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LONG DISTANCE DIRECTIONAL GROWTH OF DOPAMINERGIC AXONS ALONG PATHWAYS OF NETRIN-1 AND GDNF

C. Zhang^{1,3}, Y. Jin^{1,3,4}, K. S. Ziemba^{1,3}, A. M. Fletcher², B. Ghosh⁵, E. Truit^{1,3}, D. M. Yurek², and G. M. Smith^{1,3,5}

¹Department of Physiology, University of Kentucky, Lexington, KY 40536

²Department of Neurosurgery, University of Kentucky, Lexington, KY 40536

³Spinal Cord and Brain Injury Research Center (SCoBIRC), University of Kentucky, Lexington, KY 40536

⁴Department of Neurobiology and Anatomy, Drexel University College of Medicine, Philadelphia, PA 19129

⁵Center for Neural Repair and Rehabilitation, Department of Neuroscience, & Shriners Hospitals for Pediatric Research, Temple University, School of Medicine, Philadelphia, PA 19140-4106

Abstract

Different experimental and clinical strategies have been used to promote survival of transplanted embryonic ventral mesencephalic (VM) neurons. However, few studies have focused on the long-distance growth of dopaminergic axons from VM transplants. The aim of this study is to identify some of the growth and guidance factors that support directed long-distance growth of dopaminergic axons from VM transplants. Lentivirus encoding either glial cell line-derived neurotrophic factor (GDNF) or netrin-1, or a combination of lenti-GDNF with either lenti-GDNF family receptor $\alpha 1$ (GFR α -1) or lenti-netrin-1 was injected to form a gradient along the corpus callosum. Two weeks later, a piece of embryonic day 14 VM tissue was transplanted into the corpus callosum adjacent to the low end of the gradient. Results showed that tyrosine hydroxylase (TH⁺) axons grew a very short distance from the VM transplants in control groups, with few axons reaching the midline. In GDNF or Netrin-1 expressing groups, more TH⁺ axons grew out of transplants and reached the midline. Pathways co-expressing GDNF with either GFR α -1 or netrin-1 showed significantly increased axonal outgrowth. Interestingly, only the GDNF/netrin-1 combination resulted in the majority of axons reaching the distal target (80%), whereas along the GDNF/GFR α -1 pathway only 20% of the axons leaving the transplant reached the distal target. This technique of long-distance axon guidance may prove to be a useful strategy in reconstructing damaged neuronal circuits, such as the nigrostriatal pathway in Parkinson's disease.

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Corresponding Author: George M. Smith, Ph.D., Center for Neural Repair and Rehabilitation, Department of Neuroscience, & Shriners Hospitals for Pediatric Research, Temple University, School of Medicine, 3500 N. Broad Str., Philadelphia, PA 19140-4106, george.smith@temple.edu.

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Keywords

Axonal guidance; ventral mesencephalon (VM) transplantation; GDNF; netrin-1; lentivirus; tyrosine hydroxylase; Parkinson's disease

Introduction

Transplantation of neuronal tissue has been extensively studied both in animal models and in clinical trials, in an effort to regain function lost from neurodegenerative diseases such as Parkinson's disease (PD) (Nikkhah et al., 1994, Brundin et al., 2000, Freed et al., 2001, Agrawal et al., 2004, Braak and Del Tredici, 2008). The most commonly transplanted cell type in rodent models of PD is dopaminergic neurons from the ventral mesencephalon (VM) of developing embryos (Nikkhah et al., 1995, Gaillard et al., 2009, Thompson et al., 2009, Blanchard et al., 2010). Although most investigators have injected these cells directly into striatal targets, a few have attempted to reconstruct the nigrostriatal pathway by placing the transplants into the substantia nigra (SN). Most of these studies have used "bridging" techniques to create a growth supportive conduit extending from the substantia nigra to the striatum. Bridges have been established by injecting GDNF (Wang et al., 1996), 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 some growth of TH⁺ fibers from the SN to the striatum, coupled with improvement in amphetamine-induced rotation (a measure of symmetry of motor function), the amount of striatal reinnervation remains too low to improved spontaneous motor behavior.

To avoid introduction of extra foreign cells along the nigrostriatal pathway, it makes sense to attempt a molecular approach to create a growth supportive environment for dopaminergic axon growth. One candidate molecule for such growth enhancement is GDNF, which is known to increase survival of dopaminergic neurons and widely used in animal models and in clinical trials for Parkinson's disease (Gash et al., 1996, Grondin and Gash, 1998, Kordower et al., 2000, Kirik et al., 2004). It has also been shown to increase differentiation, fiber outgrowth and dopamine release of fetal midbrain dopaminergic neurons both in vitro and in vivo (Lin et al., 1993; Hudson 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). Viral mediated over-expression of GDNF within the striatum establishes a gradient that induces axon growth into the striatum from dopaminergic neurons within nigral transplants in both mouse and non-human primate models (Thompson et al., 2009; Kordower et al., 2000; Redmond et al., 2009). These studies were performed in partially lesioned animals, and in the mouse model complete lesions showed no growth of transplanted axons toward the striatum, indicating surviving endogenous dopaminergic axons might act as a growth supportive scaffold.

To date, GDNF has primarily been used to establish gradients or bridging pathways between the substantia nigra and the striatum, but there is no strong evidence supporting GDNF's role as a chemoattractant for dopaminergic axons. When combined with the GPI-linked glial

cell line-derived neurotrophic factor receptor α -1 (GFR α -1), however, there is an attractive guidance effect on other populations of GDNF-responsive neurons (sensory and sympathetic) (Ledda et al., 2002). Another molecule that 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). To identify if these molecules alone or in combination would enhance the outgrowth of dopaminergic axons beyond that of GDNF, we used the corpus callosal model previously established in our laboratory (Jin et al., 2008, 2011, Ziemba et al., 2008).

Material and Methods

Construction of recombinant lentiviral vectors

The coding regions of an alternatively-spliced form of rat GDNF (GDNF555, NCBI reference # S75585.1), rat GFR α 1 (NCBI reference #NM_012959, kindly supplied by Amgen) and mouse netrin-1 (NCBI reference# NM_008744, kindly 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 and a woodchuck response element (WRE). 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 or GFP, 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. The packaged virus was collected 48 hours after transfection and purification was accomplished using sucrose gradient ultracentrifugation. Viral titers were estimated using a commercially-available p24 ELISA kit (HIV-1 p24 antigen ELISA, Aalto).

Western Blots for Growth factors expression in vitro

To check lentiviral vector encoded protein expression in vitro, U373 or HEK 293 cells were infected with each lentivirus and incubated for 6–7 days. Cell supernatants were isolated and centrifuged at 500 x g to remove any cellular material. The remaining cell monolayers were rinsed three times in Hanks buffer prior to removal by scraping the cells off the plastic dish. Cell supernatants were precipitated and cell pellets were sonicated in cell homogenization buffer (100 mM Tris, pH 7.0, 150mM NaCl, 4mM EDTA, 2% BSA, 2% Triton X-100, 1.7 μ g/ml phenylmethylsulfonylfluoride, 0.5 μ g/ml antipain, 1.0 μ g/ml leupeptin, 5 μ g/ml aprotinin, benzamidine 157 μ g/ml and 1.0 μ g/ml pepstatin). Protein concentrations were measured using a BCA kit (Pierce, Rockland, IL) and the protein amount was normalized to 20 μ g/lane before loading onto SDS-PAGE gels. Western blots were performed according to the protocol from BIO-RAD. Antibodies for the growth factors are goat-anti-GDNF (1:1000, R&D systems Inc) and goat-anti-GFR α 1 (1:1000, R&D systems Inc). After incubation in these antibodies overnight, donkey-anti-goat IgG (1:10,000, Promega) was used. Chicken anti-netrin-1 (1:1000, Chemicon) was used for netrin-1 followed by anti-chicken-IgY and streptavidin AF 680.

Lentivirus injections

Adult female Sprague Dawley rats (225–250g; Harlan, Indianapolis, IN) were used for all experiments. Animals were maintained under conditions of controlled light and temperature, with ad libitum access to food and water. All procedures were conducted according to the guidelines set by the IACUC at the University of Kentucky or Temple University.

For pathway comparison, 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. All animals received an injection of 0.2 μ l lenti-GDNF at the transplant site to insure transplant survival. Animals were randomly assigned to seven treatment groups: (1) saline along the pathway, (2) lenti-GFP along the pathway, (3) lenti-GFR α -1 along the pathway, (4) lenti-GDNF along the pathway, (5) Lenti-netrin-1 along the pathway, (6) combination of lenti-GDNF and lenti-GFR α -1 along the pathway, or (7) combination of lenti-GDNF and Lenti-netrin-1 along the pathway; (n=6 for each group). Guidance pathways as shown in figure 1A were created by injecting lentivirus every 1 mm from the transplant site in the left lateral corpus callosum to the right lateral corpus callosum. Using bregma as a landmark, holes were drilled into the skull to allow injections at the following coordinates (all 0.3 mm rostral to bregma, lateral from the midline of corpus callosum in both sides, left +, right –, depths relative to dura): transplant site, +2.8 mm lateral (left side), 3.0 mm deep; pathway within the corpus callosum, $\pm$ 2.5 mm, 3.0 mm deep, $\pm$ 1.5 mm, 2.9 mm deep and $\pm$ 0.5 mm l, 3.2 mm deep. Lentivirus concentration was 1×10^6 moi/ μ l, except for combined treatment with lenti-GDNF/GFR α -1 or lenti-GDNF/netrin-1 injections, in which the concentration was reduced to 0.5×10^6 moi/ μ l with a 1:1 ratio. Injection volumes ranged from 0.2 μ l at the transplantation site (+2.5 mm), and increased along the pathway to create a gradient, with 0.4 μ l at +1.5 mm, 0.6 μ l at 0.5 mm, 0.8 μ l at –0.5 mm, 1.0 μ l at –1.5 mm and 1.2 μ l at –2.5 mm. Volumes were injected at a rate of 0.4 μ l/min using a 10 μ l Hamilton syringe with a 30-gauge beveled needle, and the needle remained in place for 2 min at the end of each injection to reduce back flow.

E14VM dissection and transplantation

Two weeks after lentivirus injections, rats received transplants of embryonic day 14 (E14) ventral mesencephalon (VM) tissue. E14 embryos were removed from a pregnant Sprague-Dawley rat and the ventral mesencephalic brain region (approximately 1 mm $\times$ 1.5 mm $\times$ 1 mm) was dissected from each fetus and kept in ice cold calcium- and magnesium-free Hibernate buffer (BrainBites, IL) until transplantation (Pruszek, 2009). Rats were anesthetized and their skulls exposed again at the transplant site (2.8 mm lateral, 0.3 mm rostral to bregma). 0.2 μ l Chondroitinase ABC (ChABC) was injected at the transplantation site prior to VM transplantation to reduce the possible overexpression of CSPG induced by damage from the transplantation needle (Jin et al., 2011). VM was implanted as a solid tissue graft (one fetus per graft) using a modified 22-gauge spinal needle lowered to a depth of –3.0 mm from the surface of the skull then pulled up to –2.8 mm before ejecting the tissue. Each tissue chunk was ejected slowly by depressing the needle's plunger 1 mm every 20 seconds. The needle was kept in place for 5 minutes after ejection, raised 0.4 mm and kept there for another 3 minutes before slowly raising it all the way out of the brain to make sure that the transplant remained in place.

As a control for endogenous TH⁺ axonal growth, combination pathways of lenti-GDNF/GFR α -1 or lenti-GDNF/netrin-1 were created (n=4 for each group) without VM transplantation. Six weeks later, rats were perfused and immunohistochemistry procedures were performed as above.

GDNF expression in vivo

In vivo GDNF expression was determined by ELISA on tissue dissected from the corpus callosum. Lenti-GDNF was stereotactically injected into the corpus callosum pathway as described above. Two weeks later, the brains were rapidly removed. A 2mm-thick coronal slice containing the injection sites was dissected using a metal rodent brain coronal section matrix (ASI instruments, Houston, TX) and tissue centered on the injection sites taken using a 1mm-diameter punch. The tissue samples were sonicated in homogenization buffer (100mM Tris, pH 7.0, 150mM NaCl, 4mM EDTA, 2% BSA, 2% Triton X-100, 1.7 μ g/ml phenylmethylsulfonylfluoride, 0.5 μ g/ml antipain, 1.0 μ g/ml leupeptin, 5 μ g/ml aprotinin, benzamidine 157 μ g/ml and 1.0 μ g/ml pepstatin). Tissue levels of GDNF were determined on homogenates using ELISA according to the supplier's recommendations (G3240, Promega).

Tissue preparation and immunohistochemistry

At four weeks post-transplantation, rats were euthanized by pentobarbital overdose and perfused transcardially with cold saline followed by 4% paraformaldehyde (PFA). The brains were removed and post-fixed in 4% PFA overnight, then cryoprotected in 30% sucrose in phosphate-buffered saline (PBS) for 2 days until brains sank to the bottom of the vials. Coronal sections were cut at 30 μ m thickness on a cryostat and collected. One in six coronal sections through the transplant area were used for immunohistochemistry. Immunohistochemistry procedures were carried out at room temperature. The free-floating sections were quenched for 30 minutes in 3% H₂O₂ and washed with PBS. Sections were blocked with 10% normal goat serum (NGS) in PBS for 1 hour and incubated overnight in a mouse primary antibody against tyrosine hydroxylase (TH; 1:4000 dilution; Chemicon), followed by a 1 hour incubation in biotinylated secondary antibody (biotin-SP-conjugated affinipure goat anti mouse IgG 1:600, Jackson). Sections were then incubated for 1 hour in a streptavidin-horseradish peroxidase complex (ABC Elite kit, Vectastin, Vector laboratories) in Tris-buffered saline-Triton (TBST, 0.04%). After that, the sections were exposed to di-amino-benzidine (DAB) (0.5mg/ml, Sigma plus 3% H₂O₂), followed by washing in Tris-buffer. The free floating sections were mounted on gelatin-coated slides and dehydrated in alcohol and CitriSol (Fisher Scientific) before being coverslipped with Permount (Fisher Scientific).

For fluorescent immunohistochemistry, the quenching step was omitted. After blocking, sections were incubated a mouse primary antibody against GFP (1:200, Molecular probe) overnight followed by a secondary antibody goat-anti-mouse FITC (1:500, Jackson ImmunoResearch). The sections were mounted in Fluoromount-G (SouthernBiotech).

Quantification and statistical analyses

For each animal, one in every six coronal sections (180 μ m apart) was quantified for surviving transplanted TH⁺ cells and TH⁺ axonal growth along the pathway by a blinded

observer. The total numbers of TH positive (TH⁺) cells within the transplant were estimated by stereology using an optical fractionator method (Stereology V 8.40.20, Bioquant). One-way ANOVA was used for comparison of TH⁺ cell counts between different treatments. To quantify TH⁺ axonal growth, TH⁺ fibers were counted manually with a 0.5mm² grid at 200X in the corpus callosum at 1, 2, 3, and 4 mm distances from the transplant. To determine whether the protein expressed along the pathway affected the number of axons at different counting points in the pathway, nonparametric statistical tests were used to allow for smaller sample sizes (smallest group, n=3) and large variances that were observed within groups. In addition, no axonal growth was observed in many of the control animals at distal locations, making it difficult to accurately perform parametric statistics. Accordingly, a Kruskal–Wallis test was used to determine whether composition of pathways affects axon counts at each path point, and Mann–Whitney U tests were subsequently used to determine which pathways were significantly different at those points. Differences were considered statistically significant if values were less than or equal to p 0.05. Correlation between the number of TH⁺ cells and the number of TH⁺ axons at 1mm distance from the transplant was analyzed using simple regression analysis.

Results

In vitro and In vivo protein expression

To verify protein expression, lentiviruses encoding either GDNF, GFR α -1, or netrin-1 were added to a monolayer of U373 astrocytoma or HEK 293 cells at a concentration of approximately 5 moi/cell. Six to seven days post infection either cell supernatants (lane S) and cell monolayers (lane M) were isolated and analyzed by Western blots. GDNF protein was mainly identified within the tissue culture supernatant, whereas netrin-1 was identified in the cell monolayer attached to the dish and GFR α -1 in both (Fig. 1C). There was no expression of any of these factors in cell supernatants or monolayers of U373 or 293 cells infected with lenti- GFP (lane C in Fig 1C). These data show that we obtained very good expression of these transgenes.

It is clear from the literature that viral expression patterns forming a gradient show the best prospects of inducing directed axonal outgrowth over many millimeters (Jin et al. 2008; Ziemba et al. 2008; Thompson et al. 2009; Chedotal and Richards 2010; Bonner et al., 2011). To examine if our injection procedure induced a gradient, we examined the expression patterns of GFP and GDNF. Lentivirus was injected into the corpus callosum every 1 mm at five sites as described in methods (Figure 1A). Two weeks after the virus injections, the rats were perfused and GFP was visualized by immunofluorescence staining against GFP. GFP appeared to be expressed by astrocytes in a gradient fashion as shown in Figure 1B. This also confirmed that the injection coordinates were accurate. Although, a GFP gradient was apparent on gross inspection, individual cells had equal fluorescence, most likely because GFP accumulates within the cytoplasm; however, the number of labeled cells increased at sites with larger injection volumes. Soluble factors can also diffuse and spread between injection points creating a more uniform gradient. To examine this possibility, lenti-GDNF was injected in an identical pattern as with GFP. Two weeks after injections, ELISA was used to examine the in vivo expression of GDNF. This data shows

GDNF expression increased as the amount of lenti-GDNF injected increased, confirming the establishment of a GDNF gradient extending across the corpus callosum.

The survival of transplants is critical in cell replacement therapy, and GDNF promotes survival of dopaminergic neurons both in vitro and in animal models (Lin et al., 1993; Yurek, 1997). For these studies, tissue pieces were used instead of dissociated cells to maintain a discrete transplant border for survival and outgrowth assessments. Previous studies show injection of dissociated E14 ventral mesencephalic neurons into the corpus callosum disperse over several millimeters with intermittent cell clusters making survival and axon outgrowth measurements difficult (Jin et al., 2011). To insure a similar level of survival for all transplants irrespective of treatment, a small amount of lentivirus encoding GDNF (1×10^6 TU/ μ l, 0.2 μ l) was injected at the transplant site in all rats at the time when corpus callosum pathways were created. Immunostaining for TH showed that TH positive neurons survived well at the transplantation site in all groups (Fig 2A–G). Counts of TH⁺ cell bodies revealed no significant differences between the treatment groups: Saline = 7410 ± 3902 ; LV-GFP = 5512 ± 3899 ; LV-GFR α -1 = 11565 ± 979 , LV-GDNF = 8306 ± 2813 , LV-netrin-1 = 9942 ± 5439 ; LV-GDNF/GFR α -1 = 6946 ± 4881 ; LV-GDNF/netrin-1 = 8831 ± 5042 . Data are means $\pm$ SEM; ANOVA $p = 0.3893$ (Fig 2H). Thus, difference in the number of TH-positive cells was related to the amount of tissue transplanted and not selective survival.

Comparison of TH⁺ axonal outgrowth and extension along different pathways

To determine the baseline potential of transplanted TH⁺ axons to grow within the corpus callosum, control tissue was examined from rats that received injections of either saline or lenti-GFP along the pathway and E14 mesencephalic transplants, as described in methods. With saline controls, although TH⁺ cells survived very well at the transplantation site, TH⁺ axons grew very short distances from the transplant. Few TH⁺ axons (5 ± 1 per animal, mean $\pm$ SEM) reached the point 1mm distal to the transplantation site and no TH⁺ axons reached the midline of the corpus callosum (Fig. 3A, Fig. 6A). With GFP control pathways, again TH⁺ neurons survived well and TH⁺ axons grew out of the transplant a short distance. About 4 ± 1 of the TH⁺ axons extended 1mm from the transplant and only 2 ± 1 per animal reached the midline of the corpus callosum (Fig. 3B, Fig. 6A). To determine if endogenous GDNF could be concentrated by pathway expression of GFR α -1 and enhance axon growth, a gradient pathway using lenti-GFR α -1 was created. As with the GFP control, only 5 ± 1 of TH⁺ axons per section extended 1 mm proximal to the transplant and 3 ± 1 per animal of TH⁺ axons reached the midline (Fig. 3C, Fig. 6A). These results indicate that very few TH⁺ axons grew out of the transplants. In addition, they did not extend long distances within the corpus callosum using endogenous growth cues.

When compared to the control groups, expression of GDNF or netrin-1 increased the number of TH⁺ axons in the corpus callosum at all measurement distances from the transplant (Fig. 3D and E). For GDNF- and netrin-1- expressing pathways, 19 ± 4 and 17 ± 4 TH⁺ axons per animal reached the midline, respectively. Some, but few, TH⁺ axons crossed the midline of the corpus callosum (Figs. 3E, F, H, I, Fig. 6A) and grew into the contralateral side. Even fewer extended 4mm in either the GDNF- or netrin-1- expressing

pathways (12 ± 2 and 14 ± 4 TH⁺ axons per animal, respectively). However, the increase in the number of TH⁺ axons is not significant ($p > 0.05$) compared to control values.

Because we observed relatively few TH⁺ axons extending along the pathways generated by individual growth factors, combinations of GDNF with either GFR α -1 or netrin-1 were created to determine if either of these combinations would enhance the growth of TH⁺ axons beyond that of a single treatment. A mixture of lenti-GDNF with either GFR α -1 (1:1) or netrin-1 (1:1) was injected to create growth supportive pathways along the corpus callosum. Four weeks post-transplantation, approximately four times more TH⁺ axons (70 ± 3 for GDNF/GFR α -1; 76 ± 7 for GDNF/netrin-1) grew out of the transplants and crossed the midline when compared to single treatment groups (Fig. 4). Interestingly, in the GDNF/GFR α -1 group, there was a dramatic decrease in the number of axons with increasing distance beyond the midline, with about 50% reaching 3mm and 10% reaching 4mm from the transplant site (Fig. 4A, Fig. 6A). Although the number of TH⁺ axons dropped dramatically on the contralateral side, the numbers of TH⁺ axons were significantly higher at 1 and 2 mm distal to the transplant (ipsilateral) compared to either GDNF- or netrin-1-expressing pathways (1mm or 2mm, $p < 0.001$ for either GDNF or netrin-1) (Fig. 4A). This result suggests that GFR α -1 augments the growth potential of GDNF to enhance initial TH⁺ axon outgrowth from the transplant, but does not support long distance growth.

Unlike the GDNF/GFR α -1 pathway, 80% of TH⁺ axons growing within the GDNF/netrin-1 pathway reached the last injection point, 4mm from the transplant (Fig. 4B, Fig. 6A). The numbers of TH⁺ axons were significantly higher at all distances compared to all of the control and single growth-factor treatment groups ($p < 0.001$ for netrin-1; $p < 0.01$ for GDNF). The number of TH⁺ axons reaching a distance of 4mm from the transplant was also significantly higher when compared to all other groups including the GDNF/GFR α -1 combination pathway ($p < 0.01$). The results showed that although both the GDNF/GFR α -1 and GDNF/netrin-1 pathways increased TH⁺ axonal outgrowth from the transplant, the pathway expressing GDNF and netrin-1 also enhanced long distance growth along the pathway. We also observed that axonal extension abruptly ended near the last injection site at the potential height of the guidance gradient. Surprisingly very few of these axons were observed to deviate from the pathway and grow into either the contralateral striatum or cortex, and appeared to remain suspended within the corpus callosum.

Initially, one of our concerns was that expression of growth factors within the corpus callosum could induce sprouting of endogenous dopaminergic axons from either the cortex or the striatum. To determine if this was the case, control tissues with pathways of GDNF, GDNF/GFR α -1 or GDNF/netrin-1 in the absence of E14 VM transplants were examined. As shown in figure 5, six weeks after the establishment of these pathways, no TH⁺ axons were observed in the corpus callosum for any of the treatment groups. This result suggests that these pathways do not cause the growth or sprouting of TH⁺ axons from endogenous sources.

Another concern was variability in the number of TH⁺ neurons within transplants, which might contribute to the extent of axonal outgrowth. To examine this possibility the total number of estimated surviving TH⁺ cells and the number of TH⁺ axons 1mm proximal to

the transplant were analyzed by single regression analysis (Fig 6B). This comparison was done at the nearest pathway injection site to the transplant, because all groups showed some growth to this point. There was no correlation between the number of surviving TH⁺ cells and the number of TH⁺ fibers associated with axonal outgrowth ($p = 0.3971$).

Discussion

Various neurotransplantation strategies have been investigated both experimentally and clinically to reduce the symptoms of Parkinson's disease (Taylor et al. 1991; Freed et al. 2001; Lindvall and Kokaia 2010; Wakeman et al. 2011). Many of these studies directly incorporate transplant tissue into the synaptic target regions of the striatum. Recently, several studies show the potential of GDNF gradients emanating from the striatum to direct growth from neuronal transplants placed within the substantia nigra. Although the best growth was observed with partial lesions, they provide very encouraging data supporting use of similar techniques to reconstruct the nigrostriatal pathway, which degenerates in Parkinson's patients. Presently, only a few molecules have been shown to support and guide the growth of dopaminergic neurons. We undertook this study to identify novel molecules to enhance outgrowth of dopaminergic neurons in the absence of endogenous guidance cues within the adult CNS. For this proof of concept, we chose to establish guidance molecule pathways across the corpus callosum, which normally does not contain dopaminergic axons.

To reduce potential immune response, we used lentivirus instead of adenovirus, which was used for our previous outgrowth studies (Jin et al., 2008; Ziemba et al., 2008; Jin et al., 2011). Lentiviruses are one of the prime candidates for gene therapy in the nervous system due their low cytotoxicity and immunogenicity, large cloning capacity, long term expression and ability to transduce both dividing and non-dividing cells (Naldini et al. 1996; Kumar et al. 2001). Very little immune response or tissue damage was observed in the brains of these animals. In addition, we sought to establish long distance gradients of these factors to direct the outgrowth of axons from transplants toward a distal target. Such gradients are known to play important roles in axon guidance (Dickson 2002; Jin et al. 2008; Ziemba et al. 2008; Thompson et al. 2009; Chedotal and Richards 2010). Graded increases in the volume of virus injected at multiple sites induced a very nice linear gradient of GFP and GDNF.

For all transplantation studies, the extent of surviving neurons could greatly influence the extent of axon outgrowth as well as innervation and functional recovery. In this study, survival of transplanted TH⁺ neurons between groups was variable but led to no significant difference in the number of axons that grew along the pathways. Differences in the number of TH⁺ neurons were likely due to variability in transplant size and not survival, since GDNF expression at all transplantation sites should reduce cell death. GDNF has been recognized as a potent neuroprotective factor and it has been used to prevent degeneration of nigral dopaminergic neurons in both rodent and primate models (Lin et al. 1993; Tomac et al. 1995; Gash et al. 1996; Kordower et al. 2000; Yurek et al. 2009). Therefore, very low level GDNF expression at the transplant site should function as a neuroprotective factor to ensure the survival of transplanted E14 VM neurons into ectopic location. The amount of GDNF expressed at the transplant site did not seem to have an effect on axon outgrowth since both saline and GFP controls showed some, although minimal outgrowth from the

transplant. The application of chondroitinase ABC just prior to transplantation did not support the growth of axons out of these transplants, since no outgrowth was observed in our pilot studies with or without the use of chondroitinase alone.

The signaling of GDNF has been shown to be dependent on binding to its co-receptor GFR α -1. This complex binds to the transmembrane receptor tyrosine kinase RET, causing autophosphorylation and activation of downstream signaling molecules (Jing, et al. 1996). In another signaling pathway independent of cRET, NCAM acts as an alternative receptor for GDNF/GFR α -1. Signaling through this pathway activates the cytoplasmic protein tyrosine kinases Fyn and FAK in cells, thus stimulating axonal growth (Paratcha et al. 2003; Nielsen et al. 2009). GDNF signaling can occur after binding with GFR α -1 on neurons themselves (*cis* mechanism), or between the neurons and substrate or target cells (*trans*) (Airaksinen and Saarma, 2002). In the *trans* configuration GFR α -1 has been shown to bind and increase the local concentration of GDNF to enhance short or long range directional guidance of axons even when in a uniform concentration (Ledda et al., 2002). This mechanism could function similar to that proposed for the capture and presentation of neurotrophins by the low affinity p75 receptor on Schwann cells to TRK-receptor on axons to mediate guidance during development or regeneration (Johnson et al., 1988). Expression of GFR α -1 alone was examined to determine if it would sequester endogenous GDNF or that released at the transplant site. Surprisingly, the expression of GFR α -1 alone did not increase initial axon outgrowth from the transplanted neurons and outgrowth was similar to controls. This indicates that very little, if any, GDNF is endogenously expressed within this region or released at the transplant site. Likewise, there was limited growth of dopaminergic axons within the GDNF expression gradient. The extent of axon outgrowth was greatly enhanced with the additional expression of GFR α -1 by glia within the corpus callosum, indicating the importance of a *trans* mechanism in long-distance dopaminergic axon guidance. This may help explain why GDNF has been shown to support relatively long distance growth of dopaminergic axons after partial but not complete lesions of the substantia nigra (Thompson et al. 2009). Surviving endogenous axons would express GFR α -1 receptors, which could concentrate GDNF along the pathway and enhance the growth of transplanted axons via one of several *trans* mechanisms (Crone and Lee, 2002; Paratcha and Ledda, 2008).

Although we observed a threefold increase in initial axon outgrowth along a pathway of GDNF/GFR α -1, the number of axons decreased with distance from the transplant, dropping significantly before reaching a distance of 4mm from the transplant site. This reduction in axon growth with distance could be due to saturation of cRet receptor complexes with high levels of GDNF and GFR α 1 toward the top of the gradient. Alternatively, unlike *cis* binding, *trans* binding of GDNF/GFR α -1 to cRET can occur outside of lipid rafts (Paratcha et al., 2001). Binding outside of lipid rafts increases the likelihood of cRET proteasomal degradation, leading to irreversible receptor loss and reduction of signal transduction (Pierchala et al., 2006). Therefore, even though glial expression of GFR α -1 increases GDNF mediated axon growth, continual *trans*-activation of cRET erodes its surface density and progressively reduces axon elongation.

Netrin-1 is the best-characterized member of the netrin family of guidance cues. It has been identified as a bi-functional guidance factor, either attracting or repelling growing axons

during the development of the central nervous system (Kennedy et al. 1994; Yee et al. 1999; Manitt et al. 2001; Lebrand et al. 2004). The attractive or repulsive actions of netrin-1 are known to depend on the activation of specific receptors or receptor complexes on the cell surface. Signaling through the deleted in colorectal cancer (DCC) receptor induces chemoattraction, whereas signaling through the UNC5 homologues (UNC5H; A–D) induce chemorepulsion even in the presence of DCC (Guan and Rao 2003; Moore et al. 2007). Down syndrome cell adhesion molecule (DSCAM) has been identified as a novel netrin-1 receptor involved in signaling axon guidance but the signal transduction mechanism has not been well defined (Ly et al. 2008; Liu, Li et al. 2009). Netrin-1 binds to DCC receptor and signals through downstream proteins that regulate cytoskeletal reorganization, thus causing growth cone extension and neurite growth (Keino-Masu et al. 1996; Lin et al. 2005; Lin 2006; Xu et al. 2010). Netrin-1 directs the assembly and disassembly of actin filaments by activation of the Rho family of small GTPases (Govek et al. 2005; Round and Stein 2007) and promotes actin polymerization in lamellipodia and filopodia by activation of Rac and Cdc42. (Luo 2002; Huber et al. 2003). In our netrin-1 expression pathway, similar to GDNF, a significant increase in axonal outgrowth was seen from the dopaminergic transplant. It is important to note that although netrin-1 has been recognized as a regulator of neuron migration (Xu et al. 2010), it did not induce migration of dopaminergic neurons from the transplant into the corpus callosum after netrin-1 expression.

The extent of axon outgrowth and extension was limited in the netrin-1 expression pathway but significantly enhanced when GDNF was expressed simultaneously within the corpus callosum. Previous studies showed that co-delivery of neurotrophic factors could enhance axonal regeneration (Madduri et al. 2009; Ma et al. 2010; Madduri et al. 2010). In addition, it has been reported that combinations of growth factors are more effective for survival of DA neurons (Sautter et al. 1998; Erickson et al. 2001). However, there are no reports for combinations of growth factors enhancing axonal growth of dopaminergic neurons in vivo. In this study, for the first time, we tested a combination of GDNF and netrin-1 expression within the corpus callosal pathway, each of them acting through distinct signaling pathways on axonal outgrowth and extension. As a result, significantly greater axonal outgrowth and extension was observed when compared to pathways expressing individual growth factors.

In pathways without E14 VM transplants, no dopaminergic axons grew in the corpus callosum. For GDNF/netrin-1 treatments this might be explained by the expression patterns of netrin-1 receptors. Deleted in colorectal cancer (DCC) receptor is widely expressed in developing brain and within dopamine neurons in the substantia nigra and ventral tegmental area of the adult (Osborne et al. 2005). However, these neurons show a dramatic decrease in the ratio of DCC to UNC5H receptor expression in dopaminergic neurons after puberty, with UNC5H receptor predominance (Manitt et al. 2010). The predominance of UNC5H receptors signaling in dopaminergic neurons reduces netrin-1 induced axon growth, thus preventing the growth of endogenous dopaminergic axons.

In summary, this study demonstrated that long-distance TH⁺ axonal growth within the adult CNS can be achieved by gradient expression of neurotrophic factors using lentiviral vectors. Moreover, axonal growth and extension is enhanced with combinations of growth factors. With the appropriate growth factors and guidance molecules, this strategy might be applied

to a Parkinson's disease model and result in behavioral improvements after cell transplantation into the substantia nigra.

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Highlights

- Little is known of what factors mediate the growth of dopaminergic axons in the adult brain.
- We examined factors that induced unidirectional growth of transplanted dopamine neurons.
- GDNF, GFR- α 1, and netrin-1 supported some long distance growth in the adult brain.
- Combinations of GDNF/GFR- α 1 or GDNF/netrin-1 supported robust growth over long distances.
- Such factors could be used to reconstruct the nigrostriatal pathway in Parkinson's models.

Figure 1.

Schematic diagram showing the location of lentivirus injection points and transplant location in the coronal plane at 0.3mm rostra to bregma in the rat brain (A). Blue circles represent lentivirus injections sites, with darker colors indicating a larger volume of viral particles injected at the end of the pathway. Brown spot represents VM (E14 ventral mesencephalon tissue chunk). (B) A sample coronal section from a brain injected with lenti-GFP, showing GFP gradient expression (green fluorescence) all along the pathway. (C) Western blot analysis of U373 astrocytoma cell line transfected with lentivirus encoding GDNF, GFR- α 1, or netrin-1. Blots showed no expression in GFP transfected controls (C). Protein was identified in tissue culture supernatants (S) and cell monolayers (M). (D) GDNF ELISA assay showing the gradient expression of GDNF after two weeks of Lenti-GDNF injection. Scale bar = 650 μ m

Figure 2.

TH+ cells at the transplant site in the corpus callosum pathway. Four weeks after transplantation, brains were harvested, sliced in coronal sections and stained with a monoclonal antibody to tyrosine hydroxylase, and developed with diaminobenzidine chromogen. Surviving TH+ cells were counted by optical fractionator method. (A – G) Low power composite photographs showing representative pictures of TH positive cell bodies at the transplant site (T), just above the striatum (St), for the different corpus callosal (cc) pathways; scale bar = 400µm. A) Saline; B) lenti-GFP; C) lenti-GFRa-1; D) lenti-netrin-1; E) lenti-GDNF; F) lenti-GDNF/GFRa-1; G) lenti-GDNF/Netrin-1. Note that there was TH+ dense fiber outgrowth at the transplant site. H) TH+ cell counting using optical fractionator method. There was no significant difference in the number of TH+ cells between the different control and experimental groups. N=6 for each control or treatment group. Each bar represents mean TH+ neuronal number $\pm$ SEM. $P > 0.05$.

Figure 3.

Single growth factors did not support long distance growth of TH positive fibers. Pathways were created along the corpus callosum (cc) with multiple injections of lentivirus encoding GFP, GFR α 1, GDNF or netrin-1 with volume increasing from left to right. All animals received chondroitinase ABC (10U/ml, 2ul) injections at the transplantation site just prior to VM transplantation. Saline and lenti-GFP pathways were created as controls. Pathways of saline (A), GFP (B), and GFR α -1 (C) show very few TH+ axons growing out of the VM transplants (T). Pathways of GDNF (D) or netrin-1 (E) some TH+ fibers grew along the corpus callosum toward the midline, but few of them grew across the midline to the contralateral side. Higher magnification shows good axon growth at the midline (D1 and E1) and the distal end of the pathway (D2 and E2). Scale bar = 500 μ m for A, B, C, D and E. Scale bar=100 μ m for D1, D2, E1, E2, for higher magnification of boxed area showing TH+ axons.

Figure 4.

Combination of growth factors promote long-distance growth of TH+ fibers. Combined treatment of GDNF and GFR- α 1 induced substantial outgrowth of TH+ axons upto the midline (A and A1); however the number of axons dissipated rapidly with distance past the midline. Surprisingly, axon growth at the distal most locations was minimal (A2), similar to that observed with GDNF alone. Combined treatment with GDNF and netrin-1 (B) led to a substantial increase in axon growth which continued past the midline (B1) all the way to end of the corpus callosal pathway on the contralateral side of the brain (B2). Transplant site (T), Striatum (St), ventricle (v). Scale bar =500 μ m in A and B. Scale bar =100 μ m in A1, A2, B1, B2.

Figure 5.

Growth factors do not promote endogenous TH+ fiber growth. Lenti-GDNF/GFRA-1(A) or lenti-GDNF/netrin-1(B) pathways were created without E14VM transplantation. There were no TH+ fibers seen in the corpus callosum, indicated no endogenous TH+ fiber growth into the corpus callosum following the expression of these growth factors. Transplant site (T), Striatum (St), ventricle (v). Scale bar =500 μ m.

A

Number of TH+ Axons from the transplant

B

Figure 6.

Quantification of TH+ axonal outgrowth across different treatment pathways. A) The number of TH+ fibers was counted and averaged over three sections in each animal by an observer blinded to the treatment. There was an increase in TH+ fiber outgrowth along the lentivirus-injected pathway in the GDNF, netrin-1, GDNF/GFRa-1 and GDNF/netrin-1 treatment groups. Of these treatments, GDNF/GFRa-1 and GDNF/Netrin-1 combinations showed good outgrowth upto the midline, 2 mm distance (* $p < 0.001$). GDNF/netrin showed the best outgrowth, showing statistical significance to all single treatment groups at all distances and GDNF/GFRa-1 at 4 mm distance (# $p < 0.01$). Each bar represents mean TH+ neuronal number $\pm$ SEM. B) There is no correlation between the numbers of TH+ cell survival and the number of TH+ axons at 1mm from the transplant.