Brain Research Bulletin 68 (2005) 31–41

BRAIN
RESEARCH
BULLETIN

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Delivery of sonic hedgehog or glial derived neurotrophic factor to dopamine-rich grafts in a rat model of Parkinson's disease using adenoviral vectors

Increased yield of dopamine cells is dependent on embryonic donor age

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Abstract

The poor survival of dopamine grafts in Parkinson's disease is one of the main obstacles to the widespread application of this therapy. One hypothesis is that implanted neurons, once removed from the embryonic environment, lack the differentiation factors needed to develop the dopaminergic phenotype. In an effort to improve the numbers of dopamine neurons surviving in the grafts, we have investigated the potential of adenoviral vectors to deliver the differentiation factor sonic hedgehog or the glial cell line-derived neurotrophic factor GDNF to dopamine-rich grafts in a rat model of Parkinson's disease. Adenoviral vectors containing sonic hedgehog, GDNF, or the marker gene LacZ were injected into the dopamine depleted striatum of hemiparkinsonian rats. Two weeks later, ventral mesencephalic cell suspensions were prepared from embryos of donor ages E12, E13, E14 or E15 and implanted into the vector-transduced striatum. Pre-treatment with the sonic hedgehog vector produced a three-fold increase in the numbers of tyrosine hydroxylase-positive (presumed dopaminergic) cells in grafts derived from E12 donors, but had no effect on E13–E15 grafts. By contrast, pre-treatment with the GDNF vector increased yields of dopamine cells in grafts derived from E14 and E15 donors but had no effect on grafts from younger donors. The results indicate that provision of both trophic and differentiation factors can enhance the yields of dopamine neurons in ventral mesencephalic grafts, but that the two factors differ in the age and stage of embryonic development at which they have maximal effects.

Keywords: Adenovirus; Viral vectors; Animal model; Gene therapy; 6-OHDA; Parkinson's disease; Dopamine; Transplant; Sonic hedgehog

1. Introduction

The primary deficit in Parkinson's disease (PD) is the progressive loss of dopamine cells in the substantia nigra pars compacta and the ventral tegmental area, resulting in a loss of dopamine innervation to the caudate-putamen (striatum) region of the brain. The loss of striatal dopamine and the subsequent disruption of striatal outputs to the globus pallidus, subthalamic nucleus and substantia nigra pars reticulata, are thought to account for the main clinical symptoms of PD which include rigidity, bradykinesia and tremor. The principal therapy for PD is the re-supply of dopamine to the striatal target area, by oral administration of the dopamine precursor L-DOPA. In the early stages of the disease L-DOPA is a highly effective treatment and is able to ameliorate clinical symptoms dramatically. However, as the disease progresses, L-DOPA treatment becomes less and less effective, in part because of the decreasing availability of residual dopamine neurons to maintain conversion of L-DOPA to dopamine. As degeneration continues, the dose of L-DOPA needs to be increased to maintain the same level of efficacy, and the duration of effective drug action diminishes. In the long term, many patients develop side effects, mainly in the form of uncontrollable limb movements (dyskinesia), which become as problematic as the disease itself. Supplementation of the drug regime with dopamine receptor agonists such as amphetamines and dopamine re-uptake inhibitors is able to prolong the

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doi:10.1016/j.brainresbull.2005.08.021

therapeutic window of L-DOPA but many patients reach an end stage, where L-DOPA treatment fails and an alternative form of treatment is required [10,45,76].

The implantation of embryonic dopamine-rich tissue to replace striatal dopamine innervation offers a complementary strategy for the restoration of dopamine function in the Parkinsonian brain. In rat models of PD, dopamine-rich grafts derived from the ventral mesencephalon (VM) of 14 days post-gestation embryos (E14) are able to completely reverse the drug induced rotational behaviours caused by a unilateral lesion of the dopamine system and to ameliorate behavioural deficits in a number of other tests [6,18,21]. In non-human primates too, VM grafts produce significant amelioration of the deficits induced by dopaminergic lesions [2,33,63]. On the basis of these animal studies, there have been a limited number of trials in patients, from which it is apparent that dopamine-rich transplants of the correct age, implanted using a well defined surgical protocol, can provide considerable therapeutic benefits in at least a subset of patients. Many such patients can reduce L-DOPA intake to a fraction of the normal dose, in some cases coming off L-DOPA altogether. However, the response is variable, with some patients showing dramatic and long-lasting improvements [43,56] and others showing little benefit. More critically, in recent clinical trials some patients have developed significant side affects, in particular ‘runaway’ dyskinesias which in some cases persisted even when L-DOPA was withdrawn fully [24,25]. These results have not yet been fully explained [32], although advances are being made in developing animal models of the neurobiological basis of L-DOPA and graft associated dyskinesias [41,66], with optimism that the interaction between chronic L-DOPA treatment and dopamine-rich grafts can be understood and subsequently managed to avoid this side effect.

One of the main factors limiting the development of VM transplantation as an effective therapy for PD is the relatively poor survival of dopaminergic neurons following implantation into the host brain. Although survival rates of up to 40% have been reported using factors to enhance dopamine cell survival, in untreated primary grafts typically only 5–10% of implanted embryonic dopamine neurons survive, both in experimental animals [9] and in human patients [39,44,52]. Poor dopamine cell survival and/or failure of the integration of the grafts into the host brain are likely to be a major factors in the incomplete recovery seen in many clinical trials. There is correlation between the number of dopaminergic cells surviving implantation and the degree of functional recovery seen in experimental animals [21]. As a result, in patients, up to six embryos per hemisphere are now considered to be necessary for optimal therapy [43], exacerbating the logistical difficulties of obtaining sufficient tissue of suitable quality and at the correct age of development. As a consequence strategies to enhance the yields of dopamine neurons in VM grafts have emerged as a major topic for research [9,64].

The aim of the current work was to investigate the potential of the dopamine cell differentiation factor sonic hedgehog (Shh) and the glial cell line-derived neurotrophic factor (GDNF), delivered using an adenoviral-vector, to promote the survival of VM grafts. We have used “second generation” adenoviral vectors with deletions or substitutions in the E1 and E3 regions of the viral genome [30,69]. The resulting virus particles are replication deficient, but capable of infecting a wide range of cell types including non-dividing brain cells. Co-workers have described the expression of β-galactosidase encoded within the recombinant Ad vectors RAD35 and RAd36 in the rat brain and these results indicated that RAd36 was capable of transducing striatal cells in vivo with almost 100% efficiency (i.e. a single viral particle is capable of transducing a cell) [30]. The therapeutic vectors used in this study encode the cDNAs for either Shh, or GDNF driven by cytomegalovirus-derived promoters.

In pilot studies, several different methods of application of adenoviral vectors to embryonic ventral mesencephalic grafts were investigated. Application of the vectors directly to the embryonic dopamine cell suspension embryo prior to implantation into the host brain, and transduction of accessory cells for co-grafting with embryonic VM, yielded only low transduction efficiencies. More effective was direct injection of the vectors into the host striatum, prior to the implantation of the dopaminergic graft. Using this method, large numbers of striatal neurons and glia take up the vector, such that the transgene product is expressed in the precisely the region of host brain into which the graft is to be placed. This was the route of delivery chosen for the current study.

2. Methods

2.1. Design

A total of 150 rats, received unilateral 6-hydroxydopamine (6-OHDA) lesions of the median forebrain bundle and, were allocated to 18 matched groups based on rotational testing. Rats then received striatal injections of either RAd/Shh, RAd/GDNF [35], RAd/LacZ [69] or physiological saline (to control for the effects of surgical trauma). Two weeks later, all rats received a VM graft derived from embryos of donor ages E12, E13, E14, or E15. To control for possible effects of viral vector expression on amphetamine induced rotation, rats in two control groups received 6-OHDA lesions followed by injections of RAd/Shh or RAd/GDNF, but received no subsequent VM grafts.

2.2. Experimental animals

Adult, female Sprague–Dawley rats were used, weighing 200–250 g at the time of first surgery. Rats were housed under standard conditions with free access to food and water. All experiments were conducted in accordance with requirements of the UK Animal (Scientific Procedures) Act 1986.

2.3. Adenoviral vectors

The construction and characterisation of the adenoviral vectors used in the work have been described previously [30,35,69]. The backbone consists of an adenovirus type 5 in which deletions or substitutions have been made in the E1 and E3 regions of the genome. The vectors are produced using a *trans*-complementing cell line established from human embryonic kidney cells HEK293 and purified by caesium chloride gradient centrifugation to titres of up to 4×10^{11} IU/ml [65]. The resulting virus particles are replication deficient, but capable of infecting a wide range of cell types including non-dividing brain cells. The therapeutic vectors used in the present work encoded the cDNAs for either sonic hedgehog (SHH-N amino terminal fragment) or glial cell line-derived neurotrophic factor (GDNF). In the control vector, the β-galactosidase gene LacZ was used. All transgenes were driven by a cytomegalovirus-derived promoters of human origin [69,72] (see Table 1).

Table 1
Summary of adenovirus vectors used

Vector	Transgene	Promoter[a]	Final titre (IU/μl)
RAd35	Lac Z	MIE*h*CMV/	1×10^7
RAd Shh	Shh-N	MIE*m*CMV/	1×10^6
RAdGDNF	GDNF	MIE*h*CMV/	1×10^7

[a] Promoters are derived from cytomegaloviruses of either human (hCMV) or murine (mCMV) origin.

2.4. Surgery

All surgery was performed under gaseous anaesthesia (60% oxygen/40% nitrous oxide containing 2–3% isoflurane). Animals were placed in a stereotaxic frame and cannula placements determined using the co-ordinates of Paxinos and Watson [54].

2.4.1. Nigrostriatal lesions

Lesions were carried out by injection of 6-OHDA (hydrobromide salt, Sigma Chemicals, UK) unilaterally into the median forebrain bundle using a 30-gauge cannula connected to a 10 μl Hamilton syringe in a microdrive pump set to deliver at 1 μl/min. The toxin was used at a concentration of 3 μg/μl (calculated as the free base weight) dissolved in a solution of 0.2 mg/ml ascorbic acid in 0.9% sterile saline. The stereotaxic co-ordinates used for injection were; $A = -4.4$ mm from bregma, $L = -1.0$ mm from midline, $V = -7.8$ mm below dura, with the nose bar set at −2.3 mm below the interaural line. Injections were carried out over 3 min with a further 3 min allowed for diffusion before slow withdrawal of the cannula from the brain, and cleaning, closure and suturing of the wound.

2.4.2. Viral vector injections

On completion of post-lesion rotational testing, rats were divided into groups, matched according to their rotation scores and received unilateral striatal injections of either a viral vector or physiological saline. Virus stock solutions were diluted to the required concentration using 0.9% sterile saline immediately prior to use (see Table 1). Final concentrations of vector were determined from pilot experiments (data not shown) and injected into the dopamine-depleted striatum at stereotaxic coordinates: $A = +0.6$, $L = \pm 3.0$, $V = -4.5$. All virus injections were 3 μl in volume and carried out using the same parameters for injection as the 6-OHDA lesions.

Ventral mesencephalic grafts. VM grafting was carried out 2 weeks post-injection of the viral vectors. The ventral mesencephalon was dissected from Sprague–Dawley rat embryos of donor ages E12, E13, E14, or E15 (post-plug). The actual sizes of the embryos used are given in Table 2. VM grafts were prepared as a cell suspension according to a standard protocol [19]. Grafts in each group were derived from at least two cell suspension preparations and two separate rat litters to control for the effects of differences in suspension preparation. Each injection consisted of 2 μl of the cell suspension containing the cells from 1 VM, injected at the same coordinates used for the viral vector injections.

2.5. Rotation

Methamphetamine induced rotation tests were carried out 2 and 4 weeks post-lesion to obtain an estimate of the extent of dopamine depletion in each animal. Rotation was assessed using an automated rotometer system based on the apparatus of Ungerstedt and Arbuthnott [70]. Following an intraperitoneal injection of methamphetamine hydrochloride (dissolved in 0.9% sterile saline) at a dose of 2.5 mg/kg of body weight, rotation test scores were recorded and are reported as net scores (ipsilateral minus contralateral) over a 90 min session. Only rats with a net rotation score of ≥600 turns per session were used in the experiment. Grafted rats were rotation tested 4 and 6 weeks post-implantation using the same method.

Table 2
Details of VM grafts

Donor age	CRL (mm)	Mean cells/μl	Cells implanted
E12	7.5–8.5	434000	868000
E13	9.5–10.5	406000	812000
E14	11–13	450000	900000
E15	13.5–14.5	270000	540000

2.6. ELISA

The levels of GDNF in rat brain following injection of RAd/GDNF were measured using enzyme linked immunosorbent assay (Promega (UK) GDNF ELISA System Kit#G3240). Briefly, two rats received a unilateral striatal injection of RAD/GDNF (as above) and were sacrificed 2 weeks post-injection of the vector and the fresh brain was removed quickly and placed on ice. A 3 mm-thick coronal slice was cut at the level of the injection and the both intact and injected striata dissected out and collected into 0.5 ml Eppendorf tubes, frozen using dry ice and stored at −20 °C overnight. Tissue samples were thawed and homogenised in protein free Eppendorfs using micro-pestles with a TRIS-based lysis buffer containing 0.9% NaCl, EDTA and 0.5% Nonidet P40 detergent. The supernatant was collected following centrifugation of the homogenate at 13,000 rpm for 30 min. A 24-well micro titre plate was coated with anti-GDNF antibody overnight and tissue samples were added with blocking solution to the plate for 6 h at room temperature. Samples of purified GDNF in serial dilutions (0–1000 pg/ml) were added to wells in the same plate to determine a calibration standard. Following washing anti-GDNF antibody (chicken) was added to the wells at a concentration of 1:500 and incubated overnight at room temperature. Following washing HRP conjugated anti-chicken antibody was added (1:5000) for 2.5 h at room temperature and then washed off. Activity was visualised using tetra-methyl-benzidine reaction for 15 min at room temperature and stopped by addition of phosphoric acid. Absorbance (490 nm) was measured in an automated plate reading system within 1 h of the reaction.

2.7. Histopathology

On completion of behavioural testing, animals were terminally anaesthetised by intraperitoneal injection of 200 mg/kg sodium pentobarbitone, and then perfused transcardially with 100 ml of phosphate buffered saline (PBS) at pH 7.4, followed by 250 ml of 4% paraformaldehyde in PBS over a 5 min period. The brains were then removed from the skull and post-fixed by immersion in the same fixative solution for 4 h, then transferred to 25% sucrose in PBS. After equilibration in the sucrose solution, coronal sections were cut on a freezing stage sledge microtome at a thickness of 40 μm into 0.1 M TRIS buffered saline pH 7.4 (TBS) and stored at +4 °C prior to staining. All stains were carried out on a 1 in 6 series of sections. One series was stained using the standard Nissl stain, cresyl fast violet. A second series was stained immunohistochemically for tyrosine hydroxylase (TH).

Immunohistochemistry was carried out on free-floating sections. All sections were stained simultaneously using the same solutions of antibodies and ensuring that incubation times and washes were the same for each brain. The following protocol was used. Sections were thoroughly washed in Tris-buffered saline (TBS). Endogenous peroxidase enzyme activity was quenched using a 10 min immersion in 3% hydrogen peroxide/10% methanol in distilled water, followed by washing and re-equilibration in TBS. After a 1 h pre-incubation in a solution of 3% normal goat serum/0.1% Triton X-100 in TBS, sections were incubated in the TH (mouse) antiserum (Chemicon 1:2000 dilution) in 1% normal goat serum/0.1% Triton X-100 for 60 h at +4 °C. A known positive control, and a negative control in which the primary antibody was omitted, were also run. After thorough washing, a biotinylated, rat-adsorbed anti-mouse, secondary antibody (Vector, 1:200) in 1% normal goat serum in TBS was applied for 3 h. The sections were then washed for 30 min before application of 10% streptavidin–biotin–horseradish peroxidase solution (Dako) in TBS for 90 min, followed by thorough washing and equilibration to 0.05 M Tris non-saline solution at pH 7.4. The horseradish peroxidase label was revealed by 10 min incubation in a 0.5% solution of diaminobenzidine tetrahydrochloride (Sigma chemicals, UK) in Tris non-saline containing 0.3 μl/ml of hydrogen peroxide. Sections were finally mounted on gelatine-coated microscope slides dehydrated in an ascending series of alcohols, cleared, and cover-slipped using DPX mountant.

2.8. Morphometry

Measurements of graft size were carried out using a PC-based image analysis system with Scion-Image (Beta 4.0.2) software (Scion Corporation, USA). Measurements of graft volume were from cross-sectional areas measured on TH-stained sections in a regular series (1 in 6) through the entire graft. Cell counts were carried out on a Leica DMRB microscope using a 10× 10 eyepiece graticule and a 20× objective on the same sections and corrected using the Abercrombie formula [1].

3. Results

3.1. Amphetamine rotations

Control groups that received injections of RAd/Shh or RAd/GDNF but no subsequent VM graft, showed no recovery of the post-lesion amphetamine-rotation deficit (Fig. 1). Instead of the reduction in rotation seen following a VM graft, the net rotation scores in rats injected with either RAd/Shh or RAd/GDNF showed the expected slight increase over the course of the experiment due to sensitisation to amphetamine. This result indicates that there is no recovery of the lesioned dopamine system, nor inhibition of the post-synaptic response to amphetamine, induced by either the adenoviral vector or the transgenes. Thus, any amelioration in the rotation deficit seen in groups of grafted animals can be attributed to a graft effect.

All of the grafted groups of animals showed recovery of amphetamine-induced rotation from a net ipsilateral rotation at 4 weeks post-lesion to a net contralateral score, 4 and 6 weeks post-grafting, the classic "over-compensatory" graft response [20]. There were no significant effects of virus pre-treatment on rotation scores. By contrast, there were slight differences between donor age groups on post-graft rotation: E15 grafts showed slightly less overcompensation at 6 weeks post-grafting compared to other graft groups (Fig. 2) but this difference was not statistically different.

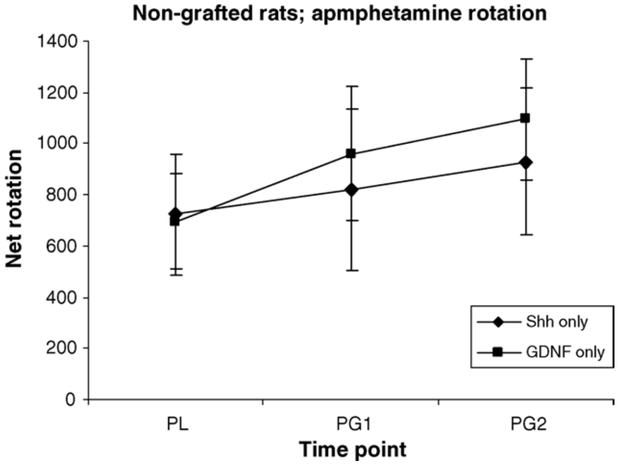

Fig. 1. Amphetamine rotation in control animals with 6-OHDA lesions and striatal injections of either RAd/Shh or RAd/GDNF. Time points are post-6-OHDA-lesion (PL), 8 weeks (PG1) and 10 weeks (PG2) post-lesion at time points corresponding to post-graft testing in the graft groups. There is no recovery of amphetamine induced rotation seen with either vector. Error bars are S.E.M.s.

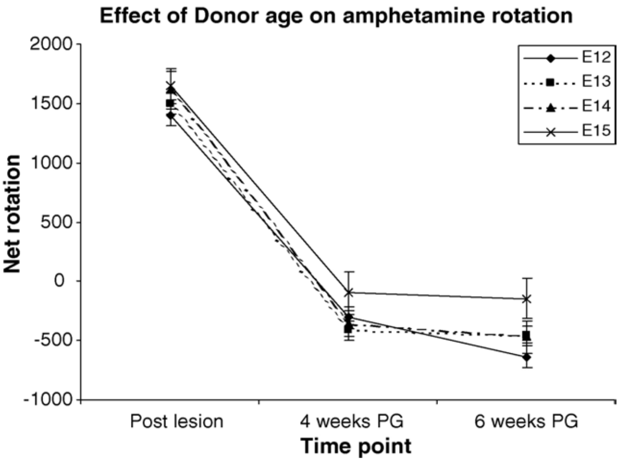

Fig. 2. Post-lesion and post-graft amphetamine rotations by embryonic donor age. There was no effect of virus pre-treatment on post-graft rotation. All graft groups showed an over-compensatory rotational response but the E15 graft group response was less than that seen with younger donor ages.

3.2. Post-mortem histopathology

Examination of cresyl fast violet stained sections revealed large surviving grafts in all donor age groups. There was no evidence of an inflammatory reaction to the injected virus or of cytotoxic tissue damage in any animal (Fig. 3).

The effectiveness of the current approach relies on successful transduction of the host striatum prior to the implantation of the VM graft. This is demonstrated in Fig. 4, which shows adjacent sections through a graft from a rat in the RAd35-E12 group stained immunohistochemically for TH and β-galactosidase, respectively. LacZ transduced cells were seen in intimate contact with the VM grafts. Similar staining patterns were seen in grafted striata transduced with either RAd/Shh or RAd/GDNF vectors and immuno-staining for the transgene products of these vectors showed staining in both the surrounding striatum and within the graft tissue itself (Fig. 5).

TH immunohistochemistry revealed healthy grafts containing many TH positive cells surrounded by a dark halo of TH reinnervation of the surrounding striatum. Fig. 6 shows representative sections from the E12 donor age group stained for cresyl violet and TH to illustrate the differences in graft morphology seen in the Shh vector treated group. Similarly, Fig. 7 shows representative sections from the E14 donor age group stained for cresyl violet and TH to illustrate the differences in graft morphology seen in the GDNF vector treated group.

3.3. Cell counts

A preliminary analysis was carried out to determine whether there was an effect of the injection of the adenoviral vector on TH cell numbers in the grafts. Analysis of variance between the LacZ injected and saline injected groups showed that there was no significant difference between saline and LacZ injected groups at any donor age ($F_{1,39} = 2.80$, $p = 0.102$, n.s.). For the purposes of clarity, the LacZ and Saline groups for each donor

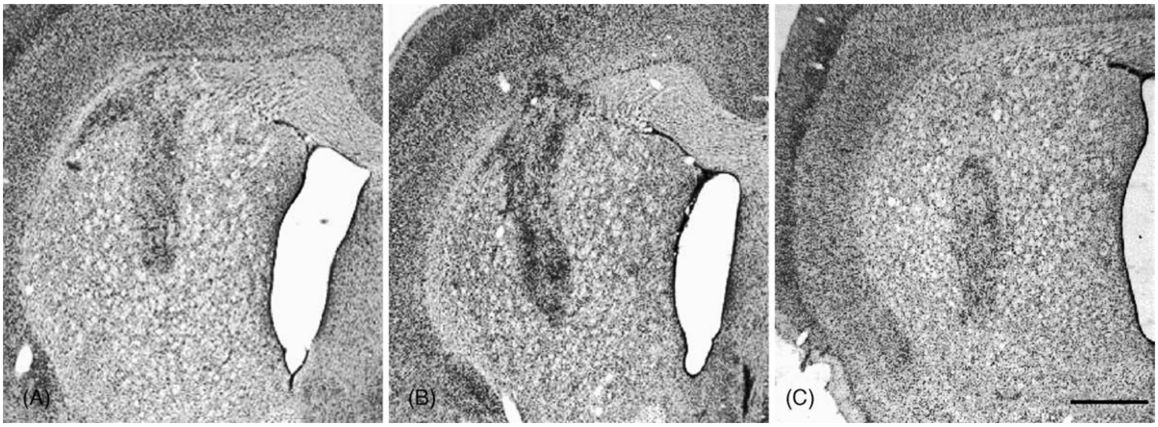

Fig. 3. Cresyl violet stained sections showing representative grafts from rats in (A) control, (B) RAd/Shh and (C) RAd/GDNF injected groups. All grafts are healthy containing large numbers of neurons. There is no evidence of inflammation either within the graft or in the surrounding host striatum. Scale bar is 1 mm.

age were combined into a single control group in subsequent analyses.

Analysis of variance between Shh, GDNF and control groups revealed significant differences in cell numbers between treatments among the different donor age groups ($F_{6,88} = 12.08$, $p < 0.001$). Post-hoc tests were undertaken using the Newman–Keuls correction for multiple comparisons and indicated that Shh pre-treatment induced a significant increase in cell numbers when compared to controls in the E12 donor age group ($t = 5.57$, $p < 0.01$) and that GDNF pre-treatment caused a significant increase in TH cell numbers with respect to the control treatments in the grafts from E14 and E15 donors ($t_{88} = 7.05$, $p < 0.01$, and $t_{88} = 2.28$, $p < 0.05$, respectively) (see Fig. 8).

Measurement of the concentrations of tissue GDNF using ELISA showed that 2 weeks post-injection of the RAD/GDNF vector, the mean level of GDNF in the injected striatum was 79 ± 35 pg/mg of tissue, over and above the background levels of GDNF detected in Rad35 injected and non-injected striata.

4. Discussion

In the current study, we report on use of adenoviral vectors to deliver the differentiation factor Shh or the trophic factor GDNF, to embryonic VM grafts in a rat model of PD. The strategy of transduction of the dopamine-depleted striatum prior to grafting was successful in delivering the protein products from both transgenes to the implanted tissue. Immunohistochemical staining using antibodies against Shh, GDNF and β-galactosidase showed widespread distribution of transduced cells around the VM grafts and in intimate contact with them. Additionally, Shh

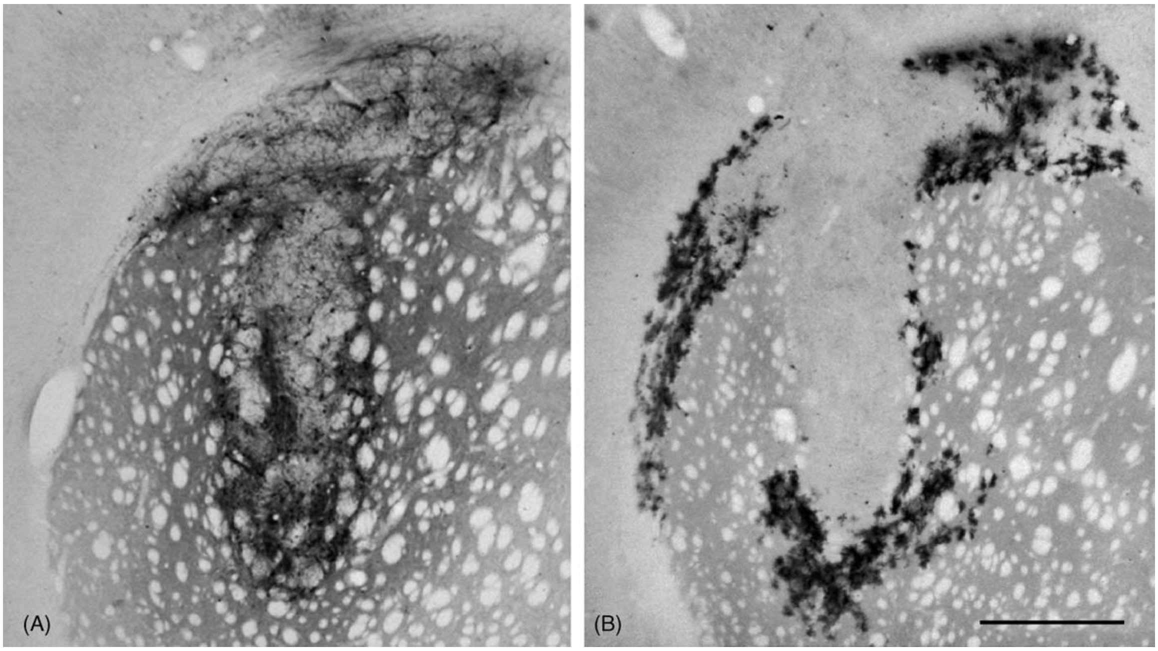

Fig. 4. Histological sections of a VM graft from the RAD35/E12 group. (A) Tyrosine hydroxylase immuno-staining shows a healthy dopaminergic graft with many TH positive neurons and displaying extensive outgrowth into the surrounding striatum. (B) β-Galactosidase immuno-staining on an adjacent section shows LacZ transduced cells in the host striatum and overlying corpus callosum are in intimate contact with the VM graft. Scale bar is 1 mm.

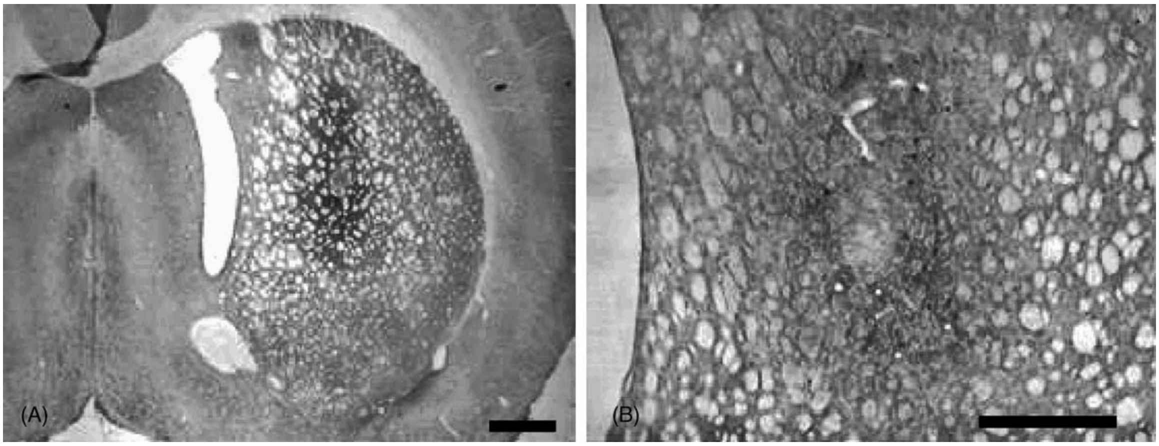

Fig. 5. Expression of transgenes in the grafted striatum following viral vector injections. (A) GDNF expression. Dense immunoreactivity around a small section of graft in a brain from the GDNF/Graft group. (B) Shh expression surrounding a graft in a section from an animal in the Shh/Graft group. Shh staining shows immunoreactive cell bodies as well as diffuse staining of the striatal parenchyma. Unlike staining for β-galactosidase, immunoreactivity for Shh and GDNF is not confined to the periphery of the graft but is also seen within the grafted tissue. Scale bar is 1 mm.

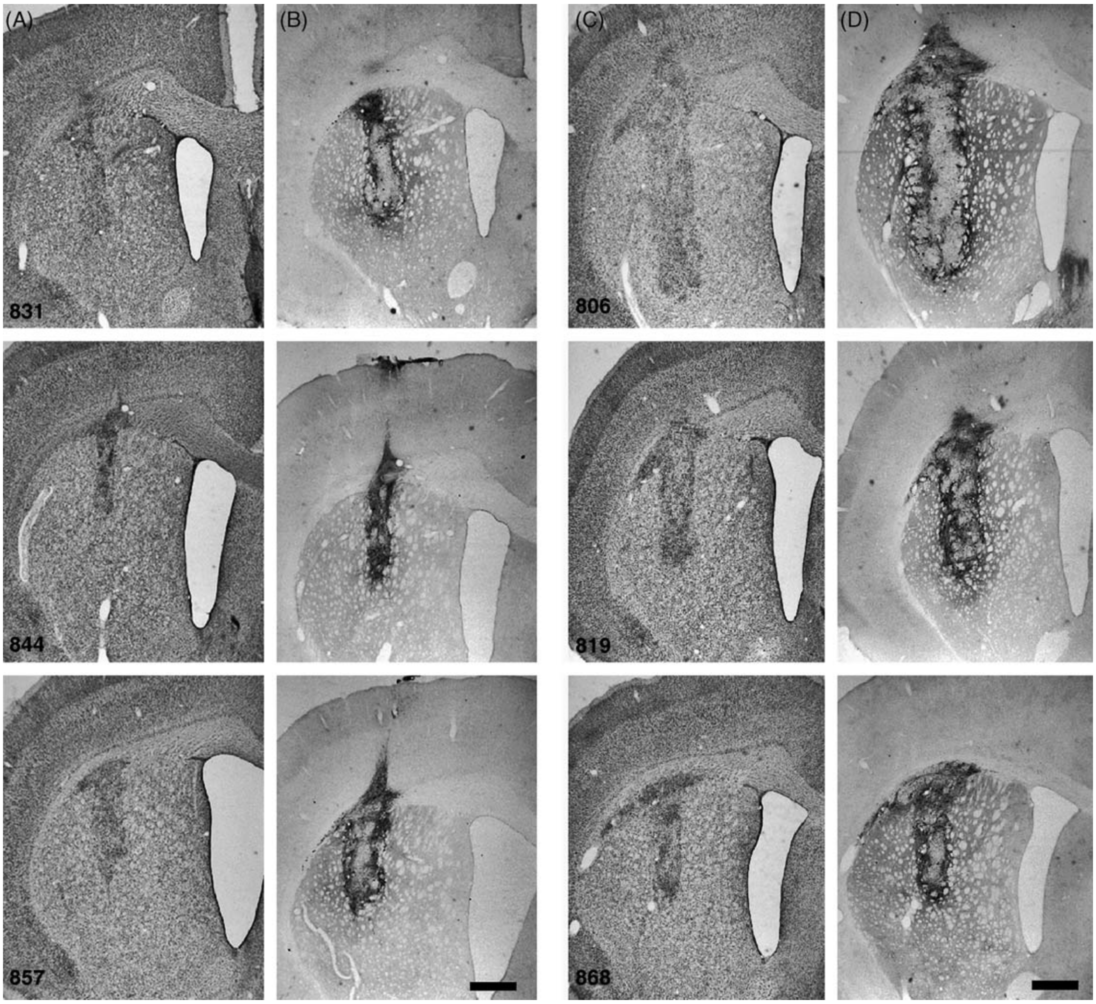

Fig. 6. Representative sections showing E12 donor age grafts from saline (rats 831, 844, 857; A, B) and Shh (rats 806, 819, 868; C, D) treated groups. Cresyl violet (A, C) and adjacent TH (B, D) stained sections. Grafts treated with the Shh vector contained significantly greater numbers of cells than the saline treated group. Numbers represent individual rat numbers. Scale bar is 1.0 mm.

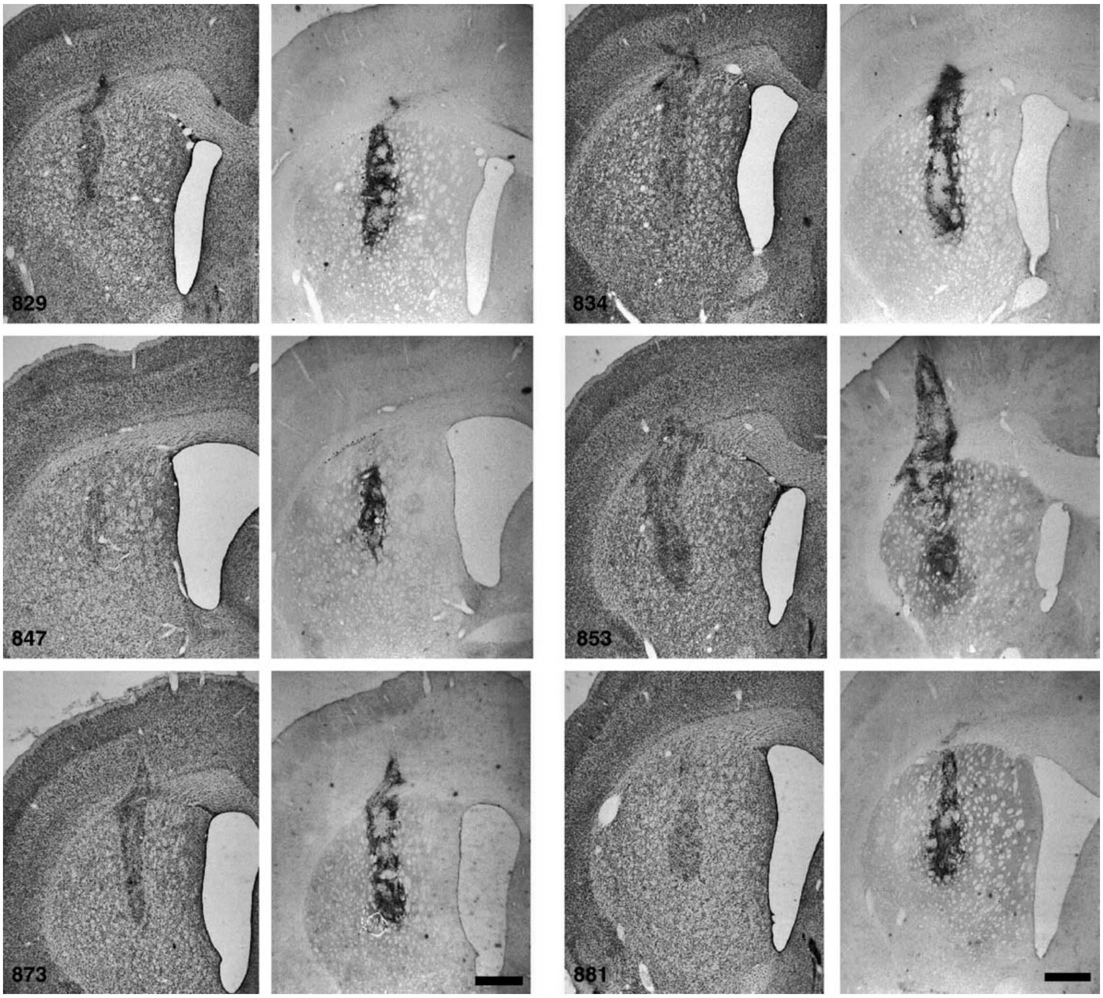

Fig. 7. Representative sections showing E14 donor age grafts from saline (rats 829, 847, 873; A, B) and GDNF (rats 834, 853, 881; C, D) treated groups. Cresyl violet (A, C) and adjacent TH (B, D) stained sections from three rats in each group. Grafts treated with the GDNF vector contained significantly greater numbers of cells than the saline treated group. Scale bar is 1.0 mm.

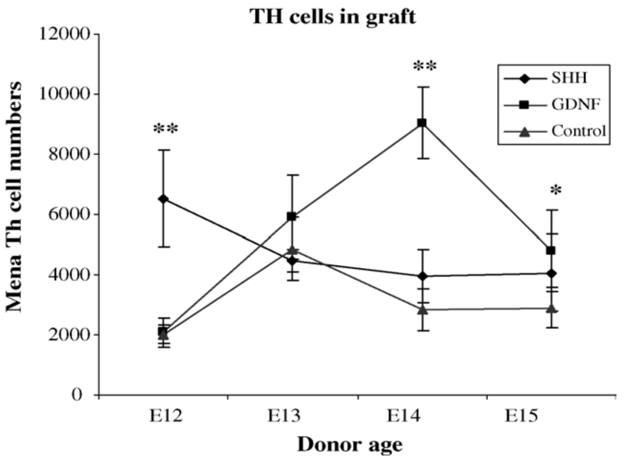

Fig. 8. Plot of estimated mean TH cell numbers in the dopaminergic grafts in each group. There was no significant effect on cell numbers at any age group in the LacZ and saline treated group. However at the E12 donor age, Shh treated grafts contained significantly more cells than all other treatment groups and at the E14 donor age significantly greater numbers of cells were seen in the GDNF treated group. Error bars are S.E.M.s.

and GDNF immunoreactivity could also be observed within the graft tissue itself, indicating diffusion of the proteins into the graft. Both RAD/Shh and RAD/GDNF were able to improve the survival of VM grafts but the effects seen with each vector were dependent on the embryonic donor age of the implants to which they were applied. RAD/Shh caused a significant increase in TH cell numbers in grafts derived from E12 embryos, whilst RAd/GDNF increased TH cell numbers in grafts derived from E14 and E15 embryos.

The use of the transcription factor Shh in the present study was influenced by work carried out in 1999 by Sinclair et al. [62], in which it was shown that virtually all of the surviving TH-positive neurons in transplants from E14 rat embryos had undergone final division in utero, prior to their excision from the embryo. By contrast neurons dividing in the grafts post-implantation, failed to express TH-positive phenotypes. One inference from this work was that the numbers of dopamine cells in E14 grafts was low because the many of the dopaminergic cells implanted had not yet differentiated. Thus, the dopamine “pre-cursor” cells in the grafts failed to develop into the dopaminergic phenotype because of an absence of the correct developmental signals in the adult host brain. In the present study we sought to investigate the provision of factors known to be important in dopaminergic differentiation to dopamine grafts using adenoviral vectors.

A number of important factors involved in the differentiation in dopamine cell have been identified. Sonic hedgehog (Shh) is involved ubiquitously in embryonic development but has been specifically identified as one of the principal factors in the determination of neuronal specification of dopamine neurons in the developing mesencephalon. Shh is expressed in the notochord and floor plate of the developing midbrain as a 45 kDa protein that undergoes proteolytic cleavage into carboxy terminal (Shh-C) and amino-terminal (Shh-N) fragments [46]. The latter Shh-N fragment contains the active signal in dopamine cell development and when applied to E9 ventral mesencephalic explants in culture can increase the yield of dopamine neurons in a dose dependent fashion and the blocking of Shh using specific antibodies is able to block this effect [36]. The mechanism of action is complex but Shh is thought to be primarily inductive for dopamine cell fate and acts in conjunction with other factors including fibroblast growth factor type 8 (FGF8) in the topographic organisation of the developing ventral midbrain [37,55]. Members of the transforming growth factor (TGF) family are also involved in the development of this region. Farkas et al. showed that both Shh and TGF-β were required for DA cell differentiation in vitro and that blocking of either of these factors restricted development of the dopaminergic phenotype. TGF-β2 and β3 and the bone morphogenetic proteins BMP-4 and BMP-7 have also been shown to be involved the development and topography of the ventral mesencephalic dopamine cell groups [23,57].

Whilst Shh is one of the principal factors determining dopaminergic cell fate, fibroblast growth factor-8 (FGF8) may be more important in determining the distribution of dopamine cells along the anterior posterior axis of the developing VM. Thus the development of dopamine neurons in both the substantia nigra and the hypothalamus is thought to rely on intersecting signals along the anterior–posterior (FGF8) and dorso-ventral (Shh axes) [67]. Neither factor alone is sufficient to determine DA phenotype in vivo. However, recombinant Shh alone is sufficient to induce ectopic DA neurons in the dorsal midbrain (outside the area where they normally develop) and it seems that Shh is the main physiological inducer of endogenous DA neurons in the ventral midbrain and ventro-rostral forebrain [73]. Thus, Shh was a logical choice as the differentiation factor chosen for use in the current study.

Expression of Shh in the developing rat embryo is seen between the ages of E9–E16 and thus precedes and overlaps the appearance of dopaminergic neurons in the ventral midbrain, which occurs between the ages of E11–E16 [8]. We might hypothesise from this that grafts derived from donor ages even younger that E12 might provide a source of cells VM grafts that could be treated with Shh or other dopaminergic differentiation factors to produce dopamine cells for transplantation and we are making investigations in this regard. Other workers have used Shh and other differentiation factors to improve dopamine cell survival in vitro [36,47,51].

Application of Shh to primary VM grafts been carried out previously by Yurek et al. who co-implanted Shh expressing fibroblasts with grafts of E14 VM and reported a doubling of TH cell numbers in the Shh treated grafts when compared to untreated controls [75]. This result is at odds with the current work in which there was no effect of Shh treatment on E14 grafts. This is most likely due to a dose effect (see below) but might be accounted for by differences in the grafting protocol used. The grafts implanted in the Yurek study were solid pieces and the overall size of grafts (500–1000 cells) was considerably smaller than those in the present study [75].

GDNF was used as the positive control in the current experiment. GDNF is not expressed in the developing ventral mesencephalon but is a known neurotrophic factor for developing dopamine neurons and is expressed in the developing striatum during embryonic development [42]. In animal models of PD, the direct administration of GDNF protein has been shown to have potent ameliorative and reparative effects, protecting against the effects of dopamine lesions and assisting the partial regeneration of injured dopamine neurons [5,26–29]. GDNF transgenes have also been delivered using gene therapy approaches. GDNF and Shh have been reported to protect dopamine cells against the effects of a dopamine lesion when delivered using AAV [16,48,71] adenovirus (AV) vectors [13,14,35,68] and LV vectors [4,11,31,38]. When administered to embryonic VM grafts, GDNF can improve cell survival, fibre outgrowth from the graft and graft induced behavioural recovery [3]. The beneficial effects of GDNF on E14 VM graft survival have been demonstrated previously by a number of workers primarily using standard E14 VM grafts and GDNF, administered either directly to the graft [22,49,58,74] or indirectly using co-grafted, genetically modified neurospheres [53] or GDNF-secreting encapsulated cells [61]. GDNF has also been shown to have beneficial effects on dopaminergic graft in human patients [50]. Thus, whilst not a novel finding, the efficacy of GDNF in the current experiment demonstrates well, both the further potential of the current vectors for in vivo gene therapy and the effectiveness of the striatal pre-loading approach used in the current study. Because of its known effects on dopamine cells and VM grafts, in the present work, RAd/GDNF was considered a useful positive control for the RAd/Shh treatment.

Adenoviral vectors have previously been investigated as gene therapy tools in animal models of PD. In rat models, adenoviral vectors containing GDNF directly injected into the brain have been shown to ameliorate the effects of 6-OHDA lesions, and reduce the amplitude of behavioural deficits caused by dopamine lesions [4,11,12,15,17,40]. In a strategy aimed at replacing dopamine synthesis in the depleted striatum, Horellou et al. used an adenoviral vector containing the TH gene to partially restore dopamine function in the hemiparkinsonian rat brain [34]. Only one study has reported the use of adenoviral vectors to modify dopamine implants in a fashion similar to that used in the present work. Sanchez-Capelo et al. delivered transforming growth factor beta (TGFβ1) to VM grafts by prior injection of an adenoviral vector into the striatum [60]. Although they found that expression of that particular transgene was detrimental to the survival of ventral mesencephalic grafts, the study was the first to demonstrate the effectiveness of this method of gene delivery. The main considerations in such an approach are that the transgene being delivered by the viral vector should produce a diffusible product, be active in the striatum at the time of implantation and should not induce an inflammatory reaction that might adversely affect survival of the graft. Pilot studies involving direct injection of the current vectors led us to choose 2 weeks post-injection of the vectors as the time point best satisfying these criteria. As in the present work, implantation of the graft at the same coordinates as the injected vector results in intimate association of the VM graft with transduced cells.

5. Conclusion

The improvement of E12 graft survival following treatment with RAd/Shh in the present study is an interesting result. Since at this age very few dopamine neurons have undergone final differentiation, the result indicates that at this stage of development, the differentiation and/or survival of implanted dopamine precursors can be increased by post-implantation exposure to Shh. This corroborates the original hypothesis on which this paper is based, namely that the poor survival of dopamine neurons post-grafting might be enhanced by the application of differentiation factors [62]. However, Sinclair et al. also hypothesised that the survival of E14 grafts might be improved by treatment with differentiation factors. In the current experiment the beneficial effect of Shh was limited to grafts of younger donor age, i.e. to dopamine precursors at an earlier stage in their lineage, and no effect was observed on grafts from donors beyond E13 days of age.

Shh is still expressed in the developing rat embryo up to E16 but at ever decreasing levels with increasing age. Thus, the dose of Shh required for appropriate signalling changes with embryonic age and perhaps it is not surprising that a Shh dose that had a functional effect on E12 grafts had no effect on E13 or E14. Further experiments might be carried out in which different doses of Shh (controlled by injection of various titres of RAd/Shh) are applied to E13 or E14 grafts to improve dopamine cell yield.

Whilst the improved survival seen in the E14 and E15 grafts following treatment with RAd/GDNF is almost certainly due to trophic support of dopamine cells in the graft, the mechanism by which RAd/Shh treatment improves the survival of E12 is not so clear. In the developing embryo, Shh affects the proliferation as well as the differentiation of dopamine precursors and may also have trophic effects on fully differentiated dopamine cells [7,35,59]. Thus, increased cell yields might be the result of enhanced proliferation of dopamine precursors; of enhanced differentiation of precursors into the dopaminergic phenotype; of trophic support of differentiated dopamine neurons; or any combination of these three mechanisms. Future experiments will investigate the characteristics of E12 VM both in vitro and in implants looking at the effects of Shh and other differentiation factors on proliferation, differentiation and survival.

Acknowledgements

This work was funded using grants provided by the Medical Research Council (UK) and the NINDS and National Institutes of Health (USA) Grants NS42893-01, NS44556-01 and U54-4NS04-5309.

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