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14. ABSTRACT
This year we have published a major paper describing how hNPC-GDNF can survive and integrate into the damaged rodent brain and release GDNF for long periods. This GDNF was sufficient to induce up regulation or sprouting of dopamine neurons. We have also established that the lesion environment is crucial for both the migration and survival of transplanted cells. In our regulation studies it is clear that the single vector tTRK system, while very efficient in vitro does not work well in vivo – possibly due to low expression levels with this construct. We have begun working with a new construct which may overcome this problem. Our imaging studies have shown that rats have terminal lesions which remain consistent over time. Finally we have shown that hNPC-GDNF can also survive in the MPTP treated Parkinsonian monkey and release GDNF which increases TH activity within targeted regions of the striatum. This is an important first step showing that this technology is able to deliver GDNF in the best model of Parkinson's disease we currently have. A further 15 animals are currently being transplanted with cells generated within the current grant. We plan to perform some imaging studies on these animals in the final year.

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Introduction

The use of GDNF for the treatment of Parkinson's disease has hit an interesting time. The recent publication of a double blind trial showing no significant effect when GDNF is delivered by a pump and catheter system has cast a shadow over the efficacy of GDNF (Lang et al., 2006). However, it is clear that the delivery system used in this trial was not the same as the one used in our original trial (Gill et al., 2003), and I have previously predicted that GDNF delivery using a wide bore catheter may not work in patient's (Basu, 2004). The whole range of issues surrounding GDNF delivery for Parkinson's has been very recently summarized by a group of us who met last year to discuss these issues (Sherer et al., 2006). However, it is clear that alternative sources of GDNF delivery other than pumps for PD and other neurological disorders are now more important than ever. Thus the goals and objectives of the current proposal have become even more crucial for the field.

This is the yearly update for our DOD award describing progress made for the third year - April 1, 2005 and April 1, 2006. Our aims for this year were to publish the preliminary findings presented in last year's update and, as quoted at the end of our 2005 report, "*In the third year we will determine GDNF regulation in vivo, test therapeutic benefits of GDNF in a rodent model of PD, and use PET imaging to monitor effect of GDNF regulation on the dopamine system. We also aim to determine the amount of GDNF needed to be effective and how long therapeutic benefits will remain if GDNF expression is turned off in vivo*".

In early 2006 we published our first set of findings from this study in *Gene Therapy*, showing that GDNF released from human neural progenitor cells (hNPC) could be regulated *in vitro*, increase dopamine neuron survival in a rat model of Parkinson's disease and that they could also survive and release GDNF in aged primates. Although regulation was achieved to some degree *in vitro*, shut of the gene was not complete, and we could not regulate GDNF production *in vivo* (Behrstock et al., 2006). The aim of this year's work was to test a new single vector regulation system described in last year's update *in vivo* to see if it was better than the two vector system. We also continued and extended our studies aiming to improve survival and integration of the cells through comparing different lesion types with regard to cell migration and survival. Finally, we performed our first transplants of hNPC-GDNF into a full MPTP monkey model of Parkinson's disease with very interesting results.

Body

***Task 1.* To produce rat and monkey neural stem cells which secrete GDNF under an inducible promoter.**

a. Assess and optimize GDNF release from rat and monkey neurospheres using lentiviral vectors (Months 1-18).

This part of the grant was summarized in last year's update. We showed that monkey and rat neurospheres were very difficult to expand in culture and shifted to human neurospheres. We have been able to now define the exact growth patterns of these cells and have shown that hNPC undergo natural senescence patterns *in vitro* (Wright et al., 2006). This is important for the current studies as it shows the cells will not spontaneously immortalize in vitro and thus provide a safe source for transplantation. However, during the expansion phase we can produce enough cells for banking and transplantation. These cells have been used in all of the studies described below.

b. Establish DOX regulation profile on GDNF secreted from rat and monkey neurospheres infected with lentivirus (Months 6-24).

Completed last year.

c. Select and characterize lentiviral clones which express GDNF at high levels following differentiation (Months 18-36).

Discussed in last year's report.

d. Produce and optimize new vectors with combinations of GDNF/GFP (retrovirus/AAV) - assess which is most efficient at GDNF production *in vitro* and compare with lentivirus (Months 6 - 48).

As explained in last year's progress report, we began studying a new tetracycline regulated promoter system termed tTRK. The new tet-on system is based on a tet-regulated tTRK transcriptional repressor and is delivered on a single virus expressing GDNF from a regulated promoter and the transcriptional repressor via an internal ribosomal entry site (IRES). This system is all on one vector construct and does not therefore require dual infection. During this past year, we have confirmed that hNPC infected with lentivirus expressing GDNF under the control of the tTRK promoter have very efficient regulation *in vitro*. GDNF expression can be repeatedly turned off and on in these infected cells by the addition or removal of doxycycline, respectively, in a tightly regulated manner. However, at the same time it is clear that the absolute produce of GDNF from these cells was around 10 times lower than that produced from the two vector tTA construct. We are currently uncertain as to how important this is for regulating dopamine neuron survival in vivo as a strict dose response curve has yet to be carried out. However, in Task 2d below we discuss the results of transplanting cells that can be regulated using this construct. These studies indicated that the released dose was perhaps undetectable, or that regulation (switching on) was not possible from these cells *in vivo*. Thus, other vector systems will be required to optimize regulated GDNF delivery *in vivo.*

Therefore, we are currently investigating other regulatable systems *in vitro*. Specifically we have a collaborative project beginning with a company called Rehogene who have produced a novel regulation system (http://www.rheogene.com/). This company has been given a major grant from the FOX foundation to produce regulated GDNF for direct gene therapy. Currently we are planning to use the Rehogene system to infect hNPC and subsequently regulate GDNF *in vitro* and *in vivo*. It will be cloned into our current lentiviral system and the switching will be determined as described in detail within our report last year. This has already been agreed upon by Rheogene and is currently going through the University of Wisconsin IP system. The advantage of this inducible system is that it utilizes a very powerful promoter and shows high expression rates in many cell types. Furthermore, the activator is currently being taken through FDA approval processes for a number of different indications and has been shown to be very inert and not toxic to humans. Preliminary data from Rheogene also suggest that the activator can penetrate the blood brain barrier. For these reasons we will continue to develop this inducible system over the final year of the grant and use it in the models outlined in the original proposal in place of the tTRK systems.

Task 2. **To protect against toxic cell death in the brain by transplanting GDNF producing stem cells into rodent and primate models of PD.**

a. Perform pilot monkey transplant study with GFP/GDNF retroviral construct using micro-PET and post mortem data to establish survival and possible function of cells (Months 6-18).

As described in last year's progress report, and our recent paper, we found hNPC survived well and expressed GDNF in the aged primate brain 3 months after transplantation (Behrstock et al., 2006).

b. Assess optimal source and preparation (FACS, pre-differentiation) of rodent neural stem cells for grafting (Months 12-48).

We have found that good migration and integration of the cells can be achieved without FACS sorting and pre-differentiation. However, there is strong evidence that hNPC are regionally specified, both from our own work and that of others (Hitoshi et al., 2002;Ostenfeld et al., 2002). This may mean that hNPC derived from different brain regions show different types of survival and migration. All of the studies done to date have been with hNPC derived from the human fetal cortex – but the region we are focusing on is the striatum for transplantation. We have recently generated lines from developing fetal striatal tissues which grow in a similar fashion to those from the cortex. We are therefore interested to learn if cells derived from the fetal striatum will incorporate more, migrate further, and have more differentiation potential upon transplantation back into the injured rat striatum compared to cortically derived cells. These experiments are currently under way and are planned for the final year.

We have also found that the state of the striatum may dictate how the hNPC-GDNF migrate and survive – but not differentiate. This came about through our recently completed studies where we have transplanted hNPC into the striatum of rats with lesions that mimic Huntington's disease (McBride et al., 2004). We noticed that in these studies, the migration and survival of the cells was greater than in the Parkinson's disease models described in the current proposal. Thus we decided to directly compare transplantation of cells into the intact striatum and different lesion models in order to better understand how the environment can affect survival and migration of the cells. Transplants of hNPC-GDNF into intact animals produced very thin transplants with little migration away from the core of the transplant (Fig. 1A). In contrast, transplants of hNPC-GDNF into quinolinic acid lesions of the striatum (HD model) resulted in large transplants that in some cases filled most of the damaged area (Fig. 1B). Transplants of hNPC into 6OHDA lesions, which do not kill cell bodies within the striatum but induce retrograde death of dopamine bodies in the brain stem showed a level of survival and migration in-between that of controls and quinolinic acid lesions (Fig. 1C). This was reflected by more total hNUC positive cells within the transplants into the quinolinic acid lesioned animals (Fig. 2). However, interestingly the phenotype of cells maturing within the transplants was not affected by the lesion type. Most retained nestin expression (>70%) while very few expressed GFAP (<10%; Fig. 2). These studies are currently being prepared for publication. However, the bottom line is that the state of the brain prior to transplantation will clearly have a significant effect on hNPC survival and migration and will be considered in the monkey studies (as described in the last section) and Parkinson's disease if and when the cells are used for patients.

c. Assess whether rodent GDNF secreting stem cells prevent neurotoxic cell death and how this relates to dopamine storage in the brain using micro-PET (Months 18-36)

As mentioned previously we switched to using only human neural progenitor cells and have shown that they can prevent cell death and induce dopamine neuron sprouting and survival. We have also been attempting to improve our PET imaging methods to be ready for the regulated systems as they are developed. Last year's progress report clearly described the benefit of the ^{11}C labeled dihydrotetrabenazine (DTBZ) tracer as it is specific to the dopaminergic nerve terminal transporter protein (VMAT). This past year we have conducted studies to test the reliability of PET imaging by performing multiple scans on the same rat over a period of 3 months. Although we have been struggling with consistent tracer production, PET scanning showed a consistent lesion in the rats over time (Fig. 3). These data suggest that PET imaging of the dopamine system using the DTBZ tracer can produce reliable results in order to assess lesion size and potential effects of GDNF expression. Progress

continues on improving radiotracer specific activity, and we recently produced a precursor used in [^{11}C]DTBZ radiochemistry at a high specific activity which will aid in consistent production of the radiotracer. Moreover, we have been altering the anesthesia equipment such that when we scan multiple rats simultaneously, the isoflurane levels can be independently adjusted for each animal. We also plan to use a Co-57 transmission source for improved accuracy in the attenuation and scatter corrections allowing for better data analysis.

d. Prove regulated delivery can be achieved, and establish effects of switching GDNF on and off on dopamine storage in the brain using micro-PET (Months 24- 48).

This was a major goal for this year and has been completed using the new tTRK single vector tet on system described in detail last year and briefly above (Task 1, Section D). Having established a robust expression profile *in vitro*, we next transplanted tTRK-GDNF infected hNPC into 6-OHDA lesioned rats. We were strictly interested in whether GDNF expression would be detected (turned on) by the addition of doxycycline to the rats' drinking water. Therefore, we did not assess any behavioral measures in any of the lesioned rats. As a positive control we transplanted constitutively expressing GDNF infected hNPC (using a PGK unregulated promoter). To determine how sensitive our method of GDNF detection was *in vivo*, we also transplanted one set of animals with a 1:1 mix of PGK-GDNF infected and wild-type uninfected hNPC to see how a 50% reduction in GDNF release would relate to detection in brain sections.

All animals had successful grafts covering a significant proportion of the striatum (Fig. 4). As shown previously (Behrstock et al., 2006), rats receiving the PGK-hNPC-GDNF had extensive GDNF expression throughout the transplant area (Fig. 4A). The rats with the 1:1 mix of infected and uninfected hNPC also had GDNF expression in the transplant area, but it was dramatically reduced compared to PGK-hNPC-GDNF alone (see Fig. 4B). None of the rats receiving the tTRK-hNPC-GDNF showed GDNF expression (see Fig. 4C) despite all animals having an equivalent number of hNPC surviving transplantation (Fig. 4D-E). These data suggest that either (i) the level of GDNF expression is too low to be detected using our immunohistological methods or (ii) doxycycline is not able to get into the brain at high enough levels to activate the tTrk promoter and switch on GDNF. The second possibility seems unlikely as Aebischer and colleagues have recently shown that the same construct can be switched on and off following direct expression *in vivo* using direct injection of the virus (Szulc et al., 2006). Therefore, our studies have shown that the single vector system expressed in human neural stem cells is not switched on, or switched on at very low levels *in vivo* below the sensitivity of our GDNF detection methods. This is further supported by the data showing that if high constitutively expressing hNPC are diluted 1:1 with wild type cells expression is reduced to close to non detectable levels (Fig. 4B). Thus the 1:10 lower GDNF release from the inducible vector may simply not be detected *in vivo (*as noted in Task 1D*)*

To add to the complexity of doxycycline regulated systems, recent evidence in monkeys suggests that the levels of doxycylcine required to achieve regulation *in vivo* is very high, and results in serious side effects (Kordower, personal communication). Thus the strategy of using doxycyline regulated systems appears to be problematic – both from the practical stand point and potentially the clinical stand point due to the high doses of doxycyline that will be required. For this reason we have now switched to a new vector system provided by a company called Rheogene (see Task 1, Section D for details). We hope this year to transplant these cells into the same model and test for the regulation of GDNF release.

In our work last year hNPC-GDNF cells were transplanted into aged monkeys. However, these monkeys were not lesioned with MPTP to induce parkinsonian symptoms. This year we have begun collaborating with another investigator at the University of Wisconsin-Madison, Dr. Marina Emborg, and

have shown in a small pilot study that constitutive PGK-GDNF infected male hNPC can survive and express GDNF in 3 MPTP lesioned monkeys. The cells survived in small but significant transplants detected using in situ hybridization to the Y chromosome (Fig. 5). They survived up to three months which was the end point for this study and continued to secrete GDNF at high levels (Fig 6A,B). This amount of GDNF was able to increase TH expression in local fibers surrounding the release site in a targeted fashion (Fig. 6C,D) showing proof of concept that GDNF released from human neural progenitor cells can increase TH expression in the MPTP monkey brain. These data are very encouraging, and we have begun a large-scale experiment to address any functional benefit provided by the expression of GDNF. We plan to perform imaging studies on these monkeys over the next final year of this grant. Although we are not sure that the regulated cells will be available, it will be of interest to see if the "hot spot" areas with the transplants show up on the PET scans.

Key research accomplishments

This year we have published a major paper describing how hNPC-GDNF can survive and integrate into the damaged rodent brain and release GDNF for long periods. This GDNF was sufficient to induce up regulation or sprouting of dopamine neurons. We have also established that the lesion environment is crucial for both the migration and survival of transplanted cells. In our regulation studies it is clear that the single vector tTRK system, while very efficient in vitro does not work well in vivo – possibly due to low expression levels with this construct. We have begun working with a new construct which may overcome this problem. Our imaging studies have shown that rats have terminal lesions which remain consistent over time. However, we have not been able to perform repeat scans in animals with transplants as the in vivo regulation has not yet been validated. This is a focus of the final year. Finally we have shown that hNPC-GDNF can also survive in the MPTP treated Parkinsonian monkey and release GDNF which increases TH activity within targeted regions of the striatum. This is an important first step showing that this technology is able to deliver GDNF in the best model of Parkinson's disease we currently have. A further 15 animals are currently being transplanted with cells generated within the current grant. We plan to perform some imaging studies on these animals over the current year of final funding.

Reportable Outcomes

1. S. Behrstock, A. Ebert, J. McHugh, S. Vosberg, J. Moore, B. Schneider, E. Capowski, D. Hei, J. Kordower, P. Aebischer, and C. N. Svendsen. Human neural progenitors deliver glial cell line-derived neurotrophic factor to parkinsonian rodents and aged primates. *Gene Ther.* 13 (5):379-388, 2006.

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Papers were presented at the Society of Neuroscience meeting. Dr. Svendsen has given over 20 invited lectures over the past year presenting aspects of the current work both in the USA and abroad.

Conclusions

We feel that good progress has been made this year towards our original goals. We conclude that human neural progenitor cells modified to release GDNF remain a potential source of tissue for new cellular therapies for Parkinson's disease. By learning more about stem cell drug delivery it may be possible to explore other therapies for war injuries in the future.

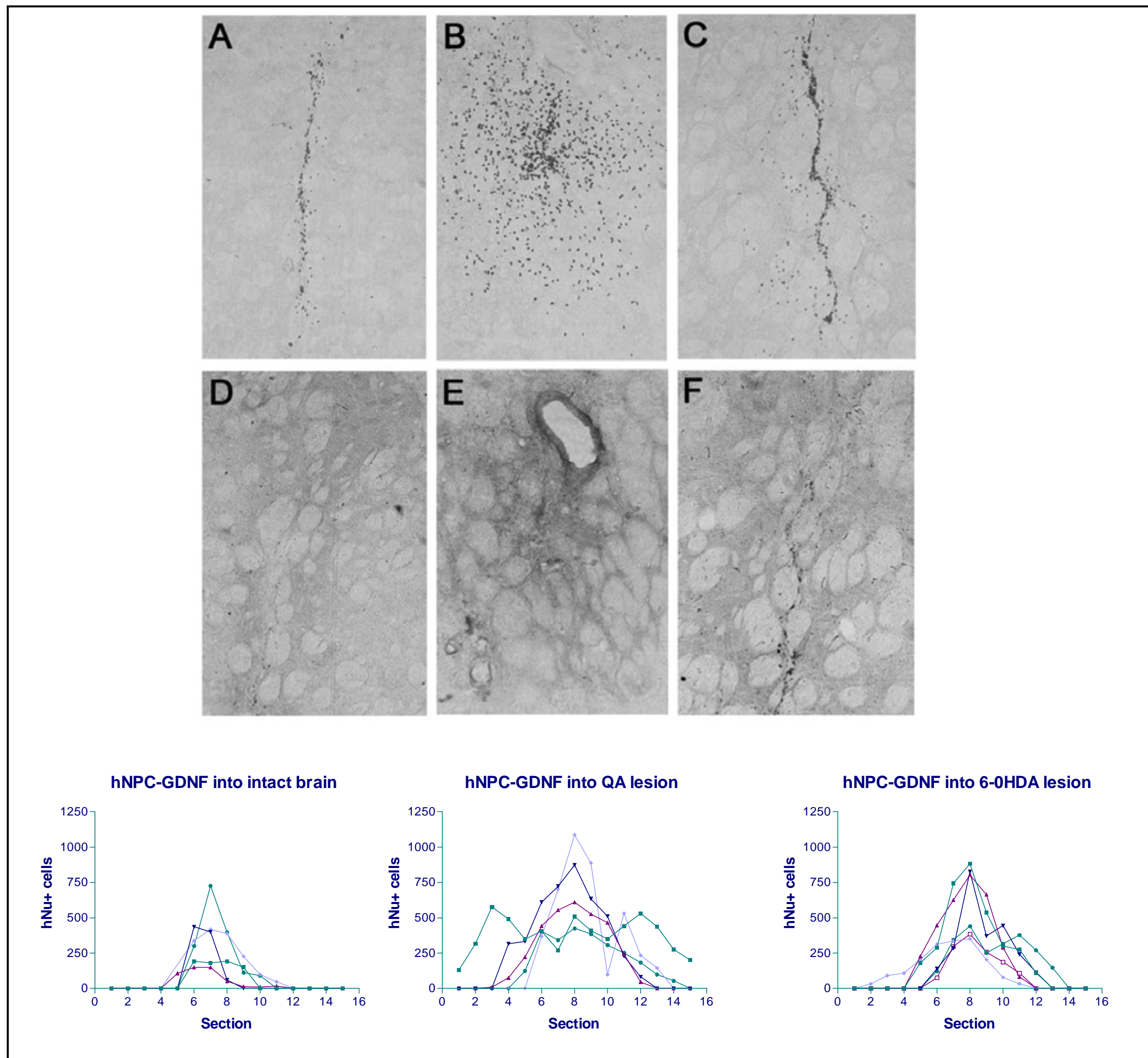

Figure 1. The lesion environment dictates hNPC-GDNF cell survival and migration. Cells transplanted into (A) the intact striatum (B) the quinolinic acid lesioned striatum (C) the 6-OHDA lesioned striatum. (D-F) represent the corresponding sections stained with GDNF. Note the larger number of cells and greater spread in the quinolinic acid lesion group. The data for 6 animals is shown graphically below the images.

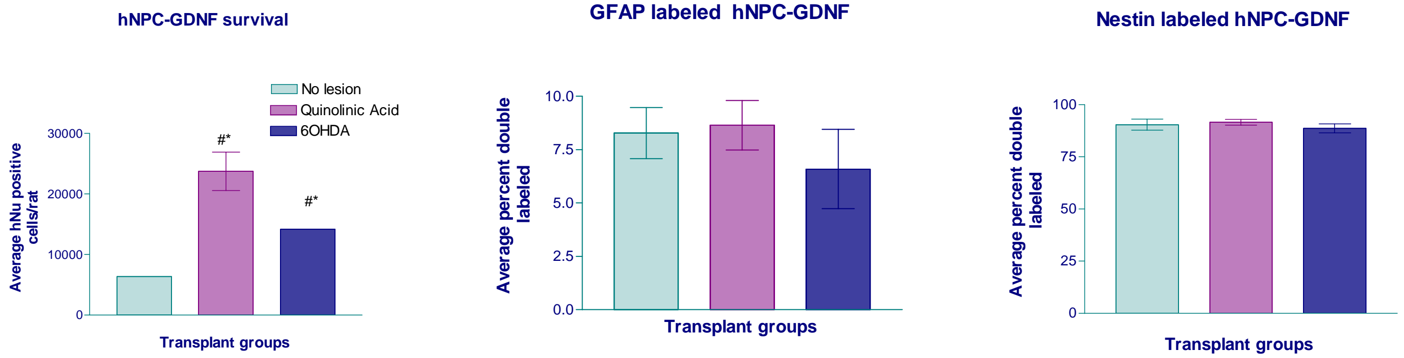

Figure 2. Graphs showing the survival of hNPC-GDNF cells in the different lesion models and maintenance as nestin positive progenitors or their differentiation into GFAP positive astrocytes. Note that while the lesion has a significant effect on the number of surviving hNPC, it does not change their differentiation profile.

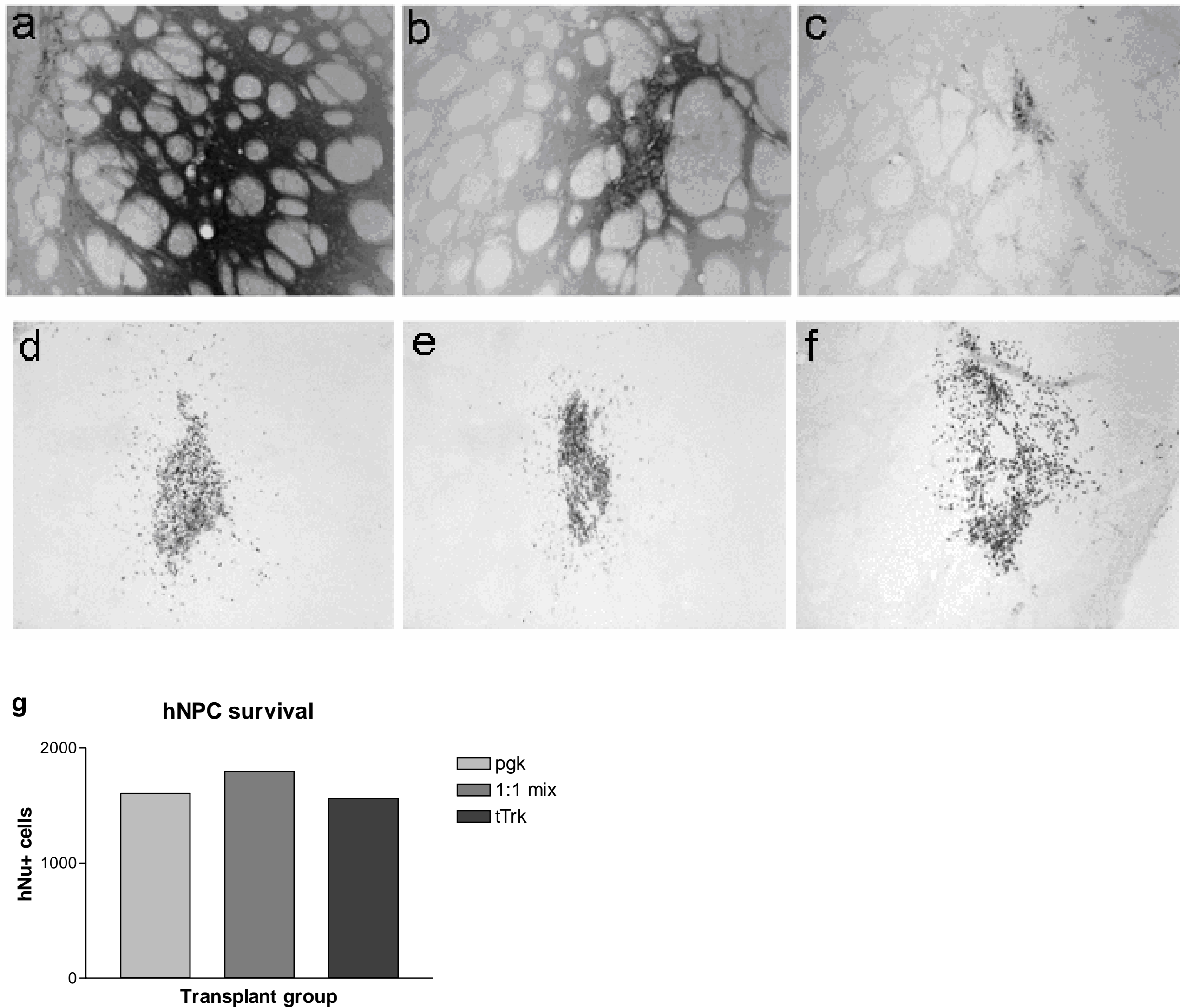

Fig. 3. Rat striatum stained for GDNF expression and hNPC-GDNF survival. Extensive GDNF expression is observed in rats transplanted with PGK-GDNF hNSC (a). A detectable level of GDNF, although drastically reduced, is observed in rats transplanted with a 1:1 mix of PGK-GDNF cells and non-transduced hNPC (b). No specific GDNF staining was observed in the rats transplanted with tTrk-GDNF hNSC (c). The staining observed in these rats is non-specific antibody binding. Staining for surviving transplanted cells for tTRK-GDNF (d), 1:1 mix (e), and PGK-GDNF (f) transplanted rats showed similar graft sizes and total cell survival, and the data are quantified in g. These data suggest that the lack of GDNF expression in the tTRK transplantation group was not due to poor graft survival.

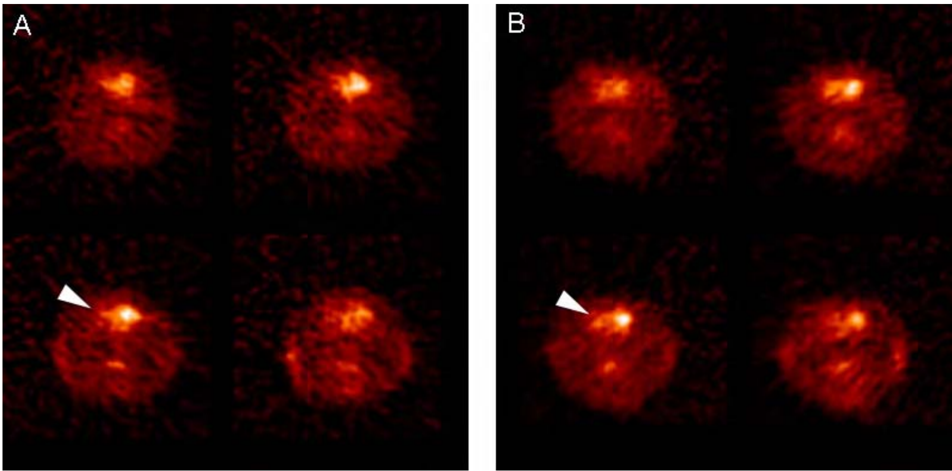

Fig. 4. [C11]DTBZ PET imaging of the rat striatum. A rat lesioned with 6-OHDA in the left medial forebrain bundle induced a dramatic loss of dopamine activity as evidenced by the lack of [C11]DTBZ binding in the left striatum (arrowhead) at 2 months (A) and 5 months (B) post- lesion.

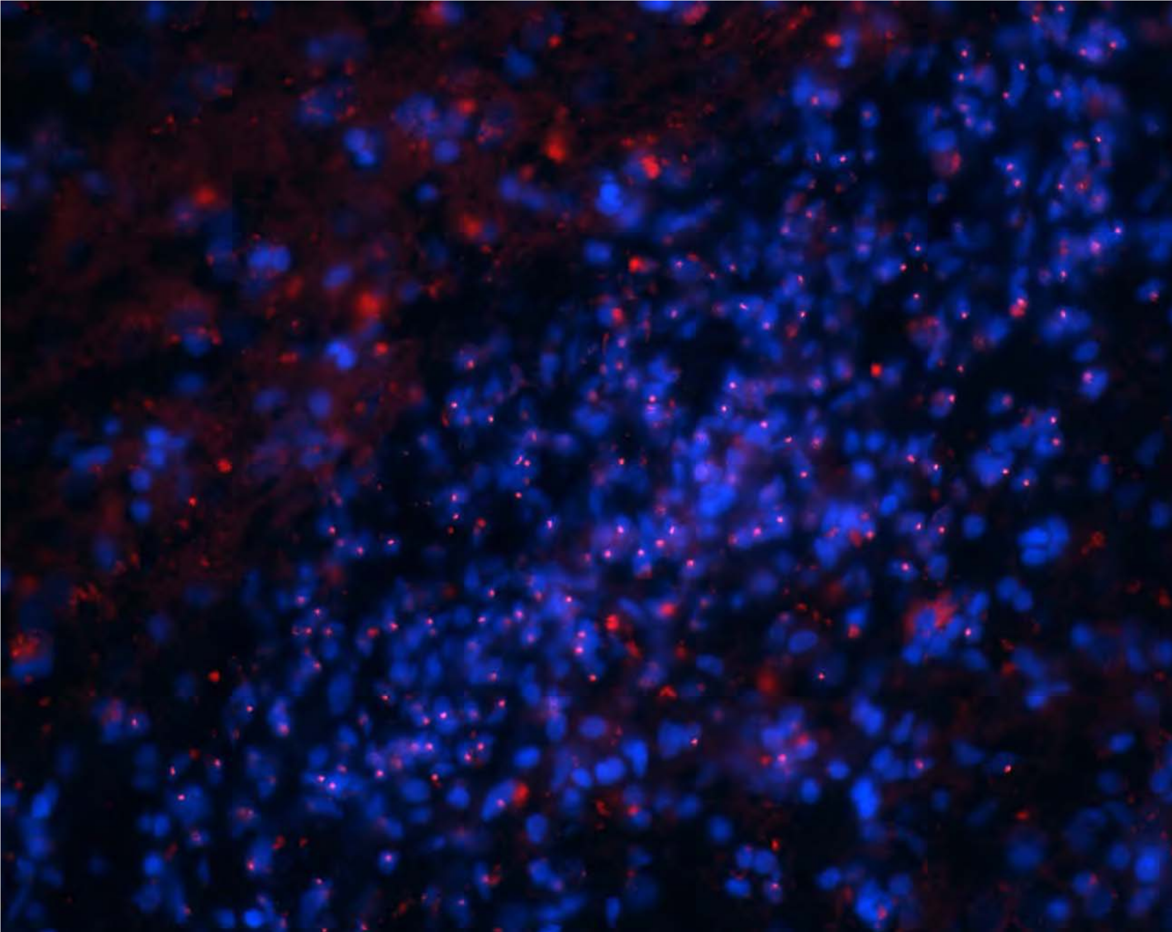

Fig. 5. PGK-GDNF hNSC survived transplantation into monkey brain. Using in-situ hybridization for the Y chromosome (shown as small red dots in the core of the transplant), hNSC were easily detectible within the transplant core. No positive cells were found outside this core region suggesting very limited migration in this graft model. A general nuclear dye (blue) was used to identify all cells, both human and monkey

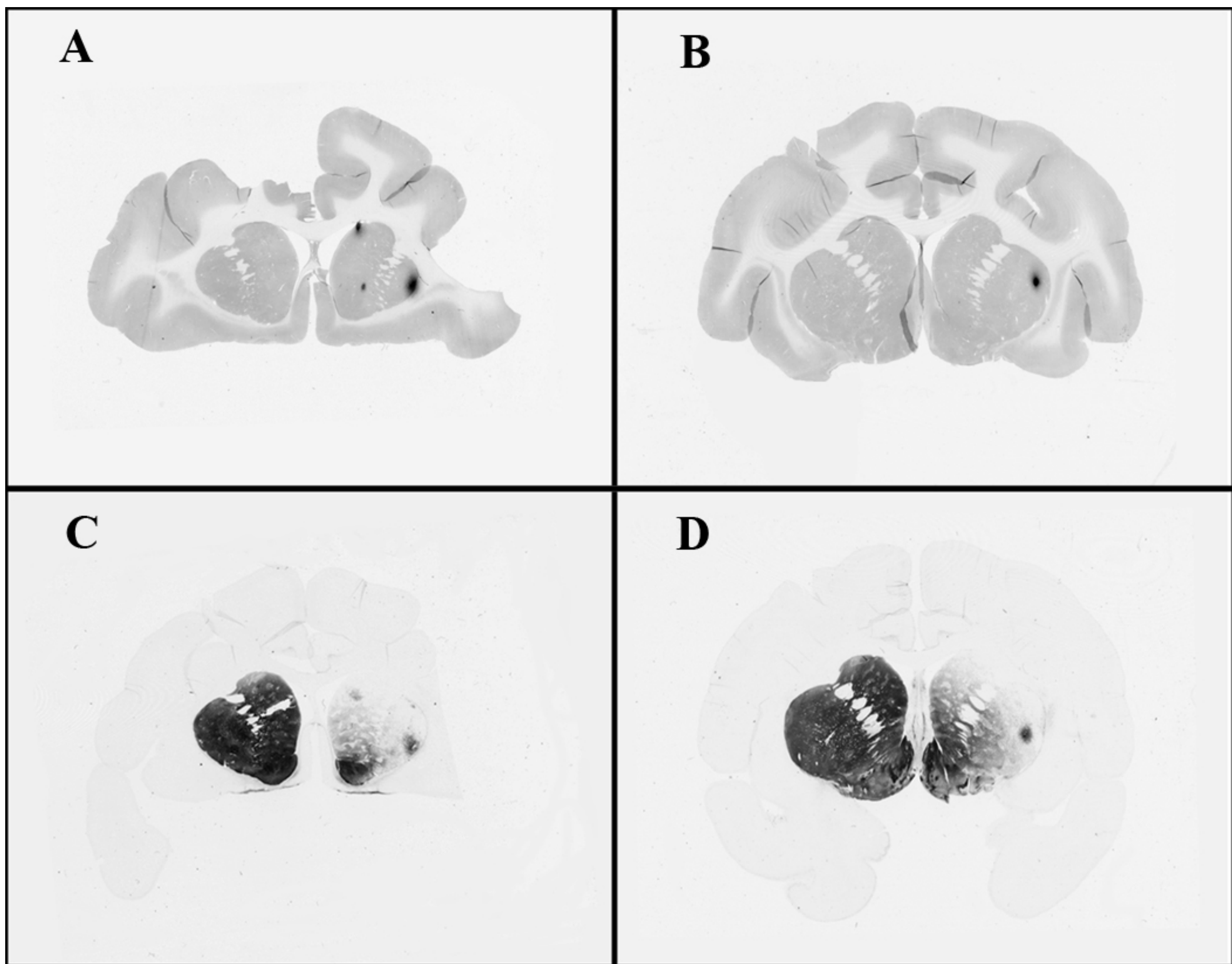

Fig. 6. Expression of GDNF by transplanted hNPC-GDNF into monkey brain and corresponding TH immunohistochemistry. Using immunohistochemistry for GDNF, distinct regions of GDNF expression were detected using immunocytochemistry in the monkey striatum 3 months after transplantation (A and B). Interestingly, in the denervated striatum, specific areas of TH staining were observed (C and D) only in areas corresponding to the transplant sites. These areas correlated exactly with the Y-chromosome in situ staining shown in Fig 4 above.

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ORIGINAL ARTICLE

Human neural progenitors deliver glial cell line-derived neurotrophic factor to parkinsonian rodents and aged primates

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Glial cell line-derived neurotrophic factor (GDNF) has been shown to increase the survival and functioning of dopamine neurons in a variety of animal models and some recent human trials. However, delivery of any protein to the brain remains a challenge due to the blood/brain barrier. Here we show that human neural progenitor cells (hNPC) can be genetically modified to release glycosylated GDNF in vitro *under an inducible promoter system. hNPC-GDNF were transplanted into the striatum of rats 10 days following a partial lesion of the dopamine system. At 2 weeks following transplantation, the cells had migrated within the striatum and were releasing physiologically relevant levels of GDNF. This was sufficient to increase host dopamine neuron survival and fiber outgrowth. At 5 weeks following grafting there was a strong trend towards functional improvement in transplanted animals and at 8 weeks the cells had migrated to fill most of the striatum and continued to release GDNF with transport to the substantia nigra. These cells could also survive and release GDNF 3 months following transplantation into the aged monkey brain. No tumors were found in any animal. hNPC can be genetically modified, and thereby represent a safe and powerful option for delivering growth factors to specific targets within the central nervous system for diseases such as Parkinson's.*
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Keywords: *GDNF; stem cell; cell therapy; neurodegeneration*

Introduction

Glial cell line-derived neurotrophic factor (GDNF) was first isolated by virtue of its neuroprotective and trophic effects on dopamine neurons that are lost in Parkinson's disease (PD).[1] Although GDNF does not penetrate the brain from the blood, studies in Parkinsonian primates showed that direct infusion into the ventricles led to a reversal of symptoms.[2] Furthermore, a series of experiments in both monkeys and rats have shown that GDNF can prevent dopamine neuron cell loss and induce fiber sprouting in a wide range of different models of PD.[3] This led to a clinical trial where GDNF was administered via bolus injections directly into the ventricles of Parkinsonian patients. The outcome of this trial was negative, with some significant side effects reported.[4,5] This may have been due to poor penetrance of GDNF from the ventricles into the deep lateral regions of the posterior putamen, a region preferentially affected in PD. In order to overcome this problem, GDNF has now been continually infused using pumps and catheters directly into the caudal putamen of patients with PD over a period of 3 years. The results of this open-label study showed that GDNF was safe and provided significant decreases in Parkinsonian symptoms, significant increases in dopamine storage within the brain and increased dopamine neuron sprouting based on post mortem data.[6–8] A second open-label study has also shown significant improvements in patients receiving similar doses of GDNF to only one side of the brain.[9] However, delivery of proteins via a catheter and pump is complex, the GDNF concentrations have to be very high, the pumps need regular refilling and delivery is to only a point source within the putamen. Furthermore, a recent double-blind trial delivering GDNF to a larger number of patients failed to show significant effects, although this study used a different delivery system and dose of GDNF to the open-label studies described above (Lang *et al.*, personal communication). Clearly, alternative methods of drug delivery to the brain need to be developed.

Direct gene therapy using live virus has emerged as one alternative method of growth factor delivery, and has been shown to work well in both rodent and primate models of PD.[10–12] Unfortunately, there are risks that transport of live virus to ectopic regions of the central

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nervous system will produce side effects.[13] Furthermore, viral infection of the diseased host nervous system may lead to extra stress within already sick neurons and glia. Modification of cells in the culture dish before subsequent transplantation is termed *ex vivo* gene therapy and increases safety since no live virus is injected into the subject and host neurons do not need to be infected. Autologous fibroblasts are an obvious choice for *ex vivo* gene therapy. They can be modified in the dish to release growth factors and survive transplantation for extended periods in the brain (for a review, see Gage[14]). Recently, this technique has been used to deliver nerve growth factor to the basal forebrain of 10 patients with Alzheimer's disease with some modest clinical effects.[15,16] While the use of autologous cells has some benefit, fibroblasts do not migrate following transplantation and so drug delivery is limited to only the immediate site of the transplant. Fortunately, autologous cells are not essential as allografts appear to be accepted in the human brain following only brief suppression based on fetal transplant studies in PD.[17]

Recently, efficient methods have been developed for the extended growth and differentiation of well-characterized human neural progenitor cells (hNPC). These cells can be isolated from post-mortem fetal brain tissue and expanded for significant periods of time in culture.[18,19] hNPC react very differently from fibroblasts or primary fetal tissue following transplantation. Rather than forming a transplant core of non-migrating cells, a single deposit can fill almost the entire striatum.[20,21] Furthermore, a number of reports have shown that hNPC can differentiate into both astrocytes and neurons following transplantation into the nervous system.[18,20,22–25]

We have previously shown that non-modified hNPC can lead to functional restoration in a model of Huntington's disease, potentially through neuroprotective effects on dying striatal neurons.[21] However, naïve hNPC alone do not have any significant functional effects in models of PD.[26] Rodent neural progenitor cells that have been genetically modified to produce GDNF are physiologically active on dopamine neurons, both *in vitro* and following transplantation.[27] In addition, a mouse immortal cell line that produces GDNF, survives transplantation in rodent models of PD and prevents degeneration of dopamine neurons when transplanted before the lesion has been generated.[28,29] While these studies highlight the potential of GDNF-secreting cells, it will be crucial to demonstrate the efficiency of cells after rather than before the lesion to be relevant to PD. Furthermore, normal rat cells or immortalized mouse cells are not appropriate for human clinical trials which will require human cells.

GDNF has potent effects not only for PD but also for other neurological disorders, such as for amyotrophic lateral sclerosis (ALS)[30,31] and for the aging process.[32] We have recently shown that hNPC engineered to produce GDNF and transplanted into the spinal cord can survive and increase motor neuron enzyme activity in a rodent model of ALS.[33] Here we use hNPC modified to release GDNF as a unique delivery option for this trophic factor to the parkinsonian rat and aged monkey brain.

Results

hNPC can be modified to release GDNF under a regulated promoter system

The inducible lentiviral construct used in this study is based on the already published non-inducible system described in detail previously[34] and is shown schematically in Figure 1. The mouse phosphoglycerate kinase 1 (PGK) promoter (strong constitutive promoter) drives the tetracycline transactivator (tTA1) in the lenti-tTA

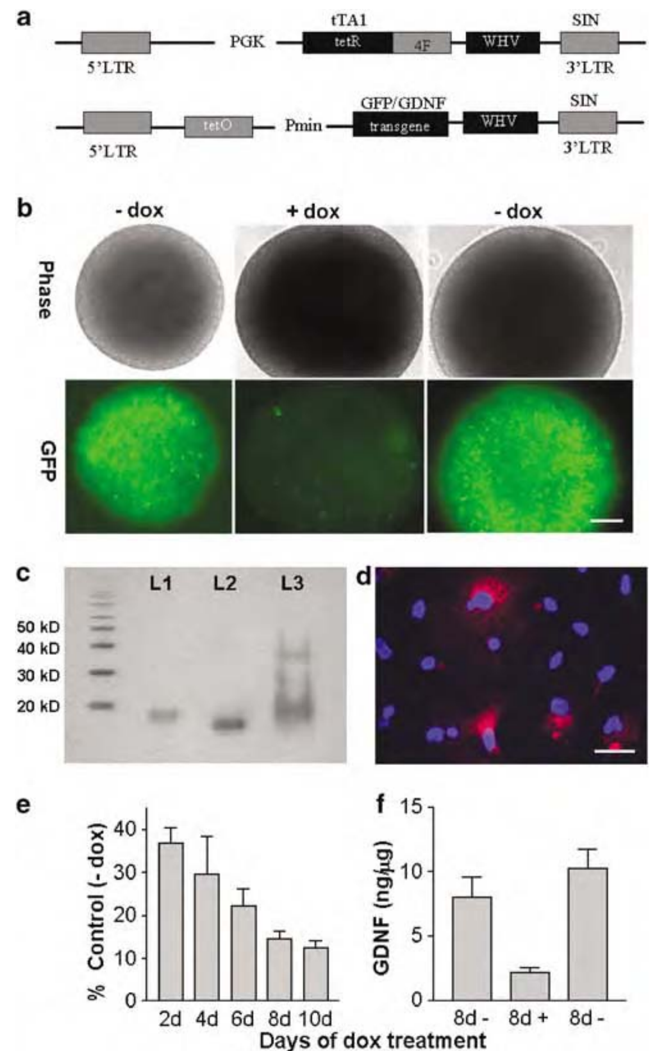

Figure 1 The Tet-off lentiviral construct provides regulated GFP and GDNF production in hNPC. Cells were coinfected with lenti-tTA1 and either [ind]lenti-GDNF or [ind]lenti-GFP (**a**). Neurospheres infected with[ind]lenti-GFP show tight regulation of GFP by the addition and withdrawal of doxycycline (**b**). Western blot demonstrates GDNF production by hNPC infected with [ind]lenti-GDNF (lane 3). Molecular weight markers are on the left, hGDNF from mouse cell line (lane 1), and rhGDNF from *E. coli* (lane 2) (**c**). Approximately 40% of cells were shown to express GDNF following infection with the inducible GDNF construct (**d**). GDNF levels in the presence of doxycycline compared to those in the absence of doxycycline decrease following doxycycline treatment for 2 days and fall to over 90% shutdown in GDNF production following 10 days exposure (**e**). This regulation was dynamic as when doxycycline was removed for an 8-day washout, GDNF expression switched on again to levels similar to those seen before switch off (**f**). Scale bars are 125 μm in (**b**) and 40 μm in (**d**).

construct. The post-translational *cis*-acting regulatory element of the woodchuck hepatitis virus (WHV) is included and has been shown to significantly enhance transgene expression.[34] In the absence of doxycycline, tTA1 will bind to the tetracycline operon (tetO) that is upstream of a minimal promoter driving the gene of interest. In this case, the gene is GDNF in the ^{ind}lenti-GDNF construct or enhanced green fluorescent protein (GFP) in the ^{ind}lenti-GFP construct. In the presence of doxycycline, the tTA is unable to activate the transgene.

The ^{ind}lenti-GFP construct (Figure 1a) was used to optimize lentiviral infection of hNPC and subsequent gene regulation. Small, intact spheres (<200 μm) were co-infected with lenti-tTA and ^{ind}lenti-GFP, resulting in a high percentage of GFP-expressing cells (Figure 1b). In the presence of doxycycline for 48 h, the destabilized form of GFP was almost entirely shut off (Figure 1b). Robust GFP expression returned when doxycycline was removed for 48 h, suggesting that tight and reversible regulation of this marker gene could be achieved (Figure 1b). Lentiviral infection of hNPC did not affect proliferation or differentiation, shown by continued neurosphere growth and expected numbers of astrocytes and neurons (data not shown).

Having optimized lentiviral infection and regulation of human neural cells using the visible GFP reporter, neurospheres were next co-infected with the lenti-tTA and either ^{ind}lenti-GFP or ^{ind}lenti-GDNF constructs (Figure 1a). Neurospheres infected with both lenti-tTA and ^{ind}lenti-GFP did not release GDNF at levels measurable with ELISA, Western blot or immunocytochemistry (data not shown). This confirms our earlier results using gene chip analysis for human neurospheres[19] and ELISA for rodent neurospheres.[27] Neurospheres co-infected with both lenti-tTA and the ^{ind}lenti-GDNF construct (hNPC-GDNF) produced large amounts of glycosylated GDNF revealed by Western blot analysis (Figure 1c, lane 3). This naturally glycosylated GDNF was similar to the glycosylated form produced by a non-human mammalian cell line (Figure 1c, lane 1) and was in contrast to the lighter-weight nonglycosylated human recombinant GDNF produced by bacteria (Figure 1c, lane 2) and used for previous clinical trials.[6] Immunocytochemistry for GDNF showed that over 40% of the hNPC following infection expressed GDNF while other cells were completely negative following acute neurosphere dissociation and plating for 1 h (Figure 1d). The protein was seen to accumulate within cells and had a punctate appearance consistent with normal production in golgi and release into the medium (Figure 1d). Addition of doxycycline to the media significantly reduced GDNF levels overtime to approximately 10% of 'on' values without doxycycline by 10 days (Figure 1e). The reason for this slow and incomplete shut off in GDNF protein levels when compared to GFP may be related to the long half-life of GDNF. hNPC-GDNF plated for 8 days were shown to release approximately 8–10 ng GDNF/μg of protein into the medium over a 24-h period (Figure 1f). GDNF release could be switched off by doxycycline treatment and on again by removing doxycycline from the medium for 8 days (Figure 1f), showing that the regulation was reversible. GDNF-transduced hNPC remained healthy, continued to expand and produced stable amounts of GDNF for at least 20 weeks in culture. Thus, hNPC can be modified to secrete naturally glycosylated and regulated human GDNF for long periods *in vitro*.

GDNF release from hNPC is physiologically active in vitro

Having shown that hNPC-GDNF release high levels of the protein, we proceeded to establish the possible functional effects *in vitro*. Primary rat dopamine neurons were cultured in basal media, supernatant from hNPC or supernatant from hNPC-GDNF. The dopamine neurons were grown under minimal culture conditions to induce stress and subsequent low survival rates. The number of primary neurons staining for tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine production, significantly increased when media from wild-type human neurospheres was added 1 h after plating. This suggests a protective effect from secreted factors produced by the hNPC ($P<0.001$; Figure 2a and c) and supports our earlier observations that neural stem cells alone can increase the survival of primary dopamine neurons.[35] Supernatant from hNPC-GDNF had effects on overall dopamine neuron number comparable to that produced from wild-type hNPC (Figure 2c). In contrast, conditioned supernatant from hNPC-GDNF significantly increased both dopamine neuron neurite outgrowth and cell body area when compared with supernatant from wild-type hNPC ($P<0.001$; Figure 2b, d and e). Together, these data show that, while conditioned media (CM)

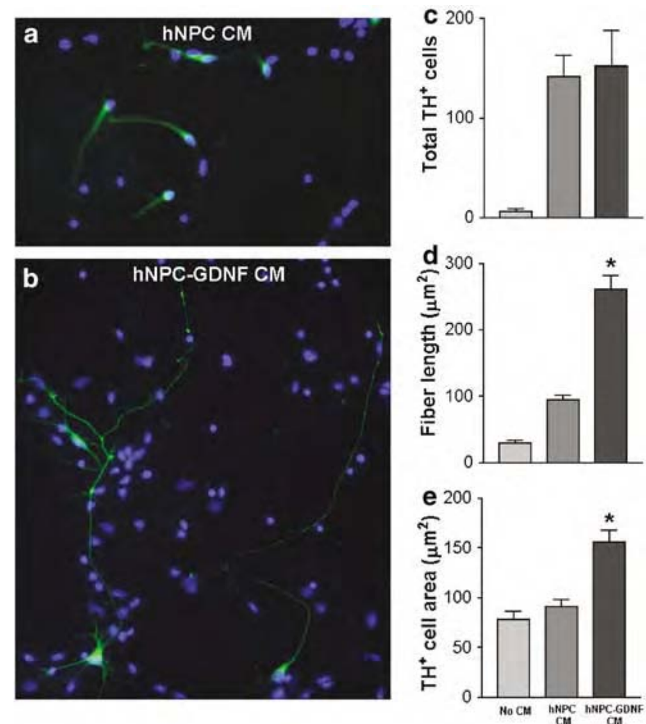

Figure 2 Functional effects of GDNF released from hNPC *in vitro*. Following plating of primary rat dopamine neurons, overall survival was significantly enhanced with either CM from wild-type hNPC or hNPC-GDNF (**a**–**c**). However, when the fiber length (**d**) or cell body area (**e**) of dopamine neurons was assessed, significant increases were seen only in response to CM from hNPC-GDNF.

from human neural cells is protective for dopamine neurons, only hNPC modified to release GDNF can achieve powerful trophic effects on dopamine fiber outgrowth and cell body area.

hNPC-GDNF induce dopamine fiber outgrowth 2 weeks following transplantation into the partially 6-hydroxydopamine (6OHDA)-lesioned rat brain

In order to establish whether the hNPC-GDNF could survive for short time periods following transplantation and modulate dopaminergic function, we grafted 120 000 cells into the striatum of rats with unilateral partial terminal lesions of the dopaminergic system pre-existing 1 week prior to transplantation. In this partial lesion, 6OHDA injected into the striatum selectively damages a proportion of dopaminergic fibers modeling the expected situation in the human striatum of a patient with mid-stage PD.[36] The results are described in Figure 3a. Animals had a reproducible partial lesion damage evidenced by loss of TH expression within the lesion core and penumbra and intact expression in distal/surrounding regions. GDNF-expressing hNPC survived transplantation into the core of the lesion and were detected using a human nuclei-specific antibody (hNUC). GDNF staining revealed a good correlation between surviving hNPC-GDNF stained with hNUC marker and GDNF expression, although there was some diffusion of GDNF away from the graft core. There was no GDNF staining in animals receiving wild-type hNPC transplants or on the non-lesioned side of the brain, demonstrating the selective release of GDNF from engineered cells and the specificity of this technique.

In every animal, regions of GDNF-expressing hNPC correlated with areas of high TH fiber intensity at 2 weeks post-transplantation (Figure 3a). Since the TH fibers in the area of 6OHDA administration are generally gone 1 week post-lesion (the time at which the hNPC were transplanted), we assume that this represents sprouting of surrounding fibers into this region, although it is also possible that this represents a protection effect. In contrast, animals receiving wild-type hNPC showed no TH fiber sprouting, suggesting that the effect was due to GDNF release from the hNPC and not simply the cells alone (Figure 3a). Cell counts of the total number of dopamine neurons surviving in each group revealed significantly more TH-positive cells on the transplanted side following hNPC-GDNF transplantation (Figure 3b; $P<0.05$). GDNF is known to be retrogradely transported from terminals in the striatum back to the mesencephalon.[37] Animals with hNPC-GDNF transplants in the striatum showed GDNF expression in dopamine neurons within the mesencephalon (Figure 3c–e), demonstrating that GDNF released in the striatum was taken up by dopaminergic terminals

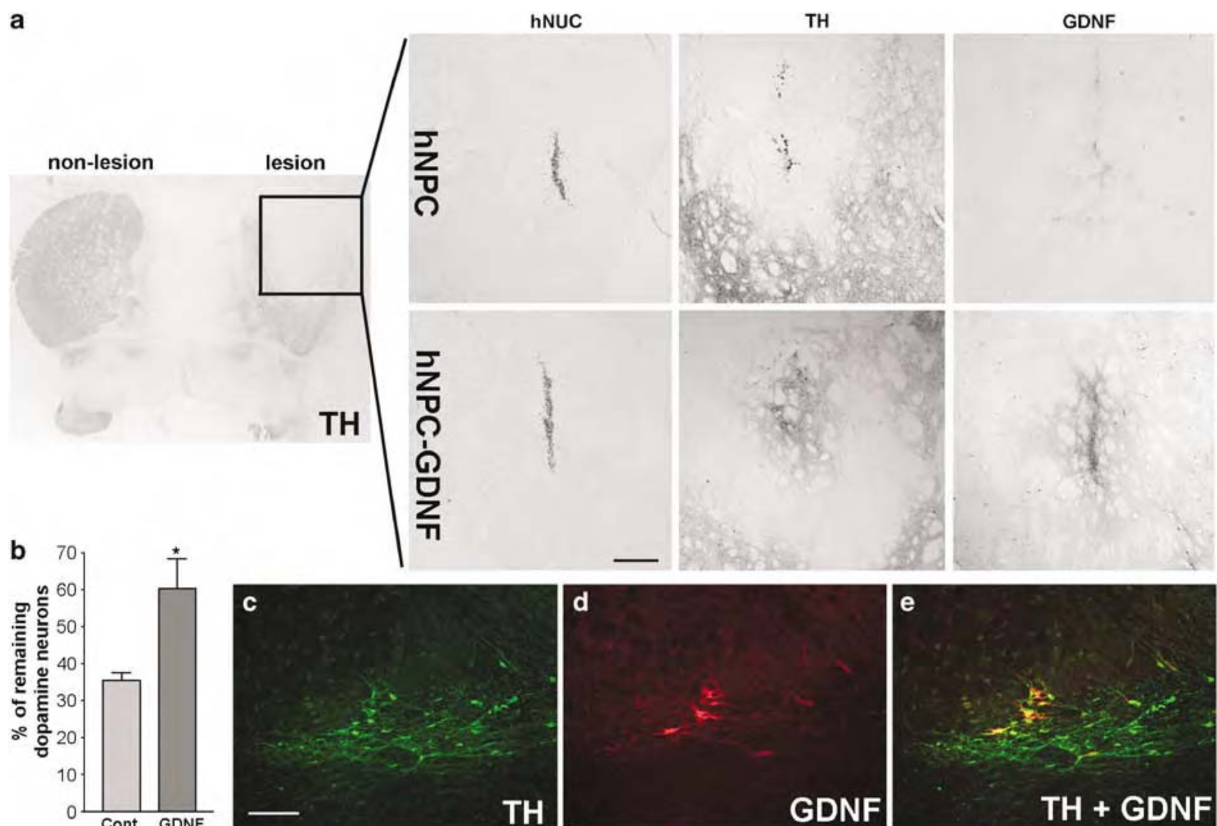

Figure 3 hNPC-GDNF survive in the partially lesioned striatum and protect dopamine neurons. Animals killed at 2 weeks show GDNF release from hNPC-GDNF, but not wild-type hNPC, and show TH fiber sprouting around the graft site (**a**). GDNF released from hNPC-GDNF transplants in the striatum increase the percentage of dopamine neurons remaining in the mesencephalon of the transplanted side (GDNF) compared to the non-transplanted side (Cont) ($P<0.05$) (**b**). The GDNF released from the hNPC in the striatum is transported back to TH-expressing cell bodies in the substantia nigra (**c**–**e**). Scale bars are 500 μm in (**a**) and 100 μm in (**c**).

and retrogradely transported to dopaminergic cell bodies.

We next asked whether GDNF released from the hNPC could be regulated *in vivo*. Transplanted animals were treated for 14 days with doxycycline, but we saw little downregulation of GDNF immunoreactivity in the grafts (data not shown). This is probably due to the incomplete downregulation of GDNF expression in this system as shown *in vitro* (Figure 1e). Thus, further modifications to the viral construct may be required to reduce GDNF *in vivo* expression levels below detection.

Long-term survival and expression of hNPC-GDNF in the rat and primate brain

To establish whether the GDNF-expressing hNPC could induce functional recovery in this partial lesion model of PD, a larger group of animals were transplanted with 240 000 cells over two sites and tested for amphetamine-induced rotation before and after transplantation (Figure 4a). Control (nontransplanted) animals showed significant spontaneous recovery over time (Figure 4b) in this partial terminal lesion model of PD. We attribute this to the daily cyclosporine treatment received by all animals, which is known to lead to sprouting and protection of dopamine neurons in this model.[38] Notably, the group with hNPC-GDNF transplants recovered faster over the first 6 weeks of testing (Figure 4b) although this did not reach significance when compared over the whole 10-week time course using ANOVA. Even though all animals showed functional recovery, there were significantly more dopamine neurons in the hNPC-GDNF transplant group (Figure 4c; $P<0.05$), suggesting a survival-promoting effect even at this late time stage.

Anatomical analysis using the human nuclei-specific antibody revealed that hNPC-GDNF survived up to 9 weeks following transplantation and migrated away from the transplant to fill a large area of the striatum (Figure 4d). Individual host neurons could be seen in the striatum decorated with GDNF based on immunocytochemical staining (Figure 4e). Further analysis of GDNF expression revealed a substantial release of GDNF covering the striatum in all transplanted animals (Figure 4f). In contrast to Figure 3a, there was no increase in TH fiber expression in these long-term animals in regions of high GDNF activity and dense cell transplants. This may be due to a downregulation of TH in response to GDNF over long but not short time periods, as shown in a previous rodent study.[39]

The graft anatomy clearly demonstrated that hNPC-GDNF had good survival and robust GDNF expression for a long period in 6OHDA-lesioned rats. To establish what types of cells had been generated, we double labeled sections with an antibody to glial fibrillary acidic protein (GFAP; astrocyte marker), nestin (progenitor marker) or NeuN (neuronal marker) along with the human specific nuclear antigen (hNUC). Immunohistochemistry revealed that very few of the cells had matured into astrocytes at this time point (<5% GFAP positive; Figure 5a) and that none of the transplanted cells appeared to differentiate into neurons. A rare example of an astrocyte-differentiated cell is shown in Figure 5b–d. Instead, the majority of the transplanted

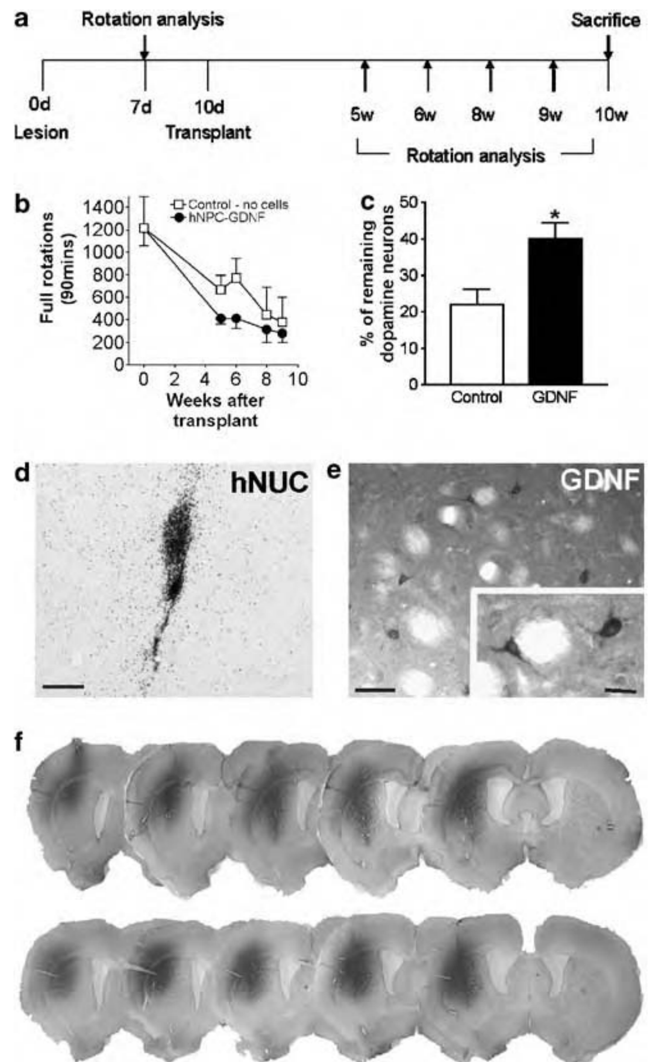

Figure 4 hNPC-GDNF express GDNF long term in the partially lesioned striatum and provide protection. Rats receiving hNPC-GDNF or no cells post-lesion were assessed for functional recovery by amphetamine rotations (**a**). Rats receiving hNPC-GDNF had a trend towards fewer rotations at 5 and 6 weeks post-transplantation compared to the control group. By 8 weeks, control rats showed similar recovery from the partial lesion (**b**). More TH-positive neurons are present in the hNPC-GDNF-transplanted rats compared to no transplant rats (**c**). Cells, identified with the human nuclear specific antibody hNUC, survive for at least 9 weeks following transplantation (**d**) and release GDNF, which is taken up by host neurons (**e**) and distributed to cover a large region of the striatum (**f**). Scale bars are 500 μm in (**d**), 100 μm in (**e**) and 30 μm in (**e** inset).

migrating cells continued to express nestin (>90% nestin positive; Figure 5e).

In a small study, three aged rhesus monkeys were transplanted with hNPC-GDNF into the putamen. One animal died due to natural aging and/or cyclosporine complications, one showed no transplant perhaps due to rejection, but the last animal had a robust transplant that expressed high levels of GDNF 3 months after grafting (Figure 6). This shows that the cells used in this study are able to survive and release GDNF in the primate brain for extended periods of time.

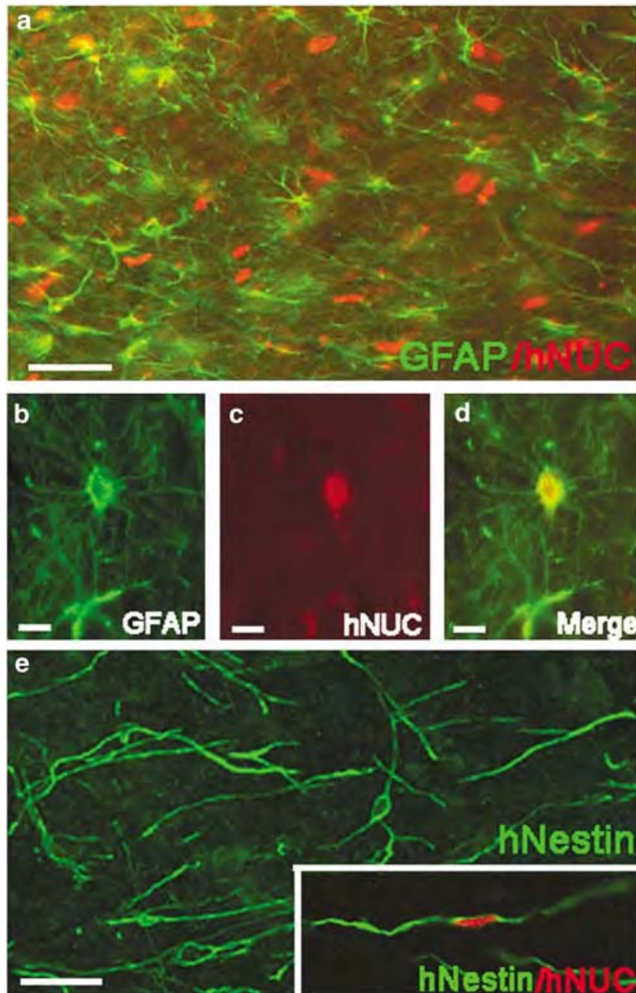

Figure 5 Differentiation of hNPC transplanted into the lesioned rodent striatum. Most of the cells migrating away from the core of the transplant did not stain for GFAP (**a**), although a few were clearly double labeled for the human nuclear protein and GFAP (**b**–**d**). The majority of migrating cells were stained for human nestin (**e**), which also colabeled with human nuclear protein (inset). Scale bars are 100 μm in (**a**) and (**e**), 50 μm in (**b**–**d**).

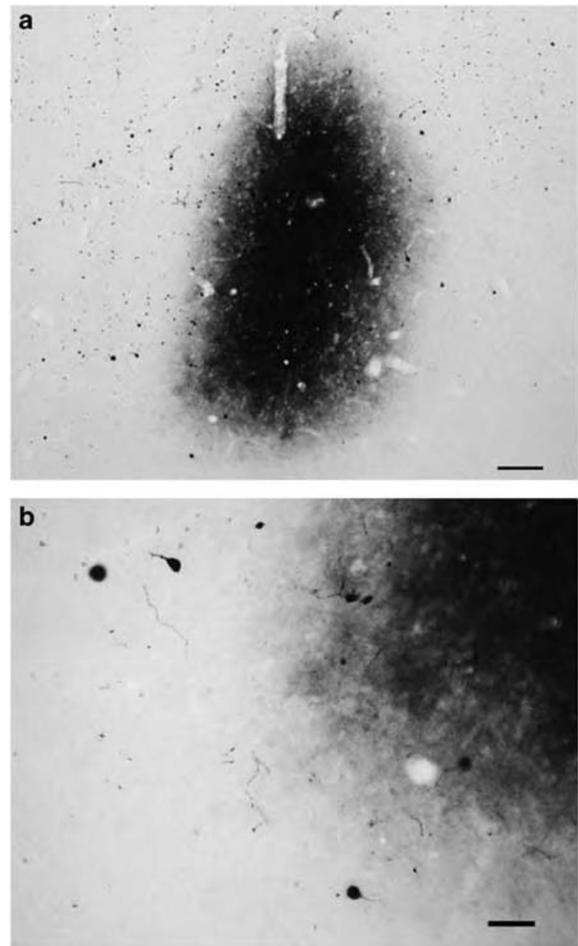

Figure 6 hNPC survive transplantation within the primate putamen 3 months after transplantation. Low magnification shows a large transplant with robust GDNF expression (**a**). Increased magnification of the brain region containing the hNPC transplant shows individual cells stained for GDNF (**b**). Scale bar is equal to 30 μm in (**a**) and 100 μm in (**b**).

Discussion

Since the discovery of nerve growth factor,[40] there has been an intense interest in the potential of growth factors for restoring the damaged central nervous system. GDNF has been a focus due to its potent effects on dopamine neurons,[1] although it has since been found to bind to receptors and exert trophic effects on a variety of neurons within the brain.[41] As PD attacks a range of neural transmitter systems in addition to dopamine, this pleotrophic aspect of GDNF may benefit multiple neurons, leading to enhanced effects in patients. However, delivery of GDNF has been a consistent challenge. A few studies have used immortal rodent cell lines to deliver GDNF before the lesion and shown protection.[28] Here we show for the first time that genetically modified hNPC can act as long-term 'mini pumps' to deliver GDNF to specific regions of the rodent and primate brain. Furthermore, we show that transplanted cells had physiological effects on dopamine neurons after the parkinsonian lesion had been administered, which is critical since there are currently no predictive factors for PD.

Engineered GDNF-secreting hNPC were remarkably stable over multiple passages and consistently released the same level of glycosylated GDNF into the medium. This allows banking and full characterization of the cells prior to transplantation to provide a level of safety required for human clinical trials. Clearly, regulation of GDNF release would be of interest for clinical trials. Inducible promoter systems driving TH expression packaged in adenoviral vectors have been shown to work with hNPC both *in vitro* and *in vivo*.[42] We constructed a vector to attempt regulation of GDNF release following transplantation. While regulation *in vitro* was very efficient for the marker protein GFP, we could not switch off GDNF production below approximately 10% of control values. Furthermore, *in vivo* we were not able to detect down-regulation of GDNF in response to doxycycline. This result is similar to other reports where inducible vectors have been used to control the release of GDNF following direct injection of viral vectors to the brain, but only met with limited

success.[43] There are a number of reasons why regulation cannot be achieved *in vivo*. Perhaps the most parsimonious of these is that GDNF may have a long-half life when compared to TH or destabilized GFP although there is limited evidence in the literature at present. Another possibility is that even if GDNF expression in the brain is reduced by 90% with doxycycline this low amount may still be detected with the sensitive immunocytochemical assay. We are currently exploring this issue further and developing new single vector systems to improve the regulated production of GDNF and achieve 100% shut-off both *in vitro* and *in vivo*.

The safety of progenitor cell transplants is always a key concern. Neural tissues derived from mouse embryonic stem cells can form teratomas in the brain.[44] In contrast, the human progenitor cells used in the current study are derived from fetal brain tissue, not human embryonic stem cells. Fetal-derived hNPC have been transplanted in hundreds of studies and never been found to form tumors to date. Our unique method of hNPC propagation permits constant cell/cell contact and eliminates exposure to serum or trypsin and stress from single-cell dissociation.[18] This technique may reduce the possibility of immortalization events seen in human embryonic stem cells associated with repetitive trypsin treatment and dissociation to single cells.[45] We have previously shown that hNPC grown as neurospheres without dissociation during passage do not express telomerase required for immortalization[46] and therefore have a finite lifespan in culture (Wright *et al*., in preparation). We have also shown that transplanted hNPC express Ki67 and divide *in vivo* for only a limited time.[46] In the current study, we confirm these observations as there was no sign of overgrowth or tumor formation. In addition, hNPC grown as neurospheres are never exposed to mouse feeder layers, reducing the possibility of contamination recently reported for human embryonic stem cells.[47] Human neural progenitors can be expanded to large numbers, genetically modified, tested for safety, banked, and may be more accepted after transplantation due to their human neural origin. Thus, these cells represent an ideal vehicle for delivering proteins to the human brain.

Transplants of hNPC-GDNF survived well in the brain for an extended time period, released GDNF at robust levels and increased the number of TH neurons and their fiber projections around the transplant site. Many of the transplanted cells (>90%) maintained an immature phenotype, as evidenced by constant nestin expression. This may reflect the long time course of maturation required for human progenitor cells when compared to rodent progenitors, or the lack of appropriate species-specific differentiation signals in the rodent brain. Naïve hNPC had no effect on either survival or TH fiber sprouting, supporting our previous findings.[26] This is in contrast to a study that suggested that naïve immortalized mouse stem cells derived from the developing cerebellum (C17.2 cells) could restore dopamine neurons and function in a mouse model of PD.[29] However, this may be due to the fact that C17.2 cells were found to endogenously release GDNF, in contrast to the hNPC described in the current study. At five and six weeks following transplantation, animals with hNPC modified to express GDNF showed a trend towards functional effects when compared to control animals. By 10 weeks, this difference was no longer present due to spontaneous recovery in the control group. This may either be due to a lack of complete lesion in these animals, or some protective effects of the cyclosporin treatment.[38] In aged monkeys, hNPC-GDNF could survive and produce GDNF for up to 3 months, showing that this method of delivery is also applicable to primates.

hNPC can be modified in culture and survive transplantation into compromised environments, including the lesioned rodent striatum, the aged primate brain and, as recently shown by our laboratory, the spinal cord of ALS rats.[33] Cells continue to act as mini-pumps for several months, with release of the therapeutic molecule reaching physiological levels. Together, these results show that combining human progenitor cell therapy with *ex vivo* gene therapy is a powerful approach to the future treatment of PD and other neurological conditions.

Materials and methods

Cell growth

Human cortical neural progenitor cells were isolated from fetal cortex between 10 and 15 weeks of gestation according to protocols approved by the NIH and local ethics committee at the University of Wisconsin Madison and by the University of Washington Birth Defects Laboratory. Following dissociation in 0.1% trypsin, cells were seeded at 200 000 cells/ml in T75 flasks containing DMEM/Ham's F12 (Gibco-BRL) supplemented with penicillin/streptomycin (Gibo-BRL, 1%), N2 (Gibco-BRL, 1%), epidermal growth factor (Sigma, 20 ng/ml) with heparin (Sigma, 5 μg/ml) and fibroblast growth factor-2 (R&D, 20 ng/ml). Neurosphere colonies rapidly formed and were expanded by chopping using an automated tissue chopper as described in detail previously.[18] At 10 weeks the cells were switched to medium containing LIF (Chemicon, 10 ng/ml) and under these conditions the cultures could be grown for at least another 30 weeks in a relatively stable form based on gene expression patterns and differentiation potential.[19]

Lentiviral infection

The lentiviral particles were suspended in 1% fetal bovine serum albumin in phosphate-buffered saline. Lentivirus infection, except for the long-term transplant experiment, was 12 h in culture medium with 25 ng of ^{ind}lenti-GFP or ^{ind}lenti-GDNF and 75 ng of lenti-tTA per three spheres. For the long-term transplant experiment, we slightly modified the infection paradigm by dissociating spheres to single cells and infecting with only 5 ng of ^{ind}lenti-GDNF and 10 ng of lenti-tTA per 10^5 cells for 24 h before the virus was diluted out with fresh medium. New spheres were then formed through aggregation and allowed to expand for at least 5 weeks prior to transplantation.

GFP regulation

Following ^{ind}lenti-GFP infection, the GFP expression in a representative neurosphere was photographed, and a phase photograph was taken at the same time. This sphere was then cultured in media with doxycycline (Sigma, 0.1 μg/ml) for 48 h and again photographed under both fluorescence and phase. Doxycycline was removed from the media for 48 h. Following this

washout, a photograph was again taken under both fluorescence and phase.

Immunocytochemistry

Neurospheres infected with ^{ind}lenti-GDNF were dissociated using accutase (PAA Laboratories) and plated onto glass coverslips coated with poly-L-lysine (Sigma, 0.01%) and laminin (Sigma, 0.001%). Cells were plated at 30 000 per coverslip in differentiation media DMEM/Ham's F12 with 2% B27 (Gibco-BRL) for 7 days. Following fixation with 4% paraformaldehyde and block in 10% normal donkey serum, cells were stained for GDNF (R&D, goat 1:500) with a cy3-conjugated secondary (Jackson Laboratories 1:1000) and hoechst.

GDNF quantification and regulation

Following ^{ind}lenti-GDNF infection, GDNF levels and regulation were assessed. Neurospheres were dissociated with accutase and equally divided into wells. Wells ($n = 3$) were maintained in B27 differentiation media and wells ($n = 3$) were maintained in B27 differentiation media with doxycycline (1 μg/ml). The plating media with or without doxycycline was collected every 2 days for 10 days and the samples were stored at -20°C for later analysis. GDNF was measured in the supernatant from ^{ind}lenti-GDNF-infected neurospheres using a GDNF Elisa Kit (Promega), according to the manufacturer's instructions. For each collection day, we report GDNF levels in the plus doxycycline groups as a percentage of the GDNF levels in the minus doxycycline groups. In addition to confirming GDNF shut off overtime, we tested the reversible regulation of GDNF by measuring the supernatant from ^{ind}lenti-GDNF-infected cells plated in B27 differentiation media for 8 days without doxycycline, then with doxycycline for 8 days and finally following doxycycline washout for 8 days.

GDNF purification

Recombinant human GDNF (rhGDNF) was purified from ^{ind}lenti-GDNF-infected hNPC media using heparin affinity chromatography. A 10 ml Heparin Sepharose (Amersham Biosciences, Uppsala, Sweden) column was equilibrated with 50 mM Na_2HPO_4, pH 7.2. A volume of 2 liters of hNPC-GDNF CM was loaded onto the column at a flow rate of 20 ml/min. The column was washed with three column volumes of equilibration buffer and the GDNF was eluted with approximately 1 column volume of 50 mM Na_2HPO_4 with 750 mM NaCl. The GDNF was collected in a 5 ml fraction and stored at -30°C.

The rhGDNF from the heparin sepharose fraction was concentrated by TCA precipitation. TCA (Sigma, St Louis, MO, USA) was added at a ratio of 1.0 g TCA to 1.0 ml GDNF fraction. The precipitation proceeded at 4°C for 30 min before centrifugation at 13 000 r.p.m. for 60 min. The precipitate was re-suspended in $1 \times$ LDS sample buffer (Invitrogen, Carlsbad, CA, USA) for SDS-PAGE and Western blot analysis.

Western blot

Western blot analysis was performed on partially purified rhGDNF from hNPC along with commercially available rhGDNF from the mouse mammalian cell line NSO (R&D Systems) and from *Escherichia coli* (Peprotech). Samples diluted in LDS sample buffer and reducing agent were heated to 70°C for 5 min. SDS-PAGE was performed using NuPAGE®, 10% Bis–Tris gels (Invitrogen) and GDNF was transferred to a PVDF membrane using the XCell SureLock™ and Blot Module. Western blot was performed using the WesternBreeze® Immunodetection Chromogenic kit (Invitrogen). GDNF was detected with a goat anti-GDNF primary antibody (R&D 1:500), followed by horseradish peroxidase (Dako 1:2000).

GDNF functional effects *in vitro*

Primary ventral mesencephalon was dissected from E14 embryos of Sprague–Dawley rats and plated onto poly-L-lysine-(0.01%) and laminin-(0.001%) coated coverslips. Cells were cultured for 7 days in either basal N2 (Gibco-BRL, 1%) media ($n = 3$), supernatant from wild-type neurospheres ($n = 3$) or supernatant from neurospheres infected with ^{ind}lenti-GDNF ($n = 3$). Following fixation with 4% paraformaldehyde and rinses with phosphate-buffered saline, cell cultures were stained for TH (Chemicon, mouse 1:200) with FITC-conjugated secondaries (Jackson Laboratories, 1:1000) and hoechst. Cells were viewed under a fluorescent microscope and four fields were analyzed from each of the three coverslips per group. Fluorescent digital images were captured with a digital video camera using the Spot camera image analysis system. The number of TH-positive cells was quantified by counting cells immunoreactive for TH in 12 randomly selected fields. The neurite length and cell body size were quantified by using metamorph to determine the μm^2 and radius, respectively, for TH-positive cells in 12 randomly selected fields.

Partial 6OHDA lesion

Adult male Lewis rats (300–350 g) were injected with a total of 21 μg of 6OHDA resuspended in 0.9% saline with 0.2 μg ascorbic acid. Injections of 7 μg/3.5 μl were made over three sites from bregma: AP +1.0, ML -3.0, DV -5.0; AP -0.1, ML -3.7, DV -5.0; AP -1.2, ML -4.5, DV -5.0.

Cell transplants

Cells were plated as neurospheres for 1 week in B27 differentiation media supplemented with cilliary neurotrophic factor (R&D, 5 ng/μl). To prepare cells for transplantation, the medium was removed, cells were rinsed and accutase was added for 15 min at 37°C to detach plated spheres. Cells were collected, centrifuged at 1000 r.p.m. for 5 min, accutase was removed and cells were rinsed. Cells were incubated in DNAase for 10 min at 37°C, centrifuged at 1000 r.p.m. for 5 min, DNAase was removed and cells were dissociated in Liebovitz/0.6% glucose (1:1) supplemented with 2% B27. Cells were counted, centrifuged at 1000 r.p.m. for 5 min, re-suspended at 66 500/μl and maintained on ice.

For the short-term (2-week) transplant experiments, hNPC ($n = 3$) or hNPC-GDNF ($n = 3$) were transplanted into one site at AP -0.3, ML -3.6, DV -5.2, 1 week post-lesion. For the long-term (9-week) transplant experiments, hNPC-GDNF ($n = 9$) were transplanted into two sites at AP +0.5, ML -3.3, DV -5.2 and AP -0.4, ML -3.7, DV -5.2, 10 days post-lesion. Control animals ($n = 4$) received lesion, but no transplant. A 10 μl Hamilton syringe was lowered from dura, left in place for 1 min before injecting 3 μl cells over 3 min and

retracted after an additional 3 min. Animals were injected with cyclosporin (i.p. 10 mg/kg) 1 day before and every day following transplantation. At 2 or 9 weeks post-transplantation, animals were perfused with chilled 0.9% saline and 4% paraformaldehyde. Brains were cryoprotected in 30% sucrose with 0.1% sodium azide, and sectioned to 40 μm using a microtome.

Immunohistochemisty

Sections were stained for human nuclei (Chemicon, mouse 1:50), GDNF (R&D, goat 1:250), TH (Chemicon, mouse 1:2000), nestin (Chemicon, rabbit 1:200) and GFAP (Dako, rabbit 1:2000). For human nuclei, sections were rinsed in Tris-HCl, incubated in 2 N HCl for 30 min at 37°C, quenched in 5% H_2O_2 and 10% methanol, and blocked in 10% normal horse serum prior to primary antibody. Biotinylated mouse secondary (Vector Labs, 1:200) and ABC (vectastain kit) were followed by DAB (Sigma) development. For human GDNF, sections were rinsed in TBS-T, quenched in 0.1 M sodium periodate and blocked in 3% NHS and 2% BSA prior to primary antibody. Biotinylated goat secondary (Vector Labs, 1:200) and ABC were followed by DAB development with nickel ammonium sulfate enhancement. For TH staining, sections were rinsed in PBS, quenched in 3% H_2O_2 and 10% methanol, and blocked in 5% horse serum. TH antibody was followed by biotinylated mouse secondary (Vector Labs 1:200), ABC, and DAB development. For double labeling with human nuclei and nestin or GFAP antibodies, sections were processed as for nuclei staining, but primary antibodies were followed by mouse TRITC- and rabbit FITC-conjugated secondary antibodies (Jackson Labs, 1:1000). For nigral sections double labeled with TH and GDNF, sections were processed using tyramide signal amplification according to the manufacturer's instructions (Invitrogen) with mouse FITC- and goat TRITC-conjugated secondary antibodies (Jackson Labs, 1:1000).

GDNF functional effects in vivo

TH-positive neurons in the substantia nigra were viewed at $\times 10$ magnification under a light microscope. Images were captured with a digital video camera using the Spot image analysis system. At 2 and 8 weeks post-transplantation, TH-positive neurons were quantified ipsilateral and contralateral to the lesion in three anatomically equivalent sections through the substantia nigra. TH-positive neuron survival was calculated as percent of TH-positive neurons remaining, and data are expressed as average percent TH neurons $\pm$ s.e.m. (paired Student's *t*-test).

Amphetamine-induced rotations (2.5 mg/kg) were assessed 1 week following a partial 6-OHDA lesion and animals with >300 rotations/1.5 h were separated into balanced transplantation groups. Cells were transplanted 10 days following the lesion, and animals were retested weekly for amphetamine-induced rotations beginning 5 weeks post-transplantation.

Monkey studies

Three aged monkeys (22, 29 and 35 years of age) served as subjects. Coordinates for transplantation of GDNF-expressing hNPC were based upon MRI guidance. Under sterile conditions, monkeys received two injections of cells placed unilaterally into the caudate nucleus (10 and 5 μl) and three injections were placed into the putamen (10, 10 and 5 μl) on the same side. One animal died from natural causes 2 months following transplantation. The remaining 2 monkeys were killed 3 months following transplantation via perfusion with saline followed by fixation with a 4% Zamboni's fixitive. GDNF staining follows the protocol described in Kordower *et al*. (2000).

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