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TARGETING AXON GROWTH FROM NEURONS TRANSPLANTEDINTO THE CENTRAL NERVOUS SYSTEM

Kristine S. Ziemba
University of Kentucky, ksziem0@uky.edu

ABSTRACT OF DISSERTATION

Kristine S. Ziemba

The Graduate School

University of Kentucky

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TARGETING AXON GROWTH FROM NEURONS TRANSPLANTED INTO THE CENTRAL NERVOUS SYSTEM

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine Department of Molecular Physiology at the University of Kentucky

By
Kristine S. Ziemba

Lexington, Kentucky

Director: Dr. George M. Smith, Professor of Physiology

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Damage to the adult mammalian central nervous system (CNS), either by traumatic injury or disease, usually results in permanent sensory and/or motor deficits. Regeneration of neural circuits is limited both by the lack of growth-promoting molecules and by the presence of growth-inhibitory molecules in the mature brain and spinal cord. The research described here examines the therapeutic potential of viral vectors and neuronal transplants to reconstruct damaged neural pathways in the CNS.

Experimental neural transplantation techniques often fall short of expectations because of limited transplant survival and insufficient neurite outgrowth to repair connections and induce behavioral recovery. These shortcomings are addressed in the current studies by virus-mediated expression of cell-specific neurotrophic and guidance molecules in the host brain prior to cell transplantation. The initial proof-of-principle studies show that viral vectors can be used to create axon-guidance pathways in the adult mammalian brain. With such pathways in place, subsequent transplantation of neurons leads to long-distance, targeted outgrowth of neurites.

Application of this technique to a rat model of Parkinson's disease demonstrates that circuit reconstruction leads to functional recovery. For this study, rats were lesioned on one side of their brain with 6-hydroxydopamine to produce a hemiparkinsonian state. The motor deficit was confirmed by amphetamine-induced rotation testing and spontaneous motor asymmetry testing. The rats were then divided into experimental groups to receive lentivirus injections along a path between the substantia nigra (SN) and the striatum to express glial cell-line derived neurotrophic factor (GDNF), GDNF family receptor alpha-1 (GFRα1), netrin-1 or green fluorescent protein (GFP, control). One group received combination injections of lenti-GDNF and lenti-GFRα1. One week after virus injections, animals received transplants of embryonic midbrain dopaminergic neurons into their SNs. They were tested for motor asymmetry every two weeks for a total of eight weeks and then brain tissue was harvested for immunohistochemical analysis. Results demonstrate that virus-induced

expression of GDNF and GFRα1 supports growth of dopaminergic fibers from cells transplanted into the SN all the way to the striatum, and these animals have a significant reduction in both drug-induced and spontaneous motor asymmetry.

KEYWORDS: Axon guidance, growth factors, neuronal transplantation, Parkinson's disease, viral vectors

Kristine S. Ziemba

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By

Kristine S. Ziemba

Dr. George M. Smith
Director of Dissertation

Dr. Ok-Kyong Park-Sarge
Director of Graduate Studies

March 1, 2007

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Chapter One: Introduction

Wherein lies that marvelous power which enables the nerve fibers from very distant cells to make contact directly with certain other nerve cells…without going astray or taking a roundabout course?...I believe that one could…think of processes like the phenomenon called…chemotaxis."

-Santiago Ramón y Cajal, 1893

Overview

Damage to the adult central nervous system (CNS) by traumatic injury or disease presents an enormous challenge to medical practitioners and researchers. The unfortunate truth is that many neurological disorders are still incurable, so neurologists are left with few options other than palliative care for their patients. While a skilled surgeon may be able to reconnect a severed hand and effect satisfactory functional recovery, a severed spinal cord leads to lifelong disability. Similarly, infarcted brain tissue does not heal after a stroke and the progressive loss of neurons to degenerative diseases such as Alzheimer's, Parkinson's, and amyotrophic lateral sclerosis is irreversible at this time. The intractability of CNS damage is due to the sheer complexity and specificity of neural circuitry as well as the lack of growth-promoting cues and presence of growth-inhibitory molecules in the adult CNS environment.

The specificity of neural connections was first elucidated as part of the *neuron doctrine* by Santiago Ramón y Cajal in the 1890's (Shepherd, 1991). In addition to describing the neuron as the basic unit of the nervous system, the synapse (a term later coined by Charles Sherrington) as the location of signal transmission between neurons, and the unidirectional flow of signal within neural circuits, Cajal's doctrine established that neural circuits are precise and predictable in their connections and activity (Cajal, 1995; Eccles and Gibson, 1979). These connections are formed as an animal develops, with axons extending sometimes over great distances to reach their unique targets. With another enormous contribution to neuroscience, Cajal first described the "steering" behavior of axonal growth cones and proposed the *neurotropic theory* to explain their directional amoeboid movement, which he compared with

leukocyte chemotaxis (Sotelo, 2002). More recently, specific molecular cues – both positive and negative - that direct axon growth during development have been identified. With a more complete understanding of the growth-promoting environment of the developing nervous system, we will be better equipped to develop regenerative and repair strategies for injured adult nervous tissues.

Development

As an embryo develops, the growth trajectories of nerve fibers are determined by physical constraints and specific molecular cues in the environment (Song and Poo, 2001). The molecular guidance mechanisms may be roughly divided into four types: contact attraction (short-range), chemoattraction (long-range, diffusion-based), contact repulsion, and chemorepulsion (Tessier-Lavigne and Goodman, 1996). If an axon must traverse a relatively long distance, its journey may be divided into shorter segments, punctuated by "choice points" where specialized cells provide attractive or repulsive cues to the growth cone. Development of neural circuits is also stepwise, with earlier-growing axons - "pioneer axons", which grow through a somewhat simpler histological landscape - providing a growth scaffold for the fibers that follow (Tessier-Lavigne and Goodman, 1996).

There is some disagreement about whether to classify certain molecules as *attractive* or *repulsive* when their effects may be more accurately described as "outgrowth-promoting" or "outgrowth-suppressing" (Baier and Bonhoeffer, 1994; Tessier-Lavigne and Goodman, 1996). This is best demonstrated *in vitro* with collagen gel media, where the effect of a molecular gradient on axon growth may be separated from all other physical and chemical factors. In such an experiment, an "outgrowth-promoting" molecule (i.e., a growth factor), presented in a gradient, will selectively accelerate and stabilize axons that are growing toward the higher concentrations. Some argue that this should not be interpreted as a *guidance* effect, even though the end result is more axon growth toward rather than away from the gradient, because the growth factor did not cause individual axons to alter their growth trajectories (Baier and Bonhoeffer, 1994). If

the resultant pattern of axon growth is most important, however, it may be unnecessary to separate attraction from growth-promotion or repulsion from growth inhibition (Tessier-Lavigne and Goodman, 1996).

Another complication with regards to classifying guidance molecules is the fact that whether a molecule is “attractive” or “repulsive” depends entirely on a growth cone’s response to it. Some molecules act as both: chemoattractants to certain neurons and chemorepellants to others. Furthermore, an individual axon may alter its response to a guidance cue, from attraction to repulsion (or vice versa), based on regulation of receptor expression within the growth cone. In fact, this is how axons are able to move toward an intermediate “choice point”, such as the midline of the developing spinal cord, and then move away from that point to their final destination on the contralateral side. If axons did not possess this plasticity, they would simply stall at the first attractive location they reached and long-distance migration would be impossible due to constraints of diffusion (Tessier-Lavigne, 2004).

Several specific axon-guidance molecules have been identified and intensely studied since the early 1990’s. With the previously-mentioned caveats in mind, these molecules may be classified as follows: 1) secreted, long-distance chemoattractants include Netrins, hepatocyte growth factor (HGF), the neurotrophins nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3 and NT-4/5, and morphogens Wnt and sonic hedgehog (Shh); 2) secreted, long-distance chemorepellants also include Netrins, plus Semaphorins, Ephrins, Slits, and bone morphogenic protein (BMP); 3) contact repellants include Eph Ligands, transmembrane Semaphorins, and some extracellular matrix (ECM) molecules like tenascins; and 4) contact attractants include members of the immunoglobulin (Ig) superfamily of cell adhesion molecules (CAMs), Cadherins, and ECM molecules such as laminin (Reviewed in Tessier-Lavigne and Goodman, 1996; Song and Poo, 2001; Tucker et al., 2001; Dickson, 2002; Tessier-Lavigne, 2004; Chilton, 2006). Each guidance molecule affects a growth cone by interacting with a unique cell-surface receptor, but the intracellular signaling cascades that are activated by different

signals may overlap - either enhancing or inhibiting one another - and the final common target is the cytoskeleton (Dent et al., 2003).

Growth cone dynamics are ultimately determined by cycles of polymerization and depolymerization of two cytoskeletal components: actin and microtubules. Within a growth cone, actin filaments exist in two different states: tight bundles of parallel filaments that extend into distal filopodia, and a looser network of filaments in the proximal, veil-like lamellipodium (Dickson, 2002). Microtubules, which are important components of the cytoskeleton all along axons and dendrites, also extend into the growth cone. During directional changes in axon growth, some microtubules extend into filopodia and align with elongating actin bundles – an interaction that may be essential for growth cone turning (Dent et al., 2003). Positive growth cues along an axon's path lead to actin polymerization and subsequent filopodial extension in the direction of those cues, while negative cues cause depolymerization and retraction of filopodia. Hence, there must be spatial asymmetry of molecular responses within a single growth cone as it navigates its environment: a front-to-back polarity is required for forward movement, and a right-to-left polarity is necessary for turning (Kamiguchi, 2006).

Axons can change their growth trajectories in response to gradients of diffusible guidance molecules as small as 0.1% across the diameter of the growth cone (Rosoff et al., 2004). The mechanism by which such subtle differences in concentration may be transduced into directional growth cone responses is still being elucidated, but probably involves receptor-containing lipid rafts in the growth cone membrane (Kamiguchi, 2006), localized changes in protein synthesis and degradation (Campbell and Holt, 2001, Gallo and Letourneau, 2002), and spatiotemporal gradients of calcium ions within the growth cone (Henley and Poo, 2004; Gomez and Zheng, 2006).

As previously mentioned, a growing axon must also be plastic in its responses to environmental cues. For instance, in order to continue growing up a long-distance gradient of attractive molecules, it must adapt to the changing concentration and grow away from an area it previously found attractive (Gallo

and Letourneau, 2002). This may occur by a process called *adaptation* (or *desensitization*), where a growth cone becomes temporarily unresponsive to a previously attractive guidance cue due to a decrease in cytosolic calcium signaling, followed by *resensitization* to slightly higher concentrations of the guidance cue, mediated by local protein synthesis and mitogen-associated protein (MAP) kinase signaling (Ming et al., 2002).

Repulsive guidance cues cause depolymerization of the actin cytoskeletal filaments and localized collapse of the neural growth cone. Semaphorin 3A (Sema3A) is one example of a primarily repulsive guidance cue, and it exerts its effect on actin filaments through activation of the small guanine triphosphatase (GTPase) RhoA. It has recently been demonstrated that mRNA encoding RhoA is transported preferentially to the growing tips of axons, and Sema3A signaling leads to local synthesis of the RhoA protein and subsequent actin disassembly (Wu et al., 2005). Furthermore, the identification of functional RNA interference (RNAi) proteins in growth cones has presented a potential mechanism for local regulation of mRNA transcripts such as those for RhoA (Hengst et al., 2006).

Once a developing axon has navigated to its appropriate target location, the final step must be the formation of functional synaptic connections. Like growth cone steering, synaptogenesis is a complex process involving cell-cell signaling, changes in protein expression, and alterations of intracellular microarchitecture. In the target area, priming factors such as fibroblast growth factors (FGFs), Wnts, cholesterol and thrombospondin are secreted by both neurons and associated glial cells, directing the initiation of synaptic contacts (Pfrieger and Barres, 1996; Mauch et al., 2001; Waites et al., 2005). More intimate contacts between axons and target dendrites result in contact signaling via cell adhesion molecules (CAMs), including members of the cadherin and immunoglobulin superfamilies (Yamagata et al., 2003). The signaling cascades that are set in motion with CAM binding lead to the development of specialized structures that define the synapse: presynaptic active zones and receptor-rich postsynaptic densities (Varoqueaux et al., 2006). Finally, neuronal activity

probably determines the stability of newly-formed synapses, with non-functional synapses facing elimination (Waites et al., 2005).

Regeneration in the Adult CNS

One idea promoted by Santiago Ramón y Cajal which turned out to be flawed was the assumption that the adult mammalian CNS is incapable of producing new neurons (Cajal, 1958). Recent research has led to the rejection of that assumption, which had acquired the status of dogma over the course of decades. It is now accepted that new neurons are continuously produced in specific regions of the adult CNS, namely the subventricular zone (SVZ) of the lateral ventricles and in the subgranular zone (SGZ) of the hippocampal dentate gyrus (Reviewed by Ming and Song, 2005; Hagg, 2005; Shivraj Sohur et al., 2006). In the healthy adult brain, neurogenesis rarely occurs outside of these two regions. After pathological insults or experimental manipulations of the CNS, however, neurogenesis may be stimulated in regions of the brain usually considered to be non-neurogenic (Magavi et al., 2000; Ming and Song, 2005; Shivraj Sohur et al., 2006). The physiological significance of this neurogenic potential remains to be determined, however, and therapeutic harnessing of the potential is in the very early stages of investigation.

Another previously pervasive assumption was that injured neurons of the adult CNS are incapable of regenerative growth. Francisco Tello, a student of Ramón y Cajal, countered that assumption by showing that adult CNS axons could sprout when peripheral nerve grafts were placed into the cerebral cortex (reviewed by Sotelo, 2002). However, it wasn't until the pivotal studies of Aguayo and colleagues in the late 1970's that the intrinsic growth capacity of some adult CNS neurons was fully appreciated. They demonstrated that injured neurons in the brainstem and spinal cord of adult rats could extend axons over distances greater than 30mm through a peripheral nerve bridge graft (David and Aguayo, 1981). Since that time, much attention has focused on the environmental obstacles that prevent CNS neurons from realizing their

regenerative potential: a paucity of growth-promoting molecules and an abundance of growth-inhibitory molecules surrounding injured neurons.

The molecules that have been implicated as inhibitory to the regeneration of mature CNS neurons at an injury site include the myelin-associated molecules Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte-myelin glycoprotein (OMgp), the repulsive guidance molecules EphA4 and semaphorin 3A, and extracellular matrix molecules such as chondroitin sulfate proteoglycan (CSPG) (Pasterkamp et al., 1999; Sandvig et al., 2004; Schwab et al., 2005; Goldshmit et al., 2006; Niclou et al., 2006; Yiu and He, 2006). The expression and up-regulation of such molecules following injury is only half of the story, however. It has also been determined that adult neurons respond differently to molecules such as MAG than embryonic neurons do. At some point during development, some neurons switch their response to MAG from one of growth promotion to one of inhibition (DeBellard et al., 1996). The secondary messenger molecule cyclic adenosine monophosphate (cAMP) has been shown to play an important role in determining such responsiveness: young neurons have higher levels of cAMP and have a positive growth response to MAG, while adult neurons have lower levels of cAMP and have a negative response (Cai et al., 2001). Another difference between adult and embryonic neurons is that the younger cells can respond to a low level of growth-promoting molecules by increasing their expression of receptors for such molecules, while adult cells normally do not (Condic, 2001). Hence, the intrinsic properties of adult neurons must still be considered when tackling the problem of abortive regeneration in the CNS (Domeniconi and Filbin, 2005).

To promote regeneration after CNS injury, experimental approaches have included blocking or removing inhibitory molecules, providing growth-promoting molecules, providing growth-supportive substrates, increasing neuronal responsiveness to growth-promoting molecules and decreasing responsiveness to growth-inhibitory molecules. Chondroitinase ABC, an enzyme that degrades the glycosaminoglycan (GAG) side chains of CSPG molecules, has been used to render injury sites in the CNS more permissive to axon growth and has led to

improved functional recovery in animal models (Barritt et al., 2006; Houle et al., 2006; Massey et al., 2006). Administration of a soluble function-blocking Nogo-66 receptor (Nogo-R) fragment has shown efficacy in vivo, increasing axonal regeneration across a spinal cord lesion site after direct and transgenic administration (Li et al., 2004; Li et al., 2005). Supporting the concept that cAMP levels determine growth responses to myelin-derived inhibitory molecules, injection of a cAMP analog into dorsal root ganglia (DRG) allows central axons of sensory neurons to regenerate into the spinal cord after a lesion (Neumann et al., 2002; Qiu et al., 2002). There is also evidence suggesting that a combinatorial approach – i.e., using cAMP and neurotrophic molecules - may be best to optimize the regenerative response following spinal cord injury (Lu et al., 2004). These studies and many others suggest that with careful molecular manipulation, injured neurons in the adult CNS may respond in a more "embryonic" way, allowing for extensive regeneration and functional recovery.

Neuronal Transplantation

Experimental transplantation of exogenous neurons into adult mammalian brains was reported in the late 1800's, but was not pursued as a potential therapy for CNS disorders until the 1970's (Reviewed by Emsley et al., 2004). Transplanted cells may confer therapeutic benefits in two different ways: 1) by forming functional, reciprocal connections within the host brain or spinal cord, essentially replacing lost neural circuits, or 2) by producing neurotransmitters or neurotrophic/neuroprotective factors to enhance survival, regeneration and function of spared host neurons (Bjorklund et al., 2000; Emsley et al., 2004). Cells used for transplantation are always immature: derived either from embryonic dissections or *in vitro* expansions of neural stem/progenitor cells. As discussed in the previous sections, their immature state means these cells are more likely to survive, grow, respond to environmental cues and successfully integrate into the host CNS.

Investigations into neural transplantation therapies are ongoing for many neurological disorders, including spinal cord injury, stroke, amyotrophic lateral

sclerosis, Huntington's disease, and Parkinson's disease. Animal models of these injuries and diseases have revealed potential for transplantation, and early clinical trials have demonstrated the safety and feasibility of cell grafting into the human CNS. The most benefit has been seen in cases where transplanted cells do not replace lost circuits, but rather can act in a supportive role, providing protective and restorative compounds to the injured tissue or producing and releasing neurotransmitters in a relatively unregulated manner. This is the simpler task for grafted cells, and is sufficient in many cases to provide functional improvement for patients. For instance, many spinal cord injuries are classified as "incomplete" – that is, there is partial sparing of ascending and descending white matter tracts after injury – so protecting these fibers and supporting new axonal sprouts from them is a reasonable job for transplanted cells (Reier, 2004). A more difficult task is to *replace* neurons that have been lost with appropriate axon growth, synaptic connections and functional integration into the host.

Parkinson's disease

Research into cell transplantation for Parkinson's disease (PD) began in the 1970s, when it was first determined (using intraocular grafts and then brain grafts) that embryonic neural tissue was best suited for surviving transplant procedures, extending neurites into host tissues, and forming synaptic connections (Olson et al., 1983; reviewed by Dunnett et al., 2001). One problem that was encountered at the time was insufficient graft survival, mainly due to a lack of blood supply to the cells within a piece of transplanted tissue. The development of a procedure to dissociate appropriate neurons into a suspension and deliver them to the host brain by stereotaxic injection helped to alleviate this problem (Bjorklund et al., 1980). The cells used for transplant in animal models of PD were – and usually still are – dopaminergic neurons from the ventral mesencephalon (VM) of developing embryos.

Most successful transplant studies, including human clinical trials, involve grafting of embryonic VM cells directly into the striatum of the host. This ectopic graft placement avoids the problem of how to direct axonal growth long distances

from the original location (the substantia nigra, SN) to the appropriate target (striatum). It has been shown in many laboratories that VM transplants into the striatum of parkinsonian animals can survive long-term, extend neurites into surrounding host tissue, make synaptic connections, respond to afferent stimulation, express enzymes for dopamine synthesis, release dopamine, and reverse some behavioral deficits (reviewed in Barker, 2002; Redmond, Jr., 2002). The survival of transplanted VM neurons is variable, however, and has been enhanced with various techniques, including repeated injections of GDNF adjacent to grafts (Rosenblad et al., 1996), co-transplant of neurospheres modified to produce GDNF adjacent to grafts (Ostenfeld et al., 2002), and *ex vivo* transduction of neurons with cDNA encoding human vascular endothelial growth factor prior to transplant (Casper et al., 2002). Graft survival and striatal reinnervation were also improved by using a multiple-site microinjection technique to distribute the cells over a wider area (Nikkhah et al., 1994b).

Success in the laboratory led to clinical trials of fetal tissue transplantation in PD patients, starting in Mexico and Sweden in 1988 (Madrazo et al., 1988; Lindvall et al., 1988). Since that time, it is estimated that 400 PD patients have received fetal tissue transplants, with 300 of those being published. Variation in methods and results makes it difficult to compare these attempts, however, and much controversy still exists regarding the efficacy and safety of fetal tissue transplants in PD patients (Redmond, Jr., 2002). Two double-blind, sham-surgery-controlled randomized trials of fetal nigral tissue transplants were published in 2001 (Denver) and 2003 (Tampa) with somewhat disappointing results in each case (Freed et al., 2001; Olanow et al., 2003). The primary outcome measure of these studies (a subjective global rating scale) revealed no significant difference between implant and sham-operated groups after one or two years. By some other measures, there was clinical improvement in implant versus sham-operated patients in the Denver study, but only in the younger (<60 years old) subgroup of patients. In the same study, 5 patients (15%) with the greatest extent of tissue engraftment in the striatum (as determined by PET scans) developed severe dyskinesias (Freed et al., 2001; Greene and Fahn,

2002; Ma et al., 2002). In the Tampa study, thirteen out of 23 grafted patients (56.5%) developed dyskinesias in during off-medication periods between 6 and 12 months after grafting. Most of the dyskinesias were considered mild, but three patients had severe dyskinesias and required corrective surgery. There was no correlation between striatal fluorodopa uptake in the striatum and appearance of dyskinesias in this study (Olanow et al., 2003).

In both animal models and human Parkinson's patients, it has been determined that grafts of fetal nigral cells into the striatum, though often functional, do not result in a complete recovery of motor function (reviewed by Winkler et al., 2000). Reinnervation and restoration of dopamine content within the striatum are not enough to repair what is broken in PD. In fact, the results of one placebo-controlled clinical trial suggest that "too much" graft-induced striatal reinnervation may cause debilitating side effects, even when striatal dopamine levels do not exceed "normal" values (Freed et al., 2001; Ma et al., 2002). This is not too surprising, given that in their natural location (the substantia nigra pars compacta, SNc), nigrostriatal dopaminergic neurons receive regulatory afferent input and release dopamine from their dendrites which then binds to other dopaminergic cells and the axon terminals of striatonigral projection neurons in the substantia nigra pars reticulata (SNr) (Cheramy et al., 1981; Robertson, 1992). These complexities of the striatal-nigrostriatal circuit are completely ignored when transplants are placed exclusively into the striatum. In addition, most cells within a piece of embryonic VM are not developmentally destined to become nigrostriatal neurons. Even some of the dopaminergic cells (A10 neurons of the ventral tegmental area) do not normally innervate the striatum, but rather project to limbic and cortical regions. Improper placement of these cells into the striatum may contribute to unwanted side effects (Isacson et al., 2003).

To explore the role of dopaminergic input to the SN itself, intranigral transplants of fetal VM cells have been carried out in 6-OHDA-lesioned rats (reviewed by Winkler et al. 2000). While early attempts at this procedure failed due to the small transplant site and traumatic transplantation procedures, Nikkhah and colleagues used a microtransplantation approach that resulted in

extensive reinnervation of the SNr and some functional recovery (Nikkhah et al., 1994a). This study showed that intranigral grafts decreased rotational asymmetry caused by injection of dopamine agonists, but not that caused by amphetamine. Other studies of intranigral VM transplantation have confirmed the attenuation of dopamine agonist-induced turning, and shown that some more complex behaviors may be partially restored (Olsson et al., 1995; Yurek, 1997). Intranigral transplants performed in MPTP-treated monkeys resulted in a "modest but detectable benefit" (Collier et al., 2002). To determine whether reinnervation of *both* the SN and the striatum could result in additive functional benefits, some groups have performed simultaneous intrastriatal and intranigral grafts. This method seems to provide more behavioral benefits that striatal grafting alone, and one pilot clinical trial has already determined the safety and efficacy of double grafts in human PD patients (Olsson et al., 1995; Mendez et al., 1996; Mukhida et al., 2001; Mendez et al., 2002).

Reconstructing the nigrostriatal pathway

To truly restore the circuitry that is lost in PD, neurons that are transplanted into the substantia nigra should extend axons to the striatum. The distance between the SN and the striatum, coupled with the inhibitory environment of adult CNS, makes it difficult to effect such axon growth (Schwab et al., 1993). Successful reconstruction of the nigrostriatal pathway was accomplished in neonatal (postnatal day 3) rat brains previously lesioned with 6-OHDA, using rat embryonic VM, but this has little clinical relevance, since PD only affects adults (Nikkhah et al., 1995). In the adult rat, only xenografts of human or porcine tissue into the SN have resulted in some unaided long distance axonal growth within the medial forebrain bundle and the internal capsule, specifically toward and then into the striatum (Wictorin et al., 1992; Isacson et al., 1995; Armstrong et al., 2002). These studies suggest that although rat brain becomes less permissive to axon growth as it ages, growth is possible given the right conditions in the host and/or the donor tissue.

Attempts to reconstruct the nigrostriatal pathway in adult rat brains using allogenic tissue have relied on “bridging” techniques, that is, creating a growth-supportive conduit between the SN and the striatum. Wang and colleagues (Wang et al., 1996) grafted fetal VM tissue into 6-OHDA-lesioned SN immediately followed by injection of 100µg GDNF along a track from the SN to the striatum. Compared to control animals (without bridge) or to animals that received a bridging injection of BDNF, the GDNF-bridged animals showed a significant decrease in amphetamine-induced rotational behavior 1-3 months after grafting. TH^+ fibers were found in the striatum at 3 months post-grafting, indicating that some transplanted neurons had extended axons to the target (Wang et al., 1996). Also in 1996, Zhou and colleagues published a study wherein they bridged the SN transplant site to the striatal target using the excitotoxic compound kainic acid. Kainic acid had been shown previously to increase the expression of growth-supportive molecules such as NGF and GDNF. In this study, creating a bridge with kainic acid did result in some axonal outgrowth from the SN to the striatum, increasing dopamine within the striatum and ameliorating some behavioral deficits (Zhou et al., 1996). Another approach to creating bridges from SN to striatum involves grafting of exogenous cells that provide a growth-permissive environment along the pathway. Examples include bridges of dissociated striatal tissue (Dunnett et al., 1989), fibroblast growth factor (FGF)-4-secreting schwannoma cells (Brecknell et al., 1996), GDNF-secreting Schwann cells (Wilby et al., 1999) and kidney tissue (Chiang et al., 2001).

While each of these bridge-graft studies reported growth of TH^+ fibers from the SN to the striatum, coupled with improvement in amphetamine-induced rotation, the amount of striatal reinnervation remains too low to result in improved spontaneous motor behavior. It has been estimated that at least 10-20% of normal striatal dopaminergic innervation (10-12,000 DA neurons in the normal rat striatum) is necessary to improve such behaviors, but only 3% is required to significantly reduce amphetamine-induced rotation (Kirik et al., 1998; Winkler et al., 2000). The average number of axons to grow through the bridge in the study

by Wilby and colleagues was about 300, or 10% of the number of surviving grafted VM neurons (Wilby et al., 1999). In order to see improved motor function that is clinically relevant, future studies will need to increase the number of grafted neurons that follow the nigrostriatal pathway and reinnervate the striatum.

Viral Vectors for CNS application

One way to therapeutically alter the adult CNS environment is to utilize viral vectors to express neuroprotective or neurorestorative molecules. The vectors may target exogenous cells to be transplanted into the brain (*ex vivo* therapy) or target the host brain cells directly (*in vivo* therapy) (reviewed by Davidson and Breakefield, 2003). Therapeutic strategies of gene therapy may include decreasing the activity of a dominant-negative mutant protein by antisense oligonucleotides or RNA interference, replacing a missing protein in recessive genetic conditions, or providing general neural protection and support with neurotrophic factors, antioxidants or anti-apoptotic factors (reviewed by Costantini and Isacson, 2000; Trulzsch and Wood, 2004). Used in conjunction with cell transplantation, viruses can alter cells to deliver molecules that enhance graft-cell survival and increase fiber outgrowth (Casper et al., 2002; Ostenfeld et al., 2002).

Several different types of viral vectors have been used successfully in experimental models to transfer genes into the CNS, including adenovirus, adeno-associated virus, herpes simplex virus, and lentivirus. Decisions about which vector is best suited to a particular situation are based on factors such as safety, specificity of targeting, efficiency of transduction, the size of the transgene, duration of expression and whether regulation of expression is required. Different vectors have different strengths and weaknesses in each of these areas and modifications may also improve characteristics such as transduction efficiency and host cell targeting (Costantini and Isacson, 2000; Davidson and Breakefield, 2003).

Many CNS disorders have been targets of gene therapy, at least in experimental animal models, including Parkinson's disease, Huntington's

disease, amyotrophic lateral sclerosis, stroke, lysosomal storage diseases, chronic pain, malignant glioma and epilepsy (Costantini and Isacson, 2000; Davidson and Breakefield, 2003). PD may be an optimal target for gene therapy due to the well-defined, localized nature of the neural degeneration (Bohn, 2000). Indeed, several studies have already focused on viral vector-mediated delivery of GDNF to protect or restore dopaminergic neurons that have been exposed to a toxic insult such as 6-OHDA (in rats) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, in primates) (Bensadoun et al., 2000; Bjorklund et al., 2000; Kordower et al., 2000; Kozlowski et al., 2000; McGrath et al., 2002; Palfi et al., 2002; Zheng et al., 2005). Molecules other than GDNF have also been investigated as targets of gene therapy for PD, including enzymes involved in biosynthesis of dopamine, such as tyrosine hydroxylase, aromatic-L-amino-acid decarboxylase (AADC) and GTP cyclohydrolase I (Shen et al., 2000; Azzouz et al., 2002; Muramatsu et al., 2002). Three Phase 1 clinical trials of gene therapy for PD patients are currently underway, each one utilizing adeno-associated virus (AAV), which is one vector known to have low immunogenicity, and is the only virus currently approved for clinical trials. The genes being delivered to patients' brains are glutamic acid decarboxylase (GAD) to the subthalamic nucleus (Neurologix), AADC to the striatum (Avigen) and neurturin to the striatum (Ceregene). So far, no safety concerns have been reported, and neither have there been reports of efficacy (Dass et al., 2006).

Aims of this dissertation research

The general goal of the experiments described in the following chapters is to develop a technique, using viral vectors and cell transplantation, to replace long neural tracts in the damaged adult mammalian CNS. The viral vectors are used to modify the host environment in order to enhance cell survival and target axon growth from transplanted cells. More specifically, my aims are:

1) To determine whether viral vectors may be used to create pathways to direct neurite outgrowth from a cell transplant site in the brain to a distant target location.
2) To determine whether such a targeting technique may be applied to a Parkinson's disease model and result in behavioral improvements after cell transplantation into the substantia nigra.

Chapter Two: Targeting Axon Growth from Dorsal Root Ganglion Neurons

Introduction

Central nervous system (CNS) damage due to disease or injury may be repaired by neural transplantation, but only if certain obstacles are overcome. Finding a safe, reliable source of cells for transplantation is both a scientific and an ethical challenge, but that is just the first step. Once cells are prepared for transplantation, a sufficient number of neurons must survive the transplantation procedure and extend fibers – sometimes over long distances – to reach appropriate targets and replace damaged circuitry. Transplantation experiments have produced promising results in animal models of spinal cord injury, amyotrophic lateral sclerosis, epilepsy, stroke, Parkinson's disease and Huntington's disease, and several clinical trials have already been conducted in these arenas (Bjorklund et al., 2000; Isacson et al., 2003). In most cases so far, cell-replacement therapy is limited to local effects on host circuitry, because axons are unable to extend over long distances in the adult CNS. This limitation is most likely due to two related issues: a lack of growth-promoting molecules and guidance cues in the adult host tissue, and the presence of inhibitory molecules, especially after injury (Aubert et al., 1995; Sandvig et al., 2004; Domeniconi and Filbin, 2005).

Since neurons used for transplantation are usually fetal or stem cell-derived, their intrinsic growth potential is high. To take advantage of that potential, the host environment must be altered to more closely resemble the permissive milieu of the developing nervous system. One way to effect such a change is to use viral vectors to overexpress growth-supportive molecules in neurons and glia of the host prior to transplantation. Recombinant adenoviral vectors have proven to be safe and effective tools for gene transfer into the CNS (Smith and Romero, 1999; Gerdes et al., 2000; St.George, 2003). We have used adenoviral vectors previously to over-express nerve growth factor (NGF) or fibroblast growth factor 2 (FGF-2) in the dorsal horns of adult rat spinal cords,

causing robust sprouting and regeneration of nociceptive sensory afferents through the inhibitory dorsal root entry zone (Romero et al., 2000, 2001). These studies support the idea that adenovirus-induced expression of growth-supportive molecules in the CNS can counterbalance inhibitory signals and encourage axon growth.

The current study seeks to determine whether adenoviral vectors may be used to create guidance pathways for axons of transplanted neurons to follow to a distant target in the adult brain. Previous work by Davies et al. showed that embryonic or adult dorsal root ganglion (DRG) neurons, when transplanted into the corpus callosum of adult hosts, could extend axons for several millimeters along the tract only if scarring from transplantation was minimal (Davies et al., 1994, 1997). The fact that axon growth in the corpus callosum is possible yet constrained makes it an ideal location to test the effect of virus-induced protein expression on axon growth and pathfinding decisions in the CNS. Since much is known about the chemoattractive and chemorepulsive molecules that influence the normal development of nociceptive sensory afferents, these neurons were chosen as our first test candidates.

Methods

Construction of adenoviral vectors

Replication-defective, temperature-sensitive recombinant adenoviruses encoding NGF, FGF-2, semaphorin3a, and green fluorescent protein (GFP) were constructed as previously described (He et al., 1998; Romero and Smith, 1998; Tang et al., 2004) . All plaque-purified adenoviruses were examined for replication-competent adenoviruses by PCR and were amplified and purified by double cesium chloride gradient ultracentrifugation. The absolute concentration of viral particles was determined by optical absorbency, and the concentration of infectious particles (pfu) was quantified by viral hexon protein expression in infected HEK293 cells using the Adeno-X Rapid Titer kit (BD Biosciences). Expression of each protein by virus-infected U373 cells was confirmed by

Western blot 72h after transfection as previously described (Romero et al., 2000; Tang et al., 2004).

Adenovirus injections

Adult female Sprague-Dawley rats (retired breeders, Harlan) were used for all experiments. Animals were maintained under conditions of controlled light and temperature, with food and water available *ad libitum*. On the day of surgery, rats were anesthetized with a mixture of ketamine (67 mg/kg i.p.) and xylazine (6.7 mg/kg i.p.) and placed into stereotactic frames. On that day and the day following, rats were given i.p. injections of monoclonal antibodies to block the T-cell receptors CD4 (W3/25, 50µg) and CD45 (OX-22, 50µg), to suppress any immune response to the adenovirus (Romero and Smith, 1998). For comparisons of guidance pathways, animals were randomly assigned to one of four treatment groups: 1) Ad-FGF-2 along the guidance pathway, Ad-NGF at the target, 2) Combination Ad-FGF-2/Ad-NGF along the pathway, Ad-NGF at the target, 3) Ad-GFP along the pathway, Ad-NGF at the target, or 4) Ad-GFP along the pathway and at the target (No NGF). Guidance pathways were created by injecting each adenovirus every 1mm from a transplant site in the left lateral corpus callosum to a 90-degree turn into the contralateral striatum (target). Using bregma as a landmark, holes were drilled into the skull to allow injections at the following coordinates, all 0.3µm caudal, depths relative to dura: transplant site, +2.8mm lateral (left side), -3.0mm deep; pathway within the corpus callosum, +/- 2.5mm lateral, -3.0mm deep, +/- 1.5mm lateral, -2.9mm deep and +/-0.7mm lateral, 3.2mm deep; pathway into striatum at -2.5mm lateral (right side), depths of 3.5mm and 4.0mm; striatal target, -2.5mm lateral, 4.5mm deep. For the semaphorin experiment, all animals received pathways of Ad-FGF-2/Ad-NGF and striatal NGF targets, then half the animals received two 0.4µl injections of Ad-semaphorin3A 1mm dorsolateral from the pathway turning point at 3.2mm lateral, 2.2mm deep and 3.6mm lateral, 2.6mm deep. For the cortical target experiment, the striatal path and target were replaced with injections at -2.5mm lateral, 2.5, 2.0 and 1.5mm deep. Adenovirus concentrations were $5x10^6$ pfu/µl,

except for combination Ad-FGF-2/Ad-NGF injections, where the Ad-NGF concentration was reduced to $1x10^6$ pfu/µl for a 5:1 ratio of Ad-FGF-2 to Ad-NGF. Injection volumes ranged between 0.2 µl (Ad-NGF at transplant site) to 2.0µl (Ad-NGF at striatal target), and increased along the pathway to create a gradient effect. Volumes were injected at a rate of 0.4µl/min using a 10µl Hamilton syringe and a 30-gauge beveled needle, and the needle remained in place for 2 minutes at the end of each injection.

DRG isolation and transplantation

One week after adenovirus injections, DRG neurons were isolated from postnatal day 1 or 2 (P1-P2) Sprague-Dawley rat pups (timed-pregnant dams from Harlan). Each pup was quickly decapitated and DRGs were removed using sterile Dumont forceps and placed into Hank's buffer containing 1% collagenase, kept on ice until all dissections were complete. After a 20-minute incubation at 37ºC, DRGs were washed then trypsinized for 10 minutes at 37ºC, treated with DNAse and washed twice more with 10% fetal bovine serum in Dulbecco's Modified Eagle Medium (FBS/DMEM), then triturated to disperse the ganglia into a cell suspension. Cells were then plated out in 10%FBS/DMEM containing 50ng/ml NGF for 45 minutes at 37ºC to allow adhesion of Schwann cells. Cells remaining in suspension were then spun down and washed in N2-supplemented media three times to eliminate FBS. The number of live neurons per microliter was determined by treatment with trypan blue and counting on a hemocytometer. The final cell suspension was supplemented with NGF (50ng/ml) and kept on ice until transplant (up to 3 hours total). Rats previously injected with adenovirus were re-anesthetized and their skulls exposed again at the transplant site (2.8mm lateral, 0.3mm caudal to bregma). 3000-5000 DRG neurons were injected with a Hamilton syringe/30-guage needle in a volume of <2µl (0.4µl/minute) into the left corpus callosum, 3mm below the dura. The needle was left in place for 10 minutes, withdrawn 0.4mm, left for an additional 5 minutes, then slowly retracted the rest of the way.

Immunohistochemistry and Quantification

Rats were killed 3 weeks after DRG transplants by pentobarbital overdose and perfusion with cold saline followed by 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde overnight, then transferred to a 30% sucrose solution for approximately two days, allowing brains to sink to the bottom of the vials before cryosectioning. One in five coronal sections (30μm thick) through the transplant area were immunostained using a rabbit primary antibody to calcitonin gene-related peptide (CGRP; Jackson Labs; 1:10,000 dilution), a biotinylated secondary antibody (goat anti-rabbit IgG, 1:600) and developed with a diaminobenzidine chromogen to visualize nociceptive neuronal cell bodies and axons. For each animal, three sections in the 1:5 series (150μm apart) with the most CGRP+ cells or fibers were quantified for transplant survival or axon growth along the pathway by a blinded observer. For cell survival, the total number of visible CGRP+ cell bodies at the transplant site were manually counted at 200x total magnification and summed over the three sections containing the most cells. Axons were counted manually at 200x total magnification at the following points along the pathway: in the corpus callosum 1 and 2mm from midline ipsilateral to transplant (mid -1mm, mid -2mm), in the corpus callosum 1 and 2mm from midline contralateral to transplant (mid +1mm, mid +2mm), in the contralateral striatum 0.5mm below the corpus callosum (c.c. -0.5mm), and in the contralateral striatum 1mm below the corpus callosum (c.c. -1mm). To correct for differences in transplant size and survival, axon counts at the more distal path points were divided by a count closer to the transplant (mid-2mm or mid-1mm). For quantification of turning success with and without semaphorin expression adjacent to the turn, animals with fewer than 10 axons at the turning decision point (average over 3 sections) were eliminated from the analysis. This led to elimination of 3 animals from each group. Axons were then counted at distances relative to the needle track from the Ad-NGF target injection: 200μm past the track in the corpus callosum, and 200μm below the corpus callosum in the striatum.

Statistical Analyses

To analyze cell survival, t-tests were used to compare groups without NGF at different time points and the +NGF group to each time point without NGF. To determine whether the molecule(s) expressed along the pathways affected the number of axons at different points along the pathway, nonparametric statistical tests were employed to allow for smaller sample sizes (smallest group, n=3) and large variances that were observed within groups. A Kruskal-Wallis test was used to determine if pathway composition affected axon count at each path point, and Mann-Whitney U-tests were subsequently used to determine which pathways were significantly different at those points. For turning analysis with and without semaphorin, sample sizes were larger (n = 8 and 9, respectively) and an F-test confirmed equal variance, so a t-test assuming equal variance was utilized to compare those two groups. Differences were considered statistically significant if p-values were less than or equal to 0.05.

Results

Transplant survival requires Ad-NGF injection at transplant site

Initial experiments followed the injection scheme depicted in Fig. 2-1*A*, either with or without inclusion of a small-volume Ad-NGF injection at the transplantation site. Expression pathways were constructed by injecting Ad-FGF-2 at several sites along the corpus callosum and down into the right striatum, where a larger volume of Ad-NGF was injected to create a target for growing axons. One week after virus injections, DRG neurons were isolated from P1-2 rat pups and injected at the transplant site in the left corpus callosum. Immunohistochemical staining in test animals one week after transplant confirmed that FGF-2 was being expressed along the corpus callosum and that there was robust NGF expression in the target striatum. Furthermore, CGRP+ axons had grown along the expression pathway, some following needle tracks that overshot the corpus callosum during virus injections leading to FGF-2 expression in the underlying striatum (Fig. 2-1 *B-E*). After allowing two weeks for axon growth,

immunostaining of brain sections through the transplant site revealed good cell survival with NGF expression at the transplant site, but no visible CGRP+ cells in animals without NGF at the transplant site. A subsequent time-course study showed the demise of most transplanted neurons within the first few days after transplant when no NGF support was provided (Fig. 2-2 *A -D*). There was a significant drop in cell survival between days 1-3 (n = 4) and days 7-11 (n = 6, p=0.04), and then no CGRP+ cells could be found at two weeks in those animals lacking NGF at the transplant site (n = 3). There was a significantly higher number of transplanted cells that survived for two weeks with NGF at the transplant site when compared to without NGF at any time point ($p<0.05$ vs. 1-3d No NGF; $p<.001$ vs. 7-11d No NGF). A statistical comparison could not be made at the 14-day time point because without NGF, the cell count was zero in all animals (no variance). In all subsequent animals in this study, a small volume of Ad-NGF was injected at the transplantation site along with the pathway injections one week before transplant to ensure survival of transplanted cells.

Axons do not cross midline without neurotrophin expression along path and/or at target

To determine whether a pathway expressing neurotrophins is necessary for growing nociceptive axons to reach the target area in the contralateral hemisphere, pathways were created by injections of Ad-FGF-2 or a combination of Ad-FGF-2 and Ad-NGF (5:1 ratio) and compared to control pathways created by injections of Ad-GFP one week prior to DRG cell transplantation. Every path included a small Ad-NGF injection at the transplant site to ensure transplant survival, and all but four GFP-pathway animals (GFP only) received Ad-NGF target injections in the contralateral striatum. Three weeks after transplant, immunohistochemical staining for CGRP revealed that pathways expressing FGF-2 (n = 4) or a combination of FGF-2 and NGF (n = 8) supported robust, long-distance axon growth toward the target (Fig. 2-3 *C, D*). All animals in these two groups had axons that grew at least 2mm beyond the midline into the contralateral hemisphere, and ten out of twelve had some turning of axons from

the corpus callosum into the target striatum (Fig. 2-3 *I-M*). In animals that received Ad-GFP injections only along the path and at the target (n = 4), no axons crossed the midline in the corpus callosum, and only two had axons grow more than 1mm away from the transplant site (Fig. 2-3 *A, E, F*). Out of three animals that had GFP pathways ending in an NGF target, only one had axons grow beyond the midline, with a few fibers turning into the contralateral striatum (Fig. 2-3*B*, *G* and *H*). In the other two animals, axon growth stopped at or before the midline. A Kruskal-Wallis test confirmed that the expression of neurotrophins significantly increased the number of CGRP+ fibers in the corpus callosum at 1mm and 2mm past the midline (mid +1mm, $p = .006$; mid + 2mm, $p = .01$) and at 0.5mm and 1mm below the corpus callosum in the target striatum (c.c.- 0.5mm, $p = .03$; c.c.-1mm, $p = .05$) after correcting for transplant size/survival (Fig. 2-3*N*). Subsequent Mann-Whitney U-tests showed significant differences in axon number ($p<0.05$) at mid+1mm between the GFP-only group and both FGF2-expressing groups and between the GFP/NGF target group and both FGF2-expressing groups. At mid+2 and at 0.5mm below the corpus callosum, significant differences were found between the GFP-only group and both FGF-expressing groups and between the GFP/NGF target group and the combination FGF+NGF/NGF target group. At the furthest point quantified, 1mm below the corpus callosum, only the combination FGF+NGF/NGF target group showed a significant increase in axon number over GFP pathways both with and without NGF targets. These results indicate that adenovirus-induced expression of FGF-2 and NGF can enhance and direct nociceptive axonal growth from transplanted DRG neurons over distances of at least 6mm, including a turn from white matter into grey matter, overcoming an otherwise inhibitory environment.

Expression of a chemorepulsive molecule adjacent to turn in path enhances axon turning

Since many axons continued to grow in the corpus callosum beyond the pathway turn (see Fig. 2-3*J* and *L*), we next tested the hypothesis that expression of a chemorepulsive molecule adjacent to the turning point could increase the number

of axons making the desired pathway choice. As shown in Fig. 2-4*A*, the adenovirus injection scheme for this experiment included Ad-semaphorin3A (Ad-sema) injections approximately 1mm from the turn in the dorsolateral direction. Control animals received the same pathway and target injections (Ad-FGF2/NGF and Ad-NGF, respectively), but did not have Ad-sema injections. As in the previous experiments, DRG transplants were done one week after virus injections and animals were killed for histological analysis three weeks after transplant. For this experiment, animals in which CGRP+ fibers did not make it as far as the pathway turning point in the contralateral hemisphere were eliminated from the analysis (three per group). In the remaining animals, an observer unaware of treatment counted CGRP+ fibers that had continued past the target needle track in the corpus callosum and CGRP+ fibers that had turned down into the target striatum, averaged over three sections per animal. The results show a significant increase in the proportion of axons that make the desired turn, from 51% to 77%, in animals with semaphorin expression adjacent to the turning point (no sema, n = 9, mean $\pm$ SEM = 51 $\pm$ 7%; with sema, n = 8, 77 $\pm$ 7%; p=.02, Fig. 2-4 *B-F*). These data support the hypothesis that adenovirus-mediated expression of chemorepulsive molecules can help to selectively target axon growth from transplanted neurons.

Cortical NGF expression leads to enhanced axon growth on brain surface

An interesting observation in some animals was that many CGRP+ fibers grew up into the cortex on the target side, following the needle track from the Ad-NGF target injection, sometimes in greater numbers than those turning toward the striatum. This is consistent with observations by Davies, et al., who described axon growth out of the corpus callosum into the host prefrontal cortex as a default pathway for transplanted CGRP+ DRG neurons (Davies et al., 1997). With this in mind, we altered the adenovirus injection scheme to see if greater numbers of fibers would choose that route if the NGF-expressing target was in the cortex rather than in the striatum. Pathways were constructed with either a combination of Ad-FGF-2 and Ad-NGF (5:1) or Ad-GFP leading to the cortical

NGF target (Fig. 2-5*A*). It was expected that with FGF/NGF pathways, axons would extend along the corpus callosum from the transplant, as before, and then turn up to reach the target area on the contralateral side. Instead, the cortical expression of NGF greatly enhanced growth of CGRP+ fibers along the brain surface in the meninges (Fig. 2-5 *F* and *K*). Many axons from transplanted cells grew upwards immediately and then along the brain surface, with some growing down again wherever there was a needle track from an injection that included Ad-NGF; that is, every needle track in the combination FGF/NGF pathways, and only the target needle track in GFP pathways (Fig. 2-5 *B-M*). Growth within the corpus callosum was limited, but in animals with FGF/NGF pathways, 5 out of 6 had some callosal axon growth all the way across to the target area (a distance of ~5mm). Only one out of three GFP-pathway animals had growth that far in the corpus callosum – in the other two, axons stopped within 1.5mm of the transplant. Axon counts within the corpus callosum were compared by Mann-Whitney U-tests and found to be significantly higher at every path point (corrected for transplant size/survival) when neurotrophins were expressed in the corpus callosum, as compared to GFP-pathway animals ($p<.05$). When growth within the corpus callosum was compared between animals with a cortical NGF target and animals with a striatal NGF target, the pathways leading to a striatal target contained significantly more CGRP+ fibers at midline +1mm, but neurotrophin-expressing pathways contained more fibers than GFP controls in both experiments (Fig. 2-5*N*). The number of CGRP+ axons reaching each NGF-expressing target area (striatal or cortical) was similar, but with cortical targets, no difference existed between animals expressing neurotrophins along the corpus callosum and those expressing only GFP (Fig. 2-5*O*). Obviously, many of the axons had followed an alternative pathway to the target, growing along the brain surface and avoiding the white matter of the corpus callosum altogether. To be sure that the CGRP+ fibers observed in the meninges were not from NGF-induced sprouting of endogenous nociceptive fibers there, three control animals received the same Ad-FGF/NGF pathway injections and Ad-NGF cortical targets, but no DRG cell transplants. In these animals, there were no

visible CGRP+ fibers at the brain surface or penetrating the cortex at any point after the same period of time (data not shown). The results of this experiment underscore the fact that virus-mediated expression of neurotrophic molecules may help to tip the balance of axon guidance in one direction or another, but endogenous cues in the complex molecular environment of the host CNS are key players in directing growth from transplanted cells.

Discussion

The results presented here support the hypothesis that directed, long-distance axon growth from transplanted neurons in the adult CNS may be achieved by virus-mediated expression of specific molecules with neurotrophic or chemotactic properties. Such growth is possible even when the host tissue has been injured by the virus-injection and transplantation procedures. As during development, the best axon targeting is achieved when positive cues are expressed along the desired pathway and in the target tissue and negative cues are expressed in adjacent areas to prevent unwanted turning or sprouting into non-target tissue. The choice of molecules for this technique depends on the specific subpopulation of neurons to be targeted: in our study, nociceptive sensory neurons from the dorsal root ganglia.

During embryonic development, growth cones on different sensory neuronal subtypes respond to different guidance cues, depending on which receptors they express. Based on both *in vitro* and *in vivo* experiments, it has been determined that NGF acts as a chemoattractant for embryonic sensory axons expressing the high-affinity receptor tyrosine kinase Trk A (Gundersen and Barrett JN, 1979; Paves and Saarma, 1997; Tucker et al., 2001). Adult sensory neurons are also able to alter their axon growth patterns in response to NGF, as we have shown previously that adenovirus-mediated overexpression of NGF in the dorsal spinal cord induces extensive sprouting of nociceptive afferent fibers throughout the dorsal horn and even into the ventral horn and lateral funiculus of adult rats (Romero et al., 2000). In the setting of injury to the dorsal root,

lesioned axons that would normally not re-enter the spinal cord were stimulated to do so by virus-mediated NGF expression in the dorsal horn (Romero et al., 2001). This probably reflects the ability of neurotrophins to increase the intrinsic growth state of neurons and overcome inhibitory signals present in the adult CNS environment (Cai et al., 1999). Virus-mediated expression of FGF-2 in the spinal cord had a similar regenerative effect on sensory afferents after dorsal root injury, resulting in growth into the cord, consistent with the known neurite growth-promoting effects of FGF-receptor activation (Williams et al., 1994; Romero et al., 2001). Based on these lines of evidence, we chose NGF and FGF-2 as our positive growth cues for transplanted postnatal DRG neurons in the adult rat corpus callosum, and both molecules improved axon targeting.

Our data support the role of NGF as both a survival-enhancing and axon-targeting molecule for postnatal nociceptive DRG neurons. Without expression of NGF at the transplant site, the vast majority of CGRP+ neurons died within one week of transplant. When Ad-NGF injections were included at the transplant site, many CGRP+ cell bodies were present at two weeks post-transplant. Axon growth away from the transplant site and along the white matter tract was also greatly enhanced by prior injections of adenovirus encoding FGF-2 or a combination of FGF-2 and NGF along the corpus callosum. With only Ad-GFP injections along the path and no NGF-expressing target in the contralateral hemisphere, no CGRP+ fibers grew as far as the midline, and only one out of three animals had contralateral fiber growth when the NGF target was present but the connecting pathway was GFP only. These results differ from those of Davies et al. (1997), who demonstrated long-distance growth of CGRP+ fibers within the adult corpus callosum after DRG transplants without accompanying overexpression of growth-supportive molecules. The discrepancy is most likely due to a difference in transplantation technique. While we used a 30-gauge needle for virus injections and transplants, the Davies group used a microtransplantation method to minimize glial scarring (Davies et al., 1997). In eventual clinical applications, it would be best to reduce the amount of tissue damage due to the cell transplantation procedure because resultant scarring

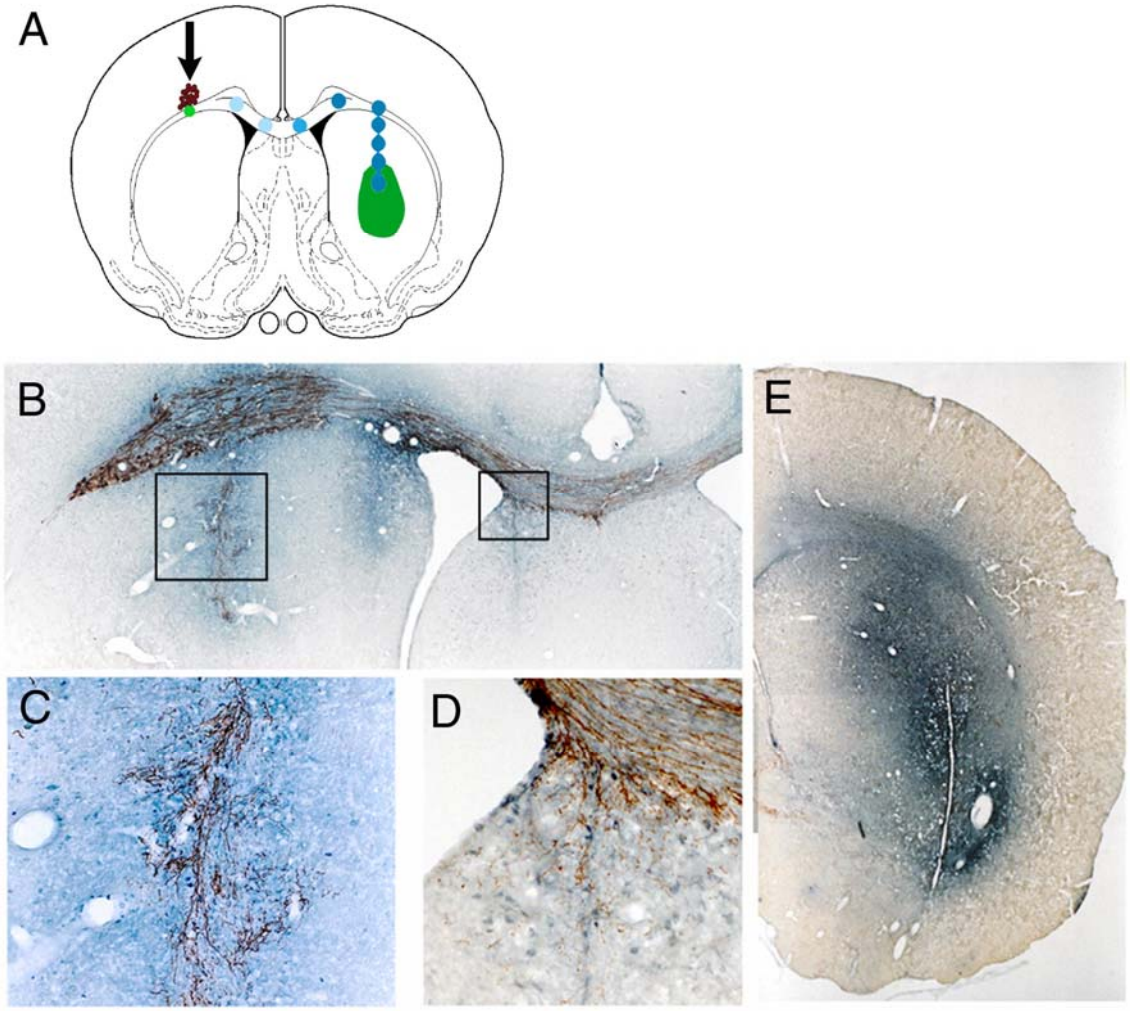
leads to expression of chondroitin sulfate proteoglycans (CSPGs) which are known to interfere with axon regeneration (Davies et al., 1999; Jones et al., 2003; Silver and Miller, 2004). In this model, however, we demonstrate the effectiveness of preformed growth pathways in directing axon growth from transplants in spite of inhibitory signals present in the host tissue, which may be a factor when transplants are made into previously injured brains or spinal cords.

With these encouraging results, we next sought to increase the proportion of axons that turned toward that target rather than continuing along the corpus callosum or growing up toward the cortex by expressing a chemorepulsive molecule, semaphorin3A, dorsolateral to the turn. An extra stimulus is necessary in this situation since white matter may support axon growth that is parallel to, but not perpendicular to the tract, and crossover between white and grey matter is usually restricted by the cytoarchitecture (Crutcher, 1989). Our choice of molecule for this purpose was again based on knowledge of sensory neuron development. Centrally-projecting nociceptive DRG axons are prevented from overshooting their destination in the dorsal-most laminae of the embryonic spinal cord by expression of sema3A in the ventral cord. (Messersmith et al., 1995). Peripheral sensory axons also have their growth pathways restricted by the presence of sema3A in surrounding, non-target mesenchymal tissue, and both central and peripheral semaphorin expression patterns are spatially and temporally regulated to coincide with the development of sensory nerve tracts (Giger et al., 1996; Masuda and Shiga, 2005). Adult nociceptive sensory fibers are similarly repelled and prevented from sprouting by sema3A (Tanelian et al., 1997; Tang et al., 2004). Consistent with these findings, the expression of sema3A dorsal and lateral to the desired ventral turn in the guidance pathway increased the proportion of axons making the “correct” turning decision from 51% to 77%. While expression of positive growth cues along a desired pathway helps to target axon growth, negative cues in the periphery prevent growth into non-target areas and thus improve accuracy in our model as in embryonic development.

An interesting observation in initial experiments was the growth of many CGRP+ axons from the corpus callosum up the needle tracks from Ad-NGF target injections, into the overlying cortex. Even though the Ad-NGF was injected into the striatum, it is likely that a small amount of virus was pulled up the track as the needle was being withdrawn from the brain, leading to some cortical NGF expression. This small amount of expression was apparently enough to encourage growth in that direction, possibly because the host environment in the cortex is inherently more growth-permissive than the striatum. In fact, previous DRG transplants into the corpus callosum by Davies, et al. resulted in CGRP+ fibers turning up into the host cortex without any induced expression of neurotrophic factors (Davies et al., 1997). Based on these observations, we altered the pathway-injection scheme to place the NGF target in the cortex contralateral to the transplantation site. Our prediction was that axons would grow along the corpus callosum as before, and then the majority of the fibers would turn up toward the target. Instead, the high cortical NGF expression directed most axon growth from the transplant site up to and along the brain surface, with some fibers diving down into cortical needle tracks where Ad-NGF had been injected. The combination of NGF availability and the highly vascular nature of the leptomeninges may have led to this pathway decision, since endothelial basement membranes are a rich source of laminin. Laminin, by way of integrin receptors, activates signaling pathways that overlap downstream with NGF signaling pathways, leading to further enhanced neurite outgrowth from sensory neurons (Liu et al., 2002; Tucker et al., 2005). The results of this experiment highlight the importance of endogenous growth cues in the host environment for determining axon trajectories of transplanted neurons.

The current study provides evidence that preformed guidance pathways created by injection of viral vectors can direct axon growth from transplanted neurons to desired target locations. Furthermore, the accuracy of targeting may be improved by judicious expression of chemorepulsive molecules in surrounding areas. This technique could potentially improve outcomes in therapeutic

transplantation paradigms requiring axon growth over long distances and/or through inhibitory host tissue.



<u>Figure 2-1</u>: Confirmation of expression of NGF and FGF-2 after adenoviral injections into the corpus callosum and striatum. A) Schematic of injection protocol – Blue circles represent injection sites for Ad-FGF2, combination Ad-FGF2/NGF or Ad-GFP (control); Green circles represent Ad-NGF injections, small volume (0.2µl) at transplant site (arrow) in the left corpus callosum and large volume (2µl) at the target site in the right striatum. Brown circles represent transplanted DRG neurons, injected one week after the adenoviral pathway. B) Double immunostaining shows CGRP+ fibers (brown) growing along a pathway of FGF-2 expression (blue). Higher magnification insets (C & D) depict axons following needle tracks from Ad-FGF-2 injections, which overshot the corpus callosum. E) Immunostaining for NGF shows widespread expression in the target striatum.

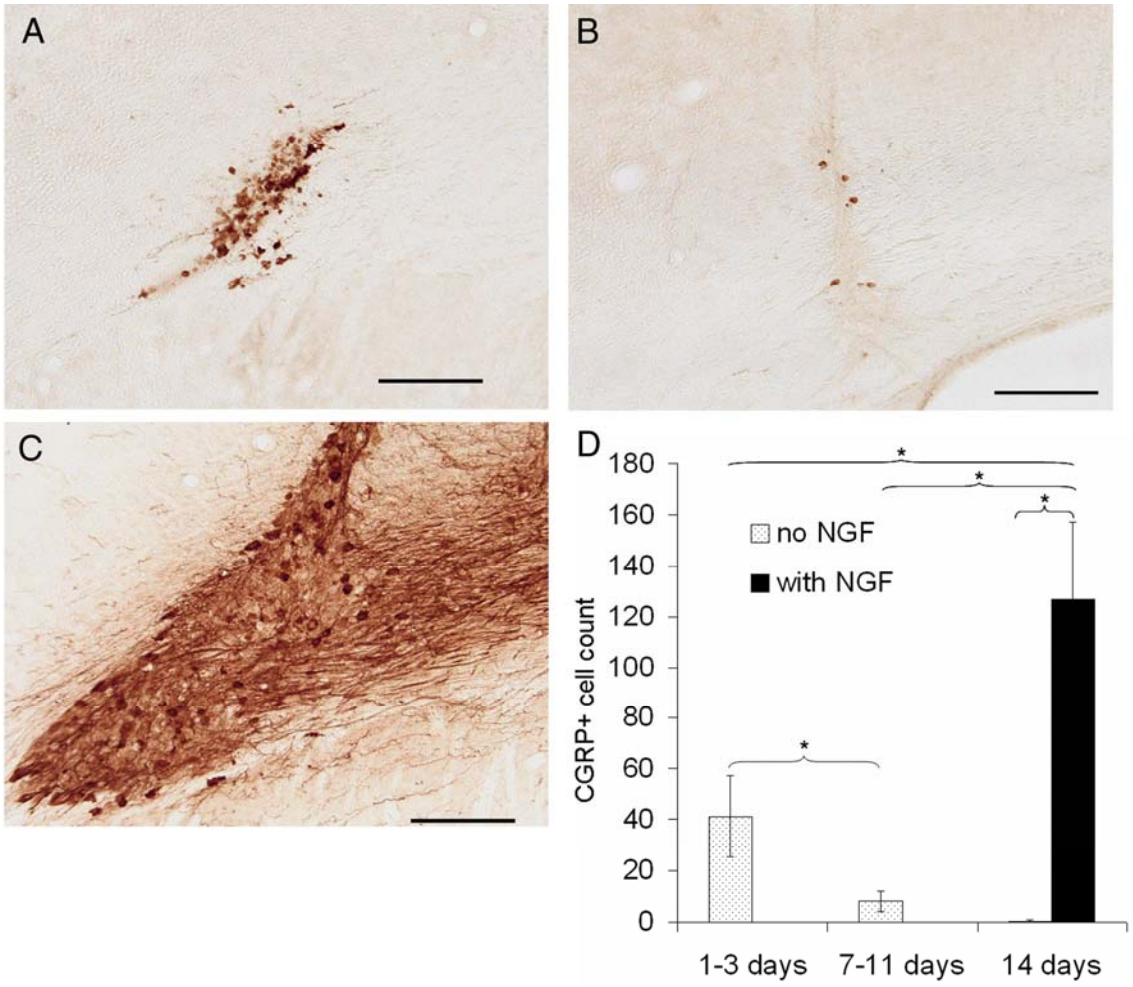
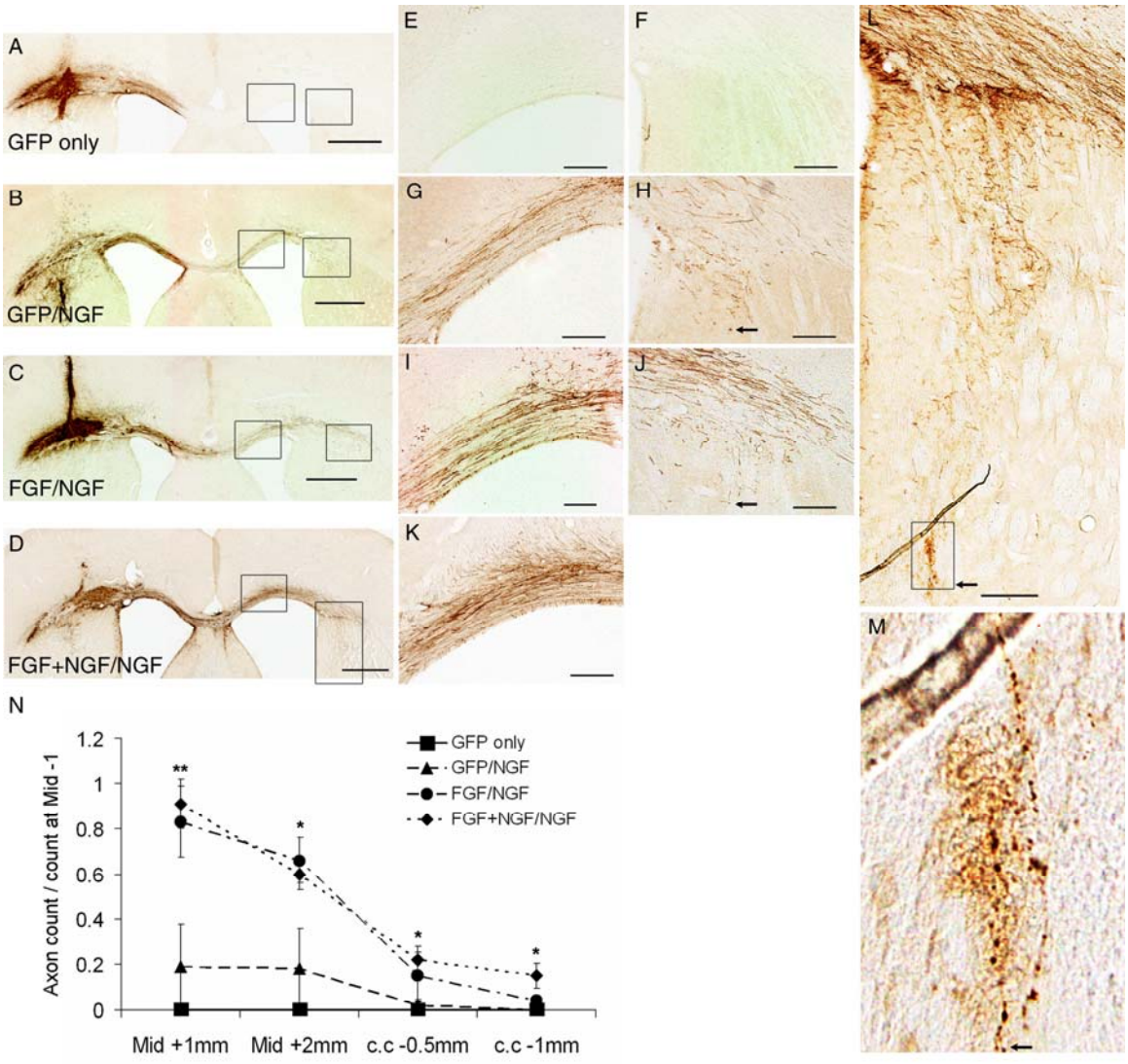
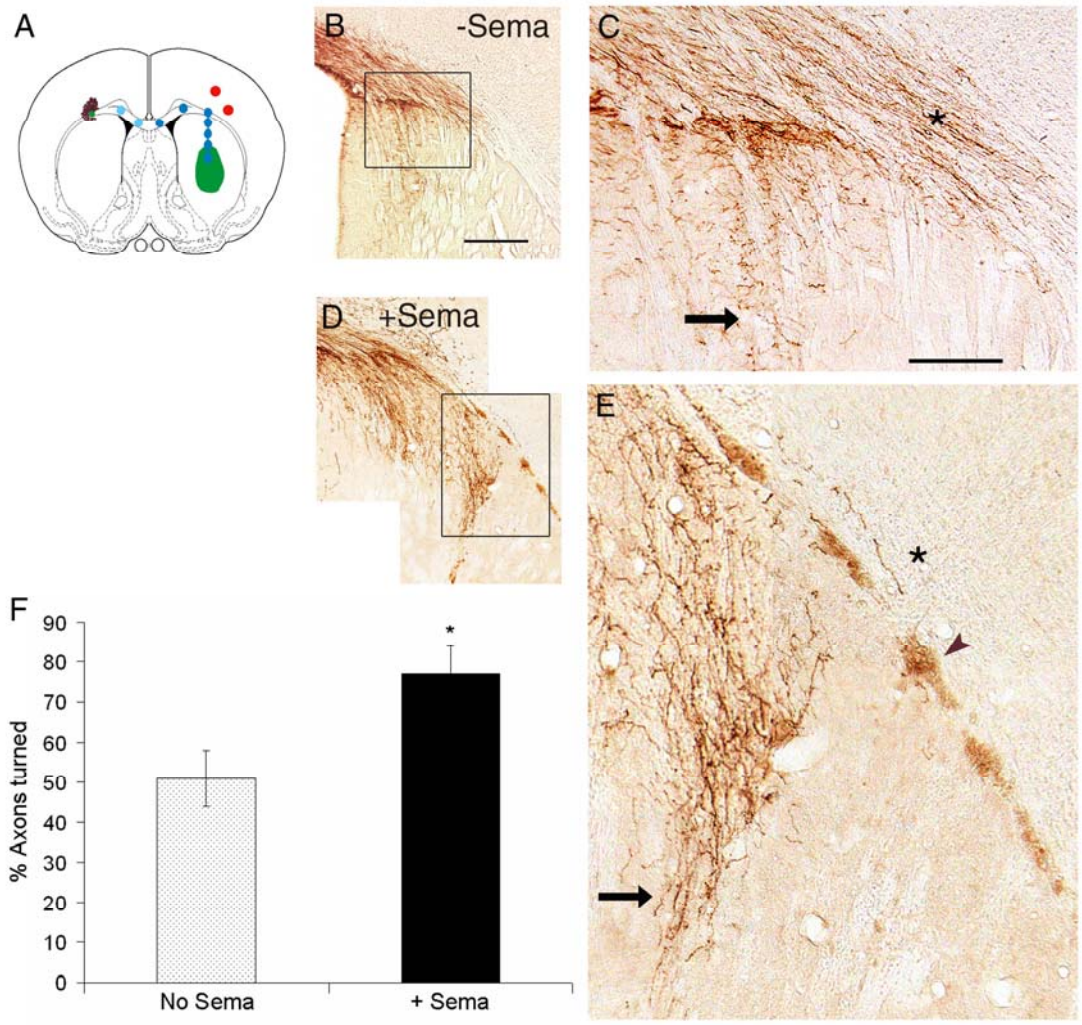


Figure 2-2: NGF expression at the transplant site is necessary for cell survival. A-C) Representative CGRP-immunostained sections through transplant sites; A) Transplant after 24h with no Ad-NGF; B) Transplant after 7 days with no Ad-NGF; C) Transplant after 14 days with Ad-NGF at transplant site. Scale bars = 200μm. D) Counts of CGRP+ cell bodies at the transplant site 1-14 days after transplantation. For each animal, cell bodies were counted in 3 sections separated by 150μm and added together (semi-quantitative method). At 14 days, there were no CGRP+ cell bodies found in any of the three animals lacking NGF expression at the transplant site – the small mark visible at that point in the graph is for illustrative purposes only. Bars represent mean ± SEM at each time point. *$p < 0.05$.



<u>Figure 2-3</u>: Virus-mediated expression of neurotrophic molecules along a pathway improves long-distance growth of axons toward a desired target. Representative CGRP-immunostained sections show nociceptive axon growth three weeks after DRG neuron transplants. *A*) GFP pathway, no target; there was no CGRP+ fiber growth beyond the midline in any animals from this group. *B*) GFP pathway, NGF target in striatum; this section is from the *single* animal that showed CGRP+ fiber growth beyond the midline in this group. *C*) FGF pathway, NGF target in striatum; there is ample CGRP+ fiber growth into the contralateral (target) hemisphere, to and beyond the pathway turning point within the corpus callosum. *D*) FGF + NGF (5:1) combination pathway, NGF target in striatum; there is robust CGRP+ fiber growth into the contralateral hemisphere within the corpus callosum, with more successful turning into the target striatum. Scale bars in *A-D* = 1mm. *E & F*) Higher magnification of boxed areas in *A*. *G & H*) Higher magnification of boxed areas in *B*. *I & J,* Higher magnification of boxed areas in *C*. *K & L)* Higher magnification of boxed areas in *D*. Scale bars in *E-L* =

200μm. Areas pictured in *E, G, I* and *K* include the path point “midline +1mm”, which is quantified in the graph. Areas pictured in *F, H, J* and *L* show the extent of CGRP+ axon growth toward the striatal target, with arrows indicating the most distal fibers. *M*) Higher magnification of boxed area in *L*. *N*) Average number of fibers counted at various distances along the pathway, divided by the number counted at 1mm left of midline (ipsilateral to transplant) to adjust for any differences in transplant size or survival. Mid = midline; c.c. = corpus callosum. Mid +1mm and Mid +2mm are on the right side, contralateral to the transplant. Symbols represent means $\pm$ SEM. *$p < 0.05$, **$p < 0.01$ (Kruskall-Wallis). GFP only, n = 4; GFP/NGF target, n = 3; FGF/NGF target, n= 4; FGF+NGF/NGF target, n = 8.



<u>Figure 2-4</u>: Expression of a chemorepulsive molecule adjacent to the pathway turning point increases the proportion of axons making the desired turn. ***A***, Schematic of injection protocol, blue circles along corpus callosum represent Ad-FGF2/NGF injections, green circles represent Ad-NGF injections at the DRG transplant site (left) and striatal target (right), red circles are Ad-semaphorin injections (only in the +Sema group), and brown circles represent transplanted DRG neurons, injected one week after the adenoviral pathway. ***B-E***, Representative sections immunostained for CGRP; without semaphorin (***B & C***), or with semaphorin ***(D & E)*** adjacent to the turn. *C & E* are higher magnifications of boxed areas in *B & D*. Scale bar in *B* = 500μm, scale bar in *C* = 200μm. Arrows indicate mediolateral location of Ad-NGF target injection, asterisks (*) are in the corpus callosum beyond the pathway turning point. Arrowhead in *E* indicates macrophages within the corpus callosum (not CGRP+ staining). ***F***, Bars represent mean percent (+/- SEM) of CGRP+ fibers making turn: 77% with semaphorin, 51% without semaphorin; t-test *p=.02; n=8 with sema, n=9 without sema.

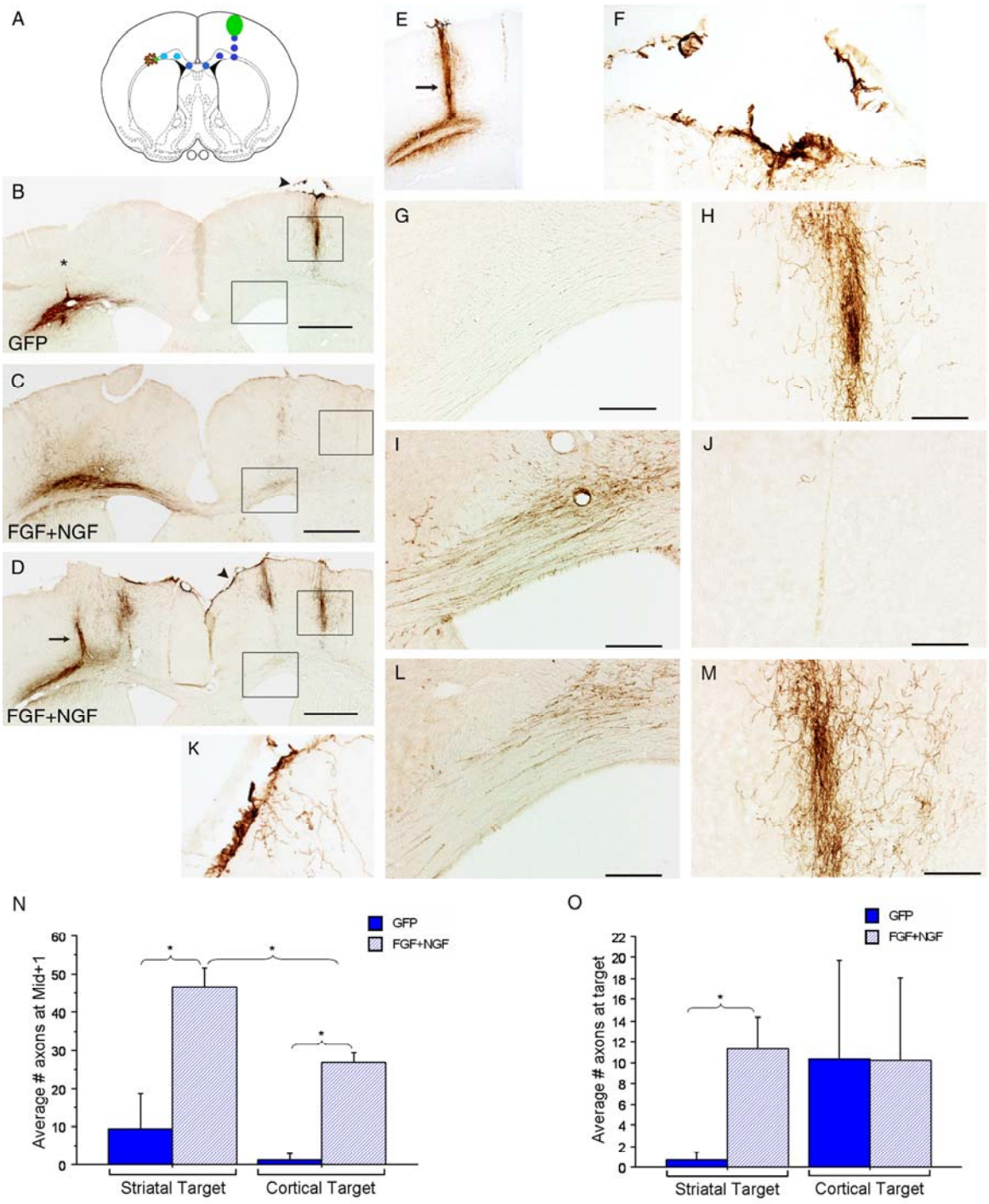


Figure 2-5: NGF overexpression in the cortex leads to axon growth along the brain surface when neurons are transplanted into the corpus callosum. *A*, Schematic of injection protocol with cortical target: Blue circles along corpus callosum represent Ad-FGF2/NGF or Ad-GFP (control) injections, green circles represent Ad-NGF injections at the DRG transplant site (left) and cortical target (right). Brown circles represent transplanted DRG neurons, injected one week after the adenoviral pathway. *B-M*, Representative coronal sections after three

weeks' growth, immunostained for CGRP. *B*, GFP pathway, NGF target in cortex; asterisk (*) indicates area where axons are found growing up toward the brain surface in a separate section, shown in *E*; arrowhead points to CGRP+ fibers growing within the meninges, which have mostly pulled away from the brain parenchyma during tissue processing. *C & D*, FGF+NGF (5:1) combination pathways, NGF target in cortex. Two different animals from this treatment group are represented to illustrate variability in axon-pathfinding decisions. The arrow in *D* points to CGRP+ axons growing up toward the brain surface, while the arrowhead in *D* points out dense fiber growth within the meninges; Scale bars in *B-D* = 1mm. *E*, A separate section from the same animal as *B*, showing where the CGRP+ axons have grown up from the transplant site toward the brain surface. *F*, Higher magnification of area indicated by arrowhead in *B*, showing CGRP+ fiber growth within the meninges. *G & H*, Higher magnification of boxed areas in *B*. *I & J*, Higher magnification of boxed areas in *C*. *K*, Higher magnification of area indicated by arrowhead in *D*, showing CGRP+ fibers within the pia and coursing down into the cortex. *L & M*, Higher magnification of boxed areas in *D*. Scale bars in *E-J* = 200μm. *N*, Quantification of CGRP+ axon growth in control (GFP) pathways and FGF+NGF pathways at 1mm beyond the midline when the NGF-expressing target was either in the striatum or in the cortex. There is significantly more axon growth in FGF+NGF pathways than in GFP pathways for both injection paradigms, but fewer axons follow the corpus callosum pathway when the target is in the cortex because they choose the alternative route along the brain surface. *O*, Quantification of CGRP+ axon growth into the target area, either halfway between the brain surface and the corpus callosum in the striatum (pictured in *H, J* & *M*), or 0.5mm below the corpus callosum in the striatum. With the striatal target, there is a significant difference between GFP pathways and FGF+NGF pathways, but with the cortical target, axon growth along the brain surface to the target eliminates the difference between pathways. Bars represent means $\pm$ SEM. Mann-Whitney U-tests at each path point, *$p < .05$. GFP/NGF, n = 3; FGF+NGF/NGF, n=4.

Chapter Three: Preliminary Studies with Nigrostriatal Dopaminergic Neurons

Introduction

Since Parkinson's disease (PD) is caused by the selective degeneration of nigrostriatal dopaminergic neurons, the potential exists for alleviation of symptoms by rebuilding the nigrostriatal circuit. This would require transplantation of healthy dopaminergic neurons into the substantia nigra (SN) and subsequent axon growth to and innervation of the striatal target. Many previous studies have focused on neuronal transplantation for PD, both in animal models and in clinical trials, but the vast majority of these studies have involved transplantation of new neurons directly into the striatum (Reviewed by Winkler et al., 2000). In this way, a new, internal source of dopamine is provided, eliminating the need for pharmacological dopamine replacement. Unfortunately, such techniques only confer mild benefits, if any, and have also lead to distressing side effects in some human transplant recipients (Freed et al., 2001; Greene and Fahn, 2002; Ma et al., 2002). These disappointing results may be due, at least in part, to the ectopic placement of the dopaminergic cells and concomitant lack of regulatory afferent input (Cheramy et al., 1981; Robertson, 1992; Baker et al., 2000; Winkler et al., 2000). In addition, fetal tissue transplants inevitably contain some cells that normally do not project to the striatum, so any connections that they make in that area will be aberrant and may cause side effects (Isacson et al., 2003). It is therefore critical to pursue transplantation techniques that more closely approximate a reconstruction of the nigrostriatal pathway.

The major challenge in attempting to rebuild this circuit is the sheer distance between the SN and the striatum, along with the difficulty that axons have in growing such distances through a mature CNS environment. Experiments addressing these issues have used the unilateral 6-hydroxydopamine (6-OHDA) lesion model in adult rats, which creates a

"hemiparkinson" state by elimination of nigrostriatal neurons on one side of the brain. To repair the damage, some groups have attempted "bridging" techniques, with dopaminergic cell transplants into the SN and various growth-supportive substrates between the SN and the striatum, including embryonic striatal tissue (Dunnett et al., 1989), glial cell-line derived neurotrophic factor (GDNF) (Wang et al., 1996), fibroblast growth factor (FGF)-4-transfected schwannoma cells (Brecknell et al., 1996), GDNF-secreting Schwann cells (Wilby et al., 1999), and kidney tissue (Chiang et al., 2001). Although some degree of graft-derived dopaminergic fiber growth along the pathway is reported in each of these studies, quantification of fiber growth and striatal innervation is minimal or absent, so it is difficult to interpret the success of each technique. Furthermore, improvement in parkinsonian motor symptoms – the desired endpoint with nigrostriatal pathway reconstruction – is not addressed beyond amphetamine-induced rotation scores in any study except Chiang's, which does report improvements in postural asymmetry but still does not analyze spontaneous motor behavior (Chiang et al., 2001). The best technique for rebuilding a functional nigrostriatal circuit, then, remains to be found.

To avoid introduction of extra foreign cells along the nigrostriatal pathway, it makes sense to attempt a molecular approach for creating a supportive environment for dopaminergic axon growth. One candidate molecule for such growth enhancement is GDNF, which is known to increase the survival, differentiation, fiber outgrowth and dopamine release of fetal midbrain dopaminergic neurons both in vitro and in vivo (Lin et al., 1993; Stromberg et al., 1993; Hudson et al., 1995; Johansson et al., 1995). GDNF has been used successfully to increase the survival of fetal dopaminergic cell transplants in the 6-OHDA-lesioned rat striatum (Rosenblad et al., 1996; Yurek, 1998; Ostenfeld et al., 2002) and shows some promise for increasing fiber outgrowth from dopaminergic cells transplanted into the lesioned SN (Wang et al., 1996; Wilby et al., 1999). So far, there is no strong evidence supporting GDNF's role as a chemoattractant for dopaminergic axons, but when combined with the GPI-linked glial cell line-derived neurotrophic factor receptor α1 (GFRα1) there is an

attractive guidance effect on other populations of GDNF-responsive neurons (sensory and sympathetic) (Ledda et al., 2002). Another molecule which acts as a chemoattractant guidance cue is netrin-1, which has recently been shown to have positive directional effects on neurite outgrowth from cultured dopaminergic neurons (Lin et al., 2005, 2006).

Direct injection of any of these molecules may have a temporary positive effect on neurite outgrowth from dopaminergic neurons transplanted into the SN, but longer-term, stable expression may be more effective and would require a viral vector approach. The study described in chapter two of this dissertation demonstrates the effectiveness of guidance pathways created by adenoviral-vector-mediated expression of neurotrophins in enhancing and directing neurite outgrowth from transplanted dorsal root ganglion (DRG) nociceptive neurons. The current study aims to apply this technique to transplanted dopaminergic neurons in the 6-OHDA-lesioned rat brain. Initial attempts to use adenoviral vectors in this model created unacceptable levels of brain damage due to the fragile nature of the lesioned striatum and the immunogenic potential of adenovirus, so this chapter describes the design and characterization of lentiviral vectors encoding GDNF, GFRα1, netrin-1 and green fluorescent protein (GFP), then describes initial experiments utilizing these vectors to direct dopaminergic neurite outgrowth in collagen-gel cocultures and along short, intrastriatal pathways in the 6-OHDA-lesioned rat brain.

Methods

Lentivirus construction and characterization

The coding regions of an alternatively-spliced form of rat GDNF (GDNF555, NCBI reference # S75585.1), rat GFRα1 (NCBI reference #NM_012959, supplied by Amgen) and mouse netrin-1 (NCBI reference# NM_008744, supplied by Marc Tessier-Lavigne and Genentech) were each cloned into a lentiviral vector plasmid containing a flap sequence, cytomegalovirus (CMV) promoter, multiple cloning site, internal ribosome entry

site (IRES) sequence followed by the eGFP coding sequence and a woodchuck hepatitis virus post-transcriptional response element (WPRE). A four-plasmid system was used to package the virus, ensuring that resultant virions were replication incompetent: 293FT cells were transiently transfected with 1) one of the above-mentioned vector plasmids, 2) a packaging plasmid encoding the HIV -1 Gag and Pol proteins, 3) an envelope plasmid encoding the vesicular stomatitis virus glycoprotein (VSV-G) to confer broad tropism and 4) a plasmid encoding the Rev post-transcriptional regulator that is required for Gag/Pol expression and virus production. Packaged virus was collected 48 hours after transfection following a poly-L-lysine (PLL)/low-speed centrifugation protocol described by Zhang, et al (2001). Briefly, cell media was collected, spun for 5 minutes at 1000g, filtered and mixed with 0.5µg PLL per 10ml media, then incubated on ice for 30 minutes. The media/PLL was then spun at 10,000g for 2 hours, the supernatant aspirated, and the virus-containing pellet was resuspended in phosphate-buffered saline (PBS) containing 3mM spermine and 0.3mM spermidine (cationic polyamines, to stabilize DNA).

Viral titers were estimated using a commercially-available p24 ELISA kit (Retro-Tek HIV-1 p24 antigen ELISA, ZeptoMetrix Corporation). Vector-mediated protein expression was confirmed *in vitro* (transduced 293FT cells) by ELISA for GDNF (Promega) and by immunocytochemistry for GFRα1 and netrin-1 (goat-anti-GFRα1, R&D Systems Inc., 1:100; chicken anti-netrin-1, Chemicon International, 1:100; donkey anti-goat Texas Red, Jackson ImmunoResearch Laboratories, 1:100; donkey anti-chicken Rhodamine RedX, Jackson ImmunoResarch Laboratories, 1:100). Expression of GDNF *in vivo* was examined after three stereotactic injections of 2µl lenti-GDNF into the left striatum of 5 Sprague-Dawley rats. The rats were killed by decapitation one week after virus injection, striatal tissue (3mm-thick slice) was harvested from both hemispheres and frozen quickly, and then proteins were extracted and subjected to ELISA for GDNF (Promega).

Because some *in vitro* immunocytochemistry revealed “clumped” expression patterns, the ability of lenti-GFP to successfully transduce 293FT

cells was tested after sonication in a water-bath sonicator to break up clumped virus. The sonicator basin was filled with water containing copious amounts of ice to prevent heating of the virus, and viral aliquots (in 0.5ml Eppendorf tubes) were sonicated for 0, 3 or 5 minutes. Virus was then added to cultured 293FT cells at ~70 confluency, and GFP expression was observed 5 days later by fluorescence microscopy. The extent of transduction and GFP expression was essentially unchanged with up to 3 minutes of sonication, so all subsequent viral aliquots were sonicated for 3 minutes prior to use.

Collagen-gel coculture experiments

Protein-expressing cell aggregates: 293FT cells were grown to ~70% confluency in 100mm cell culture dishes, and then 1 x 10^7 TU of lentivirus was added (after a 3-minute sonication of each viral aliquot to prevent clumping) to each plate as follows: 1) lenti-netrin, 2) lenti-GDNF, 3) lenti-GFRα1 and 4) lenti-GDNF *plus* lenti-GFRα1 (1 x 10^7 TU each). Each virus was mixed with 10ml of media then added to the cells. Two days after the addition of virus, cell media was removed and the cells lifted off each plate with trypsin/EDTA, spun down and resuspended in fresh media. Cells were counted with a hemocytometer and adjusted to a concentration of about one million cells per milliliter, then 3ml of each suspension was added to a sterile 10ml Erlenmeyer flask covered with a foil cap (4 flasks total). Flasks were placed on a gyrating platform shaker set at approximately 90 rpm in a 37ºC humidified incubator with 5% CO_2 for three days to allow aggregates to form.

Dopaminergic cell harvest: Embryos at day 14 (E14) were removed from a pregnant Sprague-Dawley rat and the ventral mesencephalic (VM) brain region was dissected out of each fetus and kept in ice cold, sterile, calcium- and magnesium-free buffer. The tissue chunks were rinsed in fresh buffer several times, trypsinized (0.125% trypsin) for 10 minutes at 37ºC, and then rinsed again. Cells were then dissociated by trituration in .004% DNAse, layered over sterile FBS and pelleted out by centrifugation. The resulting cell pellet was resuspended in Dulbecco's modified Eagle Medium (DMEM)/Ham's F12

(DMEM/F12, 1:1) with 5% fetal bovine serum (FBS) and 10U/ml penicillin, and cell density and viability was determined with trypan blue and a hemocytometer.

Dissociated VM co-culture: [Note: This initial experiment was done prior to construction of the lentivirus, using previously-generated adenovirus (Ad-GDNF ±Ad-GFRα1 or Ad-GFP) to transduce U373 cells in culture rather than lentivirus and 293FT cells as described above.] Each well of a 24-well, poly-D-lysine-coated culture dish was divided into equal quadrants by carefully marking the bottom of the dish. Twenty microliters of the E14 VM cell suspension (~100,000 cells) was then added to the center of each well (where the quadrant lines intersected) and cells were allowed to adhere for 4 hours at 37ºC. Collagen media was prepared by mixing equal parts concentrated (2X) DMEM/F12 media and rat tail collagen along with N-2 supplement, 5% FBS, penicillin/streptomycin, then adjusting pH with 1N NaOH. The collagen media was kept on ice to prevent gelling during preparation. The dish containing the VM cells was then removed from the incubator and 250µl collagen media was added to each well, one or two previously-transfected U373-cell aggregates (expressing GDNF ± GFRα1 or GFP) were placed in the upper-left quadrant and the gel was allowed to set at 37ºC for one hour. Extra media (DMEM/F12/5%FBS) was added to each well once the gel had set, and then the cells were allowed to grow at 37ºC for three days.

Re-aggregated VM co-culture: As an alternative procedure, dissociated VM cells were re-aggregated following a method developed by Sortwell, et al. (2004) before co-culturing with transfected 293FT-cell aggregates. Striatal oligodendrocyte-type-2 astrocyte (SO2A) conditioned media was generously supplied by Caryl Sortwell and used to resuspend VM cells for reaggregation. Three million VM cells were suspended in 2.5ml SO2A-conditioned media in a sterile 10ml Erlenmeyer flask covered with a foil cap. The flask was placed on a gyrating platform shaker set at 90 rpm in a 37ºC, humidified incubator with 5% CO_2 for three days to allow aggregates to form.

Once both VM and 293FT-cell aggregates were ready, collagen gel media was prepared as described above. 250µl of collagen media was added to each

well of a 24-well culture dish, followed by one VM aggregate and one transduced 293FT-cell aggregate positioned approximately 200-500μm apart. The gel was allowed to set for 1 hour, then DMEM/F12/5% FBS media was added to each well. Cultures were kept at 37ºC for three days to allow axon outgrowth from the VM cells.

Immunocytochemistry: After 3 days of growth, gels were fixed with an equal volume of 8% paraformaldehyde for one hour, washed, blocked with 5% normal goat serum (NGS) in PBS with 0.3% Triton-X (PBS/Tx) for 2 hours, then incubated in primary antibody to tyrosine hydroxylase (Mouse anti-TH, Chemicon, 1:1000) overnight at 4ºC. After five 30-minute washes, gels were incubated in secondary antibody (Goat anti-mouse biotin, Chemicon, 1:600) overnight at 4ºC, then developed with using an avidin-biotin-peroxidase kit (VectaStain Elite, Vector Labs) followed by diaminobenzidine (DAB) long enough to visualize TH-positive dopaminergic fibers.

Growth patterns in the dissociated VM experiment are described qualitatively due to difficulty in following individual axons from cell body to termination. For quantification of neurite outgrowth in the re-aggregate experiment, a blinded observer counted the number of TH+ fibers growing in a trajectory toward or away from the 293 aggregate, based on a visual division in to quadrants.

Intrastriatal pathway experiment

6-OHDA lesioning: Adult female Sprague-Dawley rats (225-250g) were given unilateral 6-OHDA lesions of the nigrostriatal pathway as follows. Each rat was anesthetized by inhalation of 2% halothane mixed with room air, positioned in a stereotaxic frame and the skull exposed. Two injections of 3.0μg/μl 6-OHDA in 0.9% saline with 0.2% ascorbic acid were made at a rate of 1.0μl/min for 2 min, in the vicinity of the medial forebrain bundle (AP-4.4, ML 1.2 relative to bregma, 8.4mm deep from skull level) and the rostral substantia nigra pars compacta (AP -5.3, ML 2.0, 8.4mm deep). This procedure has been shown to produce complete lesions, with near complete dopaminergic denervation of the

ipsilateral striatum. This denervation was confirmed 4-5 weeks after lesioning by amphetamine-induced rotation testing (>450 ipsilateral turns in 90 minutes).

Pathway injections and transplants: Five weeks after lesioning, animals with verified lesions were given five injections of lentivirus encoding either GDNF (LV-GDNF, 6.72 x 10^5 TU/µl, n=5) or GFP (LV-GFP, 2.8 x 10^5 TU/µl, n=5) at 2.8mm lateral to bregma, and depths from -5mm to -3mm (every 0.5mm) relative to the dura in the lesioned striatum. Volumes of virus injected at the five sites, deepest first, were: 0.6µl, 1.0µl, 1.4µl, 1.8µl and 2.0µl. One week after virus injection, ventral mesencephalon (VM) tissue was dissected from embryonic day 14 (E14) rats and implanted as whole tissue chunks by a modified 22-guage spinal needle at the same coordinates as the viral pathway, except at 5.5mm deep relative to the dura (0.5mm below the deepest virus injection). Two weeks after VM transplants, animals were killed and brains removed for IHC analysis. Coronal slices (30µm, cryostat sectioned) were stained using a mouse anti-TH monoclonal antibody at 1:4000 dilution (Chemicon), a biotinylated secondary antibody (goat anti-mouse IgG, Chemicon, 1:600), and developed with a diaminobenzidine chromogen. Pictures of stained sections were taken at 20x total magnification with constant filter/exposure settings and analyzed using MetaMorph imaging software. All pictures were thresholded at the same settings to highlight TH+ cells and fibers. Three square regions (500µm/side) were placed (1) just below the corpus callosum (cc), (2) just above the cc, and (3) at the top of the cortex, and one 2.5mm^2 oval region was placed at the transplant site. The percent thresholded (TH+) area within each region was averaged over three sections per animal and then compared between treatment groups.

Results

Characterization of lentivirus

Viral titers, as determined by p24 ELISA assay, were as follows: lenti-GDNF, 1.2 x 10^6 TU/µl; lenti-GFRα1, 2.75 x 10^5 TU/µl; lenti-netrin-1, 8.3 x 10^5 TU/µl; lenti-GFP, 5.5 x 10^5 TU/µl. Every lentivirus also carried the gene for GFP

(following the IRES sequence), and GFP expression by lentivirus-transduced 293FT cells was confirmed by fluorescent microscopy. GDNF expression was confirmed by ELISA of media collected from 293FT cells transduced with lenti-GDNF, after allowing one week for full expression of the GDNF protein. To quantify *in vivo* expression of GDNF, 2µl lenti-GDNF was injected into 3 different spots in the left striatum of five unlesioned rats. After allowing 10 days for protein expression, the rats were killed by decapitation, brains were collected fresh and both right (control) and left striata were subjected to protein analysis by GDNF ELISA. An average of 0.5 ng GDNF/g brain tissue was detected in the right (uninjected) sides, and 0.73 ng GDNF/g in the left (lentivirus-injected) sides, with an average 51.6% increase in GDNF expression detected. Lentivirus-induced expression of both netrin-1 and GFRα1 was confirmed by immunofluorescent staining of transduced 293FT cells in culture and in virus-injected brain tissue, but was not quantified. Sample images are presented in Figure 3-1.

Collagen gel co-cultures

In order to determine the chemotropic influence of GDNF (±GFRα1) and netrin-1 on primary dopaminergic neurons, cells expressing each protein and VM cells from E14 embryos were co-cultured in collagen-gel media. Due to the non-homogenous distribution of TH+ dopaminergic neurons in intact VM tissue pieces, dissected VM tissue was dissociated into a cell suspension before culture. Preliminary experiments involved plating a drop of VM cell suspension into each well of a poly-D-lysine-coated 24-well plate and then overlaying the collagen gel media with an aggregate of virally transduced cells in one corner of each well once the VM cells had adhered to the plate bottom. After allowing three days for axon growth, the cultures were fixed and stained for expression of TH. Quantification of directional TH+ axon growth proved impossible, however, because many axons grew toward other TH+ cells and intertwined with those fibers, making it difficult to analyze their ultimate growth trajectory. In a few instances where long TH+ axons could be traced, there was evidence of a

directional influence of cells producing GDNF (with or without GFRα1; Figure 3-2).

To avoid the problem of determining the starting point of each TH+ axon, the next experiment utilized E14 VM cells that had been dissociated and then re-aggregated into spheres approximately 500μmm in diameter. A VM aggregate was positioned about 500μm from a previously-transduced 293FT aggregate within collagen gel media and allowed three days for axon outgrowth. The 293FT cells were expressing GDNF, GFRα1, netrin or both GDNF and GFRα1. Quantification of TH+ axon outgrowth relative to the position of the 293FT cell aggregate is summarized in figure 3-3. While there was a tendency for more TH+ axons to grow toward targets secreting netrin or GDNF with GFRα1, the difference in direction was not statistically significant for any treatment group (Figure 3-3D; Paired t-tests for proximal vs. distal: netrin, $p=0.2$, $n=4$; GDNF, $p=0.8$, $n=6$; GFRα1, $p=0.5$, $n=3$; GDNF + GFRα1, $p=0.2$, $n=5$).

Striatal pathway

In order to determine the feasibility of *in vivo* targeting of axon outgrowth from transplanted dopaminergic cells, short intrastriatal pathways were constructed by multiple stereotactic injections of lentivirus encoding GDNF or GFP (controls) in animals previously lesioned with 6-OHDA to eliminate endogenous TH+ striatal fibers (Figure 3-4A and B). One week after virus injection, E14 VM tissue was transplanted below the expression pathway in the ventral striatum. After allowing two weeks for fibers to grow out of the transplants, brain tissue was harvested and immunostained for TH to determine the density of dopaminergic fiber growth along each pathway (Figure 3-4C and D). Quantification of TH+ staining density in the transplant area and at 3 different points along the vertical expression pathway revealed that GDNF-expressing pathways contained significantly more TH+ fibers more distal points than GFP pathways. To be certain that the observed effect was due to increased fiber growth along the pathway, and not simply due to increased survival of grafted

cells, TH+ fiber density at each path point was divided by the amount of TH+ staining within the transplant area (a reflection of dopaminergic cell survival and transplant density; Figure 3-5).

Discussion

Viral vectors are useful tools which may be used to modify the environment of the adult CNS to make it more hospitable to neurite outgrowth. Such modification is critical to the success of therapeutic strategies that include neural transplantation for repair of CNS injury. In the previous chapter, our lab's development of viral vector strategies to support and target long-distance axon growth from transplanted cells is described. Growth-supportive pathways created by multiple virus injections were shown to enhance axon growth from transplanted neurons from one side of the brain to the other, and even to steer the axons around a 90-degree turn from white matter into grey matter. The work presented in this chapter serves as a bridge between development of that technique and a clinically-relevant application: reconstruction of the nigrostriatal pathway in Parkinson's disease (PD). Previous cell-replacement strategies for PD have involved transplantation of dopaminergic cells into the striatum rather than into the substantia nigra (SN), where the cells normally reside (Winkler et al., 2000; Freed et al., 2001). The distance between the SN and the striatum presents a huge obstacle to complete reconstruction of the circuitry that breaks down in PD – but this obstacle may be surmountable with the help of viral vectors.

In our early attempts at using viral vectors in the 6-OHDA-lesioned rat brain, we injected adenovirus encoding the GDNF protein. This vector proved to be too damaging to the already-stressed brain, so we switched to a lentiviral vector system, which is known to be less damaging to the CNS (Jakobsson and Lundberg, 2006). We produced three different lentiviruses utilizing a vector plasmid which contained a multiple cloning site (MCS) followed by an internal ribosome entry site (IRES) sequence and the coding sequence for green

fluorescent protein. We separately cloned the coding sequences for GDNF, GFRα1, and netrin-1 into the vector, then generated and tested the lentiviruses for expression in vitro and in vivo. GDNF and netrin-1 were chosen due to their previously-demonstrated neurotrophic and/or chemotropic effects on dopaminergic midbrain neurons (Lin et al., 1993; Stromberg et al., 1993; Hudson et al., 1995; Johansson et al., 1995; Lin et al., 2005). GFRα1 was chosen for its potential to bind GDNF and present it *in trans* to growing axons expressing the RET receptor tyrosine kinase, creating a more directional effect than GDNF alone (Ledda et al., 2002).

The results of our collagen-gel coculture experiments fell short of statistical significance, but show a trend toward directional effects of netrin-1 and a combination of GDNF and GFRα1. The lack of statistical significance may be due to technical difficulties with placement of the aggregates at consistent distances apart, insufficient expression of the transgenes by cell aggregates (especially since cells in the center of the aggregates may not survive well), and small sample sizes. The experiment is currently being repeated to perfect the technique and increase sample sizes.

To quickly determine the effect of GDNF expression on dopaminergic neurite outgrowth *in vivo*, we generated short, vertical expression pathways with multiple lenti-GDNF injections in 6-OHDA-lesioned striata one week prior to transplanting fetal VM tissue below each path. Our data indicate that the expression of GDNF increases the amount of TH+ fiber growth along the pathway at points 3-5mm away from the transplant when compared to control pathways created with lenti-GFP. The question remains as to whether this is a true chemotropic effect, as axons naturally tend to grow along needle tracts in the brain – the expression of GDNF in the tract may only *increase* growth rather than *steer* it. The ultimate conclusion, however, is unaffected: virus-mediated expression of GDNF along the pathway results in much greater dopaminergic axon growth in that direction.

When trying to re-build pathways that nature builds during development, it makes sense to take advantage of natural cues. For instance, fetal midbrain

dopaminergic neurons are built to respond to growth cues in the developing forebrain, and are repelled by cues in the hindbrain (Nakamura et al., 2000). Specific subpopulations of dopaminergic neurons – A9 neurons of the SN and A10 neurons of the ventral tegmental area – respond to different cues and therefore project to and make synaptic connections with different forebrain targets (Becq et al., 1999; Yue et al., 1999). There is some evidence that such directional cues, while downregulated, still exist in the adult brain and can direct axon growth from certain types of transplanted fetal neurons to distant targets (Wictorin et al., 1992; Isacson et al., 1995; Isacson and Deacon, 1997; Armstrong et al., 2002). So far, however, long-distance growth is limited to transplants from large, slowly-maturing animal tissue donors (human or pig) into adult animal hosts with relatively small brains, such as rats. To elicit such growth from allogeneic transplants, some additional "push" is needed, but the framework is there to support growth in the proper direction. Based on the preliminary data presented here, we propose that lentiviral-mediated expression of a growth-supportive molecule such as GDNF (with GFRα1) or netrin-1 may provide the needed push to reconstruct the nigrostriatal pathway in adult parkinsonian brains.

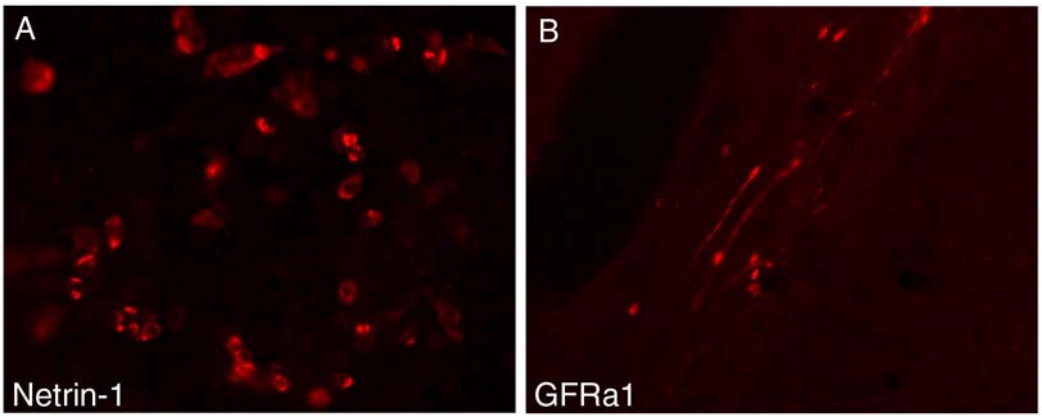
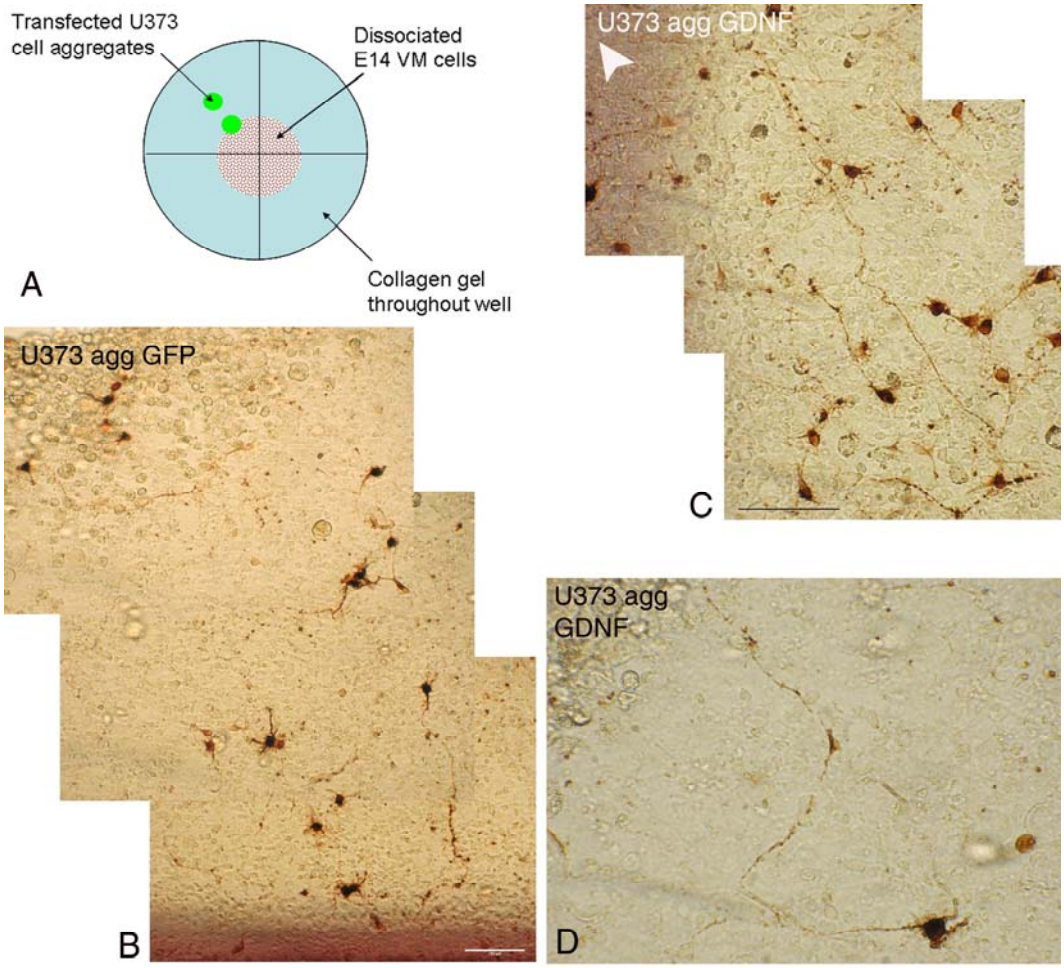


Figure 3-1. **Lentivirus-mediated protein expression *in vitro* and *in vivo***. A) 293FT cells transduced with lenti-netrin and immunostained using chicken anti-netrin-1 and donkey anti-chicken Rhodamine Red-X. B) Slice of brain tissue from animal injected intracerebrally with lenti-GFRa1, stained with goat anti-GFRa1 and donkey anti-goat Texas Red (stained cells are within the corpus callosum).



<u>Figure 3-2</u>. Collagen gel co-cultures of transduced U373 aggregates and dissociated E14 VM cells, stained for tyrosine hydroxylase after 3 days of growth. A) Schematic representation of cell placement within each well. B) Well with GFP-expressing cell aggregate in the upper left and several TH+ VM cells with scant, random axon growth. C & D) Two different wells with GDNF-expressing aggregates toward the upper left and evidence of directional influence on TH+ fiber growth. Scale bars = 100μm.

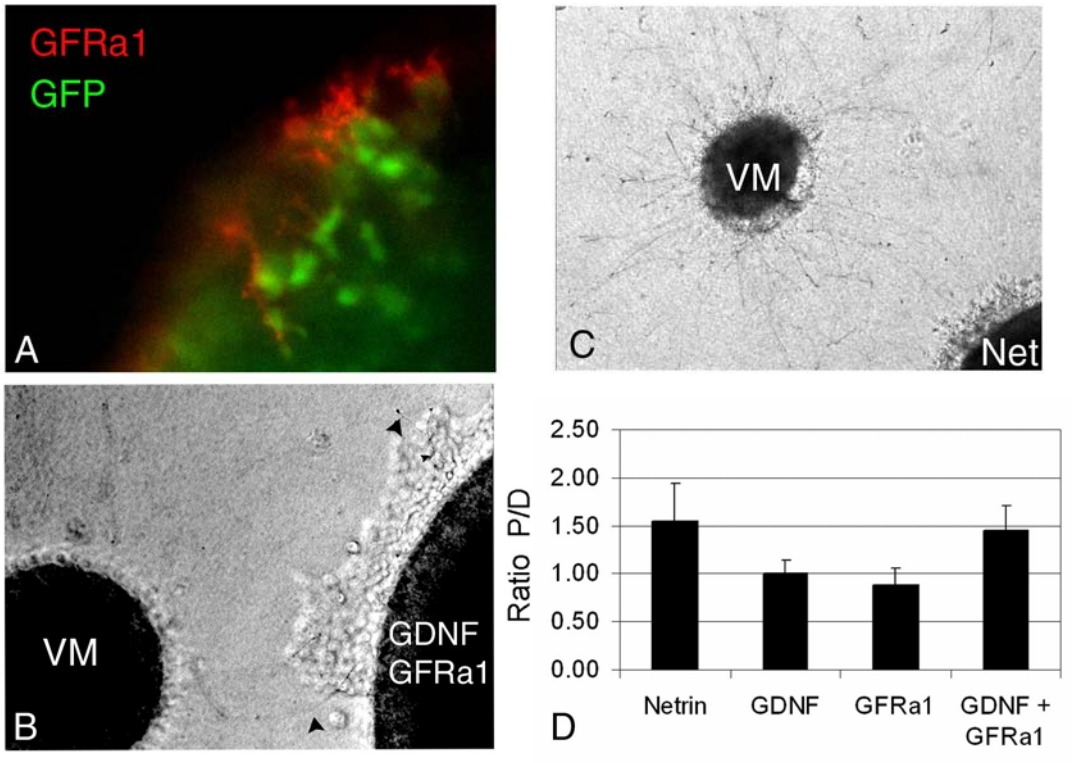


Figure 3-3. Collagen gel co-cultures of transduced 293FT cell aggregates and re-aggregated E14 VM cells after three days of growth. A) Immunofluorescent staining of a 293FT aggregate transduced with lenti-GFRα1-IRES-GFP, stained for GFRα1. B) Sample gel stained for TH with VM aggregate near 293FT aggregate expressing GDNF plus GFRα1. Arrowheads indicate TH+ fibers that have turned toward the target cells. C) Sample gel stained for TH with VM aggregate near 293FT aggregate expressing netrin-1. D) Quantification of TH+ neurite outgrowth presented as ratio of fibers in the proximal vs. the distal quadrant relative to the target aggregate location (P/D).

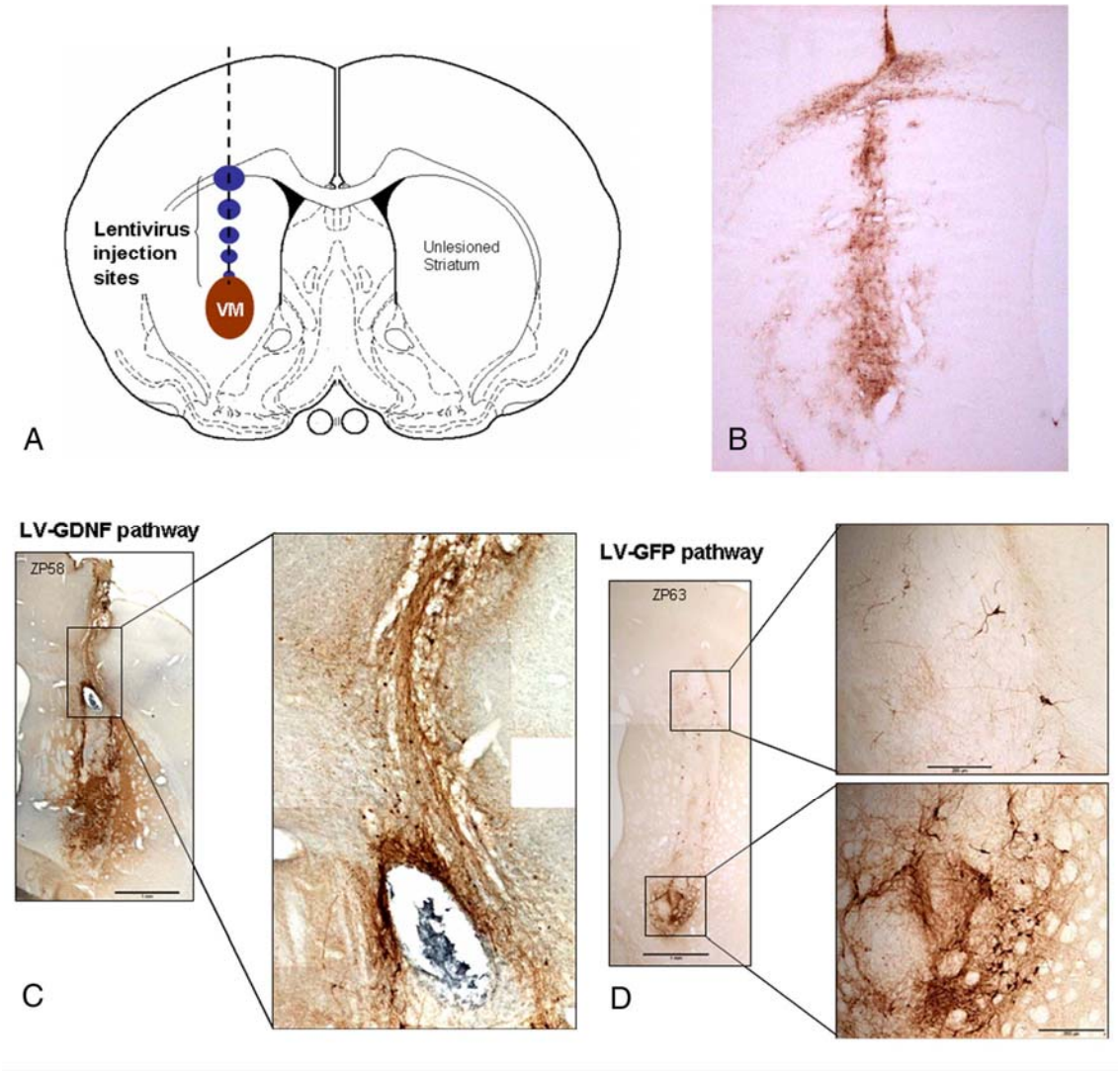


Figure 3-4. **Striatal pathway injections and dopaminergic fiber growth**. A) *Injection schematic* – Lentivirus (encoding either GFP or GDNF) was injected along a vertical pathway as indicated in the 6-OHDA-lesioned striatum. An E14 ventral mesencephalon (VM) tissue chunk was transplanted below the pathway 1 week later. B) Expression pathway as revealed by immunostaining for GFP (peroxidase/DAB). Note the spread of expression within the corpus callosum. C) Immunostaining for tyrosine hydroxylase (TH) reveals growth of dopaminergic fibers up the expression pathway in an animal injected with LV-GDNF prior to transplant. D) Same staining as in C, this time in an animal injected with LV-GFP. TH+ cells survived and sent out fibers, but the fibers remained relatively localized to the transplant area.

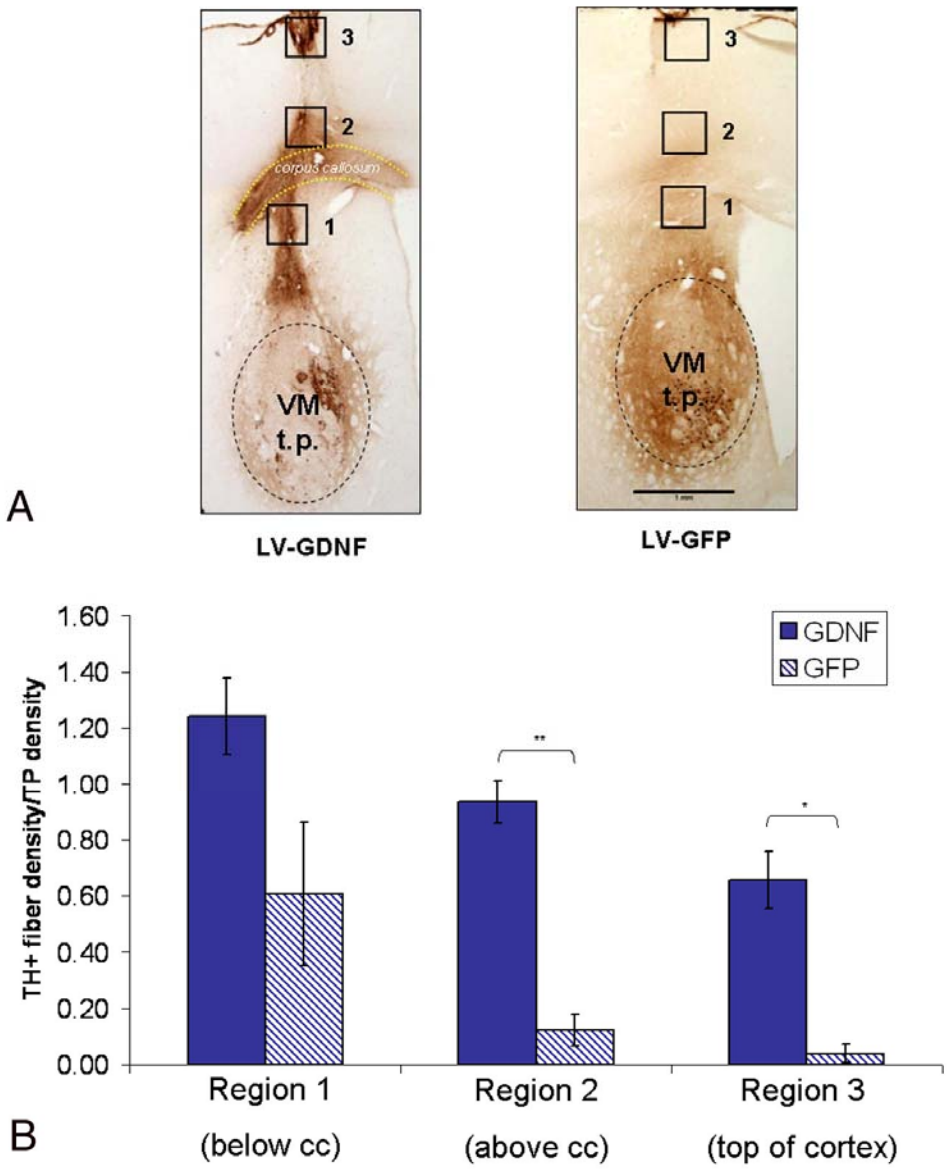


Figure 3-5. **Quantification of TH+ fiber density along GDNF or GFP-expressing pathways.** A) Representative section from a LV-GDNF animal, immunostained for tyrosine hydroxylase (TH), with illustrations of regions quantified for TH+ staining. B) Representative section from a LV-GFP animal. To compensate for any differences in transplant quality/survival, % values for regions 1-3 were divided by % values within the transplant region (t.p.). Paired t-tests (assuming unequal variance) reveal a significant increase in TH+ fiber density in regions 2 and 3 with GDNF treatment compared to GFP. *p<.05; **p<.001; (n=5 each group).

Chapter Four: Reconstruction of the Nigrostriatal Pathway

Introduction

Neuronal transplantation is a potential treatment for neurological disorders caused by the damage or degeneration of specific neural circuits in the central nervous system (CNS). Parkinson's disease (PD) is one such disorder, which affects approximately 1% of the population over the age of 65 and increases in prevalence with increasing age (Reviewed by Lang and Lozano, 1998; Dawson and Dawson, 2002). The symptoms of PD, including bradykinesia, akinesia, rigidity, and resting tremor, are due to the progressive degeneration of the dopaminergic neurons of the nigrostriatal pathway. The precise mechanism by which dopaminergic neurons are targeted and destroyed in PD has not yet been elucidated, in part because there are several subtypes of the disease – it may be heritable or sporadic, early or late onset, and slowly or rapidly progressing. Among the heritable forms, transmission of PD may be autosomal dominant, autosomal recessive, maternal, or may show anticipation through successive generations (Mouradian, 2002). Mutations in three different genes have been identified thus far in familial PD, and the functions of these genes suggest one final common pathway for dopaminergic cell death: accumulation of toxic proteins due to inefficiency of the ubiquitin-proteasomal pathway (Chung et al., 2001; Mouradian, 2002). In the more common, sporadic form of PD, substantial evidence implicates oxidative stress as a general mechanism of pathology, but the details remain to be worked out (Oertel and Hartmann, 1999; Maguire-Zeiss et al., 2005). In the future, preventative therapies may be developed that target the products of causative genes or otherwise prevent oxidative damage and apoptosis.

By the time a patient presents with parkinsonian symptoms, at least 80% of striatal dopamine has been depleted, and current therapy is aimed at replacing that dopamine to alleviate symptoms. The gold standard for treatment of PD patients is administration of the dopamine precursor levodopa. While levodopa

can profoundly improve a patient's quality of life, its effectiveness tends to decrease over the course of the illness and the medication is often associated with debilitating side effects such as motor fluctuations ("on-off" and "wearing-off" phenomena), dyskinesias, and hallucinations (Lang and Lozano, 1998). The side effects can be lessened by carefully monitoring the levodopa dosage and making corrections as necessary, but managing PD becomes increasingly difficult as more dopaminergic neurons succumb to the disease (Metman and Mouradian, 1999). Other drugs, such as monoamine oxidase B inhibitors, anticholinergic agents, and dopamine agonists have also been used to treat parkinsonian symptoms. The side-effect profiles of these drugs are less severe than levodopa's, but they are also less effective in the long run, and usually require supplementation with levodopa (Lang and Lozano, 1998; Nyholm, 2006).

Given the prevalence of Parkinson's disease and the shortcomings of clinical treatments currently available, research into potential neuroprotective and neurorestorative therapies continues to be important. Advances in basic research are made with the help of animal models of PD such as 6-hydroxydopamine (OHDA)-lesioned rats. 6-OHDA is a catecholamine neurotoxin that is transported into dopaminergic and noradrenergic neurons. Once inside these cells, it inhibits mitochondrial complexes I and IV, causing oxidative injury and cell death (Deumens et al., 2002). Direct injection of 6-OHDA into the nigrostriatal pathway causes a selective, severe loss of these dopaminergic neurons. Unilateral lesioning of the pathway leads to "hemiparkinsonian" motor deficits and results in predictable rotational behavior upon systemic injection of the stimulant amphetamine (ipsilateral rotation) or the dopamine receptor agonist apomorphine (contralateral rotation) (Deumens et al., 2002). Quantification of rotational behavior provides an estimate of severity of the 6-OHDA lesion and can be used to determine success of neurorestorative techniques. The unilateral nature of 6-OHDA lesioning also provides an internal control for histological comparisons.

Many investigators have already explored neural transplantation as therapy for PD, both in the lab and in the clinic, but in most cases, dopaminergic

neurons are transplanted directly into their target – the striatum – rather than into their true origin, the substantia nigra (SN). This leads to an incomplete recovery of function both in animal models and in human PD patients, because the transplanted neurons provide a dopamine source to the striatum but do not reestablish the degenerated neural circuit (Montoya et al., 1990; Winkler et al., 2000). In animal models only, some groups have attempted "bridging" techniques, with dopaminergic cell transplants into the SN and various growth-supportive substrates between the SN and the denervated striatum, including embryonic striatal tissue (Dunnett et al., 1989), glial cell-line derived neurotrophic factor (GDNF) (Wang et al., 1996), fibroblast growth factor (FGF)-4-transfected schwannoma cells (Brecknell et al., 1996), GDNF-secreting Schwann cells (Wilby et al., 1999), and kidney tissue (Chiang et al., 2001). Although some degree of graft-derived dopaminergic fiber growth along the pathway is reported in each of these studies, quantification of fiber growth and striatal innervation is minimal or absent, so it is difficult to interpret the success of each technique. Furthermore, improvement in parkinsonian motor symptoms – the desired endpoint with nigrostriatal pathway reconstruction – is not addressed beyond amphetamine-induced rotation scores in any study except Chiang's, which does report improvements in postural asymmetry but still does not analyze spontaneous motor behavior (Chiang et al., 2001). The best technique for rebuilding a functional nigrostriatal circuit, then, remains to be found.

The current study examines the hypothesis that lentivirus-induced overexpression of growth-supportive molecules along a path between the SN and the striatum will help to guide the growth of axons from dopaminergic neurons transplanted into the SN of the 6-OHDA lesioned rat brain, resulting in a reestablishment of the nigrostriatal pathway and an amelioration of parkinsonian symptom*s*. We compare pathways created with lentivirus encoding glial cell-line derived neurotrophic factor (GDNF), GDNF family receptor α1 (GFRα1), a combination of GDNF and GFRα1, netrin-1, and green fluorescent protein (GFP) for their effectiveness in improving dopaminergic neurite growth along the path, increasing innervation of the striatum and decreasing asymmetry in spontaneous

motor behavior. In spite of high variance between animals and some brain damage from viral injections, the data show a significant increase in dopaminergic fiber growth from transplanted neurons along the created pathway when a combination of GDNF and GFRα1 was expressed there. Further, asymmetry in spontaneous motor behavior was significantly decreased in transplanted animals that expressed either netrin-1 or a combination of GDNF and GFRα1 along the nigrostriatal pathway. These data suggest that functional restoration of the nigrostriatal pathway is possible with intranigral grafting following creation of growth-supportive expression pathways between the SN and the striatum.

Methods

Lentivirus construction

Detailed methods for the construction and characterization of lentiviral vectors used in this experiment are presented in chapter three of this dissertation. Briefly, the coding sequences for rat GDNF, rat GFRα1, and mouse netrin-1 were each cloned into a lentiviral vector plasmid containing a flap sequence, cytomegalovirus (CMV) promoter, multiple cloning site, internal ribosome entry site (IRES) sequence followed by the eGFP coding sequence and a woodchuck hepatitis virus post-transcriptional response element (WPRE). Control lentivirus was constructed with the same vector plasmid but without additional gene insertion. A four-plasmid protocol and 293FT cells were used to package the lentivirus, and purification of virus was accomplished using a poly-L-lysine (PLL)/low-speed centrifugation protocol described by Zhang, et al (Zhang et al., 2001). Viral titers, as determined by p24 ELISA assay, were as follows: lenti-GDNF, 1.2 x 10^6 TU/μl; lenti-GFRα1, 2.75 x 10^5 TU/μl; lenti-netrin-1, 8.3 x 10^5 TU/μl; lenti-GFP, 5.5 x 10^5 TU/μl.

Animals

Adult female Sprague-Dawley rats (225-250g) were used for this study and were housed two animals to a cage with ad libitum access to sterile food and water in the University of Kentucky's Division of Laboratory Animal Resources facility.

6-OHDA lesioning

Rats were given unilateral 6-OHDA lesions of the nigrostriatal pathway as follows. Each rat was anesthetized by inhalation of 2% halothane mixed with room air, positioned in a stereotaxic frame and the skull exposed. Two injections of 3.0µg/µl 6-OHDA in 0.9% saline with 0.2% ascorbic acid were made at a rate of 1.0µl/min for 2 min, in the vicinity of the left medial forebrain bundle (AP-4.4, ML 1.2 relative to bregma, 8.4mm deep from skull level) and the left rostral substantia nigra pars compacta (AP -5.3, ML 2.0, 8.4mm deep). This procedure has been shown to produce complete lesions, with dopaminergic denervation of the ipsilateral striatum.

Amphetamine-induced rotation

Four and five weeks after lesioning, rats were injected with 2.5mg/kg D-amphetamine in 0.9% saline (i.p.). Immediately after injection, each animal was placed in a harness and enclosed in a clear plastic cylinder as part of the Rotamax-8 apparatus (Columbus Instruments, Columbus, OH). The number of partial and full clockwise and counterclockwise rotations made by each rat in a 90-minute test period was automatically recorded on an attached computer with Rotacount version 2.0 software. Only animals that made at least 450 complete counterclockwise turns – equal to 5 turns/min – were used for the experiment, as this indicated a complete lesion of the ipsilateral nigrostriatal pathway.

Cylinder tests

Animals determined to have complete lesions were further tested for limb-use asymmetry during spontaneous motor behavior using the cylinder test

described by Schallert and Tillerson (1999). Rats were placed individually into a clear plastic cylinder and videotaped for 3-5 minutes – as long as it took each one to touch the cylinder wall with a forelimb at least 20 times during spontaneous exploration of the environment. Videotapes were analyzed by a person blinded to experimental design, and the number of wall touches made with the left, right or both forepaws were tallied. A limb use asymmetry score was determined by dividing the number of touches with the left paw by the total number of wall touches (left, right, or both paws together). A score of one would indicate complete asymmetry with use of left forepaw only.

Virus injections

Rats that met the amphetamine-induced rotation inclusion criterion and had baseline cylinder tests completed were randomly assigned to receive lentiviral injections along the internal capsule between the SN and the striatum: lenti-GDNF (n=6 with transplant, n=4 without transplant), lenti-GFRα1 (n=3 with transplant), lent-GDNF + lenti-GFRα1 (n=7 with transplant, n=4 without transplant), lenti-netrin-1 (n=6 with transplant), or lenti-GFP (control, n=5 with transplant). Each rat was anesthetized by 2% halothane inhalation, placed in a stereotaxic frame, and its skull was exposed. Holes were drilled at three anteroposterior (AP) locations along a line 2.4mm lateral to bregma on the animal's left side: -4.0mm, +0.2mm, and +4.6mm relative to bregma. The hole at +4.6mm (most anterior) was elongated in the rostral direction in order to accommodate the angled needle. Aliquots of virus were sonicated in an ice-water bath for three minutes, stored on ice, and mixed by pipet trituration immediately prior to injection to minimize clumping of viral particles. In order to follow the curved path of the internal capsule, three needle insertions were made at different angles relative to vertical measured from bregma with the needle set at the given angle: 0º (vertical) at -4.0mm AP, 17º at +0.3mm AP, and 49º at +5.8mm AP. The latter two AP distances differ from the drill coordinates because they correct for the angle of the needle (the drill was always in the vertical position) and the curvature of the skull. The depth (relative to the dura)

and volume of virus injections were as follows: Starting at -4.0 AP (0º), one injection was made at -8.0mm deep; Starting at +0.3mm AP (17º), 1µl at -5.3mm deep, 1.2µl at -4.3mm deep, and 1.4µl at -3.3mm deep; Starting at +5.8mm AP (49º), 0.6µl at -9.3mm deep, 0.8µl at -8.3mm deep, 1.0µl at -7.3mm deep, 1.2µl at -6.3mm deep, 1.4µl at -5.3mm deep, and 2.0µl at -4.3mm deep. A diagram of this injection scheme is shown in Figure 4-1A.

VM tissue harvest and transplantation

One week after virus injection, animals received transplants of embryonic day 14 (E14) ventral mesencephalon (VM) tissue. Immediately prior to transplantation, embryos at day 14 (E14) were removed from a pregnant Sprague-Dawley rat and the ventral mesencephalic (VM) brain region (approximately 1mm x 1.5mm x 1mm) was dissected out of each fetus and kept in ice cold, sterile, calcium- and magnesium-free buffer until transplant. Once each rat was anesthetized, a hole was drilled at -5.2mm AP and 2.4mm ML on the left side relative to bregma, and VM was implanted as a whole tissue chunk using a modified 22-guage spinal needle lowered to -8.6mm from skull level then pulled up to -8.4mm before ejecting the tissue. Each tissue chunk was ejected slowly by depressing the needle's plunger ~1mm every 20 seconds for a total distance of ~10cm (~3 minutes total). The needle was kept in place for 5 minutes after ejection, raised 0.4mm and kept there for another 5 minutes before slowly raising it all the way out of the brain to be sure the transplant remained in place.

Animals were kept alive for 8 weeks after transplant, with amphetamine-induced rotation and spontaneous limb-use asymmetry (cylinder test) measured as described above every two weeks. After the 8-week behavior tests, rats were over-anesthetized with pentobarbital and perfused with ice-cold saline followed by 4% paraformaldehyde (PFA) in phosphate buffer. Brains were carefully removed and post-fixed overnight in 4% PFA at 4ºC then placed in a 30% sucrose solution at 4ºC until sinking to the bottom of the vial (2-3 days). Brains were then halved in the sagittal plane, embedded in Tissue-Tek OTC compound,

frozen on dry ice, and sliced at 30μm parasagittally with a cryostat. Brains were divided into 5 sets of serial sections for subsequent immunostaining and anatomical analysis.

Immunohistochemistry

To visualize dopaminergic fiber outgrowth from transplanted neurons, brain sections were immunostained with a mouse monoclonal antibody to tyrosine hydroxylase (TH; Chemicon;1:4000) and a biotinylated secondary antibody (goat anti-mouse IgG; Chemicon; 1:600), and developed using an avidin-biotin-peroxidase kit (VectaStain Elite, Vector Labs) followed by diaminobenzidine (DAB) chromogen alone or DAB with nickel enhancement to better visualize fine fiber branching. Some sections were also stained for the guidance molecule netrin-1 using a chicken anti-netrin-1 primary antibody (1:200, Chemicon International) and a biotinylated secondary antibody (goat anti-chicken, 1:300, Chemicon), developed with the VectaStain Elite kit and NovaRed substrate (Vector Labs).

Quantification and statistical analyses

All quantification was carried out by an observer blinded to treatment. To quantify transplant cell survival and axon outgrowth, three sections were chosen per animal which met the following criteria: 1) contained transplanted TH+ cell bodies and 2) either had visible needle tracts from viral pathway injections OR were located in a similar anatomical location based on identifiable landmarks between 1.9 and 2.9mm lateral to bregma according to the rat brain atlas (Paxinos and Watson, 1986). Sections that included the septum (too medial) or the entorhinal cortex/parasubiculum (too lateral) were not included in the analysis. For cell survival, TH+ cell bodies were counted manually at 200x total magnification using a tally counter and an ocular grid to move methodically through the transplant area. For axon outgrowth, the number of identifiable TH+ fibers was tallied at a distance of 2mm from the rostral edge of the transplant.

Cell counts and fiber counts were averaged over the three sections for each animal.

For comparisons of striatal dopaminergic innervation, digital photographs were taken that included the entire striatum in each section (20x total magnification) with a Nikon Coolsnap camera and MetaVue imaging software (Universal Imaging Corporation). Initially, microscope, camera and threshold settings were chosen that were best for highlighting TH+ staining without including background noise. Once the settings were chosen, they were strictly adhered to throughout the quantification process. For each picture (still 3 sections per animal), the striatal area was traced with the freehand drawing tool in MetaVue and the inclusive threshold setting was applied. Measurements of region area, threshold area, and threshold area percent were logged to an Excel file. The threshold area percent value was averaged over the three sections per animal.

Results

Transplant placement and survival

Animals were killed and brain tissue harvested 8 weeks after transplant and after all behavioral analyses were completed. With immunostaining for tyrosine hydroxylase (TH), transplants were located in all but one animal (in the GDNF + GFRα1 group). In that animal, the transplant may have been pulled up with the needle during the transplant surgery, so it was excluded from all analyses. In the remaining animals, transplanted TH+ cell bodies were visible in the midbrain region, except in two cases where the whole tissue chunk had slipped in the rostral direction 1-2 mm – probably due to placement that was too deep, with the tissue slipping forward under the base of the brain and then adhering in a different location. Counts of TH+ cell bodies revealed no significant differences between treatment groups for cell survival: lenti-GFP = 59 ± 9.7; lenti-GFRα1 = 98 ± 24.1, lenti-GDNF = 105 ± 19.6, lenti-GDNF + lenti-GFRα1 = 86 ± 30.7. Data are means ± SEM; ANOVA $p = 0.66$.

Protein expression and dopaminergic fiber growth along NS pathway

Lentivirus-induced expression of the various proteins was confirmed in the experiment described in chapter three of this dissertation. Figure 4-1B shows the pattern of GFP fluorescence after lenti-GFP injections along the path of the internal capsule. This confirms that the calculated injection coordinates and angles are fairly accurate at targeting the internal capsule between the substantia nigra and the striatum at 2.4mm lateral to bregma.

Dopaminergic fiber outgrowth from transplants was variable, even within treatment groups, and there was evidence of tissue damage along the path of virus injection in many animals. Even with damage, however, there was long-distance TH+ fiber outgrowth in some animals, spanning the distance between the substantia nigra and the striatum. Figure 4-2 shows TH+ fiber growth along the expression pathway in a lenti-netrin-1 treated animal. The total distance of fiber growth is greater than 5mm. To confirm that the netrin-1 protein was expressed along the virus-injected pathway, adjacent sections were immunostained with antibodies to netrin-1 (Figure 4-3), or double-immunostained for TH (nickel-enhanced DAB) and netrin-1 (NovaRed; Figure 4-4). A brain injected with lenti-GDNF and lenti-GFRα1 is pictured in figure 4-5, immunostained for TH with nickel-enhanced DAB. The nickel enhancement allows for better visualization of fine TH+ fibers, and the section in figure 4-5 shows extensive axon growth and branching into the striatum in spite of a significant amount of tissue damage from the virus injections. Quantification of axon growth along the nigrostriatal path was done by manual counting at a distance of 2mm from the rostral edge of the transplant averaged over three sections per animal. Data are presented graphically in figure 4-5, with means +/- SEM. Differences between groups are statistically significant only for the lenti-GDNF plus lenti-GFRα1 with transplant group compared to either lenti-GFP with transplant (t-test $p = 0.04$) or lenti-GDNF plus lenti-GFRα1 without transplant (t-test $p = 0.03$). The lenti-GDNF with transplant group has, on average, almost as many TH+ fibers at 2mm as the combination group with transplant (40 for lenti-

GDNF and 43 for lenti-G&G), but due to high within-group variance, the increase compared to the other treatment groups is not statistically significant.

Striatal innervation

Reinnervation of the target striatum with dopaminergic fibers was quantified on sections stained for TH only, developed with nickel-enhanced DAB chromogen. MetaVue image analysis software was used to threshold striatal regions and quantify TH+ staining. Brain sections from two unlesioned (normal) animals were also stained for TH expression, revealing an average of 39% of the area of the striatum filled with TH+ fibers. The highest group average for post-treatment striatal innervation was in the lenti-netrin-1 group, with 5.0 ± 3.4% TH+ area (13% of normal). An animal in the lenti-netrin group had the highest score, with 22% TH+ area in the striatum, as averaged over three sections (56% of normal). Differences between groups are shown in figure 4-7 as mean ± SEM, and do not reach statistical significance due to high within-group variance.

Behavior tests

Animals were tested for amphetamine-induced rotational asymmetry both prior to transplant and every two weeks following transplant. All animals made a minimum of 450 ipsilateral turns the 90-minute test period (5 turns/minute) prior to treatment. Pre- and 8-week post-transplant rotation scores are shown graphically in figure 4-8. The only treatment group that showed a significant decline in amphetamine-induced rotation after 8 weeks was the lenti-GDNF + lenti-GFRα1 group (paired t-test $p = .05$). Decreases in rotational asymmetry between pre-treatment and 8 weeks post-treatment are correlated to the amount of TH+ innervation in the striatum: the higher the innervation, the greater the decrease in asymmetry, when data are pooled across treatments (Spearman rank correlation $p = .007$; figure 4-9).

Spontaneous motor behavior was also tested before and every two weeks after treatment using the cylinder test of Schallert and Tillerson (1999). Asymmetry in forelimb use during environmental exploration within a clear plastic

cylinder was quantified by an observer blinded to treatment. Results for all treatment groups, including those with no transplants, are shown in figure 4-10 as mean asymmetry ratio (left paw/total wall touches) ± SEM. Within each treatment group, pre-treatment asymmetry scores were compared to 8-week post-treatment scores by a student's t-test. Significant decreases in asymmetry are seen in the netrin-1 + transplant group ($p < .05$) and in the GDNF + GFRα1 + transplant group ($p < .01$). All other differences are statistically non-significant.

Discussion

Cell replacement therapy for Parkinson's disease has been the focus of many studies since about 1980, with transplantation of fetal midbrain cells making it as far as clinical trials (Bjorklund et al., 1980; Winkler et al., 2000; Freed et al., 2001). In the majority of these studies, the cell grafts have been placed directly into the striatum to serve as a source of dopamine in that denervated target. The goal of providing dopamine is achieved by such methods, but improvement in motor behavior in lab animals and clinical improvement in human transplant patients has been minimal. It is likely that the ectopic placement of dopaminergic cells is partly to blame for this limited success, as the normal circuitry of the basal ganglia - including dopaminergic innervation of the substantia nigra (SN) - is not restored.

Some attention has been paid to this shortcoming of PD transplantation methods, and attempts have been made to restore connections within the SN and subthalamic nucleus (STN) as well as the striatum, with double- and triple-graft approaches (Mendez et al., 1996; Mendez and Hong, 1997; Mukhida et al., 2001; Mendez et al., 2002). These studies demonstrated the importance of dopaminergic reinnervation of the other basal ganglia regions to achieve more complete behavioral recovery. While intrastriatal transplants were necessary and sufficient for significant decreases in amphetamine-induced rotation scores in test animals, only animals receiving double (striatum + SN) or triple (striatum + SN + STN) grafts showed improvement in more clinically-relevant forelimb

akinesia tests (Mukhida et al., 2001). While this work underscores the importance of dopaminergic connections *within* targets other than the striatum, it does not address the need for connections *between* those locations.

True reconstruction of the neural circuitry that is decimated in PD requires cell transplantation into the SN and subsequent neurite outgrowth and formation of synaptic connections in the distant striatal target. The current study demonstrates the feasibility of such an approach using lentiviral vectors to create growth-supportive pathways between the SN and the striatum prior to intranigral fetal VM transplantation. We provide evidence that this method can result in significant dopaminergic axon growth between the SN and the striatum, functional reinnervation of the striatum (assessed by amphetamine-induced rotation), and improvement in spontaneous motor behavior that have not been seen previously with bridging techniques.

The molecules that we expressed between the SN and the striatum were chosen because previous studies suggested that they had growth supportive and/or guidance effects on dopaminergic midbrain neurons. GDNF has been shown to increase the survival, differentiation, fiber outgrowth and dopamine release of fetal midbrain dopaminergic neurons both *in vitro* and *in vivo* (Lin et al., 1993; Stromberg et al., 1993; Hudson et al., 1995; Johansson et al., 1995). GDNF has been used successfully to increase the survival of fetal dopaminergic cell transplants in the 6-OHDA-lesioned rat striatum (Rosenblad et al., 1996; Yurek, 1998; Ostenfeld et al., 2002) and shows some promise for increasing fiber outgrowth from dopaminergic cells transplanted into the lesioned SN (Wang et al., 1996; Wilby et al., 1999). So far, there is no strong evidence supporting GDNF's role as a chemoattractant for dopaminergic axons, but when combined with the GPI-linked glial cell line-derived neurotrophic factor receptor α1 (GFRα1) there is an attractive guidance effect on other populations of GDNF-responsive neurons (sensory and sympathetic) (Ledda et al., 2002). With this in mind, we chose to create pathways that combined expression of GDNF and GFRα1 between the SN and the striatum. Finally, we tested netrin-1 because it has recently been shown to have positive directional effects on neurite outgrowth

from cultured fetal or stem-cell derived dopaminergic neurons (Lin et al., 2005; Lin and Isacson, 2006).

Our data suggest that a combination pathway expressing GDNF and GFRα1 is the best choice for nigrostriatal circuit reconstruction, with netrin-1 also showing some promise. The combination GDNF/GFRα1 group had significantly higher counts of TH+ fibers along the pathway, as well as significant functional improvement as evidenced by spontaneous motor behavior tests at 8-weeks post-transplant. The netrin-1 group also had significantly improved spontaneous motor behavior at the 8-week time point, but TH+ fiber outgrowth was no different than controls. Amphetamine-induced rotation scores did not change in any group except the GDNF/GFRα1 group, and in that case the decrease just reached statistical significance ($p=.05$). Correlation analysis of rotation scores vs. TH+ fiber density in the striatum suggests that higher striatal reinnervation leads to greater reductions in amphetamine-induced rotational asymmetry. Previous studies have shown that amphetamine-induced rotational asymmetry can be eliminated with intrastriatal VM transplants, but is not changed by intranigral transplants, while scores of forelimb function are better improved by intranigral transplants (Mukhida et al., 2001). Our results suggest that *both* of these functions may be improved with intranigral transplants and reconstruction of the nigrostriatal pathway.

In creating a guidance pathway between the SN and the striatum, we chose to follow the course of the internal capsule rather than making a straight line between the two points. This approach was chosen because white matter tracts in the adult CNS have been shown to provide good pathways for long-distance axon growth due to their physical properties, as long as the chemical environment is also supportive to such growth (Davies et al., 1994; Davies et al., 1997; Raisman, 2004). Previous studies in our lab have confirmed this, using the corpus callosum as a highway for axon growth between the two brain hemispheres (see Chapter 2). Our preliminary experiments with intranigral VM transplants also showed that TH+ fiber outgrowth tended to follow the course of the internal capsule rather than other possible routes (data not shown). The

lentiviral injection coordinates used in the current study attempted to follow the internal capsule's curved path, but were not always successful at doing so. In some animals, the needle tract from lentiviral injections was obviously rostral and ventral to the internal capsule (see Figures 4-2 through 4-4). It is possible that modification of the injection protocol for future studies will further improve outcome.

While the results of this study are exciting and show great potential, there are some problems that need to be addressed. First and foremost, the amount of brain damage seen in most animals secondary to virus injection is unacceptable. This was determined to be due to carryover of poly-L-lysine into the viral suspension during the concentration step, and has since been eliminated with a modified virus production protocol. Additionally, we see a significant amount of TH+ fiber sprouting within lesioned brains injected with lenti-GDNF (with or without lenti-GFRα1) in the absence of VM transplants. In the current study, we did not determine the source of this sprouting – it may be coming from dopaminergic nuclei that are located more medially, such as the ventral tegmental area, or else from the contralateral hemisphere. Future studies will determine the source of sprouting by applying retrograde tracing techniques, and will determine optimum placement and concentration of virus injections to minimize undesired fiber growth. Improvement may be seen with a decreased ratio of lenti-GDNF:lenti-GFRα1 in combination pathways, but we will need to take care to not compromise fiber outgrowth from transplanted cells.

The results presented here, while still in preliminary in nature, show great potential for a lentivirus-based method of nigrostriatal pathway reconstruction. Using this approach, it is possible to reverse multiple behavioral deficits that occur in a rat model of Parkinson's disease by restoring dopaminergic input in an anatomically-correct manner. With continued refinement of the technique, it could provide a real therapeutic option for patients with Parkinson's disease.

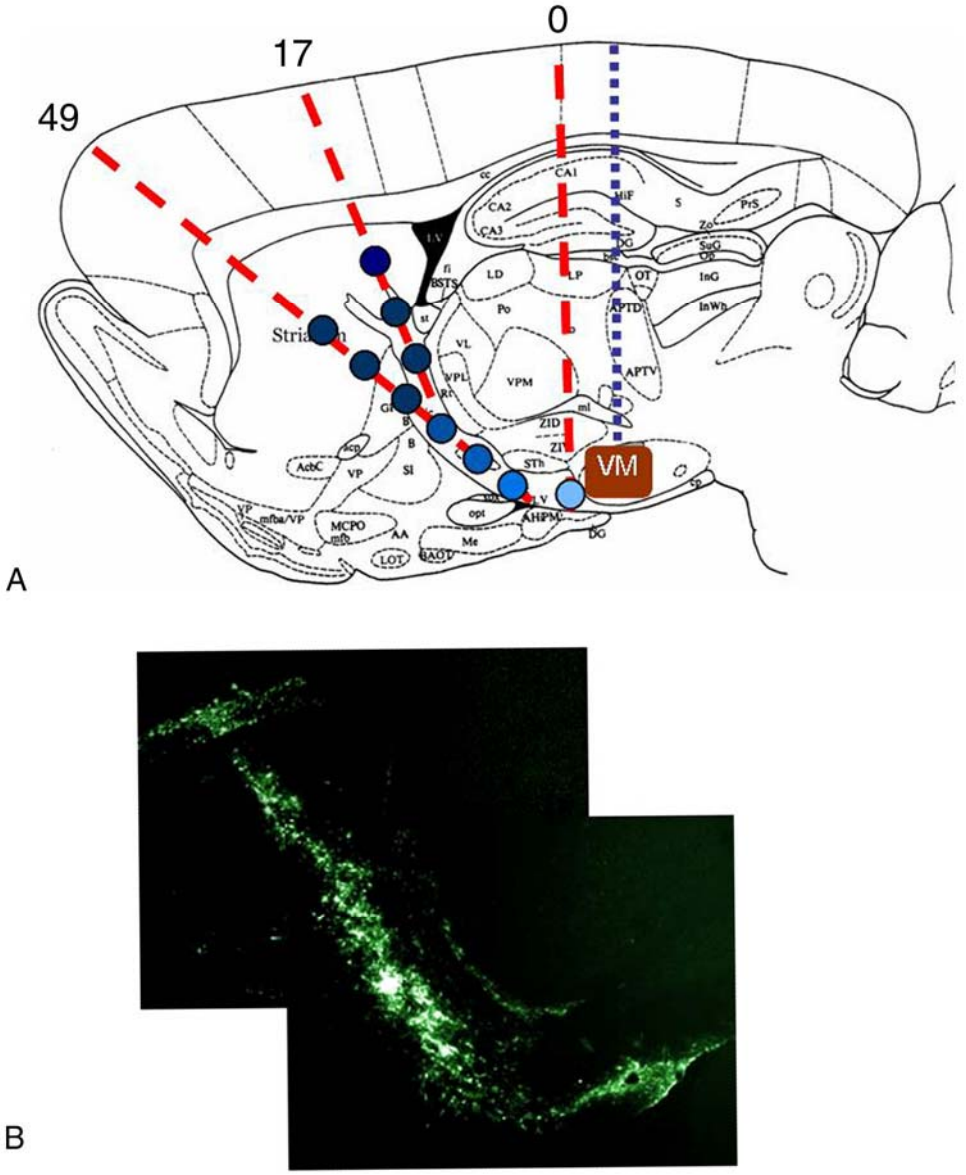
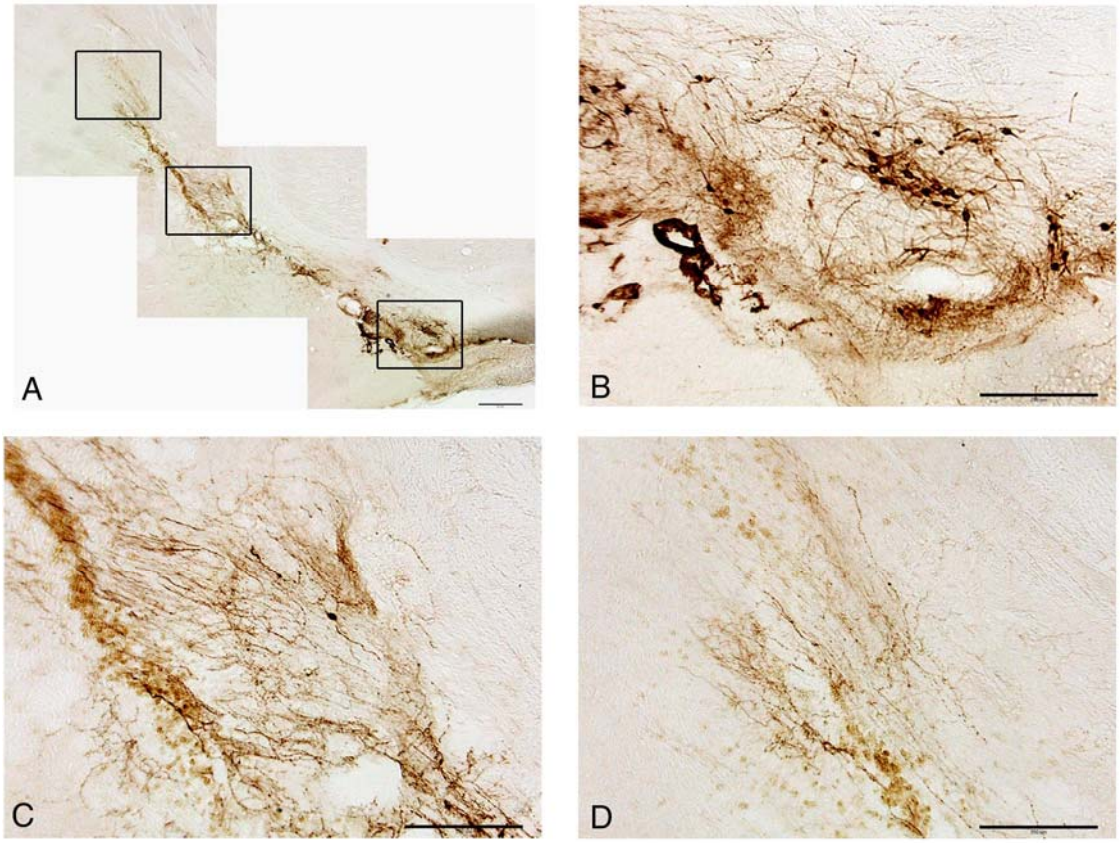


Figure 4-1: Lentivirus injection scheme. A) Schematic diagram showing the location of lentivirus injection points and transplant location in the sagittal plane at 2.4mm lateral to bregma in the rat brain. Background map from stereotaxic rat brain atlas of Paxinos and Watson (1986). Blue circles represent lentivirus injections, with darker colors indicating a larger number of viral particles injected at the striatal end of the pathway. VM = E14 ventral mesencephalon tissue chunk. B) A sample sagittal section from a brain injected with lenti-GFP, showing GFP expression (fluorescence) all along the pathway between the midbrain (lower right) and the striatum (upper left), with some spread of expression within the corpus callosum (top).



<u>Figure 4-2</u>: TH+ fibers extending between transplant site in the SN and the target striatum along a pathway created with LV-netrin-1. 8 weeks after transplant, brains were harvested, sliced in parasagittal sections and stained with a monoclonal antibody to tyrosine hydroxylase, developed with diaminobenzidine chromogen. A) Low power composite photograph shows entire pathway; scale bar = 500μm. B) Higher magnification of lower box in A, showing TH+ cell bodies and dense fiber outgrowth at the transplant site; scale bar = 200μm. C) Higher magnification of middle box in A, showing TH+ fibers (and one TH+ cell that has migrated) along the nigrostriatal pathway; scale bar = 200μm. D) Higher magnification of top box in A, showing TH+ fibers that have made it to the striatum; scale bar = 200μm

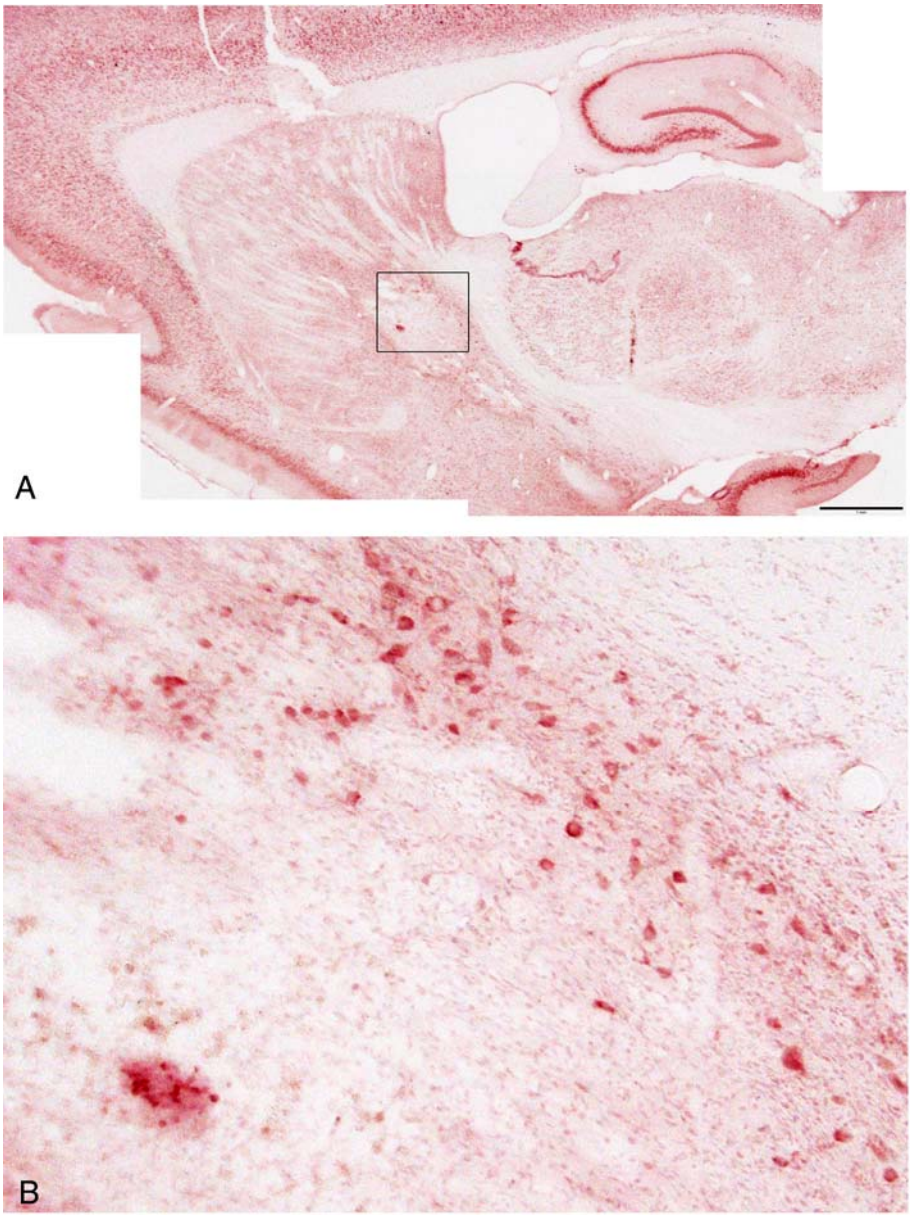
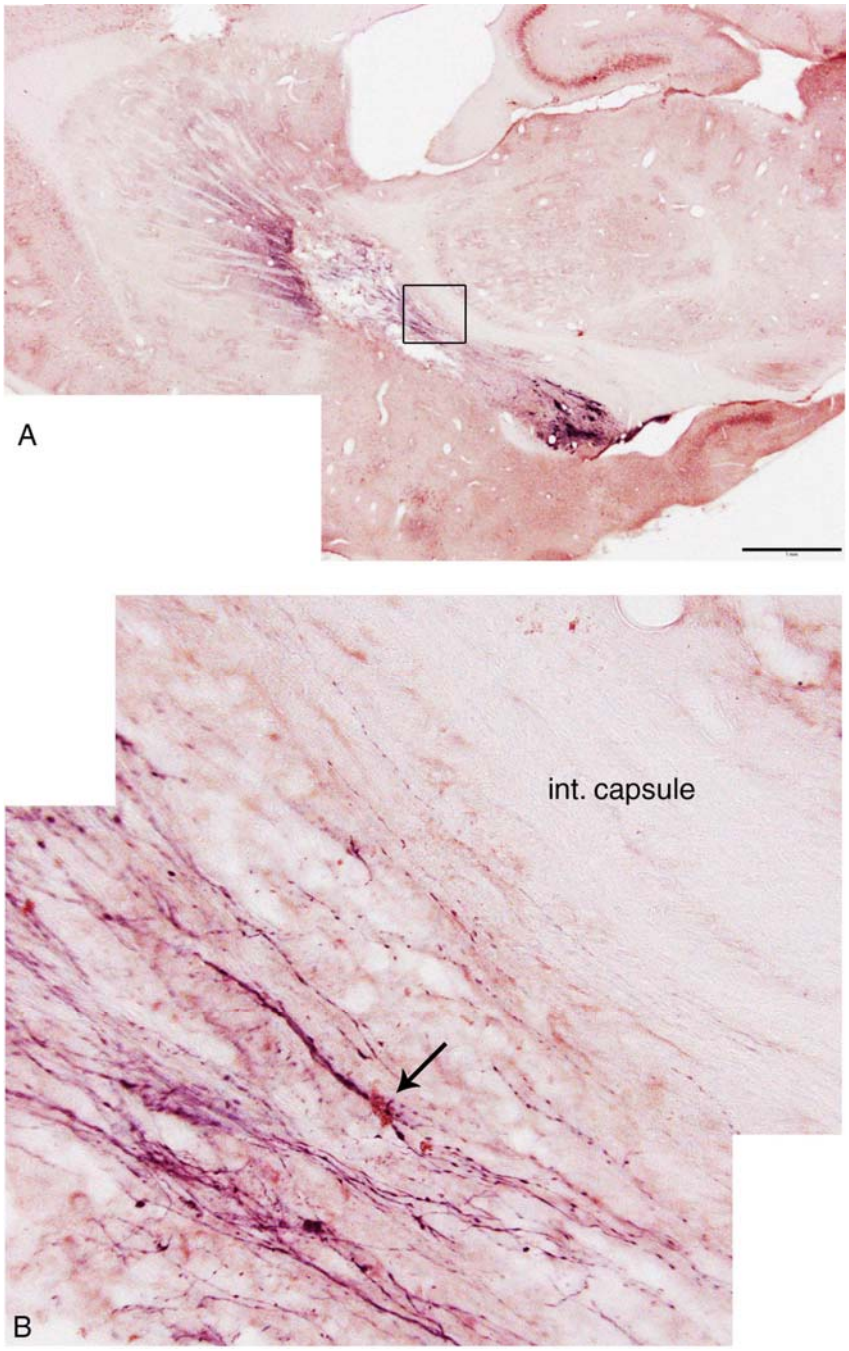


Figure 4-3: Netrin-1 expression along the virus-injected pathway confirmed by immunohistochemical staining. This is an adjacent section from the same brain shown in figure 2 stained with an antibody to netrin-1 and developed with NovaRed. A) Low magnification image showing most of the sagittal section, with evidence of endogenous netrin-1 expression, especially in the cortex and hippocampus, as well as along the LV-netrin-1 injected pathway (just rostral/ventral to the internal capsule). Scale bar = 1mm. B) Higher magnification of the boxed area in A. The red spot in the lower left is probably due to some clumping of the virus on injection, but many netrin-1+ cells are also seen just adjacent to the internal capsule (white matter in upper right of photo).



<u>Figure 4-4</u>: Double immunostaining showing TH+ fibers and netrin-1 expression along the nigrostriatal pathway. This is an adjacent section from the same brain shown in figures 2 and 3, stained with antibodies to both TH and netrin-1, then developed with DAB + Nickel enhancement (purple color, TH) and NovaRed (pink color, netrin-1). A) Low magnification image, scale bar = 1mm. B) Higher magnification of boxed area in A; arrow points to a cell with high expression of netrin-1 with a thick bundle of TH+ axons growing over it.

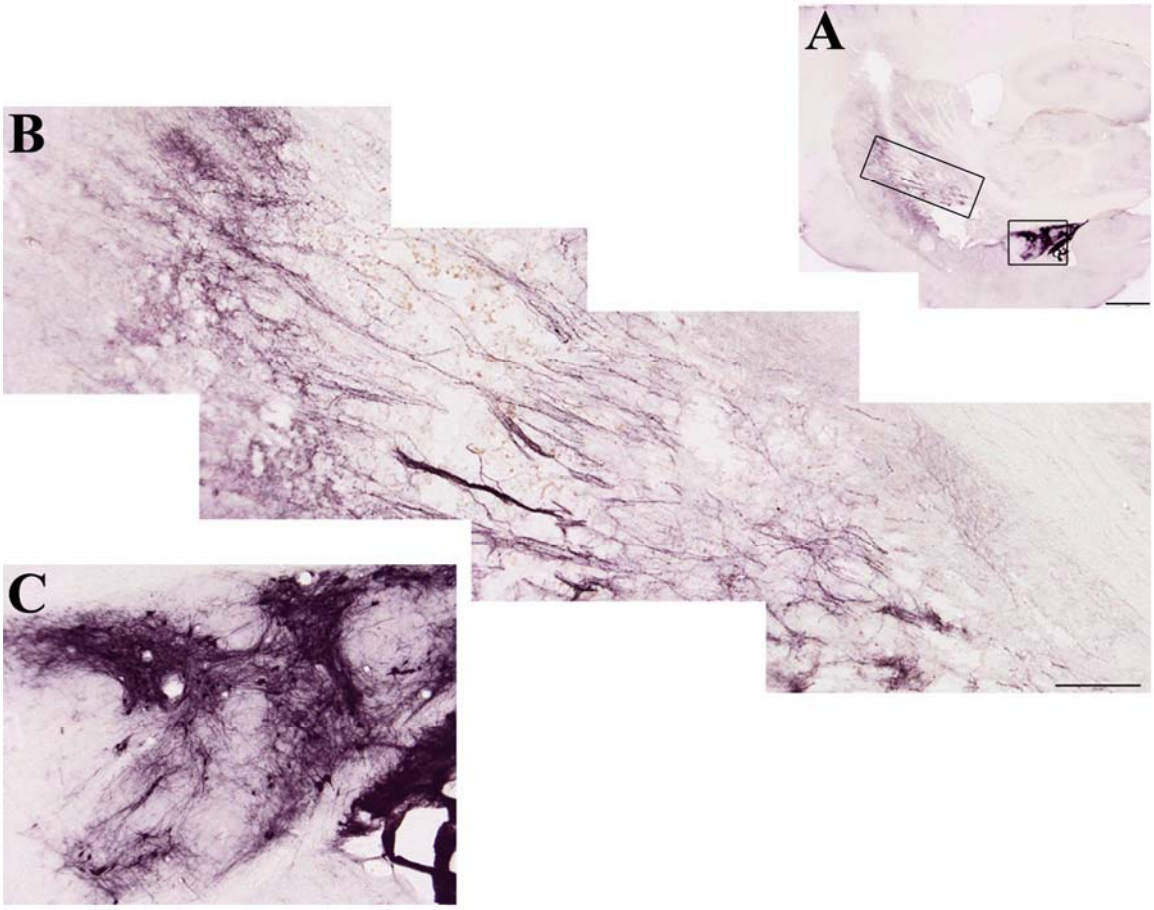


Figure 4-5: TH+ fibers extending between transplant site in the SN and the target striatum along a pathway created with LV-GNDF & LV-GFRα1. 8 weeks after transplant, brains were harvested, sliced in parasagittal sections and stained with a monoclonal antibody to tyrosine hydroxylase, developed with Nickel-enhanced diaminobenzidine. A) Low magnification shows entire pathway, with evidence of tissue damage induced by the lentiviral injections, and some slippage of the transplant in the rostral direction. However, there is robust TH+ fiber growth within the transplant, along the pathway and into the striatum. Scale bar = 1mm. B) Higher magnification of the long box in A, showing extensive TH+ fiber growth even through damaged brain tissue. Scale bar = 200μm. C) Higher magnification of small box in A, showing dense TH+ fiber growth within the transplanted tissue chunk.

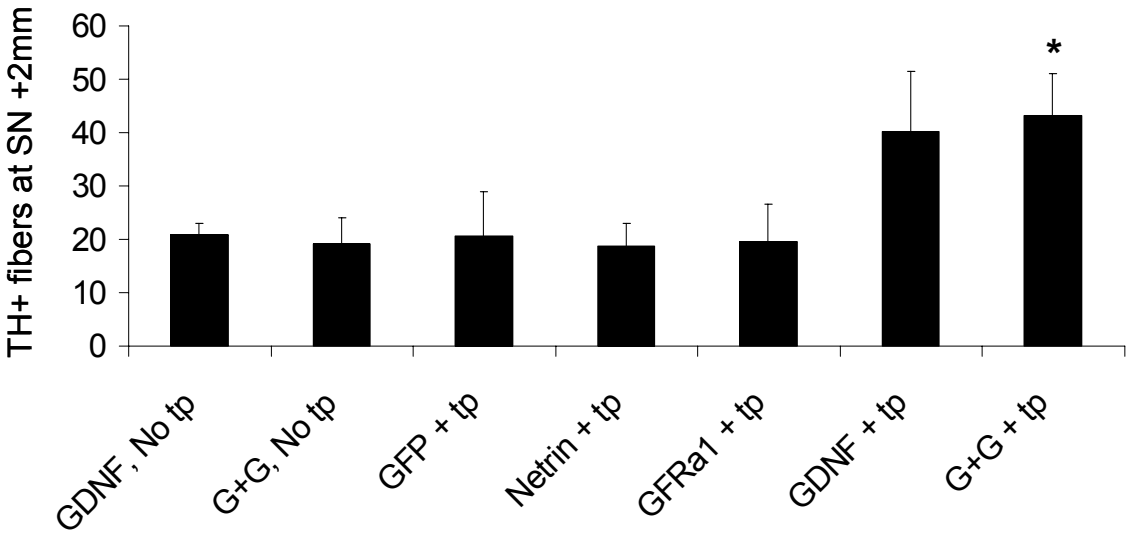


Figure 4-6: Quantification of TH+ neurite outgrowth 2mm distal to the transplant site. The number of TH+ fibers was counted and averaged over three sections in each animal by an observer blinded to treatment. There is an increase in TH+ fiber growth along the lentivirus-injected pathway in both the GDNF and the GDNF plus GFRα1 treatment groups, but the increase is significant only in the combination group ($p=.05$ compared to without transplant and compared to GFP with transplant). tp = transplant; G+G = lenti-GDNF plus lenti-GFRα1. Data are means ± SEM.

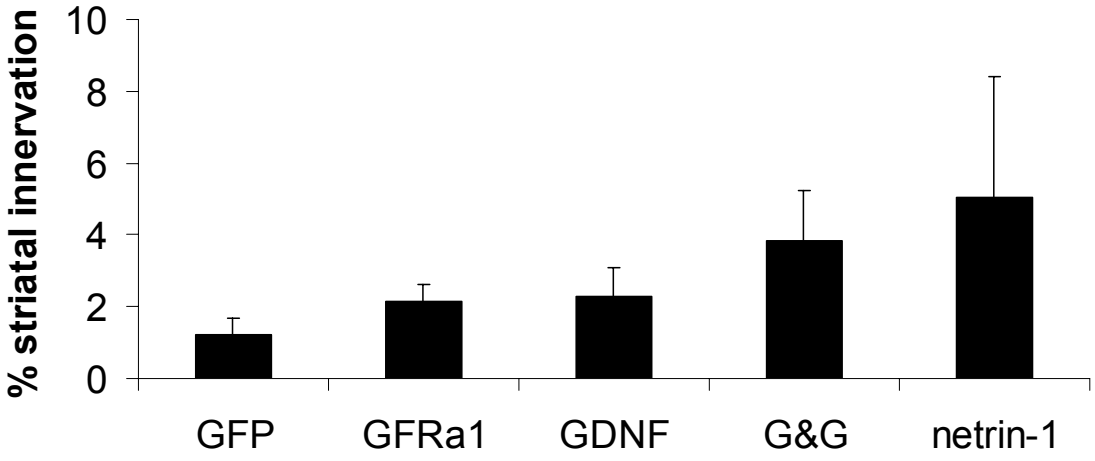


Figure 4--7: Quantification of dopaminergic striatal re-innervation. Dopaminergic innervation of the striatum was determined by thresholding images of brain sections immunostained for tyrosine hydroxylase. The area of the striatum was outlined and the percent of that area filled with TH+ staining was measured and averaged over 3 sections per animal. While there is a trend toward higher striatal innervation with lenti-GDNF plus lenti-GFRα1 and with lenti-netrin-1, differences between groups are statistically non-significant due to high within-group variation. G&G = lenti-GDNF plus lenti-GFRα1. Data are means ± SEM.

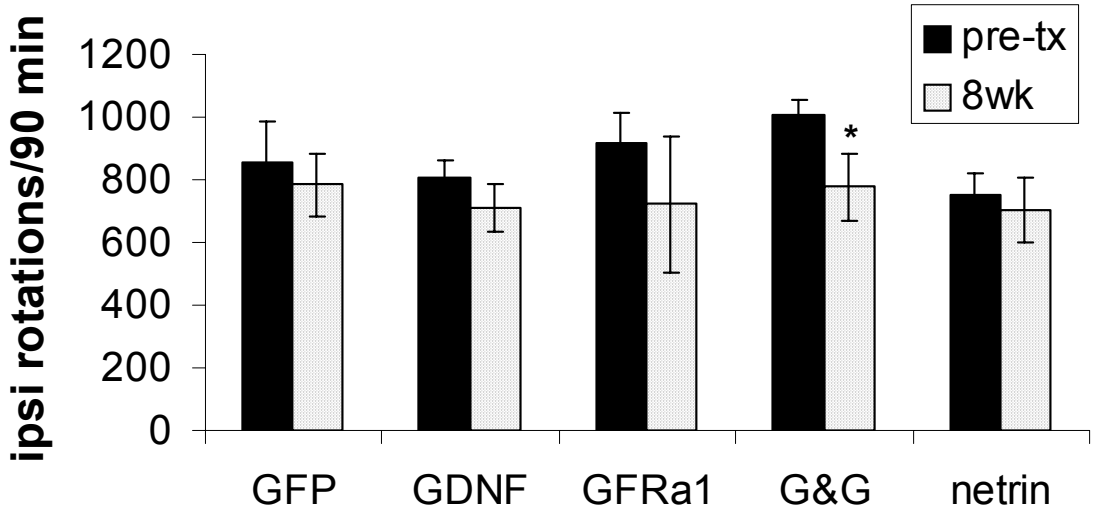


Figure 4--8: Quantification of amphetamine-induced rotation in the five treatment groups with transplants. Pre-treatment and 8-week post-treatment scores are presented for the number of ipsilateral (counterclockwise) rotations in 90 minutes after injection with 2.5mg/kg D-amphetamine in saline (i.p.). The only significant change between the two time points is in the lenti-GDNF plus lenti-GFRα1 group (G&G; paired t-test *p = .05). Data are means ± SEM.

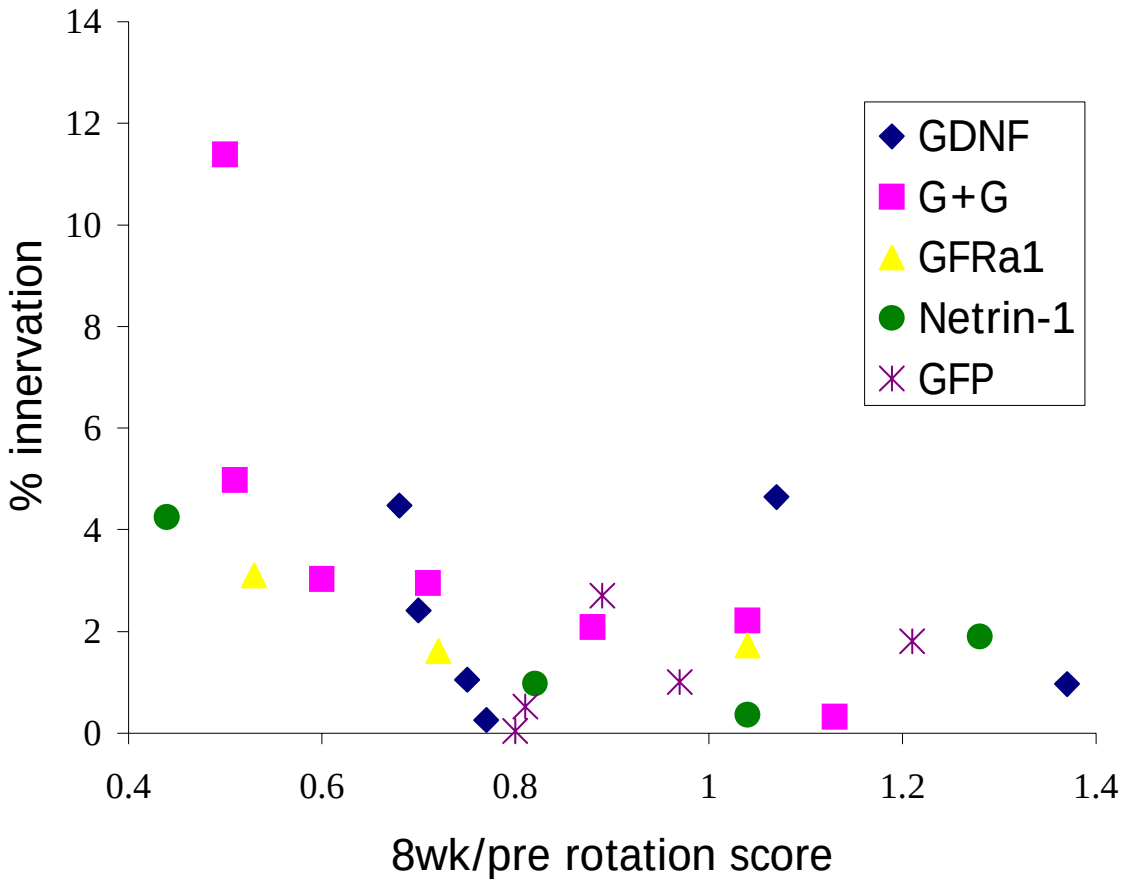


Figure 4-9: Negative relationship between amount of striatal reinnervation and amphetamine-induced rotation scores. There is a significant negative rank correlation between the amount of striatal innervation and the ratio between the 8-week rotation score and the pre-treatment rotation score across all groups. This confirms that a more successful re-innervation will likely result in further decreases in post-transplant rotational asymmetry. G+G = lenti-GDNF plus lenti-GFRα1.

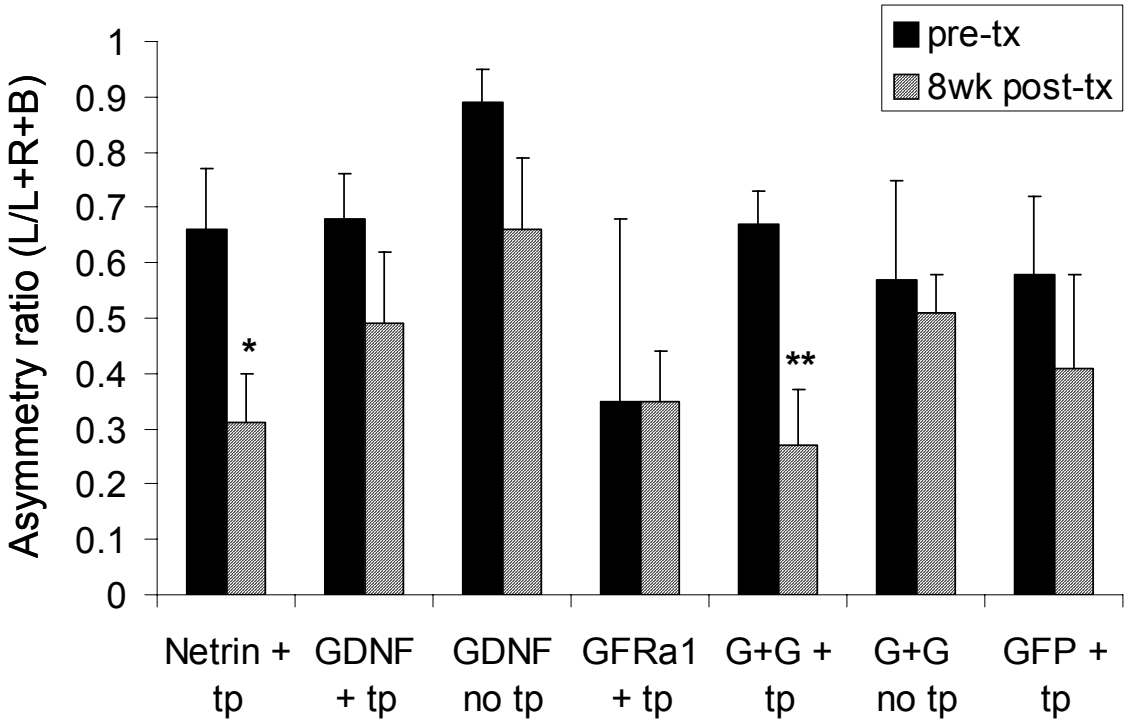


Figure 4-10: Quantification of spontaneous limb-use asymmetry in all seven treatment groups pre-treatment and 8 weeks post-treatment. Limb use asymmetry in spontaneous cylinder wall-exploration was calculated by dividing left paw touches by total wall touches (right, left or both paws) in a 3-5 minute test period. An asymmetry score of 1 would indicate 100% left paw use. A significant decrease in asymmetry was found 8 weeks post-treatment in the netrin-1 + transplant group (*p<.05) and the GDNF + GFRα1 + transplant group (**p<.01). G+G= lenti-GDNF plus lenti-GFRα1; tp = transplant. Data are means ± SEM.

Chapter Five: General Discussion and Future Directions

The data presented in this dissertation represent an important step toward the goal of circuit reconstruction in the adult mammalian central nervous system (CNS). By most measures, repair of CNS tissue that is destroyed by disease or injury is still impossible – a frustrating fact for patients who suffer strokes, spinal cord injuries, or the ravages of neurodegenerative processes, as well as for the neurologists who diagnose and care for them. The body's natural healing process is effective for most other injured tissues: dead cells are removed, new cells are produced, and tissue architecture is reassembled sufficiently for recovery. For more severe damage, surgical intervention – including organ transplantation – may be required. Amazingly, even foreign parts can integrate successfully into the complex system of the human body, and transplant recipients may live near-normal lives in the care of competent physicians. The CNS, however, functions on a different set of principles.

Neuroscience research over the past two decades has shed light on the capacity of the central nervous system to adapt to change (neural plasticity) and even to produce new cells in certain brain regions (neurogenesis) (Bjorklund and Lindvall, 2000b; Kuhn et al., 2001; Curtis et al., 2003; Nudo, 2006). Unfortunately, however, new cells are not produced in sufficient quantities or in an effective enough manner to compensate for significant CNS damage. Consequently, cell-replacement strategies have been pursued as therapeutic options for conditions such as Parkinson's disease, Huntington's disease, stroke, amyotrophic lateral sclerosis and spinal cord injuries (Whittemore, 1999; Bjorklund and Lindvall, 2000a; Fricker-Gates et al., 2001; Bregman et al., 2002; Kondziolka et al., 2002; Bjorklund et al., 2003; Isacson, 2003; Reier, 2004; Emsley et al., 2004; Winkler et al., 2005; Goldman, 2005). For neural circuit reconstruction – which would be necessary for complete functional recovery in most of these cases – transplanted cells must survive and integrate effectively into the host CNS: receiving afferent input and providing efferent signals, sometimes separated by long distances. Since the adult mammalian CNS is

generally inhospitable to axon growth, circuit reconstruction that requires such growth is usually not successful, and is often not even attempted. One of the main goals of our laboratory is to provide a technique for directing axon growth from neurons transplanted into the CNS to distant target locations, reestablishing functional neural circuits and allowing behavioral recovery.

The basic concept behind the studies presented here is that local genetic manipulation of the host CNS may transform it into a permissive substrate for axon outgrowth from transplanted neurons. Stereotactic injections of viral vectors are used to express specific molecules which act as neurotrophic, chemotropic or chemorepulsive signals in such a pattern as to precisely target neurite growth. In chapter two, we prove the effectiveness of this approach in supporting axon outgrowth over distances of several millimeters in the adult rat brain, and in directing a 90-degree turn of the axons out of a white matter tract and into a pre-determined target location. In this proof-of-concept study, we transplanted early postnatal dorsal root ganglion (DRG) sensory neurons into the corpus callosum of adult rats. DRG neurons were chosen due to prior knowledge of neurotrophic and chemotropic molecules that support and direct the growth of those cells during normal development (Gundersen and Barrett, 1979; Phillips and Armanini, 1996; Paves and Saarma, 1997; Tanelian et al., 1997; Dontchev and Letourneau, 2002; Masuda and Shiga, 2005). We constructed adenoviral vectors encoding such molecules (nerve growth factor, NGF; basic fibroblast growth factor, FGF-2; and semaphorin 3a) to create our axon-targeting pathways. The adult corpus callosum was chosen as the transplantation site for ease of visualization of immunostained sensory axons and because prior work by Davies and colleagues showed that the white matter tract could support long distance axon growth from transplanted DRG neurons if non-damaging injection protocols were strictly adhered to (Davies et al., 1994, 1997). Our data showed that by expressing a combination of NGF and FGF-2 all along the corpus callosum and NGF in the contralateral striatum we could direct sensory axon growth from DRG neurons transplanted into the corpus callosum on the left side of the brain, across the midline, and out of the corpus callosum

into the striatum in the right hemisphere. This growth occurred in spite of some scarring due to the virus injections (confirmed by staining for chondroitin sulfate proteoglycans; data not shown) and axon targeting was improved by expressing semaphorin 3a adjacent to the pathway turn. These last two points highlight the significance this study and how the results differ from the earlier work by Davies, et al: first, we demonstrate the power of viral vectors to alter the host brain along a specific trajectory and support axon growth where inhibitory signals would otherwise prevent it; and second, we show that axon growth may be targeted more specifically with judicious expression of chemorepulsive molecules in the surrounding, non-target area.

Chapters three and four of this dissertation describe our application of the virus pathway/transplantation targeting method to a rodent model of Parkinson's disease (PD). As previously mentioned, cell-replacement strategies for PD have already been attempted by many groups – in fact, such therapies have made it to the level of clinical trials for PD patients, but with unsatisfactory results (Freed et al., 2001; Olanow et al., 2003). Since most previous studies (including both clinical trials) involved transplantation of fetal dopaminergic neurons directly into the striatum rather than into their ontogenetic niche in the substantia nigra (SN), it reasonable to attribute unfavorable results, at least in part, to this ectopic cell placement. Cell grafts in the striatum do produce dopamine and can theoretically produce the same kind of behavioral improvement that pharmacological dopamine replacement (L-dopa) provides. Similar to L-dopa administration, however, striatal cell transplants can cause severe motor side effects called dyskinesias which significantly decrease a patient's quality of life (Freed et al., 2001; Greene and Fahn, 2002; Ma et al., 2002; Olanow et al., 2003). The combination of minimal functional recovery and unwanted side effects associated with intrastriatal dopaminergic cell transplants urges the development of an alternative grafting strategy with the goal of true restoration of the nigrostriatal pathway. Our data so far suggest that such a goal is not out of reach.

This lab is not the first to attempt nigrostriatal pathway reconstruction in a rodent model of PD, but previous attempts have left much room for improvement

(Dunnett et al., 1989; Wang et al., 1996; Brecknell et al., 1996; Wilby et al., 1999; Chiang et al., 2001). Importantly, other studies of "bridging" methods (transplants in the SN and various growth-supportive materials between the SN and striatum) quantified only amphetamine-induced rotation as a reflection of functional improvement, and minimally or qualitatively demonstrated dopaminergic fiber growth between the transplant site and the target. Our results include three significant positive findings: 1) expression of glial cell-line derived neurotrophic factor (GDNF) plus the soluble GPI-linked receptor GDNF family receptor alpha-1 (GFRα1) between the SN and the striatum significantly increases the number of dopaminergic fibers that grow out of transplanted cells and along the pathway, terminating in the striatum; 2) The same combination of molecules between the SN and the striatum (GDNF + GFRα1) with intranigral transplants leads to a significant decrease in amphetamine-induced rotation, indicating some functional reinnervation of the striatum; and 3) Transplants into the SN, when combined with lentivirus-induced expression of either GDNF + GFRα1 or netrin-1 along the nigrostriatal pathway, lead to recovery of spontaneous motor behavior, demonstrated by a decrease in limb-use asymmetry. To our knowledge, this is the first study to show improvement in *both* types of behavior (drug-induced and spontaneous) with transplants only in the SN.

Given these promising initial data, our lab is currently working to improve certain aspects of the experimental protocol. First, the lentivirus-production method described in chapter three has been modified to eliminate possible carryover of contaminants into the viral suspension, which may have been responsible for considerable tissue damage in experimental animals. Additionally, we are working to determine the source of dopaminergic fiber sprouting in brains overexpressing GDNF in the absence of dopaminergic tissue transplants. Such aberrant sprouting may interfere with behavioral improvement in transplanted animals and even cause unwanted side effects if functional synapses form randomly. There are a couple of strategies that we could employ to decrease this sprouting: either decrease the concentration of lenti-GDNF

injected close to the SN to prevent diffusion into the adjacent ventral tegmental area (one possible source of wayward dopaminergic fibers), or express an inhibitory molecule in the adjacent regions to prevent fiber sprouting. A likely candidate molecule for this approach would be slit-2, which has been shown to repel and inhibit dopaminergic axon growth *in vitro* (Lin et al., 2005; Lin and Isacson, 2006). Finally, we may need to adjust the viral vectors in order to temporally control expression of GDNF, turning it off once reestablishment of the nigrostriatal pathway is complete to avoid potential metabolic side effects of overexpression such as down-regulation of tyrosine hydroxylase (Rosenblad et al., 2003; Sajadi et al., 2005; Winkler et al., 2006).

With continued refinement, the methods described here have far-reaching potential. As an evolutionarily "advanced" vertebrate species, humans do not have the ability to regenerate CNS tissue as our "primitive" fish and amphibian brethren do (Ferretti et al., 2003). Developmental cues that direct the initial construction of our neural circuitry are no longer present in the same patterns in adults, so reestablishment of damaged circuitry cannot proceed in an orderly fashion without help (Harel and Strittmatter, 2006). As we have demonstrated, such help can be in the form of patterned expression of guidance cues established by viral vector injections followed by transplantation of neurons with intrinsic growth potential. For clinical applications, there are several possible sources of such neurons, including embryonic stem cells and neural precursor cells which may be differentiated and/or genetically modified prior to transplantation (Correia et al., 2005; Conti et al., 2006; Muller et al., 2006). Perhaps the most intriguing possibility for CNS repair, however, is the elimination of transplantation altogether in favor of manipulation of endogenous precursors to rebuild neural circuits.

The adult mammalian brain, while relatively inefficient at self-repair, is now known to be capable of neurogenesis – contrary to almost a century of scientific dogma (Gross, 2000). New cells are constantly produced in the anterior aspect of the subventricular zone (SVZ) of the lateral ventricles and in the subgranular zone (SGZ) of the hippocampus, providing new neurons to the olfactory bulb and

potentially contributing to structural plasticity and associative learning and memory (Ming and Song, 2005). Interestingly, neural precursor cells have been found in many other brain areas – they just do not normally become neurons *in situ* due to restrictions imposed by the local microenvironment (Palmer et al., 1995; Hagg, 2005; Sohur et al., 2006). If isolated and given the right molecular cues *in vitro*, however, these precursors can give rise to mature astrocytes, oligodendrocytes and neurons. Furthermore, *in vivo* manipulations such as focal induced injury stimulate formation of new, functional neurons in "non-neurogenic" brain regions (Magavi et al., 2000; Arlotta et al., 2003; Parent, 2003; Mitchell et al., 2004),.

Using tools like viral vectors, one could imagine altering brain microenvironments to take advantage of endogenous neurogenesis for brain repair. For Parkinson's disease, there are conflicting reports regarding the presence of neural precursor cells in the substantia nigra (Lie et al., 2002; Frielingsdorf et al., 2004). But even if the neurogenesis occurs at a distance from the required location of neuronal replacement, one could, in theory, target the migration of neural precursor cells as we have targeted axon growth in the studies described here. The same concepts apply: young cells with growth potential are equipped to respond to directional cues – in fact, many of the cues are redundant, controlling both neuronal migration and growth cone advancement by signaling mechanisms that affect cytoskeletal dynamics (Song and Poo, 2001). Provide molecular signals in the proper locations, and the cells will respond accordingly.

We are at an exciting time for neuroscience: new secrets of the brain are being revealed and new technologies are being developed at an accelerated pace, so that the "holy grail" of CNS repair is closer to our grasp. There are multiple avenues being explored, including gene therapy, cell transplantation, electrical stimulation, and neuropharmacology – any one, or a combination of which may hold answers to clinical challenges in neuroscience. Just focusing on transplantation, sources of cells for CNS repair may be embryonic stem cells, bone marrow cells, umbilical cord blood or the damaged brain itself (Svendsen et

al., 1999; Newman et al., 2004; Sohur et al., 2006; Zhang et al., 2006). The extent of brain damage and success of treatment may be tracked by increasingly sophisticated imaging techniques that distinguish fine alterations in brain metabolism (Zhang et al., 2006; Roberts et al., 2007). With all the tools at our disposal, and the brain's capacity for plasticity and self-renewal, nothing seems impossible.

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Curriculum Vita

Kristine S. Ziemba
DOB 06/21/1970, Wauwatosa, WI

Education

2000-2008, University of Kentucky College of Medicine, Lexington, KY
M.D./Ph.D. Program – M.D. anticipated May 2008.
Ph.D. completed March 1, 2007 (Department of Physiology).
Dissertation: *Targeting axon growth from neurons transplanted into the central nervous system*.
1995-1996, Arizona State University, Tempe, AZ
Post-Baccalaureate Teacher Certification in Secondary Education, Biology
1993-1995, Arizona State University, Tempe, AZ
Master of Science, Zoology
Thesis: Morphological correlates of sexual dimorphism in eye size in the desert hackberry butterfly, *Asterocampa leilia*. (December 1995)
1987-1991, Arizona State University, Tempe, AZ
Bachelor of Science, Zoology, Summa Cum Laude

Employment

1999-2000: *Senior Lab Technician* Department of Internal Medicine, University of Kentucky
1996-1999: *Science instructor*, *Highland High School*, Gilbert, AZ.
1997-1998: *Adjunct faculty*, *Math/Science Division*, Chandler-Gilbert Community College, Chandler, AZ.
1996: *Instructor, Center for Academic Precocity*, Arizona State University.
1993-1995: *Graduate Teaching Assistant*, *Department of Zoology*, Arizona State University.

Awards/Support

2004-2008: Predoctoral (MD/PhD) NRSA grant 1 F30 NS048716-01, "Reconstruction of the Nigrostriatal Pathway" funded by NIH/NINDS.
2004-2006: Two-year sponsored membership, AAAS/*Science* Program for Excellence in Science.
2004: Student Travel Award for the eleventh annual conference of the American Society of Neural Transplantation and Repair, May 6-9.
2003-2004: NIH T32 trainee, Cellular and Molecular Neuroscience of Sensory Systems, grant #NIH-T32-DC-00065.
2000: M.D. / Ph.D. Scholarship, University of Kentucky,

Publications

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