 9 participants from Group B who were consented and enrolled in the study, only one subject failed to follow up at a 12-month time point. This group of participants had on average an improvement in their motor scores by 8.25 points ± 11.46 one year after the surgery (Table 5.2). Although the motor outcome profile of Group A was similar to that of Group B, the latter group scored 1.62 points less at six months following the surgery (UPDRS OFF Change: -9.44 vs. -7.82 points) (Figure 5.3).

We further explored the UPDRS part III data by performing subgroup analysis of Group A 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 implant placement (ipsilateral vs. contralateral). Analysis of the lateralization effect of the tissue implants showed a significant overall reduction in the UPDRS III motor on the side contralateral to the cell implants vs the ipsilateral side ($p= 0.0273$; see Figure

5.4). Subgroup analysis of the parkinsonian motor signs demonstrated significant improvements in tremor and bradykinesia and minor improvements in rigidity and axial symptoms (Figure 5.5).

Correlation between Levodopa response at baseline and motor outcome

The Levodopa response was determined by the difference between the UPDRS III OFF and ON medications state. 16 subjects of group A, who completed their two-year follow-ups underwent the levodopa challenge test. Participants were assessed after 12–14 h without anti-parkinsonian medication and then given their usual prescribed dose of levodopa/carbidopa tablets and they are re-scored 2 hours afterwards. In Figure 5.6, the analysis revealed that the motor performance postoperatively had a significant positive correlation with the levodopa response at baseline ($r = 0.6794$, $p<0.0038$). Participants with higher Levodopa response at the baseline tended to score less at two years after the surgery. We also intended to assess how each participant scored clinically relative to the thresholds for clinically important difference (CID). 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. In total, 12 out of 16 participants (75%) scored about the threshold for minimal CID.

Discussion

The results of this study support the concept of combining cellular therapy with DBS to alter the progression of PD. This hybrid approach of merging a direct delivery of

biological therapy with the standard DBS surgery is anticipated to expand our understanding of the disease pathology and introduces a new era for disease-modifying treatments (Rowland et al., 2016). Research evaluating two main strategies for cell-transplants in PD, using fetal nigral grafts or induced pluripotent stem cells, are ongoing in the TRANSEURO trial and GForce-PD initiative (Barker, 2019; Z. Chen, 2018). Their concept is based on replacing the degenerating dopaminergic neurons by implanted neurons, which might be able to synthesize dopamine *in vivo*. Nevertheless, these strategies have faced serious ethical and safety obstacles like the formation of teratomas, and treatment-induced dyskinesia (Christophersen et al., 2006; Knoepfler, 2009; Sonntag et al., 2018) as well as some frustrating motor results (Graff-Radford et al., 2006). In comparison, the cell-based therapy used in our DBS Plus trial involves a neurorestorative approach to repair the nigrostriatal system. This research investigates the use of autologous peripheral Schwann cells as a source of regenerative growth factors to restore and protect the degenerating dopaminergic cells rather than replacing them. The main advantage of our approach is that the patients will be providing their autologous cells, which are harvested and implanted simultaneously with the standard DBS symptomatic treatment.

This Phase I clinical trial was initially designed to test the safety and feasibility of implanting autologous peripheral nerve tissue into the substantia nigra in combination with DBS in patients with PD. Since the ethical requirement of beneficence renders the exclusion of the standard of care (DBS) unethical, we could not enroll in our clinical study, a control cohort of PD patients. Nonetheless, we compared the motor outcome of our study participants to retrospective cohort patients collected from the PPMI database. This

retrospective cohort included age and baseline-matched subjects with PD, who have received no surgical intervention, including DBS.

The DBS procedure is approved for patients who have had PD for at least four years and whose symptoms are adequately responsive to medications. This surgical intervention alone has turned out to be a relatively safe surgical procedure to treat advanced PD symptoms. The adverse events of the combined therapy reported in this study were similar to the DBS surgery without transplantation (Bratsos, Karponis, & Saleh, 2019). Only one long-term adverse event, local cellulitis, was related to our implantation procedure and it resolved with oral antibiotics. Additionally, brain mapping and MRI imaging post-op showed accurate targeting of the tissue implants in the substantia nigra with no evidence of edema, hemorrhage, or signal abnormality along the graft trajectory. Additionally, we did not observe neither off nor on-therapy dyskinesias in our participants throughout the follow-up visits. Although these patients have received DBS, we argue that implant-induced dyskinesia is not masked by the electrical stimulation since the DBS target (GPi) is distant (≥5 mm) from the implant location (SNc) and the patients are evaluated 12 hours off stimulation.

In regards to the motor outcome, there was an overall decline in UPDRS III scores (-7.82, ± 9.1) during the first six months of the surgery in participants who received DBS plus the five pieces of sural nerve tissue. This reduction appears to be slightly greater (-9.44, SD ± 9.3) in participants who received the double dose of tissue implants. Interestingly, it looks as though the improvement in UPDRS III scoring tends to be relatively stable for two years after surgery. In comparison, the disease continued to progress in the control PD

group who scored an increase of 6.53 points (SD ±10.6) at the two-year follow up similar to what was reported in another study (Vavougios et al., 2018).

A comparison between ipsilateral versus contralateral UPDRS III motor scores showed improvements on both sides but a statistically significant difference in the scores contralateral to tissue implant location. The mild ipsilateral improvement, which has been reported by other surgical interventions for PD (Walker, Watts, Guthrie, Wang, & Guthrie, 2009), might be due to regeneration of some non-decussating fibers connecting the midbrain and the striatum at the implanted side.

In depth analysis of the UPDRS motor subcomponents showed that the most significant improvement was in the tremor and bradykinesia domains. We do not anticipate this improvement could be due to DBS therapy as DBS was turned off for 12 hours before motor testing. This should be sufficient time for most therapeutic effects of the DBS electrophysiological stimulation to dissipate (Temperli et al., 2003).

To assess the importance of changes in UPDRS III scores clinically we evaluated our results relative to the CID thresholds as described by Shulman and his team (Shulman et al., 2010). Out of 16 participants who received bilateral DBS plus a single unilateral cell implant, 11 (69%) improved clinically above the minimal CID, three were stable, and 2 participants scored higher (worse).

Lastly, we analyzed the change in UPDRS III OFF score after two years in correlation with the absolute value of Levodopa response at baseline. The acute motor response to Levodopa following a minimum of 12 hours washout has been used as a guide in managing medical or surgical decisions in PD (Ganga et al., 2013). However, recent data have demonstrated that the amplitude of motor response to Levodopa tends to increase with

increasing disease duration and progression (Pieterman, Adams, & Jog, 2018). In the group of participants who completed the two year follow up, there was a significant positive correlation between the amplitude of Levodopa Response at baseline and the reduction seen in the UPDRS III Off score. This analysis was consistent with the clinical improvement recognized in those subjects who scored beyond the threshold of the minimal clinically important difference. The results of this correlation may help us to better determine those participants who are expected to respond to this cellular therapy in future enrollments.

One limitation of our study is the lack of a DBS only treatment group. It is difficult to recruit patients who receive the standard DBS surgery and to consent them for testing while being off stimulation and off medication for 12 hours. Another limitation is the lack of *in vivo* functional data to better assess the viability of the implanted SCs and their biological effects. The current FDA guidelines restrict any kind of *in vitro* manipulation or labeling of the cell implant tissues. However, an ongoing collection of post-op DaTscans should further help us evaluate the impact of the implants on regenerating the dopaminergic projections to the striatum.

Conclusion

In summary, this clinical study is exceptional because it provides prospective data on using cellular therapy in combination with DBS treatment in humans. Besides, this is the first study to investigate the influence of peripheral nerve cell implants in modifying the disease progression in PD. The results support the clinical trial's primary aim, which is to deliver pro-regenerative PN cells to the substantia nigra of patients with PD.

Throughout the analysis, we have demonstrated a favorable profile of extended safety and tolerability of the combined therapy. Our results have also shown the potential efficacy of the pre-conditioned implants in reducing the severity of the motor impairment in PD.

There was a remarkable restoration of motor function, mainly on the side contralateral to the implant location, as assessed by the standard UPDRS scale part III. Most importantly, this restoration of function was not temporary, and it persisted up to two years post-intervention. Overall, the findings presented in this study worth further clinical investigation to better understand how PN cell implants were able to interact with the degenerating central neurons and restore the structures and functions of the nigrostriatal system.

Table 5. 1 Demographic characteristics of the study subjects.

Age, Baseline scores, and duration of diagnosis are represented as Mean ± SD.

	Group A **DBS Plus Single-Unilateral Implant (N=18)**	**Group B** **DBS Plus Double Dose of Unilateral Implant (N=9)**	**Control PD (N=80)**
Male	13	7	_
Female	5	2	_
Age (year)	62.9 (± 8.1)	61.9 (± 6.7)	59.4 (± 7.9)
Baseline UPDRS III Off	38.4 (± 11.3)	42.8 (± 15.7)	34.4 (± 4.2)
Duration of Diagnosis (year)	9.3 (± 4.4)	9.3 (± 4.11)	> 5 years

Table 5. 2 Change from the baseline of UPDRS III OFF scores

Comparison of the change of UPDRS III OFF scores from the baseline of DBS plus a single dose of unilateral cell implants (group A) vs Control subjects with Parkinson's disease. (Mean ± SD)

Follow Up **TIMEPOINT (months)**	**Control PD**	**DBS Plus** **(Group A)**	**P-Value**
6	0.46 (± 6.7)	-7.82 (± 9.1)	0.0129
12	3.42 (±10.4)	-8.33 (± 9.3)	<0.0001
18	3.23 (± 9.6)	-7.00 (± 9.1)	0.0013
24	6.53 (± 10.6)	-9.56 (± 10.7)	<0.0001

Figure 5. 1 3D Mapping of the implant location

Coronal and anterior 3D view of the basal ganglia. The implantation trajectory and the final target of the tissue implants into the unilateral substantia nigra (in yellow) was mapped in Lead-DBS software. Globus Pallidus external segment (GPe), Globus Pallidus internal segment (GPi), Red Nucleus (RN), Substantia Nigra (SN), Subthalamic Nucleus (STN).

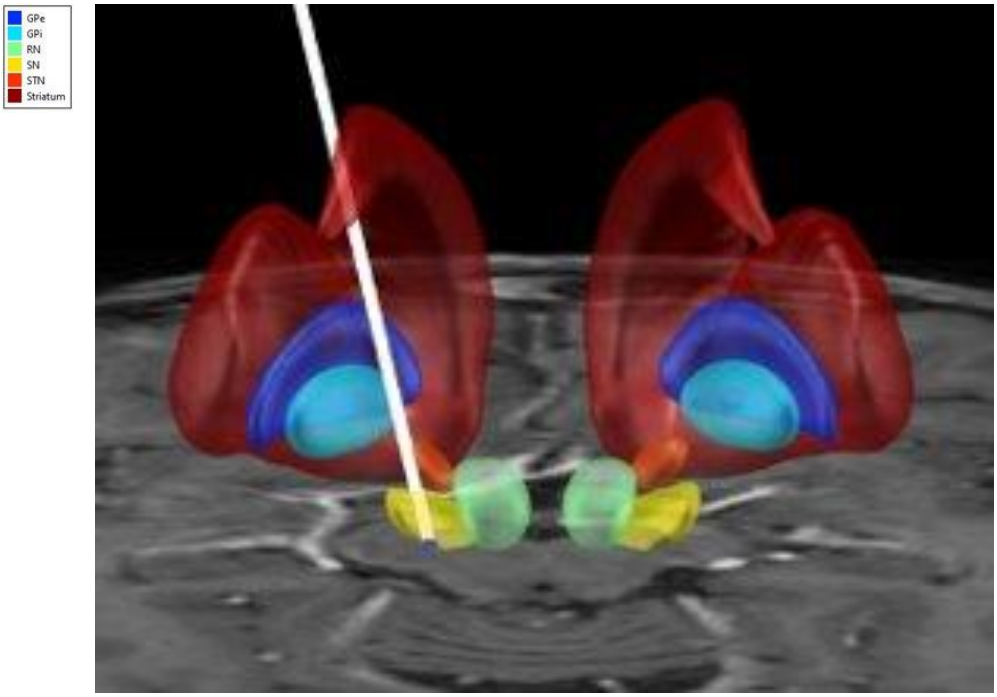


Figure 5. 2 Motor Outcome

Motor evaluation of the study participants who received DBS Plus single dose of cell implants into SNc (group A) in comparison to Control PD. (*<0.05. **<0.01, ****<0.0001).

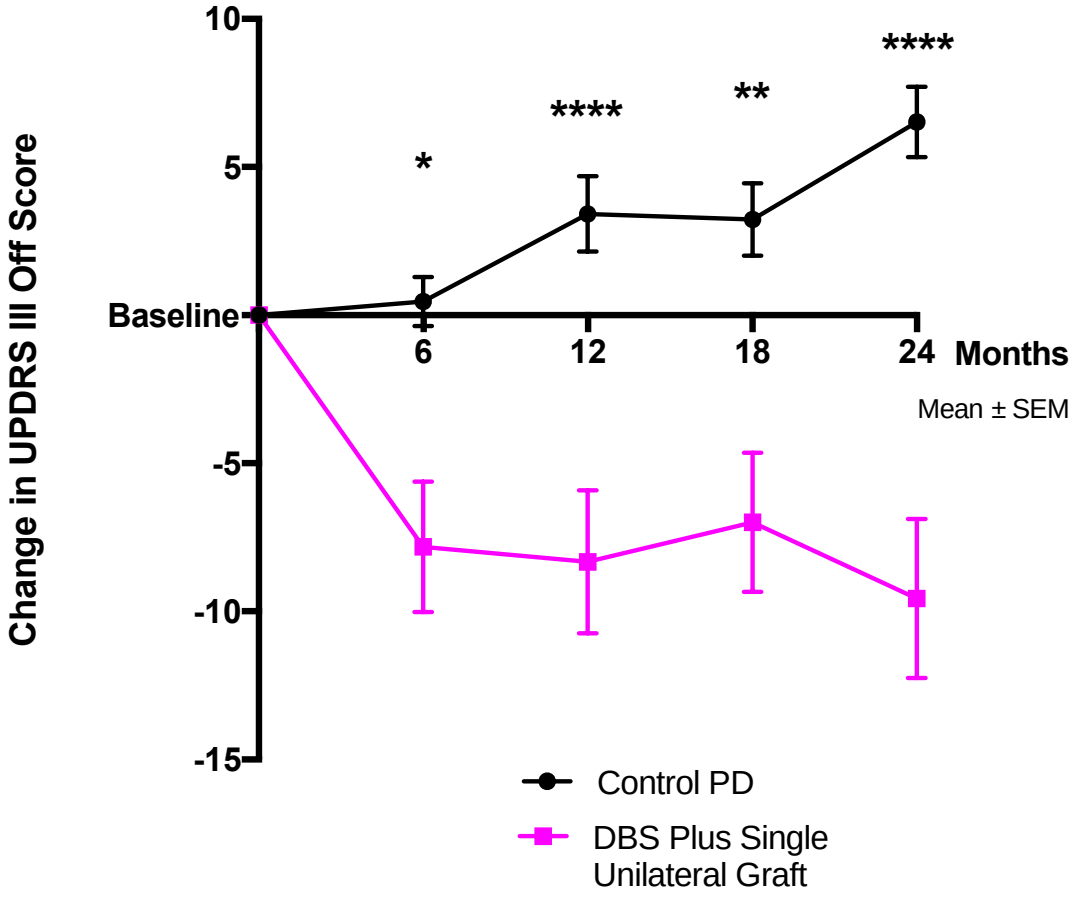


Figure 5. 3 Implant Dosage Motor Outcome

Motor Evaluation of Study participants who received GPi DBS plus double dose cell implants into the substantia nigra unilaterally.

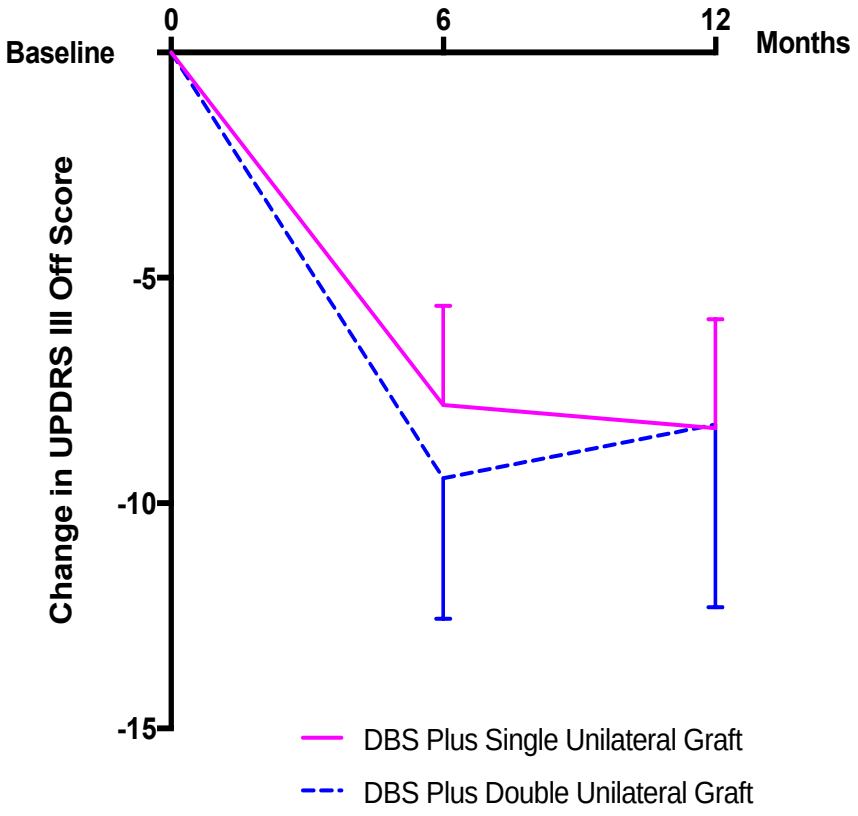


Figure 5. 4 Lateralization response to the cell implants.

Analysis of the Change in UPDRS III Off scores in participants who received bilateral GPi DBS plus single cell implants showed a significant improvement on the side contralateral to the cell implants vs the ipsilateral side (*t*-test (n=16); p-value=0.0273).

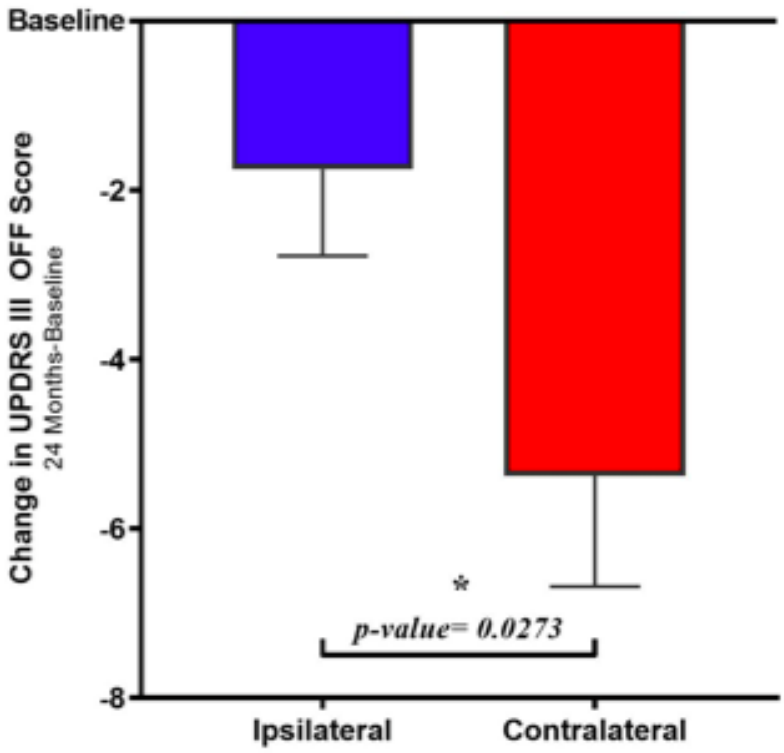


Figure 5. 5 Parkinsonian Subcomponents Analyses

Analysis of the motor subcomponents of UPDRS III scale in 16 participants of group A at two-year follow up. The significant improvements in the OFF scores were in Tremor and Bradykinesia, whereas Rigidity and Axial impairments showed some mild changes.

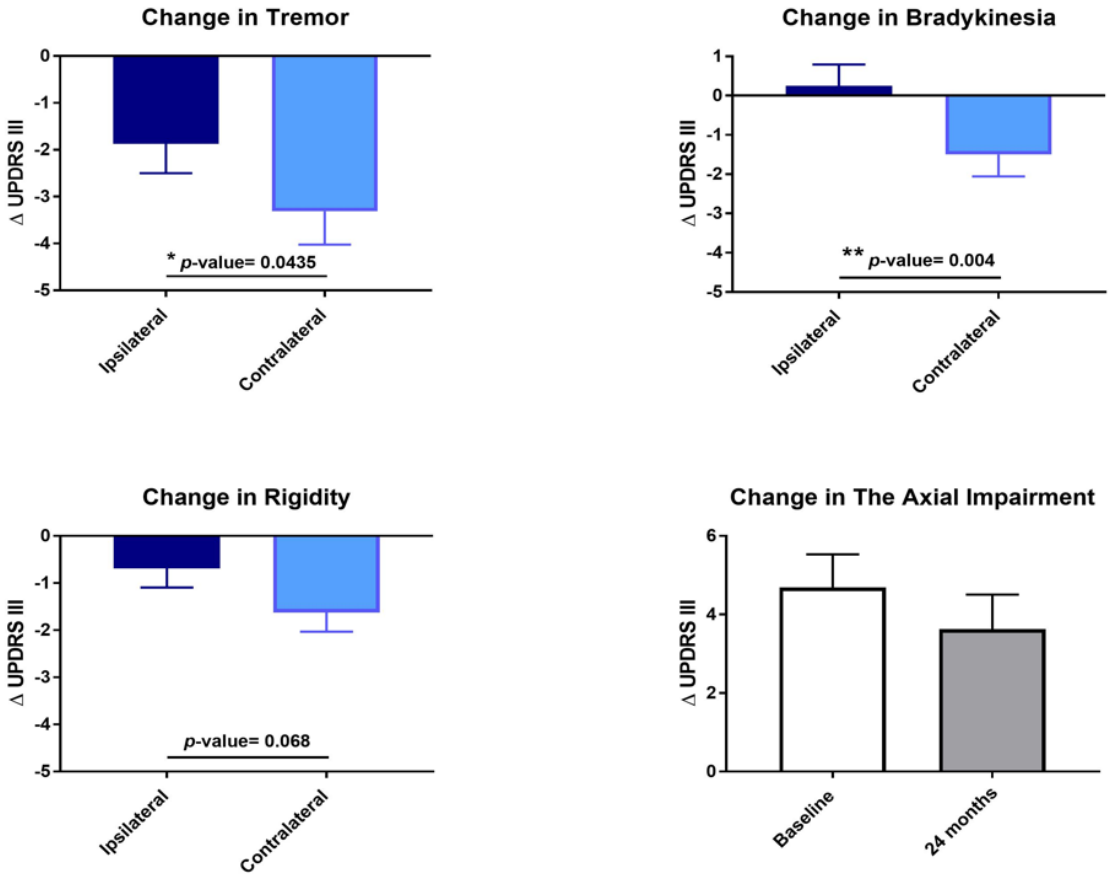
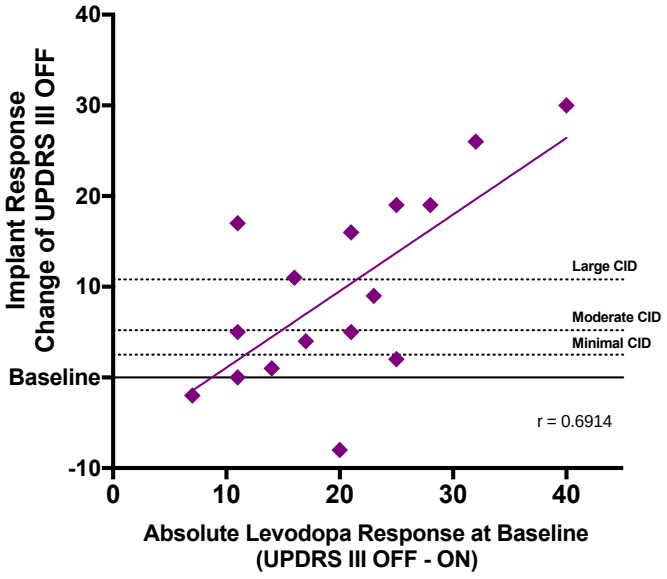


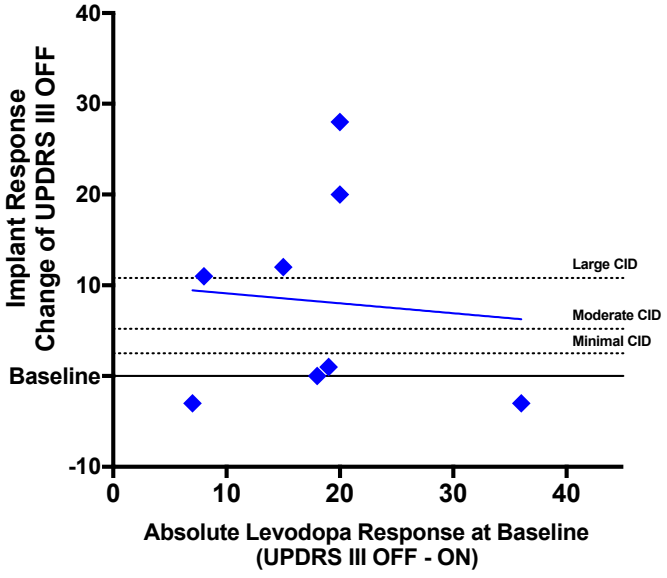
Figure 5. 6 Correlation between Implant Response and Levodopa Response at baseline

Correlation between the Levodopa Response at baseline and the Change in the motor scores at: **A)** 24 months of participants who received DBS and a single dose of nerve implants in the substantia nigra **B)** 12 months of participants who received DBS and a double dose of nerve implant. CID: Clinical Important Difference. Minimal (2.5 points), moderate (5.2 points), and large (10.8 points) CIDs are indicated. Each symbol represents a different participant.

A)



B)



DISCUSSION AND FUTURE DIRECTIONS

PN tissue as a source of cell-based therapy for neurodegenerative diseases has been investigated experimentally over the last four decades, and the past few years have shown renewed progress in its use in clinical trials. The research work demonstrated through this dissertation aimed to test how manipulating PN tissue can deliver vital therapeutic agents to CNS neurons to stop, slow, or reverse degenerative processes. Hence, PN tissue may have the ability to alter the progression of neurodegenerative diseases and contribute in the future to a true "disease-modifying therapy". The peripheral nervous sytsem retains the ability to repair itself, unlike the CNS, and likely may guide us toward new therapies for neurodegenerative diseases and CNS trauma.

Our extensive transcriptomic analysis described in Chapter Three provides robust scientific evidence of how a conditioning injury to the peripheral nerve drives the trans-differentiation of Schwann cells into "repair cells". The trans-differentiation process concurs with highly orchestrated changes in the levels of growth factors and repair signaling molecules. Some of those released growth factors, such as GDNF, have already shown efficacy in restoring and rescuing the structure and function of the dopaminergic neurons in preclinical studies. Overall, PN tissue appears to have the capacity to promote CNS plasticity and axon regeneration after an injury. We are aware that our transcriptional analysis needs to be replicated and validated by other genomic and proteomic studies. Nevertheless, we believe that our RNA-Seq analysis has helped to unmask key signaling cascades that can promote the repair and restoration of the PNS. How we can utilize the

capabilities of PN cells to minimize neuronal loss and induce axonal regeneration in neurodegenerative diseases like PD now becomes a central question.

In Chapter Four, we detailed the establishment of the xenotransplantation animal model, the Neuro-Avatar project. This project aimed to help us evaluate the neurobiology of the sural nerve implants post-implantation in deep brain areas. We also reported the long-term viability of the conditioned sural nerve implants in comparison to injury-naïve PN implants. Survival and infiltration of the implanted human cells into the host brain were assessed by staining for human nuclear antigen. We also employed proton MRS technique to determine the brain reaction to the PN implant. Interestingly, we noticed an increase in the neuronal integrity marker (NAA) around the implant. The main advantage of the *in vivo* MRS study is that it can be safely translated to the DBS Plus clinical trial to evaluate better the local effects of the implant on the metabolism and regeneration of the surrounding midbrain neurons.

While the clinical trials that have investigated implanting PN cells/tissue into the CNS are limited, those that have gone on to be published have demonstrated safety and feasibility outcomes with some suggestion of potential clinical improvement. In the last chapter of this dissertation, we tried to address the question of how the neurobiology of the conditioned sural nerve implant will correlate with clinical outcomes. Up to two years post-implantation, the overall safety profile of combining DBS and autologous sural nerve implantation into the substantia nigra is similar to that of the DBS surgery alone. None the less, the preliminary analysis of the clinical outcome demonstrated very promising improvements in the parkinsonian motor symptoms in response to the implant. An

undergoing effort to enroll and collect UPDRS III data of participants who received only the DBS procedure will help us better validate those preliminary clinical results.

In summary, the research described in this dissertation attempts to connect the dots between basic science and clinical trials in the neurodegenerative fields. The findings of these studies will significantly contribute to future translational studies in the areas of PNS injury and CNS diseases. We believe that the cell-based therapies using transplantation of autologous PN cells could become helpful in patients in the earlier stages of neurodegenerative diseases when the CNS retains a reserve of functional cells and higher "regenerative capacity."

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VITA

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Education

PhD	USA
University of Kentucky	Jan, 2017- August, 2020
Clinical and Experimental Therapeutics	
Departments of Pharmaceutical Sciences, Neuroscience	
Brain Restoration Center	
MD	Lebanon
Lebanese American University School of Medicine	2014
(Harvard Medical School International Affiliate)	
BS, **Biology**	Lebanon
Lebanese University, Faculty of Sciences	2009

Honors and Awards

Travel Award, American Society for Neural Therapy and Repair (ASNTR)	2020
Peter Glavinos Graduate Scholarship, University of Kentucky	2020
First Place in Graduate Student Poster Presentation, Todd Symposium	2019
Winner of Elevator Talk Competition Presentation, Todd Symposium	2019
Pharmaceutical Sciences Excellence in Graduate Achievement Fellowship	2019
American Neurological Association Travel Award	2019
Best Poster Award, 51st PGSRM meeting, University of Wisconsin	2019
Peter Glavinos Graduate Scholarship, University of Kentucky	2019
Research Excellence Award, The American Society for Neural Therapy and Repair	2019

First Place in Poster Presentation, Therapeutics, Outcomes, Discovery & Delivery Symposium, University of Kentucky	2018
First Place in Poster Presentation, Society of Postdoctoral Scholars Annual Research Symposium	2018
Third Place in Elevator Speech Presentation, Therapeutics, Outcomes, Discovery & Delivery Symposium at University of Kentucky	2018
Graduate Program Student Travel Award, University of Kentucky	2018
First Place in Elevator Pitch Presentation, Drug Discovery & Development and Natural Products Consortium Symposium, University of Kentucky	2017
Leadership Award, Lebanese American University	2013
Dean's List, Lebanese University	2009

Professional Experience

Penn State University Neurology Resident	Hershey, USA 2020
Harvard Medical School-Brigham and Women's Hospital Clerkship in Neurology and Functional Neurosurgery	Boston, USA July-Aug 2019
University of Kentucky Graduate Research Assistant Supervisor: Greg Gerhardt	Lexington, USA 2018-Present
Teaching Assistant, UK College of Pharmacy Course: PHR 926	Lexington, USA 2018
Helios Hospital Intern in Neurosurgery	Erfurt, Germany 2015-2016
Lebanese American University Medical Center General Surgery Residency	Beirut, Lebanon 2014-2015

Research Experience

University of Kentucky, Departments of Neuroscience and Pharmaceutical Sciences — 2018-June 2020

PhD Thesis (Mentor: Dr. Greg Gerhardt, PhD; Co-mentor: Dr. Craig van Horne, MD, PhD):
"Combination of investigational cell-based therapy and deep brain stimulation to alter the progression of Parkinson's disease"

University of Kentucky, Department of Neurosurgery — Nov, 2017

Clinical Study: The role of chemokines expression after an acute ischemic stroke.

University of Kentucky, Department of Pharmaceutical Sciences — Jan-Aug, 2017

Restoration of blood brain barrier dysfunction in epilepsy by targeting the COX and LOX pro-inflammatory pathways.

University of Kentucky, Sanders Brown Center on Aging — Aug-Dec, 2016

- Study of Aβ amyloid-induced disruption of the CNS neurovascular integrity.
- Identification of a novel strategy to enhance Aβ amyloid brain clearance by upregulating the P-glycoprotein efflux transporter.

Publications

Nader El Seblani, Andrew Welleford, George Quintero, Craig van Horne, Greg Gerhardt (2020). "Utilizing Peripheral Nerve Regenerative Elements to Repair Damage in the CNS". Journal of Neuroscience Methods, 108623.

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Dhanunjaya Rao Ginjupally, MD; **Nader El Seblani**, MD, PhD Candidate; George Quintero, PhD; Zain Guduru, MD; Craig Van Horne, MD, PhD. "Left STN DBS and Right CZi DBS in a patient with tremor-predominant Parkinson's Disease: Case report". *MS in preparation.*

Andrew S. Welleford, **Nader El Seblani**, Francois Pomerleau, Victoria Thompson, George E. Quintero, Craig van Horne, and Greg A. Gerhardt. “Neuro-Avatar: A Reverse Translational Model of an Ongoing Cell Therapy Trial for the Treatment of Parkinson’s Disease”. *MS in preparation.*