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Improving the survival of dopaminergic grafts in a rat model of Parkinson's disease

This dissertation is submitted for the degree of Doctor of Philosophy at the University of Cardiff

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I dedicate this thesis to my mother and father,
Pamela and Eduardo Torres.

(They must have done something right)

Thesis Summary

This thesis contains five original manuscripts on the subject of "Improving the survival of dopaminergic grafts in rat models of Parkinson's disease." The use of embryonic dopamine transplants for the treatment of Parkinson's disease is severely hampered by the poor survival of the implanted cells and over the last 10 years, and a great deal of research effort has been carried out worldwide into enhancing the survival rate of such transplants. The biggest advances have been in improvements of the techniques of harvesting, dissection and preparation of the embryonic donor tissue and in the provision of trophic support to the implanted cells.

The current work is based on the hypothesis that embryonic grafts contain immature dopamine neurons that fail to mature in the host brain, but that might be induced to do so by the provision of differentiation factors to the graft. Adenoviral vectors have been used to successfully deliver the differentiation factor sonic hedgehog (Shh) to dopamine grafts, and to greatly enhance the numbers of dopamine cells seen in the grafts. An exciting finding has been that dopamine grafts derived from very young embryonic donors (1-2 days younger than any previously investigated) produce dopamine grafts that, on average, contain five times more dopamine cells than conventional grafts. This finding supports the original hypothesis, as increased survival is thought to be due to the presence in the graft of cells capable of providing differentiation factors to the developing dopamine neurons.

The remaining three manuscripts deal with issues directly related to graft survival and the use of gene therapy and animal models of PD, looking at the dynamics of viral vector gene expression in the pathological brain, an investigation of the two-layer staining obtained on immunohistochemical stained sections, and a re-assessment of the amphetamine induced rotational response of dopamine grafted animals.

A note on the format of this thesis

Following a request from the thesis supervisor Professor Stephen Dunnett, to the tutor for Bioscience graduate students, Dr. Roy Richards and the assistant registrar Amanda Rose, it was agreed that the present work could be submitted as a collection of self-contained manuscripts which have either been published, are in the process of being published or are being prepared for publication. This format is used by other European countries and has several advantages. Firstly, it encourages planning and execution of experiments in a format that is suitable for publication. Secondly, any published work contained in the thesis is citable so that future experiments arising from or related to the work are able to make proper reference to it. The present format exposes the candidate to the submission procedure for scientific publications with the associated rigors of peer review and provides valuable experience in this regard. The scientific process involves research leading to new knowledge that must be communicated. The normal route for this is through scientific publication and it might be considered that scientific training should involve not only the conduct of experimental work but its analysis and communication through publication also.

The general introduction and discussion are presented in the conventional thesis format but are somewhat reduced in length as both the detailed methodologies and discussions are included in each of the manuscripts. The introduction also contains a section summarizing the work contained in this thesis.

Because of the nature of both the work, and the process of scientific reporting, all of the manuscripts herein have multiple authors. The introduction to each chapter contains an additional section outlining specifically which sections of the work contained in each manuscript are the work of the main author and which are derived from the input of others.

Finally, following the manuscript section is an appendix which contains a transcript of a book chapter; "Gene therapy for Parkinson's disease" written by the author, with the help of colleagues and which is in press at the time of writing.

Acknowledgements

First and foremost, my thanks go to my supervisor Steve Dunnett who has been both friend and mentor, not just for the duration of this thesis, but for many years previously. It was his move to Cardiff University that created the opportunity for me to join his team once again and it was he who encouraged me to engage in study for a doctorate after years of conducting scientific study without one. My thanks also to my advisor Rose Fricker-Gates for her steady guidance and friendship, once again over years extending beyond the brief interval of my PhD study.

Thanks to Christelle Monville who has been my closest collaborator over the last three years and without whose help and advice my work would have been much the poorer. Thanks also to my colleagues and office companions Simon Brooks, Eilis Dowd and Rike Zietlow, who have provided support, advice and inspiration when it was required, and to the entire Brain Repair Group who have provided an exciting and stimulating atmosphere in which to work. I am also extremely grateful to Pedro Lowenstein and Maria Castro who provided the adenoviral vectors used in the current work together with guidance and encouragement in the experiments that were carried out with these.

Last but definitely not least, thanks to my wife Charlotte, daughter Georgia and son Matthew, my loving family who helped in a hundred different ways but particularly by keeping the noise down.

E. M. Torres
April 2005

Contents

Summary of papers presented in this thesis

Summary of additional publications

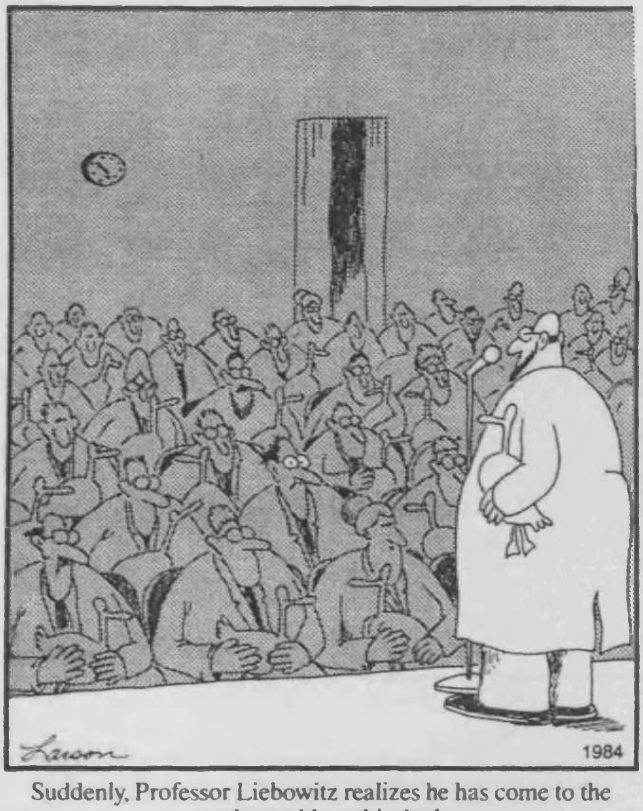
Because of the nature of working within a close-knit scientific research group, the author has been involved in numerous projects, outside the main work of this thesis. A number of publications have arisen from this work in addition to those listed above, and these are shown below.

Spatially and temporally restricted chemo attractive and chemo repulsive cues direct the formation of the nigro-striatal circuit.
Gates MA, Coupe VM, Torres EM, Fricker-Gates RA, Dunnett SB.
Eur J Neurosci. 2004 Feb; 19(4):831-44

Latency associated promoter transgene expression in the central nervous system after stereotaxic delivery of replication-defective HSV-1-based vectors.
Scarpini CG, May J, Lachmann RH, Preston CM, Dunnett SB, Torres EM, Efstathiou S.
Gene Ther. 2001 Jul;8(14):1057-71.

HSV vector-delivery of GDNF in a rat model of PD: partial efficacy obscured by vector toxicity.
Monville C, Torres E, Thomas E, Scarpini CG, Muhith J, Lewis J, Finn J, Smith C, Cai S, Efstathiou S, Howard K, Dunnett SB.
Brain Res. 2004 Oct 22; 1024(1-2): 1-15.

Neural Transplantation. (Invited Chapter in "Handbook of Experimental Neurology: Methods and Techniques in Animal Research")
Stephen B Dunnett, Eduardo M Torres, Monte A Gates
and Rosemary A Fricker-Gates
T Tatlisumak & M Fisher (eds)
Cambridge University Press, 2004.



Suddenly, Professor Liebowitz realizes he has come to the seminar without his duck.

When compiling this thesis I was tempted to do as many others have done before, and insert inspirational quotations from famous people. Somehow that didn't feel right and I plumped instead for the above cartoon. Gary Larson is one of the greatest cartoonists ever. The above cartoon is not only one of my favourites but also says something about the way I sometimes feel in the world of academia, surrounded by clever, brilliant people and thinking "what am I doing here?"

Chapter 1

General Introduction

1.1 Parkinson's Disease

In 1817, John Parkinson M.D. published his first and as it transpired, only medical paper, entitled "An Essay on the Shaking Palsy." [1] Almost 200 years later, the shaking palsy, now known universally as Parkinson's disease, is well characterized and understood. Parkinson's disease (PD) is a degenerative disease of the central nervous system generally (but not exclusively) associated with advancing age. It is a progressive disease in which the main symptoms increase in severity with time and become increasingly difficult to treat. Clinically, the symptoms are primarily motor in nature, involving bradykinesia (an inability to initiate movement), muscle rigidity and tremor of limb muscles when at rest. (For a comprehensive review see Poewe 1996).[2] These motor abnormalities are associated specifically with a loss of dopamine (DA) innervation to the striatal area of the brain (consisting of the putamen and caudate nucleus), as a result of loss of dopamine cell bodies in the afferent substantia nigra compacta (SNc). It is known that, because of compensatory mechanisms (mainly the up-regulation of DA synthesis and an increase in both the numbers and sensitivity of DA receptors in the striatum), clinical symptoms are not manifest until the cellular pathology is relatively advanced. By the onset of clinical symptoms patients have typically lost 80-85% of dopaminergic neurons in the SNc and have a 60-80% reduction in striatal dopamine levels.[3,4]

The aetiology of Parkinson's disease is less well understood. 80-90% of cases are "idiopathic," that is, the underlying cause of the disease is unknown. In the remaining cases it is possible to identify either underlying genetic components (so called "familial parkinsonism") or environmental causes such as exposure to toxins. In the late 1970s for example, a number of Los Angeles drug users were accidentally exposed to 1-methyl, 4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a contaminant in the synthetic heroin they were using. The result was an acute and severe form of Parkinsonism, resulting from massive DA cell loss in the SNc of these patients. More recently, a related toxin rotenone, which is commonly used in household pesticides, has been implicated epidemiologically with the degeneration of dopaminergic neurons. In animal studies, rats exposed to chronic levels of rotenone develop not only degeneration of the SNC dopamine neurons but other Parkinson's disease related histopathology such as Lewy bodies.[5] Evidence from other animal studies using MPTP led to a theory of mitochondrial dysfunction in PD. MPTP toxicity is effected via an

active metabolite 1-methyl-4-phenylpyridinium (MPP+) which inhibits mitochondrial NADH-linked oxidation.[6] Post-mortem studies have since confirmed the presence of mitochondrial dysfunction in PD patients.[7] Increased levels of lipid peroxidation have also been demonstrated in PD brain, implicating free radicals in the pathology of dopamine cell death.[8] Failure of the endogenous mechanisms for the scavenging of free radicals may be implicated in the disease aetiology and decreased levels of the free radical scavengers catalase and glutathione have been demonstrated in PD patients.[9] It has been shown that tobacco smoking and high caffeine intake are associated with a lower risk of PD,[10-12] though the mechanisms underlying this protective effect are unclear.

Mitochondrial abnormalities have been found in PD patients, specifically defects in the mitochondrial DNA-encoded complex 1 activity of the electron transport chain.[13] Other genetic abnormalities have been associated with PD, most notably α-synuclein upon which much research is now focused, but also Parkin, UCHL1 (ubiquitin carboxy terminal hydrolase L1) and Tau protein.[14]

Whilst there are clearly many aspects to the aetiology of Parkinson's disease, the precise disease process has yet to be determined. However, the relatively circumscribed outcome of the disease pathology, namely the loss of DA neurons in the SNc, makes Parkinson's disease a good candidate for therapeutic intervention. The discovery in the 1950s that administration of the dopamine precursor L-3, 4-dihydroxyphenylanaline (L-DOPA) could reverse a similar dopamine-denervation syndrome in experimental animals,[15] rapidly led to the clinical application of L-DOPA. When administered orally, together with the peripheral decarboxylase inhibitor carbidopa, L-DOPA proved an effective therapy for this previously untreatable condition. Subsequent innovations have increased the potency and efficacy of L-DOPA therapy, such as the development of slow releasing forms of the drug, as well as use of other dopamine agonists such as bromocriptine. Since the mid nineteen seventies, countless thousands of Parkinson's sufferers have benefited from the treatment and to this day, L-DOPA remains the mainstay of treatment for PD.

However, despite the obvious benefits, pharmacological treatment of Parkinson's patients has serious limitations. It is not curative and the underlying disease progresses inexorably with time. In the early stages of the disease, a proportion of the degenerating dopamine neurons still survive and these contain the enzymes

necessary to synthesize dopamine from L-DOPA once it arrives at the target area. In addition, there are intrinsic mechanisms which compensate for the progressive decrease in dopamine levels.[16] There is up-regulation of the numbers of striatal dopamine receptors and an increase in dopamine receptor sensitivity. There is also down-regulation of dopamine degradation in nerve terminals. Together, these compensatory mechanisms have the overall effect of increasing the efficacy of smaller and smaller amounts of endogenous dopamine as the levels decrease with time. However, as the disease progresses, the effective window of therapy progressively narrows and patients need increasing and more frequent doses of L-DOPA to obtain therapeutic benefit. Eventually, the ameliorative properties of the drug become complicated by the development of debilitating side effects, mainly in the form of drug-induced dyskinesias and on-off fluctuations in drug response.[17] These typically develop after 5-10 years of drug therapy and arise from the interaction of the increasing drug load, in particular the peak and trough nature of drug levels in the blood, with the increased dopamine-sensitivity of the brain. Continuous intravenous delivery of low dose L-DOPA has been shown to dramatically reduce drug induced dyskinesias, but is in itself not a practical therapy for a predominantly elderly out-patient population.[18] In most cases, patients reach an end-stage of the disease when the side effects of therapy come to outweigh the benefits and drug treatment becomes increasingly problematic. In the latter stages of the disease patients can find themselves having to choose between being "off," rigid and immobile, in the absence of L-DOPA or coping with severe dyskinesia in the "on" phase following L-DOPA administration. L-DOPA treatment can be enhanced or prolonged by treatment with dopamine agonists such as apomorphine, but once again, the therapeutic window of these drugs is limited by the progress of the disease. Because of this, there is an urgent clinical need for treatments that are alternative and/or supplementary to conventional drug therapies.

1.2 Neural Transplantation and Parkinson's disease

One of the most promising and most studied alternative therapies has been the replacement of lost dopaminergic neurons by the implantation of dopamine-producing cells derived from the embryonic ventral mesencephalon (VM). This approach relies on the assumption that if Parkinson's disease pathology is primarily attributable to degeneration of the dopamine neurons of the substantia nigra, then transplantation of replacement dopamine neurons into the affected area should result in recovery of dopamine dependent function. The first grafts of embryonic dopamine cells into rat brain used solid pieces of VM tissue implanted into cortical cavities.[19-21] Survival of the implanted dopamine cells in the host brain was demonstrated, together with graft-derived behavioural recovery in simple tests of motor asymmetry. Shortly after, techniques for preparing the embryonic VM as a cell suspension, prior to implantation were developed, allowing implantation of the tissue directly into the de-afferented striatum.[22] In these studies functional recovery was shown to be dependent on the topographical placement of the graft tissue as well as the reinnervation of the host striatum by the implanted dopamine cells. It was shown that the implanted dopaminergic cells, mature and send out axons into the host tissue.[23] They make appropriate synaptic connections and more importantly, deliver dopamine to the target tissue.[24-26]

Embryonic dopamine tissue allografts into human subjects were first carried out in the mid 1980s with limited success,[27-29] but improvements in grafting techniques yielded substantial improvements in the therapy and with the first cases of significant graft survival and functional benefit reported in 1990 [30] several clinical centres have succeeded in producing long-lasting functional recovery in Parkinson's patients following embryonic dopamine cell implants.[31,32] There have been failures as well as successes and the technique has had both detractors and supporters. However, there is little doubt that, in a definable subset of patients, transplants derived from donor embryos of the correct age and implanted using a robust surgical protocol, are able to provide considerable therapeutic benefits. In successful cases the implanted dopamine cells provide a continuous low dose of dopamine to the denervated striatum in a manner that eliminates the peak-trough regime of orally administered L-DOPA. Patients are able to reduce their intake of L-DOPA and other drugs dramatically to a

fraction of the previous dosage and in some cases drug treatments can be stopped altogether. The associated side affects are much reduced or eliminated altogether. Long term studies of these patients have shown that transplantation therapy is long lasting and that it has the potential to extend treatment of PD patients well beyond the therapeutic window provided by L-DOPA.[33,34] Clinical effects take the form of improvements in motor test of 30-50%, more time spent in the "on-phase" without debilitating motor symptoms and in many patients, a reduction in the doses of drugs needed to control the symptoms.[35-38]

One high profile study has thrown doubt on the potential use of dopamine transplantation for routine treatment of Parkinson's disease. In a double blind-placebo controlled study, Freed and colleagues reported limited benefits of grafts and a subgroup of patients that developed graft-induced dyskinesia, independent of L-DOPA therapy and related to high levels of dopamine from the grafted tissue.[36] Whilst the importance of the reported side effects should not be underplayed, dyskinesias were seen in a minority of patients (5 out of 33), chiefly of young age. The nature of the dyskinesia problem is still not clearly understood and others have challenged the conclusions of the report, suggesting that factors other than the amount of dopamine supplied to the brain might be responsible.[39] However, a second double-blind placebo controlled clinical trial reported no significant effects of dopamine grafts over placebo controls and also reported the development of "off-medication" dyskinesias.[40] Clearly, we need to understand more fully the origin of graft-induced dyskinetic effects [41] and a good models in rats has recently been described.[42] Moreover we will need a greater understanding of which patients are likely to be susceptible, so that in the short term, better criteria for inclusion and exclusion in clinical trials can be developed and in the long-term therapies designed that maximize the benefit and avoid the causes of the side effects in each individual patient.

Despite its successes, transplantation therapy is not widely or routinely used as a therapy for PD. The reasons for this are less to do with efficacy of the treatment itself and more to do with the complexity of the ethical, logistical and quality control problems that surround the use of foetal donor tissues. [43,44] These issues arise in part from one of the major, and long-standing problems in the use of foetal transplants in PD, namely the relatively poor survival of the implanted, dopaminergic neurons in the

host brain. In animal models, although survival rates of 20% and even 40% have been reported (in experiments where trophic factors have been used to enhance graft survival), typically only 5-10% of implanted embryonic dopamine neurons survive in the host brain.[45]

This problem can be overcome in animal models by the use of increased amounts of donor tissue, and a clear correlation has been shown between the number of dopaminergic cells surviving implantation and the degree of functional recovery in these models.[46] However, in the clinic, the necessity for donor tissue from multiple embryos imposes problems, which are greatly limiting the use of this form of therapy. Up to six embryos per patient, per side of the brain are now considered to be necessary for optimal therapy.[47] The moral, ethical and logistical issues of obtaining and using such tissue are obvious and are currently limiting the potential availability of transplantation as a therapy for PD.

1.3 Improving graft survival

Theoretically, if the survival of implanted dopamine cells could be routinely boosted from 10% up to 50% or more, then it is conceivable that optimal therapy might be achieved using a single embryo per patient, per side and the problems surrounding the use of human embryonic tissue for the treatment of PD would be much diminished. To this end, laboratories throughout the world are working to improve dopamine graft survival, using a variety of different approaches. Brundin et al reviewed the broad range of current work on improving the survival of grafted dopaminergic neurons, [45] and it is worth summarizing this work here. When addressing the question "when do transplanted neurons die?" they suggested four likely time points:

1. Excision of the embryo from the uterus. At this time the donor tissue is removed from it's maternal blood supply and hypoxic and hypoglycaemic events might lead to oxidative trauma
2. Excision of the ventral mesencephalon from the embryo. At this time afferent axons are severed and there is inevitably trauma associated with the dissociation of the tissue into a single cell suspension.

3. Implantation of the donor tissue into the host environment. This is known to be a period in which many of the implanted DA neurons disappear. Likely causes could be stresses caused to the host environment due to surgical trauma and the possible expression of inflammatory cytokines at this time.
4. Once implanted, it is possible that the trophic support necessary for the survival and maturation, growth or integration of implanted DA neurons is absent in the host environment.

Based on this four-phase hypothesis of DA cell death Brundin et al went on to suggest four strategies for the improvement of graft survival.

1. Changes in the techniques of preparation of DA grafts. In fact, the history of DA cell transplantation has seen much improvement in this regard. The move towards more physiological and nutritional media when handling the donor tissue has been one such advance. Changes in the methods of dissociation and of the enzymes used in this process have also led to better graft survival.[48-50]
2. Changes in the methods of implantation of dopamine cell suspensions. The conventional method of grafting in rat models of Parkinson's disease involves implantation of the cell suspension, using a 20 gauge Hamilton syringe, as a single placement in the striatum. Nikkhah et al developed a technique they called micro grafting in which glass micropipettes were used to implant the dopamine cell suspension as multiple small deposits (micro-grafts). They showed apparently higher rates of survival and a greater degree of innervation of the host striatum than that seen in conventional, single placement grafts. [4]
3. Targeting of cell death mechanisms using anti-apoptotic (e.g. caspase inhibitors), or antioxidant (e.g. lazaroids) approaches, or by alleviation of excitotoxicity (e.g. using calcium channel antagonists).
4. Provision of trophic factors to the implanted cells. The most successful of these has been GDNF, but FGF, NT4/5, and GDF5 have also been shown to be effective.

The current thesis also considers an observation that is not covered in the above synopsis. Sinclair et al,[51] demonstrated that the surviving population of dopamine

neurons in transplants from E14 rat embryos consisted almost entirely of neurons which had undergone their final cell division prior to excision from the embryo. Sinclair and co-workers used the marker of cell division bromo-deoxyuridine (BrdU) to label dividing cells in the rat, embryonic ventral mesencephalon at different time points. Injection of BrdU into either the pregnant dam or into the graft host after implantation resulted in labeling of cells that were undergoing division at the time of injection. They then studied DA implants derived from these tissues and determined the proportion of BrdU and tyrosine hydroxylase positive cells present in the grafts (see figure 1).

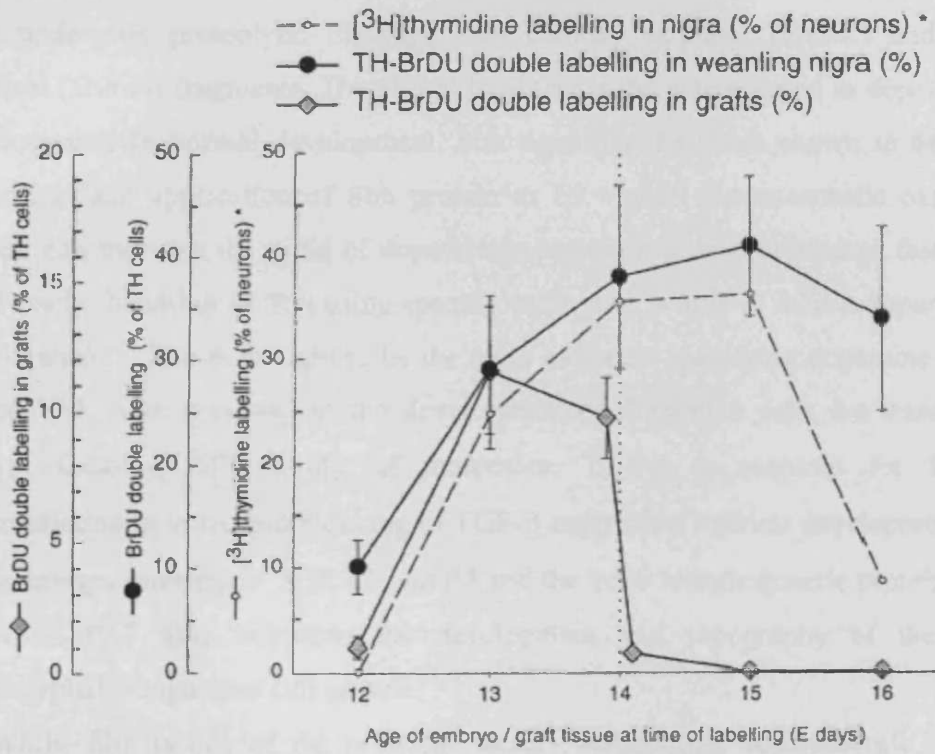


Figure 1. Birth dates of dopaminergic neurons in the developing substantia nigra or in E14 nigral grafts, as determined by TH-BrdU double labeling (from Sinclair et. al. 1999). Birth dates of DA cells during normal development (black circles) were assessed by in-utero injection of BRDU at E12 to E16 days gestation. Birth dates of grafted cells (grey diamonds) were assessed by labeling some grafts in-utero (E12-E14) and by labeling grafts in situ in the host brain after implantation (equivalent ages E14-E16). Each plot depicts the mean proportion (%) of TH neurons, which were BrdU positive. Dashed line represents previously published data of birth dates of DA neurons assessed by in-utero thymidine labeling (Altman et al 1981).[5]

The main conclusion drawn from this work was that the numbers of dopamine cells in such grafts were low, not just because dopamine cells failed to survive implantation, but because the majority of the dopaminergic cells implanted had not yet differentiated, and were unable to find the correct developmental signals in the host brain or to develop into the dopaminergic phenotype. If this hypothesis is correct then

in theory, it should be possible to provide implanted dopaminergic cells with the differentiation factors needed to enable them to complete their differentiation.

When considering which factors might be the best candidates for application to dopamine grafts, there are a number that have been specifically identified as differentiation factors for dopamine neurons. Chief among these is Sonic Hedgehog (Shh), which 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 floor plate of the developing midbrain. It is synthesised as a 45kd protein, which then undergoes proteolytic cleavage into carboxy terminal (Shh-C) and amino-terminal (Shh-N) fragments. The Shh-N fragment is the active signal in dopamine cell development. In normal development, Shh signalling has been shown to be contact dependent and application of Shh protein to E9 ventral mesencephalic explants in culture can increase the yield of dopamine neurons in a dose dependent fashion.[52,53] Conversely, blocking of Shh using specific antibodies is able to inhibit dopamine cell development.[54] Shh is thought to be the main molecule specifying dopamine cell fate in the VM. Also involved in the development of dopamine cells are transforming growth factor (TGF) family of molecules. TGF-β is required for DA cell differentiation in vitro, and blocking of TGF-β expression restricts development of the dopaminergic phenotype. TGF-β2 and β3 and the bone morphogenetic proteins BMP-4 and BMP-7 also influence the development and topography of the ventral mesencephalic dopamine cell groups.[55,56]

Whilst Shh is one of the principal factors determining dopaminergic cell fate, fibroblast growth factor-8 (FGF8) is involved in determining the pattern of distribution of dopamine cells along the anterior posterior axis of the developing VM, and development of dopamine neurones in both the substantia nigra and the hypothalamus relies on intersecting FGF8 and Shh signals along the anterior-posterior and dorso-ventral axes respectively,[57] although 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 and Shh is the main physiological inducer of endogenous DA neurons in the ventral midbrain and ventro- rostral forebrain.[58] For this reason Shh was the differentiation factor chosen for investigation in the current thesis.

Also used in the current work was the dopamine active neurotrophin glial derived neurotrophic factor (GDNF). This factor is not involved in the development of the ventral mesencephalon but is a proven neurotrophic factor for developing dopamine neurons and is expressed during embryonic development in the developing striatum.[59] GDNF has been used in animal models of PD where direct administration of the protein has potent ameliorative and reparative effects.[60-62] Using gene therapy methods, GDNF transgenes have also been used in PD models. GDNF protects dopamine cells against the effects of a dopamine lesion when delivered using adeno-associated virus (AAV) vectors,[63,64] adenovirus (AV) vectors[65,66] and lentivirus (LV) vectors.[67-70] It has also been implicated in dopamine graft survival and when administered to embryonic dopamine grafts, cell survival, fibre outgrowth from the graft and graft induced behavioural recovery are all enhanced.[71] In the present thesis, RAd/GDNF was considered a useful positive control to assess the efficacy of the adenoviral vectors and to provide a suitable baseline against which to compare the effects of RAd/Shh treatments.

1.4 Gene therapy for Parkinson's disease

PD presents a tantalising target for gene therapy mainly because the core pathology arises from the degeneration of a single group of cells the consequences of which are manifested in a circumscribed and well-characterised neural circuitry. Although the aetiology of the disease is unknown, the root cause of the clinical pathology is known to be the death of dopamine containing cells in the substantia nigra pars compacta (SNc) and the subsequent loss of dopamine innervation to the principal target area, the striatum. The failure of the dopaminergic input to the striatum results in up-regulation of the gamma-amino-butyric acid (GABA) output from this nucleus to the globus pallidus pars externa (GPe). As a consequence, there is down-regulation of the GABA output of the GPe to both subthalamic nucleus (STN) and the substantia nigra pars reticulata (SNr), resulting in disinhibition of both these nuclei. The resulting up-regulation of the glutamatergic output of the STN causes over excitation of efferent areas including the SNr and globus pallidus pars interna (GPi). It is the up-regulation of these two nuclei in particular which is thought to account for the characteristic symptoms of PD, in particular tremor, rigidity and bradykinesia.[72] (See PD affected areas in Fig. 2)

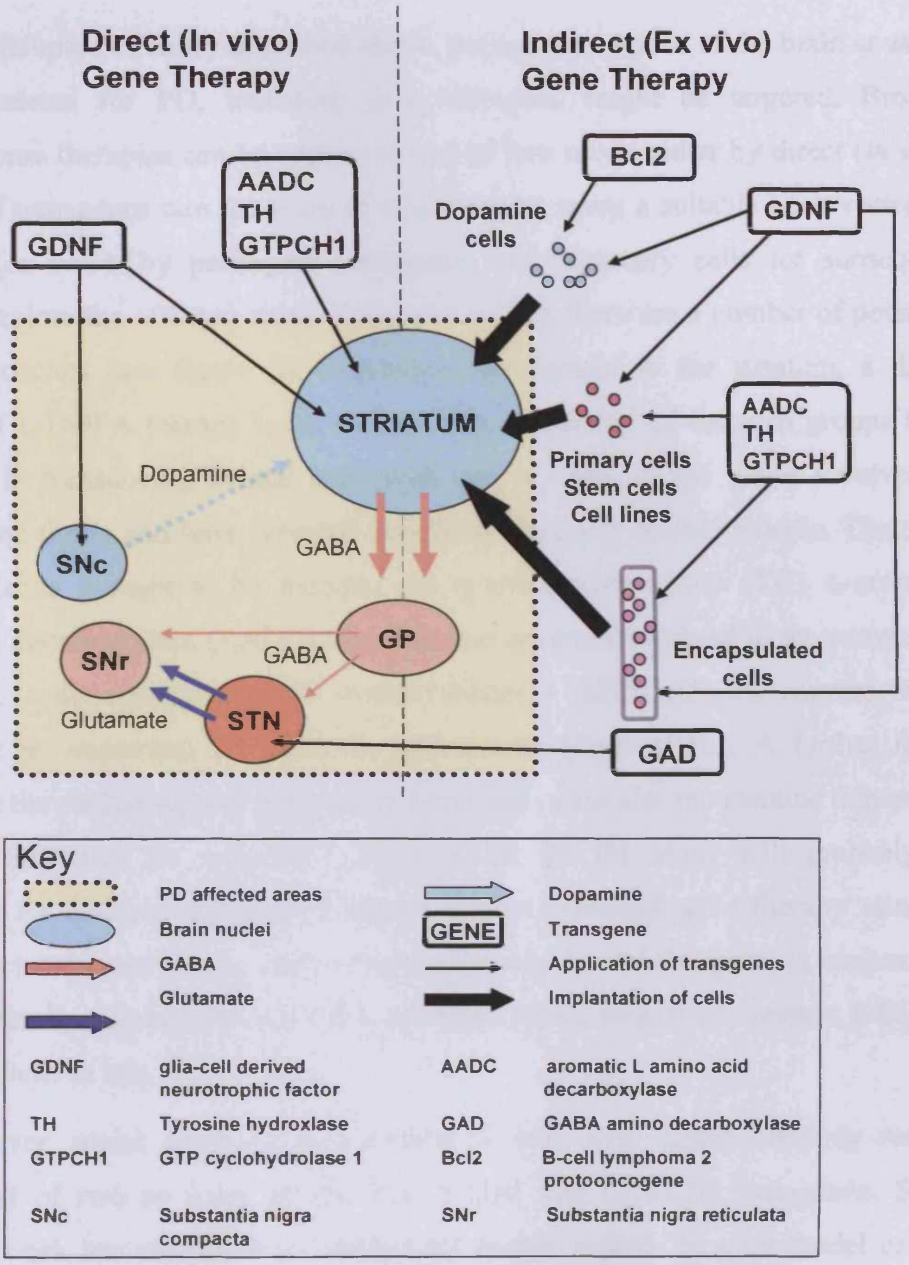


Figure 2. Targets for gene therapy in PD. Brain areas affected by PD are shown in the yellow panel. There is a degeneration of the nigrostriatal dopamine pathway (dashed blue arrows) and up regulation of GABAergic output from the striatum to the external segment of the globus pallidus (double orange arrows). This causes down regulation of GABAergic output from this nucleus (thin orange arrows) and in turn, up regulated glutamatergic output from the STN to the SNr and Gpi (pink arrows). Strategies for CNS gene therapy (shown outside the yellow panel), may be 'direct' (left), where transgenes are injected directly into the host brain; or 'indirect' (right), where transgenes are introduced into accessory cells before implantation into the host brain. Genes of interest might be targeted at a number of affected areas. Direct application of neurotrophic genes such as GDNF to either the striatum or SNc could be used to rescue the degenerating dopamine system. Alternatively, dopamine synthesis might be established in the striatum by the introduction of genes for TH, AADC and GTPCH1. Another direct approach might be to down-regulate the overactive STN by the introduction of a gene for GAD to enable synthesis of GABA in this nucleus. Indirect approaches fall into two main categories. Modification of primary cells or modification of cell lines. The survival of primary dopamine grafts might be enhanced by the introduction of GDNF or of anti-apoptotic genes such as Bcl2. GDNF and the dopamine synthetic enzymes TH, AADC and GTPCH1 might be introduced into primary cells such as host astrocytes, stem cell lines or cells contained within polymer capsules prior to implantation into the host striatum.

The disrupted circuitry described above, defines the regions of the brain at which novel treatments for PD, including gene therapies, might be targeted. Broadly speaking, gene therapies can be applied in one of two ways: either by direct (*in vivo*) injection of transgenes into the brain or blood stream using a suitable gene vector, or indirectly (*ex vivo*), by packaging transgenes into accessory cells for subsequent implantation into the affected areas. In the case of PD, there are a number of potential direct approaches (see figure 2). Dopamine replacement in the striatum, a direct correlate of L-DOPA therapy is an obvious one. A number of research groups have succeeded in transducing striatal cells with one or more of the genes involved in dopamine synthesis and have reported beneficial effects in animal models. The three principal genes thought to be required are tyrosine hydroxylase (TH), L-aromatic amino acid decarboxylase (AADC) (the first two enzymes involved in the conversion of tyrosine to dopamine) and GTP cyclohydrolase 1 (GTPCH1) the enzyme which synthesises an important TH cofactor, tetrahydrobiopterin (BH_4). A further factor involved in the packaging and transport of dopamine, vesicular monoamine transporter (VMAT), may also be required.[73] Transduction of TH alone will probably be insufficient for de-novo dopamine synthesis *in vivo*, although gene therapy using an adeno-associated virus (AAV) vector containing only the AADC gene, in conjunction with peripherally administered L-DOPA has been shown to partially reverse 6-OHDA induced deficits in rats. [74,75]

However, novel synthesis of L-DOPA *in vivo* will almost certainly require transduction of two or more of the TH, AADC and GTPCH1 transgenes. Some promising work has already been carried out in this regard. In a rat model of PD, Mandel and colleagues showed that injection into rat striatum of AAV vectors containing genes for both TH and GTPCH1 led to demonstrable L-DOPA production *in vivo* over long periods of time, and was able to ameliorate the deficits produced by the 6-OHDA lesions.[76] Kirik et al used a TH-containing AAV vector in combination with BH_4 in a partial lesion model to show good recovery on a range of behavioural tests.[77] Transduction of TH and AADC using a Herpes simplex (HSV)-based vector was carried out by Sun et al who succeeded in partially reversing apomorphine induced rotation and showed up to 10,000 TH/AADC positive cells in the striatum seven months after treatment.[78]

Transduction of TH, AADC and GTPCH1 in combination will likely be the optimal approach for dopamine replacement therapy, and several methods of gene delivery have already been investigated. AAV vectors have been used to introduce genes for TH, AADC and GTPCH1 into rat [79] and monkey [80] striatum, resulting in amelioration of lesion induced deficits. Azzouz and colleagues used a tricistronic lentivirus vector (containing 3 transgenes in sequence) in a rat model with comparable results.[81,82]

An alternative to the dopamine replacement strategy is the provision of neurotrophic factors to the Parkinsonian brain in an attempt to halt or reverse dopamine cell degeneration. Glial cell line derived neurotrophic factor (GDNF) is a prime candidate for a PD gene therapy. Direct administration of GDNF protein has been shown to have potent ameliorative and reparative effects in both rodent and primate models of PD,[60-62,83] and a report of efficacy in a pilot clinical study has attracted particular interest.[84] In animal models, GDNF gene therapy has been reported to protect dopamine cells against the effects of a dopamine lesion when delivered using AAV,[63,64] AV,[65,66] and LV vectors.[67-70] GDNF has also been used in combination with the anti-apoptotic Bcl-2 delivered using an HSV vector and found to be effective in protecting against the effects of 6-OHDA.[85] Given the progressive nature of PD, it is likely that any growth factor therapy will need to be administered continuously over a long period of time in order to sustain dopamine neuron survival and function in the long term. Moreover, since GDNF does not cross the blood-brain barrier and GDNF receptors are widely distributed throughout the body, delivery will need to be directly into the brain. Because the primary cellular pathology of PD is confined mainly to the dopaminergic neurons of the substantia nigra, supplying therapeutic genes to these neurons by injection of viral vectors into localised sites in the forebrain is the most obvious strategy. Recent trials involving direct infusion of GDNF into the brain of PD patients, via implanted cannulae, have shown mixed results. GDNF treatment showed initial promise in both pre-clinical and clinical studies,[86] However a subsequent double-blind, placebo controlled study was unable to demonstrate clinical effects,[87] As a result of which a third clinical trial was halted.[88] However a post mortem examination of a patient from the study (who died of unrelated causes) showed neuronal sprouting that was attributable to the GDNF treatment and further trials are under consideration.[89]

An innovative strategy by Luo and colleagues targeted the deep brain areas at which electrical stimulation has been directed. They used AAV vectors to introduce the GABA-synthetic enzyme glutamic acid decarboxylase (GAD) into the subthalamic nucleus in an attempt to inhibit the output of this region. Following success in animal models, the go-ahead has now been given for a clinical trial using this approach in patients.[90]

In the *ex vivo* gene therapy approach, accessory cells are used as vehicles for the introduction of transgenes into the brain. Vectors similar to those used in direct therapies can be used to engineer primary cells, cell lines or stem cells, which can then be implanted into the target areas. There are several advantages to this approach. Firstly, the host tissues are not directly exposed to potentially harmful viral vectors and so safety and toxicity issues are of less concern, in particular since the cells can undergo detailed safety assessment in man. Secondly, with few exceptions, implanted cells stay at the site at which they are injected and, unlike some viral vectors, are not transported to other sites in the brain, or via the blood stream to peripheral tissues. Thirdly, in the event of problems arising, some cell-based therapies are reversible. Encapsulated cells can be withdrawn from the brain or drug-induced suicide genes can be introduced into the implanted cells.[91-94] In the case of xenografts (implantation of cells from other species) implanted tissues survive only by continuous administration of immunosuppressant drugs and if necessary, death of the implant might be effected by the removal of immunosuppression.

The targets for *ex vivo* therapies in PD remain the same, namely the replacement of dopamine in the striatum or the delivery of neurotrophic factors to the degenerating dopamine neurons (see Fig. 2). The optimal cell to use for *ex vivo* therapies has yet to be determined. Several cell types have already been investigated. Primary rat fibroblasts or immortalised astrocyte cell lines, transduced with genes for TH and GTPCH1 have been implanted successfully into the striatum of hemi-Parkinsonian rats.[95,96] The same genes have also been shown to have functional effects when delivered using rat bone marrow stromal cells.[97] *Ex vivo* GDNF protection against 6-OHDA lesions has also been demonstrated using adrenal chromaffin cells,[98] astrocytes,[99] and neural stem cells.[100]

A final PD gene therapy strategy could be the enhancement of dopamine cell transplantation. Gene therapy techniques might be used not just to engineer cells for

use as gene delivery vectors, but also to modify primary embryonic cells to enhance their survival or promote their differentiation. As outlined previously, a major issue for primary cell transplantation is how to achieve higher yields of dopamine cells for transplantation. More than 90% of the implanted dopamine cells (or cells which have the potential to become dopamine cells) die, or never develop a dopaminergic phenotype following implantation into the host brain.[45] Poor survival has also been confirmed in human patients,[101-103] and is considered a major factor in the incomplete recovery seen in many clinical trials. In theory, if the survival of implanted dopamine cells could be routinely boosted from under 10% up to 50% or more, then an optimal therapy might still be achieved using a single embryo per patient, and the problem of limited availability of primary foetal tissue for PD therapy would be greatly reduced. To this end, several centres are exploring a variety of different approaches to reduce the processes involved in cell death and promote neuronal survival in tissues destined for transplantation.

1.5 Viral vectors for Parkinson's disease gene therapy

Whilst it might be argued that the potential targets for PD gene therapy have largely been established, the optimal means of delivery of therapeutic genes into the brain is still far from decided and is the subject of vigorous research effort. The issue is complex, as evidenced by the wide range of vector systems that are currently being investigated. In the race to find the best vector for gene delivery for the central nervous system, AAV, AV, LV and HSV-based vectors appear to be the main contenders. Each vector system has benefits and drawbacks and we shall not go into a detailed comparison here (For review see Fawcett et al 2001).[104] Ultimately, in the search for the best CNS gene therapy vector, there may be more than one winner. It is likely for example, that the optimal vector for *in vivo* delivery of one growth factor to the striatum may not be the one best suited for *ex vivo* delivery of the same or another factor to embryonic dopamine cells, and efficient delivery of the genes for dopamine synthesis may well be best achieved using a third vector. There are a number of issues to be considered when choosing the best vector for delivery of a particular transgene. Common to all is the issue of toxicity. The vector must not itself be cytotoxic, nor cause cell death by initiating a cycle of virus replication in host cells. As a consequence, all virus-based vector systems have genes deleted to reduce cytotoxicity and render them replication-deficient. In addition the vector, and the process used to

produce it, must be non-immunogenic such that no potentially damaging immune response is induced in the host. The use of *ex vivo* gene therapy is one way of avoiding this.

Tropism of viral vectors is also an issue. For direct application, the vector must be able to infect non-dividing cells and transduce them successfully. Transduction relies both on successful delivery of the transgene to the cells of interest and on successful activation of the gene promoter, which may be cell specific. For example, genes driven by the glial-fibrillary-acidic-protein (GFAP) should express only in glial cells, whilst a neuron-specific-enolase promoter will only be switched on in neuronal cells. Control of promoter expression is more problematic. Viral gene promoters are prone to switching off after a short period of time. On the other hand, promoters which drive gene expression indefinitely may have unforeseen consequences and many think that for gene therapy in humans, some form of controllable expression may well be required. The use of the gene promoters linked to tetracycline responsive elements, that enable gene switching by systemic administration of tetracycline, has already shown some promise.[105-107]

1.6 Adenoviral vectors and dopamine graft survival

One of the themes explored in this thesis is the potential of recombinant adenoviral vectors (RAd) for improving the survival of dopaminergic grafts, work made possible by collaboration with Pedro R. Lowenstein and Maria G. Castro of the Gene Therapeutics Research Institute, Cedars Sinai Medical Centre, in California.

The viral vectors used in this work are second-generation vectors in which deletions or substitutions have been made in the E1 and E3 regions of the viral genome. The vectors are produced using a *trans*-complementing cell line established from human embryonic kidney cells. The resulting virus particles are replication deficient, but capable of infecting a wide range of cell types including non-dividing cells. Co-workers have described the expression of the LacZ recombinant AV vectors RAd35 and RAd36 in rat brain and have suggested that RAd36 is capable of transducing striatal cells *in vivo* with almost 100% efficiency (that a single viral particle is capable of transducing a cell).[108] The therapeutic vectors obtained for initial consideration contained genes for either Shh, or GDNF. The transgenes were driven by promoters derived from cytomegalovirus (CMV) of either human or murine origin (see Table 1).

Table 1. Summary of Adenovirus vectors used in these experiments

Vector	Backbone	Transgene	Promoter*
RAd35	TypeV/-E1,-E3	Lac Z	MIE*h*CMV/
RAd36	Type V/-E1,-E3	LacZ	MIE*m*CMV/
RAd Shh	Type V/ -E1,-E3	Shh	MIE*m*CMV/
RAdGDNF	Type V/ -E1,-E3	GDNF	MIE*h*CMV/

* ***m*** = promoter derived from murine CMV, h = promoter derived from human CMV

Methods of application of viral vectors to dopamine transplants

A number of different methods of application of adenoviral vectors to dopamine transplants were considered in the current work (see figure 3). Firstly, the vectors might be applied directly to the embryonic dopamine cell suspension prior to implantation into the host brain, the most likely method of application should such procedures ever be developed for clinical application. In pilot experiments carried out in vitro (data not shown) the efficiency of transduction of primary cells was low (<5%) and gene expression was seen in non-neuronal cells (chiefly astrocytes). In fact, the CMV promoters used in these experiments do not switch on in neurones. Whilst this might be a useful route of administration for transgene products that are released from the cell such as Shh or GDNF, other factors such as transcription factors would need to be expressed in the target neurons or neuron precursors. Secondly, the vectors could be used to transduce accessory cells such as astrocytes for co-grafting together with embryonic dopamine cells, either as a mixed suspension or in separate grafts, distinct in location and/or in time from the dopaminergic grafts. Thirdly, vectors might be injected into the donor embryos *in utero*, prior to excision of the ventral mesencephalic tissue used for grafting, a difficult procedure but one that would be possible using existing in-house expertise. Finally, the vectors could be injected directly into the host striatum prior to implantation of the dopaminergic graft so that the transgene product would be expressed in the region of the host brain into which the graft is to be placed. Because of pilot experiments in which striatal transduction was seen to be highly efficient, and because of the problems encountered in transducing primary VM tissue, this was the route of administration chosen for our experiments with these vectors.

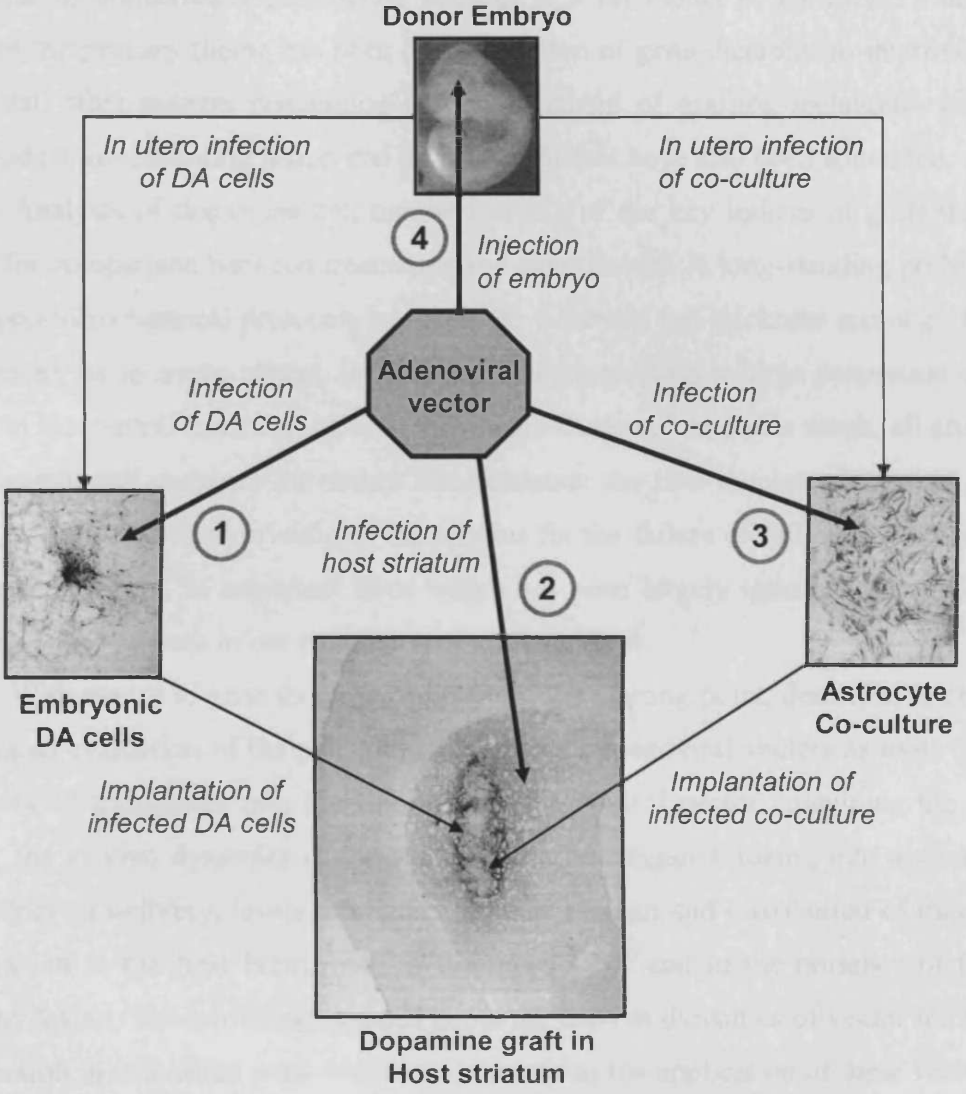


Figure 3. Diagram to show potential routes of application of AV vectors to dopamine grafts. **1.** Transduction of primary dopamine neuron suspensions with neurotrophic genes or genes important in DA cell differentiation. **2.** Delivery of the same transgenes to the host striatum prior to grafting. **3.** Transduction of co-cultures of cells (e.g. astrocytes) for co-grafting with dopamine grafts. **4.** Delivery of transgenes to the donor embryo in-utero prior to excision of graft tissue.

1.7 Aims of this thesis

The principal aim of this thesis was to investigate methods to improve the survival of implanted dopaminergic neurons in a rat model of Parkinson's disease. Whilst the primary theme has been the application of gene therapies to improve graft survival, other matters concerning the improvement of grafting techniques and the methods of investigating lesion and transplant models have also been addressed.

Analysis of dopamine cell numbers is one of the key indices of graft survival used for comparison between treatments and experiments. A long-standing problem in immuno-histochemical protocols has been the failure of full thickness staining. In our laboratory as in many others, in antibody stained sections a large proportion of the cells in the central thickness of sections remain unstained and as a result, all attempts to quantify cell numbers inevitably underestimate the true number. Chapter 2 deals with extensive work to investigate the reasons for the failure of full thickness staining in tissue sections, an important issue which has been largely ignored, but which has serious consequences in our evaluation of graft survival.

With regard to gene therapy applications, the starting point, described in chapter 3, was an evaluation of the potential of the current adenoviral vectors as tools for the delivery of transgenes into the CNS. Using the control vector containing the LacZ gene, the in vivo dynamics of the vectors were investigated, taking into account the efficiency of delivery, levels of toxicity, and the amount and distribution of transgene expression in the host brain, both in the intact CNS and in the presence of the 6-OHDA lesion. The knowledge gained about the in-vivo dynamics of vector transgene expression in this initial work was then used to plan the application of these vectors to dopamine grafts in subsequent investigations.

Chapter 4 describes the application of adenoviral vectors containing Shh or GDNF to dopamine transplants in vivo using a range of ages of the embryonic donor tissues. The ability of these therapeutic vectors to transduce cells in vivo and deliver both Shh and GDNF to dopamine grafts is clearly demonstrated by improved, vector-induced graft survival in this experiment. An unexpected result from this work was the observation that dopamine grafts derived from embryos of very young donor ages produced grafts several times larger than the E14 donor age that is conventionally used. A systematic re-evaluation of the donor age of tissues for dopamine transplants is presented in Chapter 5 in which E12 donors were confirmed as producing much

larger grafts than E14 donors and similar recovery on lesion induced rotational deficits.

Finally amphetamine-induced rotation, the most widely used method for the evaluation of dopamine lesions and grafts is examined in closer detail in chapter 6. The time course of lesioned and grafted animals is shown to be very different to that of lesion only animals and this is discussed in terms of the mechanisms involved and the implications of this for the evaluation of implanted dopaminergic grafts.

1.8 General Methods

Detailed methodologies are included in each of the manuscripts in this paper however, as many of the methods used are common to many of the experiments a summary is included here

Experimental animals

In all experiments adult, female Sprague-Dawley rats were used, weighing 200-250g 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.

Adenoviral vectors

The construction and characterisation of the adenoviral vectors used in this thesis have been described previously.[108-110] 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 were 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.[111] 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 or murine origin.[109,112]

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 were determined using the co-ordinates of Paxinos and Watson.[113] Following all surgery, wounds were cleaned and sutured using a 5/0 needle and silk thread. Animals were administered 5ml of physiological glucose/saline subcutaneously and allowed to recover in a heated recovery cage before a final health check and return to the home cage. Analgesia in the form of paracetamol (1mg/ml) was administered via the drinking water for 3 days post-operatively during which time rats received daily health checks.

Nigrostriatal lesions.

Dopamine lesions were carried out by injection of 3µl of 6-OHDA hydrobromide (HBr) at a free base concentration of 3.0-3.5mg/ml. All surgery was conducted under isoflurane/nitrous oxide anaesthesia in a Kopf stereotaxic frame, with the nose bar set 2.3mm below the interaural line, The 6-OHDA was administered either as a single injection into the median forebrain bundle (MFB) (A = -4.4, L = -0.1, V = -7.8) or using the 4-site striatal injection paradigm based on that described by Kirik and colleagues (A= +1.3, L= -2.6; A= +0.4, L= -3.0; A= -0.4, L= -4.2; A= -1.3, L= -4.6; all injections at V= -5.0).[114]

Viral vector injections.

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 3µl in volume delivered at a rate of 3ml/min with 3 min allowed for diffusion before removal of the cannula.

Ventral mesencephalic grafts.

The ventral mesencephalon was dissected from Sprague-Dawley rat embryos of donor ages E11, E12, E13, E14, or E15 (post plug). VM grafts were prepared as a cell suspension according to a standard protocol. [49] Briefly, the ventral mesencephalon was

dissected from each embryo. After washing in Hank's balanced salt solution (HBSS), the dissected pieces were placed in a solution of 0.1% trypsin in 0.05% DNAase in Dulbecco's minimum eagle medium (DMEM) for thirty minutes then washed in two changes of DMEM containing 0.05% DNAase. Dissociation of the dissected pieces was carried out in 200µl of DMEM containing 0.05%DNAase by trituration with Gilson pippettors. Initial trituration was carried out with a 1ml (blue) pipette tip (15-20 triturations) then a finer 100µl (yellow) pipette tip was used (15-20 triturations) to achieve the final, single cell suspension. The suspension was then spun in a bench-top micro centrifuge at 2000rpm for 3 minutes. The supernatant was removed and the pellet of cells resuspended in DMEM containing 0.05% DNAase at a concentration of 1 VM per 2µl. Grafted rats received 2µl of the cell suspension injected into the dopamine depleted striatum at stereotaxic coordinates: A = +0.6, L = $\pm$3.0, V = -4.5 (with nose bar set at –2.3mm) using a 30 gauge cannula attached to a 10µl Hamilton syringe, driven by an electronic syringe pump. After injection of the graft material, the cannula was left in place for 3 minutes to allow the diffusion of injected material, prior to careful removal of the cannula from the brain. To control for variability between suspension preparations, grafts in each group were derived from at least two cell suspension preparations and two separate rat litters. Each injection consisted of 2µl of cell suspension containing the cells from 1 VM injected at the same coordinates as the viral vector injection.

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.[115] Following an intraperitoneal injection of methamphetamine hydrochloride (dissolved in 0.9% sterile saline) at a dose of 2.5mg/kg of body weight, rotation test scores were recorded and are reported as net scores (ipsilateral minus contralateral) over a 90-minute session. Only rats with a net rotation score of $\geq$600 turns per session were used in the experiment. Grafted rats were rotation tested 4 weeks and 6 weeks post-implantation using the same method.

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 3mm-thick coronal slice was cut at the level of the injection and both the intact and injected striata dissected out and collected into 0.5ml 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 13000 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 hours at room temperature. Samples of purified GDNF in serial dilutions (0-1000pg/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 hours 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 (490nm) was measured in an automated plate reading system within 1 h of the reaction.

Histopathology

On completion of behavioural testing, animals were terminally anaesthetized 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 minute period. The brains were then removed from the skull and post-fixed by immersion in the same fixative solution for 4 hr, 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 40μm into 0.1M 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) (see figure 4).

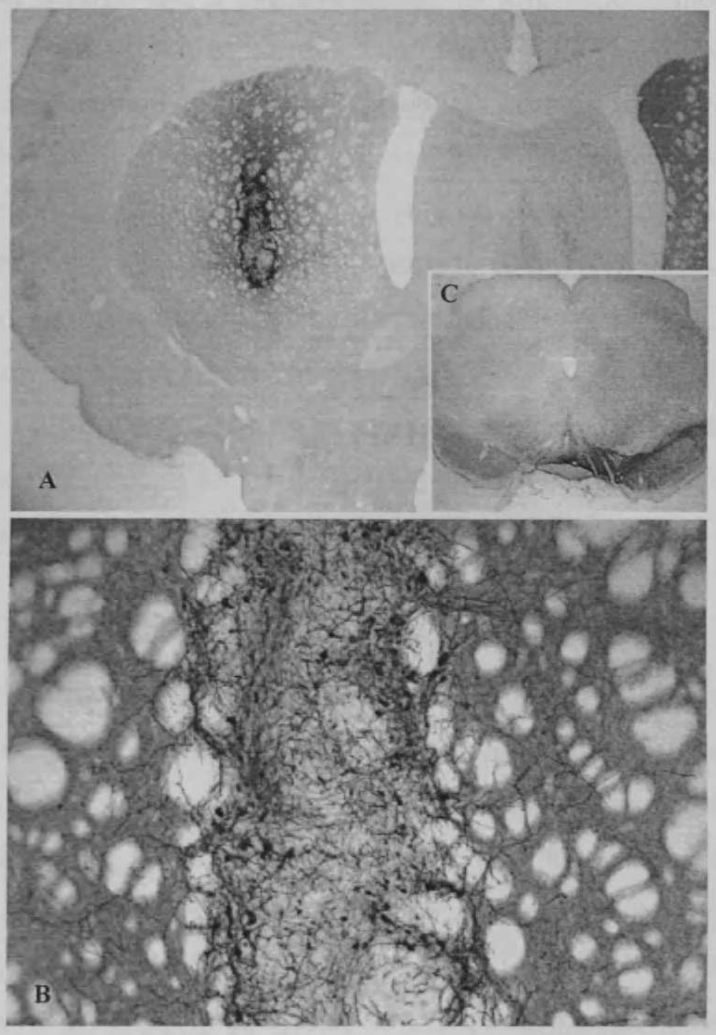


Figure 4. Typical appearance of lesions and dopamine grafts in the rat model of PD
A. Appearance of an E14 derived graft implanted into the dopamine-depleted striatum, as visualised by TH immunohistochemistry. The graft contains many TH positive cells that re-innervate the surrounding striatum (dark halo around the graft). **B**. Higher power photograph of the graft showing TH cell morphology and TH processes within and radiating from the graft. **C.** *(Inset)* TH staining of the substantia nigra showing loss of dopamine cells unilaterally on the side of the lesion injection.

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 hr 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 hr. 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 0.05M 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 0.3ul/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.

Morphometry

To determine graft survival, various measurements of graft size were carried out using either a PC-based image analysis system with Scion-Image (Beta 4.0.2) software (Scion Corporation USA) or a dedicated Olympus stereology system using CAST-GRID software. Graft volumes were estimated from measurements of the cross-sectional areas of the grafts measured on TH-stained sections in a regular series (1 in 6) through the entire striatum. Estimates of cell size were made either from measurements of cell diameter (for Abercrombie corrections of the cell counts) or of cell volume using the stereology software. Because of the relatively small number (in stereological terms) of cells in both the grafts and the lesioned substantia nigra compacta, stereological methods were not used for the estimation of cell numbers. The two-layer staining artifact, which is the basis of manuscript 1, was another reason why use of these methods was inappropriate. Cell counts were carried out manually on a Leica DMRB microscope using a 10 x 10 eyepiece graticule and a x20 objective and corrected using the Abercrombie formula.[116]

Statistics

Statistical analyses were undertaken by multi-factorial analysis of variance (Genstat 5.3, Rothampsted, UK) reporting the respective main effects and interactions for each relevant comparison.

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Chapter 2

The failure of full thickness, immuno-histochemical staining in thick brain sections.

2.1 Introduction and supplementary information

2.2 Manuscript:
The failure of full thickness, immuno-histochemical staining in thick brain sections.
E. M. Torres, Alicia Meldrum, Deniz Kirik, Stephen B. Dunnett. (2005)
(To be submitted for publication to the journal of Neuroscience.)

2.1 Introduction and supplementary information

Post-mortem histological examination of brain tissues is a critical part of investigations using animal models of Parkinson's disease. Central to the histological methodology is immuno-histochemical staining of frozen sections for tyrosine hydroxylase (TH), which is used to identify dopamine cells and processes in the striatum and substantia nigra as well as in the implanted dopaminergic tissue.

TH staining is used both to assess the extent of dopamine depletion caused by the Parkinsonian lesion and to estimate the numbers of surviving dopamine neurons in dopamine grafts. Both rely on counts of TH stained cells made in a regular series of sections. Unfortunately, we observed that in all our TH stained sections, staining was limited to the upper and lower surfaces of the section and the central thickness of the section contained no stained cells. It was later shown that this staining artefact was common to all immuno-histochemical staining.

Because this issue is so central to the investigation of dopamine graft survival we thought that it was important to try to eliminate the staining artefact to allow accurate cell counts to be carried out in all experiments. The following manuscript is an investigation of the causes of the failure of full thickness staining in immunohistochemically stained sections.

Initial pilot studies for the present study were carried out with Dr. Alicia Meldrum, a former post-doctoral scientist with Professor Dunnett. Subsequently, all of the experiments presented herein have been carried out solely by the author of this thesis. My supervisor Steve Dunnett, and Deniz Kirik, a collaborator based in the University of Lund in Sweden have been helpful with ideas and discussions throughout.

Research Report

The failure of full thickness, immuno-histochemical staining in thick brain sections.

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Abstract

Immunohistochemical staining of routinely prepared, thick sections of central nervous system tissue, suffer from an artefact; namely, that the distribution of stained cells through the thickness of such sections is not uniform. Immuno-reactive cells are seen only near the section surfaces, and the central thickness of the section is poorly stained or contains no stained cells at all. This presents a problem for cell counting but in particular for three dimensional stereology, which relies on the use of exclusion zones at the top and bottom surfaces of the section, where most of the stained cells are to be found and counts in the central thickness of the section where staining is poor or absent. We report here on experiments showing that the failure of full-thickness, immuno-histochemical staining is not a failure of the methodology *per se*. Rather, the problem originates from the fixation of tissues using a standard 4% paraformaldehyde-based protocol, which renders elements in the tissue impermeable to one or more of the solutions used. We show that this impermeability can be affected either by the inclusion in the fixative of membrane disrupting agents or by a reduction in the concentration of paraformaldehyde. We hope that the present investigations are a first step towards the development of new fixation protocols optimised for use in both immuno-histochemical methods and stereological analyses.

Key words: Fixation, paraformaldehyde, immunohistochemistry, penetration, stereology

Introduction

In our laboratory, we use relatively thick sections of rat brain (40 or 60μm) cut from paraformaldehyde-fixed tissue using a freezing stage microtome. This method of sectioning allows reliable production of multiple series of sections through the whole of the brain and supports the use of a wide range of histochemical and immuno-histochemiçal staining methods. In theory, such sections should lend themselves perfectly well to stereological cell counting using the "optical dissector" methodology (Gundersen 1986) in which zones of inclusion and exclusion need to be easily identified in the Z (depth) axis as well as in the X (horizontal) and Y (vertical) axes and thickness is an asset in this regard. Once dehydrated, the 60μm thick sections shrink to a thickness of around 20μm allowing the user to identify 2-4μm guard zones at the top and bottom of the section and still retain a comfortable depth in which to sample for cell counting.

Acknowledgements: This work was supported by grants provided by the Medical Research Council (UK). Out thanks also to Professors Hemmings and Greengard for the kind donation of their antibody to DARPP32.

However, in our hands, immunohistochemical staining using a wide range of antibodies using a standard protocol (see below), does not produce full thickness staining of tissue sections. Rather, the staining has a two-layered distribution with the upper and lower thicknesses of the section being well stained and the central thickness of the section containing little or no immunoreactivity. Other workers have reported this phenomenon (Calhoun et al. 1996; Dorph-Petersen et al. 2001; Van de Berg et al. 2003; Jinno et al. 1998). Van de Berg et al analysed sections of rat brain, stained immunohistochemically for parvalbumin, by counting cells at five different depths in the section thickness. They reported that the central thickness (33%) of the section contained no stained cells. They went on to use an optical dissector method of counting using only the stained portions of the section, in a similar fashion to that used by Calhoun et al

In our laboratory, we observe two-layered staining on immunohistochemically stained sections using antibodies against a wide range of antigens, including tyrosine hydroxylase (TH), glial fibrillary acidic protein (GFAP), choline-acetyltransferase (ChAT), dopamine and cyclic AMP-regulated phosphoprotein, molecular weight 32 (DARPP32), neuronal nuclear antigen (NeuN) and β-galactosidase. The problem is not encountered in sections stained using conventional histochemical methods such as cresyl fast violet, or haematoxylin and eosin, where cells are seen throughout the full depth of the section, regardless of the section thickness. The protocols used in our laboratory for fixation, cutting and immuno-histochemical staining of brain tissues (see table 1 below) are typical of those used in many of the laboratories working with animal models of neurodegeneration and examples of similar protocols are widespread in the literature. (Deacon et al 1998); Karlsson et al 2002; Kirik et al. 2000; Sortwell et al 2001; Choi-Lundberg et al 1997; Mandel et al 1997; Deglon & Aebischer 2002; Palfi et al 2002)

Personal communications with other workers in the field reveal that the two-layer artefact is observed in many laboratories working in the same field. In practical terms, this means that quantitative analysis of cell numbers in such sections is hampered by the presence of an unknown and variable portion of the sections which is unstained. This is a major problem for all methods of cell counting, which will inevitably underestimate the true number of cells in the section. It is a particular problem for stereological analysis using the optical dissector method, which relies on partitioning of the section thickness for random sampling in three dimensions. Other workers have found ways to apply sterological counting methods in partially stained sections by treating the top and bottom stained portions of the section as two separate entities for counting purposes (Van de Berg et al. 2003; Jinno et al. 1998; Calhoun et al. 1996; Ungerstedt & Arbuthnott 1970). However, the reason for the lack of full thickness staining is as yet unknown, and until the nature of the problem is investigated we cannot be sure that accurate cell counts are possible in these sections, using even the most stringent approach.

The present work investigates the possible causes of the failure of full thickness, immunohistochemical staining in thick sections. The investigation has been systematic and extensive and the results from each experiment have determined the direction of subsequent investigations. For this reason, the methods and results are presented in step-wise fashion in chronological order and are summarised later.

General Methods

Experimental animals

Adult, female Sprague-Dawley rats were used for all experiments. All rats weighed 200-250g at the time of first surgery. Animals 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.

Fixation and histology

The following methodology describes the standard procedures used by the Cardiff and Lund groups for the fixation and processing of rat brain for histological examination. Subsequent experiments involve modifications to the standard procedures and full details of any such modifications are given in the descriptions of individual experiments.

Rats were terminally anaesthetized by intraperitoneal injection of 1mg/kg sodium pentobarbitone, then perfused transcardially with 100ml of phosphate buffered saline (PBS) at pH 7.4, followed by 250ml of 4% paraformaldehyde in PBS delivered over 5 minutes. The brains were then removed from the skull and post-fixed by immersion in the same fixative solution for 4 hours, 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 60μm into

0.1M, 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 of sections from each brain was stained using the general neuronal stain cresyl-fast violet as follows. Sections were mounted onto gelatine coated microscope slides and allowed to dry at room temperature overnight. Slides were then dehydrated by five minutes immersion (with agitation) in an ascending series of alcohols; (70%, 95%, and 100% ethanol), then 30 minutes immersion in a 50/50 mixture of chloroform and ethanol. Slides were re-equilibrated to water via five minutes immersion (with agitation) in 95%, and 70% ethanol then distilled water. Staining was carried out by 5 minutes immersion in cresyl fast violet solution (5% in 0.1M sodium acetate buffer, pH3.5). Differentiation of the stain and dehydration was carried out in an ascending series of alcohols (70%, 95%, and 100% ethanol) before clearing in xylene and coverslipping using DPX mounting medium.

Immunohistochemistry was carried out on free-floating sections using the protocol outlined in table 1. The primary (monoclonal) antibodies and dilutions used were: β-galactosidase 1:6000 (Promega); NeuN 1:5000 (Chemicon), Tyrosine hydroxylase 1:2000 (Chemicon) and DARPP32 1:30,000 (the kind gift of Professors Hemmings and Greengard, Cornell University). Positive control tissue, and a negative control in which the primary antibody is omitted, were also run.

Morphometry

Because of the gross nature of the staining artefact observed, analysis of the distribution of staining within the thick sections was made by estimating the proportion of the section thicknesses which contained stained cells or not, an approach similar to that used previously by Anderson and Gunderson (Andersen & Gundersen 1999). Using a microscope with a stepping stage, cells were counted at different levels of focus, stepwise through the depth of the section. In this way it was easy to identify whether or not portions of the section thickness contain stained cells. We used a Leica (UK) DMRE microscope (Leica UK) equipped with a motorised stepping stage. In each of five sections, twenty fields were selected randomly by delineation of the structure to be counted and stepwise movement within the selected area to obtain a representative sample. Cells were counted using a high power (x100) objective and a 10x 10 eyepiece graticule counting grid. Starting at the surface of the section, the number of stained cells was counted. The stage was then raised 1μm at a time and at each step the number of stained cells was recorded. Another method of assessing penetration of staining was to use the stepping stage and to make a simple tally count of the number of steps through the section that contain stained cells. In each of five sections, a single field, (usually striatum) was randomly selected and examined using the high power (x100) objective. Starting at the surface of the section, the stage was raised 1μm at a time and at each step the presence or absence of stained cells in focus was noted. These data were then expressed as the percentage of the tissue thickness at the top and bottom of the section that contained staining and the proportion of the section in the middle which did not.

Adenoviral vectors

The recombinant, LacZ containing adenoviral vector (RAd36) used in this work has been previously described. (Gerdes et al. 2000). Briefly, the vector is a second generation, replication defective, E1, E3 deleted, type 5 adenovirus vector into which has been inserted the Escherichia coli LacZ gene. The inserted gene is driven by a 1.4kb fragment from the major-immediate-early, murine cytomegalovirus promoter (MIEmCMV) (Zermansky et al. 2001). Vectors were grown using a complementing HEK-293 cell line (derived from human embryonic kidney) and purified by caesium chloride gradient centrifugation to a titre of 4 x 10^{10} IU/ml. (Southgate et al. 2001)

Surgery

All surgery was performed under gaseous anaesthesia (2-3% isoflurane in Oxygen/ Nitrous oxide). Animals were placed in a stereotaxic frame and cannula placements carried out using the co-ordinates of Paxinos and Watson. Viral vectors RAd36 containing the marker gene LacZ was delivered via a 30-gauge cannula connected to a 10μl Hamilton syringe in a micro drive pump set to deliver at 1μl/minute. A volume of 3μl was delivered over 3 minutes with 3 minutes allowed for diffusion of the injected solution into the brain before careful withdrawal of the cannula. The RAd36 was used at a titre of 1 x 10^6 IU/μl. The stereotaxic co-ordinates were A=+0.6, L= ±3.0, V= -4.5, with the nose bar set at -2.3mm below the interaural line. (Paxinos 2003)

Table 1. General protocol for tissue preparation and immunohistochemistry

Step	Method	
Fixation	Sacrifice of the animal using lethal dose of pentobarbitone	
	Perfusion via the ascending aorta with 50-100ml of 0.1M phosphate buffered 0.9% saline pH7.4 (PBS) followed by 250ml of 4% Paraformaldehyde in PBS over 5 minutes.	
	Removal of the brain and post-fixation for 4-24 hours in the same fixative.	
	Cryo-protection by immersion in 25% sucrose in PBS	
Cutting	40μm-60μm sections, using a freezing stage microtome	
	Sections collected and stored in 0.1M Tris Buffered saline pH 7.4 (TBS)	
Immuno- Histochemistry		
	Free floating sections in TBS	
	3% hydrogen peroxide/ 10% methanol in distilled water	5 min
	Wash in TBS	3 x 5 min
	3% normal goat serum (NGS) in TBS with 0.2% Triton X-100:	1 hour
	Primary antibody in TBS with 1% NGS/ 0.2% TX-100:	16-72 hours
	Wash in TBS	3 x 5 min
	Biotinylated secondary antibody in TBS with 1% NGS:	3 hours
	Wash in TBS	3 x 5 min
	Streptavidin/biotin/Horseradish peroxidase complex in TBS with 1%NGS	2 hours
	Wash in TBS	3 x 5 min
	0.5mg/ml Diaminobenzidine (DAB) in 0.1M Tris Buffer pH7.4 (TB) with 0.03% hydrogen peroxide 5-10 min	
	Wash in Tris buffer	
Mounting	Sections mounted onto gelatine coated microscope slides, air dried overnight	
	Dehydrated in an ascending series of alcohols: 70%. 95%, 100%,	5 min each
	Clearing in 100% xylene	
	Cover-slipping using DPX mountant	

Experiment 1.
Demonstration of two-layered staining

Rationale

To demonstrate the artefact in an objective manner, 40μm and 60μm sections of perfusion-fixed rat brain were stained and then analysed to determine the distribution of stained cells in the depth of the section.

Method

Using the standard fixation and histology protocols described above, 6 normal rat brains were cut into 60μm sections (n=3) or 40μm sections (n=3) and three adjacent series of sections from each were stained using cresyl fast violet, and immunohistochemically for DARPP32 and NeuN respectively. The numbers of stained cells through the depth of the section was counted in the striatum of each animal as described above using twenty randomly selected fields from five sections from each animal.

Results

Figure 1 shows the two-layer staining effect in rat striatum on DARPP32 stained sections at a relatively low magnification. The DARPP32 antibody stains the cell soma, dendrites, axons and terminal fields of GABAergic output neurons and has a characteristic staining pattern. The axon bundles that make up the internal capsule in the rat are unstained with the antibody and where these pass through the section, the two-layer effect can clearly be seen.

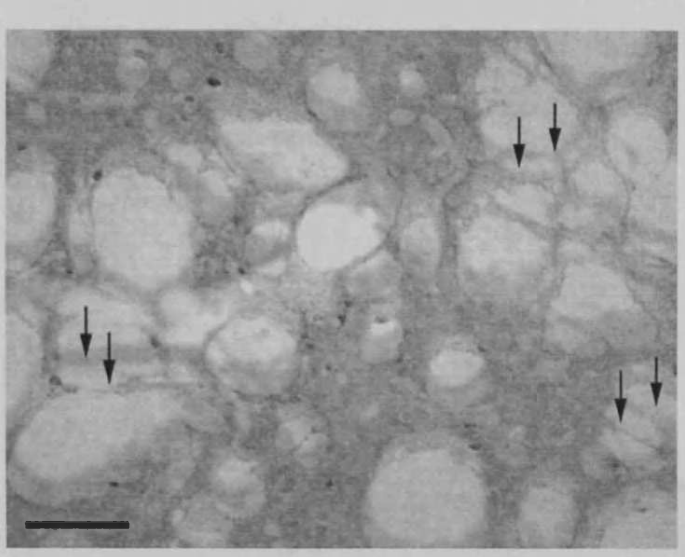


Figure 1. Micrograph of a 60μm thick section of rat striatum from experiment 1, stained immunohistochemically for DARPP32. The top surface of the section is well stained, showing DARPP32 cell bodies and their dendritic fields. The bottom surface of the section (out of the plane of focus) can also be seen. Where the unstained axon bundles, which make up the internal capsule, pass through the section, the unstained middle thickness of the section can be clearly seen as a gap between the top and bottom layers of staining. Arrows indicate areas of clear two layer staining. Scale bar is 0.1mm

Figure 2 is a plot of cell counts made through the section thickness and shows that superficial, DARPP32 staining was not limited to 60μm sections, but was also present in thinner (40μm) sections. In both, cell counts had a bi-phasic distribution through the section thickness. In 60μm thick sections, roughly half of the tissue depth contained no stained cells. In 40μm thick sections the middle third of the tissue thickness was unstained. The depth of penetration of staining was the same in both 40μm and 60μm sections; stained cells were observed only within 5-6μm of the surface, indicating that the depth of penetration of the immunostaining is not directly related to the section thickness. Allowing for the tissue shrinkage that occurs during dehydration of the sections for coverslipping, this corresponds to a penetration of staining into the original section of just 10-15μm from each surface, independent of the section thickness.

Examination of sections stained for NeuN showed a similar bi-phasic distribution of stained cells, whereas counts of sections stained histochemically with cresyl fast violet showed an even distribution throughout the section (fig. 3).

Discussion

The bi-phasic distribution of DARPP32 and NeuN stained cells obtained in such sections means that the use of optical dissector method counting through the entire section thickness is impractical, as this requires that volumes at the top and bottom surfaces of the sections be excluded from sampling. Whilst stereology might be carried out in the upper and lower stained surfaces separately, the nature of the staining artefact is unknown and it is possible that even these portions of the section contain unstained cells.

The results clearly show that the cell surfaces contain most of the stained cells and that in the middle of the section, there is little or no staining. In our laboratory, all antibodies produce this characteristic two-layer staining in thick sections. The comparison of the distributions of NeuN and cresyl fast violet stained cells in figure 3 is informative in this regard. Two-layer staining is clearly seen on NeuN stained sections, as illustrated by the biphasic distribution of cells through the section thickness. This is not seen in cresyl violet stained sections, in which cells are seen to have a monophasic distribution. Cresyl fast violet stained cells are seen throughout the thickness of the section and have a uniform distribution with depth. This shows that the problem of stain penetration is specific to the immunohistochemical method. Note that the depth of penetration of NeuN staining (figure 3) is slightly greater than that seen with the DARPP32 antibody (figure 2). In fact, most antibodies have slightly different penetration depths. This appears to be related to cell size, with larger stained cells appearing to have greater depths of penetration.

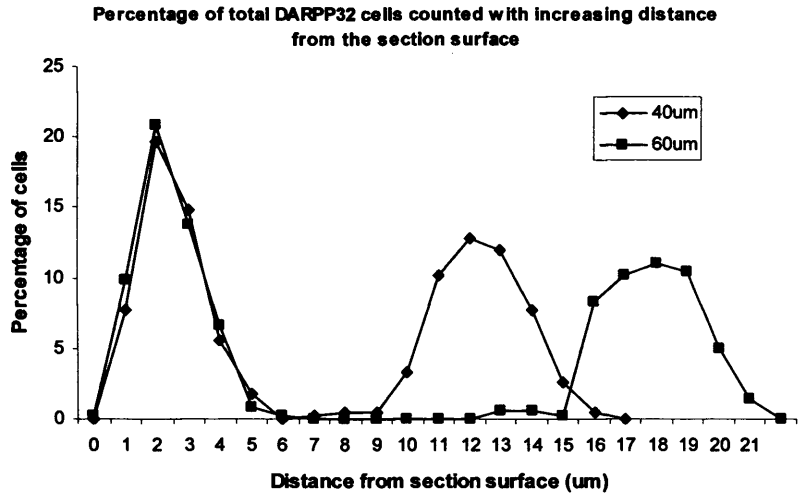


Figure 2. Counts of DARPP32 positive cell bodies in the striatum of a normal rat from experiment 1. Cells were counted at 1μm depth intervals through the thickness of the sections. The data are represented as the percentage of the total number of all cells counted which were found at each depth interval. In both 40μm and 60μm thick sections, stained cells are seen only within a thickness of 5-6μm from the section surface and only the extent of the unstained portion varied with section thickness. (Note that, due to dehydration in alcohol, there was shrinkage of the 40μm and 60μm sections to 15-17μm and 19-21μm respectively.) The broad spread of the distal peaks (on the right hand side of each plot) relative to the proximal peak (on the left hand side of each plot) is an artefact resulting from counting from one surface, in samples of slightly varying thickness.

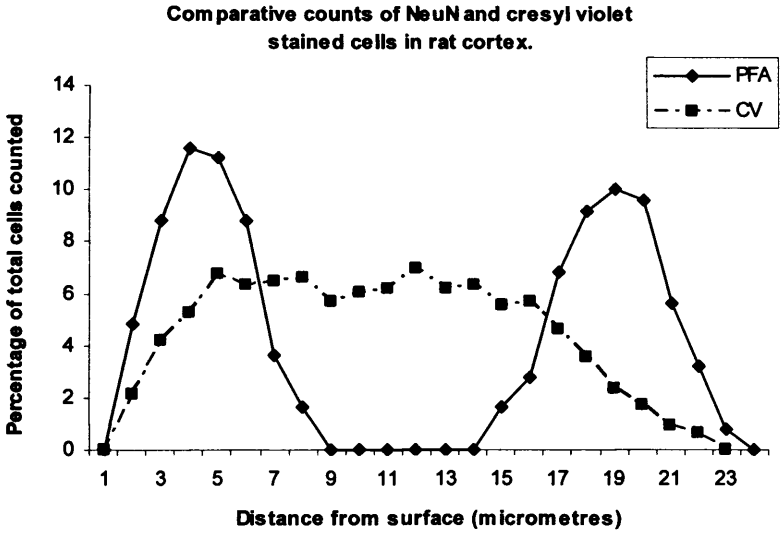


Figure 3. Counts of NeuN and cresyl violet stained cells in 60μm sections of the prefrontal cortex of normal rats from experiment 1. Cells were counted at 1μm depth intervals through the thickness of the sections. The data are represented as the percentage of the total number of all cells counted which were found at each depth interval. NeuN stained cells showed the characteristic two-peak distribution with depth, whilst the cresyl violet positive cells were distributed uniformly throughout the section

When starting to investigate the problem of full thickness staining in immuno-histochemistry it seemed a reasonable hypothesis that poor penetration of staining of immuno-histochemical stains was the result of the poor interaction of thick sections of tissue with one or more of the staining solutions involved. Our sections are routinely cut from standard, paraformaldehyde-fixed rat brain and in histological terms, are relatively thick at 40-60μm thickness. Such tissue is rich in lipids and by nature hydrophobic, so it would be no surprise if the poor penetration of aqueous solutions into such tissue was the source of the problem. On the basis of this hypothesis, there were several steps in the standard IHC protocol where poor penetration of solutions into the section might have been occurring:

1. Poor penetration of antibodies.
Antibodies and antisera are applied either in a detergent (Triton X-100) or a blocking serum (e.g. normal goat serum), or a combination of both. The detergent and the serum both have surfactant properties, which aid penetration of solutions into the section. Compared to other steps in the immuno-protocol the incubation times of antibody solutions are relatively long (3-72 hours).

2. Poor penetration of the Strepavidin/ Biotin/ HRP complex.
In the standard protocol, this is applied in the presence of normal serum but without triton X-100. Incubation time is 2 hours.

3. Poor penetration of the Di-Amino-Benzidine (DAB) solution.
The demonstration of the horseradish peroxidase (HRP) label is carried out in a buffered solution containing the substrate hydrogen peroxide, and the chromogen DAB. The solution is aqueous and contains no surfactant. The incubation time is short (5 minutes).

The following experiments were based on modifications to the standard immuno-histochemical protocol, outlined in Table 1.

Experiment 2.
Modification of the DAB protocol

Rationale

This was the first step to be investigated. Three modifications were used to try to improve the penetration of the DAB solution into the sections. Firstly, the addition of a surfactant (Triton X-100) to the DAB solution. In theory, this should aid the penetration of the aqueous DAB solution into the sections.

Secondly, dilution of the DAB solution was attempted, so that the DAB incubation time could be prolonged, thereby allowing a greater amount of time for the solution to penetrate into the sections. Thirdly, a pre-incubation of sections in "non-activated" DAB solution (containing no hydrogen peroxide) was introduced, prior to the addition of the hydrogen peroxide substrate, to allow a greater length of time for the DAB molecules to penetrate into the section.

Method

Free-floating, 60μm sections from three rat brains were each stained immunohistochemically for DARPP32 using the standard protocol up to, but not including the DAB step. Next, the DAB reaction was carried out using one of the following modifications to the standard protocol.

No modification

Condition 1)
Standard DAB solution 0.5mg/ml DAB in 40ml Tris buffer + 12ml H_2O_2 -5 min

Addition of a surfactant

Condition 2)
DAB solution with 0.05% Triton X-100 added. *(Final wash prior to the DAB in TB containing 0.05% Triton X-100).* -5 min. reaction time.

Dilution of the DAB solution to enable extension of the reaction time:

This was done by preparing the standard DAB solution, including hydrogen peroxide, then diluting to the required concentration using Tris buffer.

Condition 3) x 2 dilution -10 min.

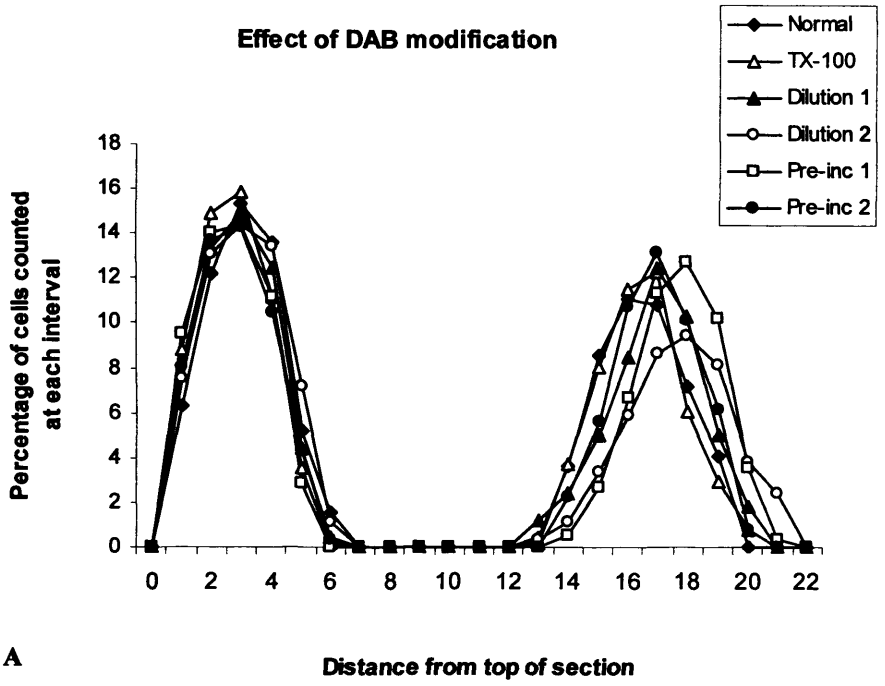
Condition 4) x 5 dilution -25 min.

Pre-incubation of sections in "non-activated" DAB (without H_2O_2)

After pre-incubation in DAB solution containing no hydrogen peroxide, sections were incubated in the standard DAB solution.

Condition 5)
5 minutes pre-incubation -5 min.

Condition 6)
20 minutes pre-incubation -5 min.



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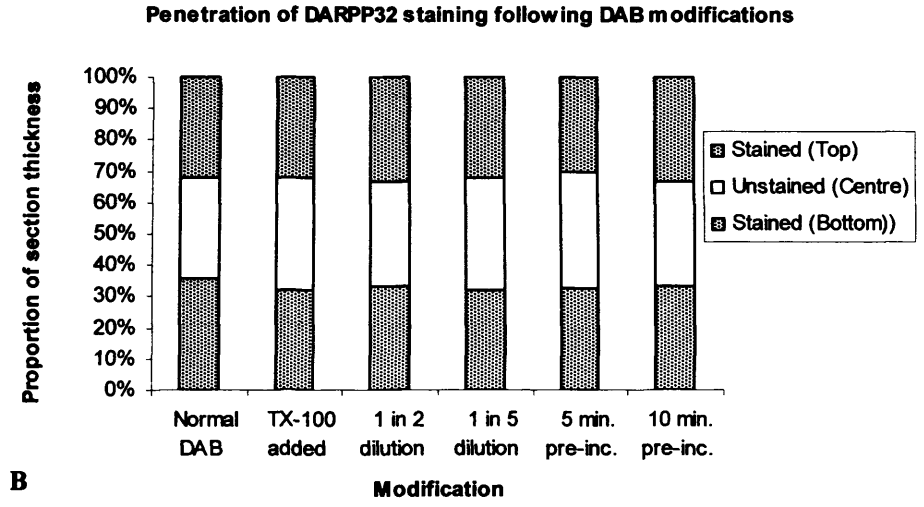

B

Figure 4. A. Counts of DARPP32 positive cell bodies in the striatum of a normal rat, from experiment 2. Cells were counted at 1μm depth intervals through the thickness of the sections as in experiment 1. Results clearly show that none of the modifications to the DAB staining protocol was able to modify the distribution of IHC staining through the section thickness. B. Data from the same sections expressed as the proportion of the section thickness containing stained cells for each condition. Each bar can be viewed as a representation of the staining showing the relative depth of stain penetration from each surface (grey) and the amount of unstained tissue in the central thickness of the section (white).

Results

Figure 4 shows the distribution of stained cells throughout the section thickness for the different conditions of staining used. The results show that for all conditions of DAB, the distribution of DARPP32 positive cells within the thickness of the tissue sections was similar and was unaffected by any of the modifications used. Neither extension of the DAB pre-incubation time, nor of the DAB reaction itself, produced a change in the penetration of staining. Nor did the addition of the surfactant TX-100 which undoubtedly would have improved penetration of the solutions themselves into the section.

Discussion

The DAB step of the immuno-histochemical protocol was an obvious candidate for investigation as it is the shortest step in the procedure and the one which contains least surfactant compounds in the solution. The failure of any modification of this step to alter the two layer staining effect is striking. Neither the extension of the DAB incubation times nor the addition of TX-100 as a surfactant produced any change in the depth of staining seen in the section. It is clear that whatever is causing the two-layer staining problem occurs at an earlier stage in the protocol.

Experiment 3.

Modification of the secondary antibody and streptavidin-biotin-complex steps

Rationale

The next hypothesis to be tested was that the problem of penetration might be caused by a failure of penetration of either the secondary antibody or the streptavidin-biotin-complex (ABC). Whilst the primary antibody incubation has a minimum incubation time of 16 hours (routinely 72 hours), the standard secondary antibody incubation time is only 3 hours and that of the ABC step only 2 hours. In the following experiment either the secondary antibody incubation time, or the ABC incubation time, or both were extended to see if staining penetration could be improved.

Method

Free-floating, 60μm sections from 4 rat brains were used. The brains were from animals which had received injections into the striatum of a viral vector containing the marker gene LacZ (see methods above) and which from previous staining were known to contain many β-galactosidase expressing cells. Striatal sections from each animal were stained immuno-histochemically for β-galactosidase using the standard protocol, with modifications, as outlined below. Condition 1 represents the standard protocol

Standard protocol

Condition 1) Overnight incubation in the primary antibody; 3 hours in the secondary antibody; 2 hours in the ABC solution.

Extension of the secondary antibody incubation time

Condition 2) Overnight incubation in the primary antibody; overnight incubation in the secondary antibody; 2 hours in the ABC solution.

Extension of the ABC incubation time

Condition 3) Overnight incubation in the primary antibody; 3 hours in the secondary antibody; overnight incubation in the ABC solution.

Extension of both the secondary antibody and ABC incubation times

Condition 4) Overnight incubation in the primary antibody; overnight incubation in the secondary antibody; overnight incubation in the ABC solution.

Results

Figure 5 shows the depth of staining observed in experiment 3 as the proportion of the total section thickness at the top and bottom surfaces of the section which contained positive cells, and the proportion of the section thickness in the middle of the section which was unstained. None of the modifications used produced an effect on the depth of stain penetration observed.

Discussion

The depth of penetration of the β-galactosidase staining in the striatum in this experiment was less than that observed with DARPP32 in experiment 2. As previously mentioned, all antibodies exhibit a clear two-layer artefact but there are slight variations in the depth of staining seen with each. The cells

demonstrated in the β-galactosidase stained sections were almost exclusively smaller glial cells. DARPP32 stains the medium sized, output neurons of the striatum, and this may account for the increased depth of staining seen when using this antibody. There was one observation of particular note in experiment 3. In some sections there were blood vessels in which the vascular endothelium had been transduced by the viral vector. In all cases where this was observed, β-galactosidase staining had the same distribution in the blood vessels as in the striatal parenchyma itself (figure 6) i.e. staining was seen only in the top and bottom 20% of the thickness of the section. Thus, the two-layer artefact was present in blood vessels, despite the fact that all of the solutions used in the immuno-histochemical protocol had full access to the lumen of the blood vessel.

Extension of the incubation times of either the secondary antibody or the ABC step, or both did not increase the depth of penetration of the IHC staining. This result, together with the observed distribution of β-galactosidase staining in the blood vessels, indicates that the problem of full thickness staining (FTS) is not a penetration problem *per se*. That is, the problem does not arise from a failure of one or more solutions used to penetrate through a thick section of tissue. If this were the case we would expect to see the vascular endothelium staining through the full thickness of the section, as there is no barrier to penetration of the staining solutions in the lumen of the vessels.

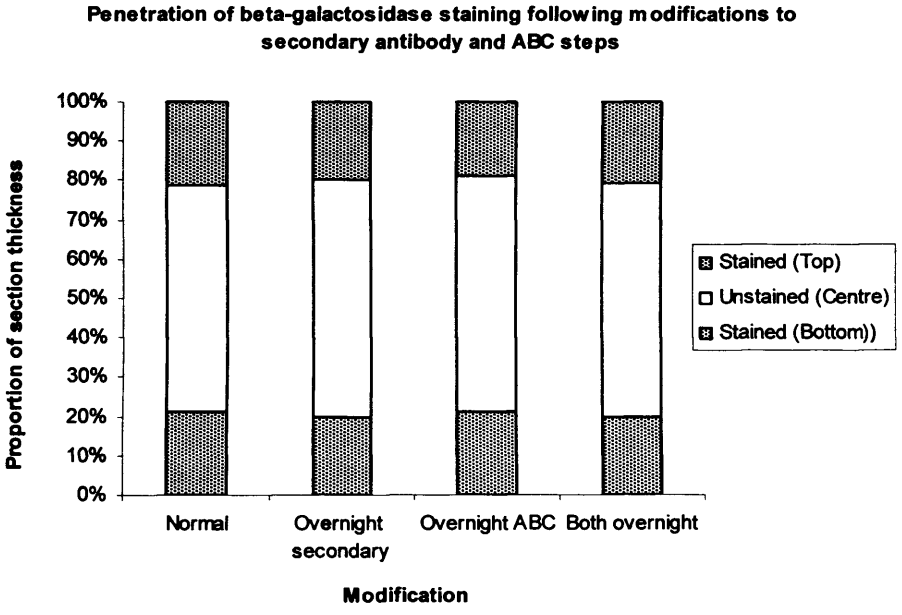

Figure 5. Data from experiment 3 showing the proportion of the section thickness containing β-galactosidase stained cells following modifications of the secondary antibody and/or ABC steps of the IHC protocol. Each bar can be viewed as a representation of the staining showing the relative depth of stain penetration for each surface and the amount of unstained tissue in the central thickness of the section. None of the modifications to the protocol were able to affect the depth of stain penetration.

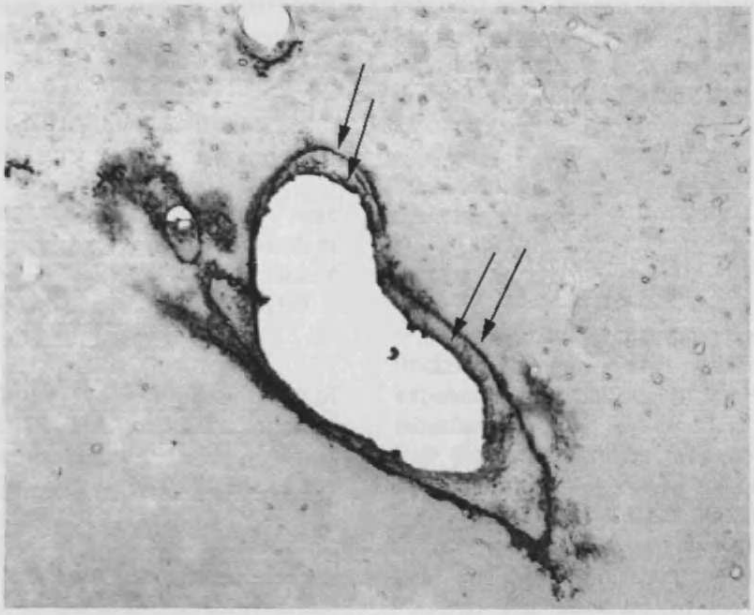

Figure 6. β-galactosidase staining of blood vessel in the ventral striatum adjacent to the corpus callosum from experiment 3 (60μm section). The vascular endothelium has been transduced with the LacZ bearing adenoviral vector. However, the endothelium is stained only in the top and bottom surfaces of the section (arrows) and that the vascular endothelium in the central thickness of the section is unstained, despite full access to the staining solutions.

If the barrier to FTS is not the section thickness itself then it must be some component within the tissue, which is impermeable to at least one of the steps in the IHC protocol. The cell membranes are an obvious candidate and the distribution of β-galactosidase staining in the blood vessels could be explained if only those cells near the section surface in which the membrane was physically cut during sectioning were able to take up the immunohistochemical stain. This hypothesis is consistent with our observations so far that:

a) Cresyl violet (CV) and other histochemical stains give full thickness staining (figure 3). Not only are the CV molecules small, relative to the immunoglobulins used in the IHC, but sections for CV are routinely de-lipidised by dehydration and immersion in chloroform, a step which is known to break down cell membranes.

b) In β-galactosidase stained sections we see two-layer staining of blood vessels, despite the full access of all solutions to the vascular endothelium. The only barrier to penetration in these cells is the cell membrane itself.

c) Slight differences are seen, in the depth of penetration depending on the antibodies used, probably due to differences in the size, shape and orientation of the cells being stained. sectioning so that cells penetrate more deeply into the section from the cut surface.

d) In areas of the section where there is two layer staining of cell bodies, we occasionally see full thickness staining of axons which pass through the section presumably because the ends of the axons have been cut at both ends during sectioning.

Experiment 4. Post sectioning permeabilisation of cell membranes.

Rationale

Working on the hypothesis that the barrier to staining might be the cell membrane itself, the following modifications were carried out to investigate whether or not post-sectioning treatment of sections, using known membrane permeabilising agents, could be used to obtain full thickness staining.

Method

Free-floating, 60μm sections from four rat brains fixed using the standard protocol were used. As in experiment 3, the animals had received injections into the striatum of a viral vector expressing the marker protein β-galactosidase. Striatal sections from each animal were stained immunohistochemically for β-

galactosidase using the standard protocol following treatment with known membrane permeabilising agents.

Condition 1)
No pre treatment. Standard IHC protocol.

Condition 2)
Pre treatment with Acetone. Sections were washed in distilled water, and then placed in 100% acetone for 10 minutes. 3 x 5 minute washes in water before re-equilibration to TBS.

Condition 3)
Pre-treatment with the Tween 20. A solution of 0.2% tween 20 (v/v) in TBS for 1 hour, followed by replacement of the TX-100 in the blocking and primary antibody steps with 0.2% Tween 20.

Condition 4)
Heat treatment. Microwave treatment of sections in a solution of phosphate buffered saline. Sections were brought to the boil on high power, then kept boiling for 5 minutes (low power), and cooled to room temperature.

Condition 5)
Delipidising the sections with Chloroform ethanol. This is our standard delipidising method for histochemical methods. Sections were mounted onto gelatin coated microscope slides and dried overnight. Dehydration in an ascending series of alcohols to 100% ethanol, then 30 minutes in a solution of 50% Chloroform/ 50% Ethanol before rehydration in descending alcohols to water then TBS. Immunohistochemistry carried out on slide mounted sections.

Results

Figure 7 shows the proportion of the section thickness stained for each condition observed in experiment 4. None of the free-floating modifications (2-4) had an effect on penetration. Full thickness staining was observed on the slide-mounted, delipidised sections. However, the quality of the staining following delipidisation was massively reduced, affecting both the distribution and the intensity of the staining. The thickness of the slide mounted sections was also reduced to about 60% of the thickness of the free-floating sections further reducing their usefulness for stereological methods. Delipidisation of sections prior to immunohistochemical staining in this way would not be an effective method for sections destined for either microscopic or stereological analysis.

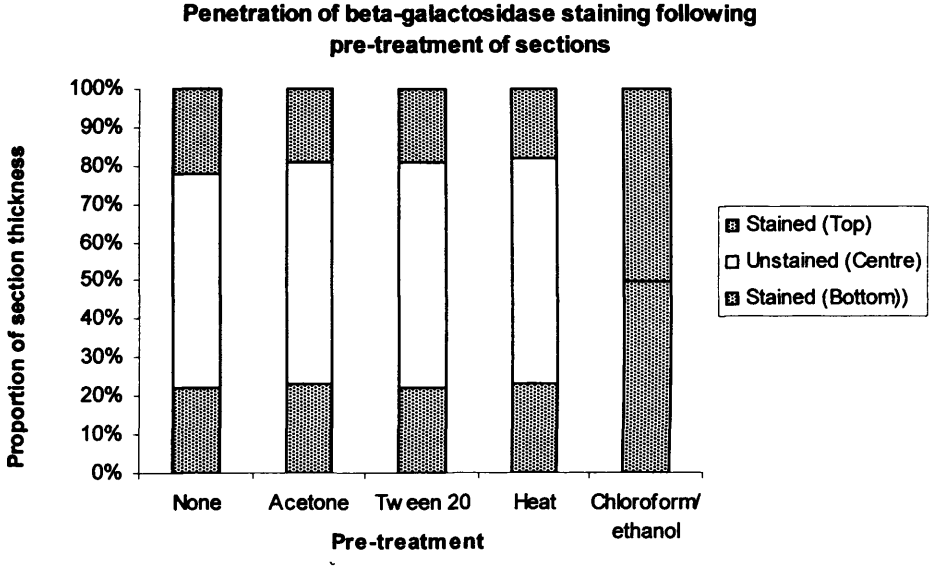

Figure 7. Data from experiment 4 showing the proportion of the section thickness containing β-galactosidase stained cells following pre-treatment of sections with membrane permeabilising agents. Each bar can be viewed as a representation of the staining showing the relative depth of stain penetration for each surface and the amount of unstained tissue in the central thickness of the section. Only the chloroform methanol modification of the protocol was able to affect the depth of stain penetration.

Discussion

It appears that using the standard protocol for tissue fixation, cell membranes are preserved in such a way that only dehydration and delipidisation of the tissue was effective at breaking down the membrane structure.

Nevertheless, the full thickness staining seen following de-lipidisation in condition 5 supports the hypothesis that cell membranes are the barrier to penetration of staining and that disruption of the cell membranes can allow penetration of the IHC solutions into cells and give full thickness staining.

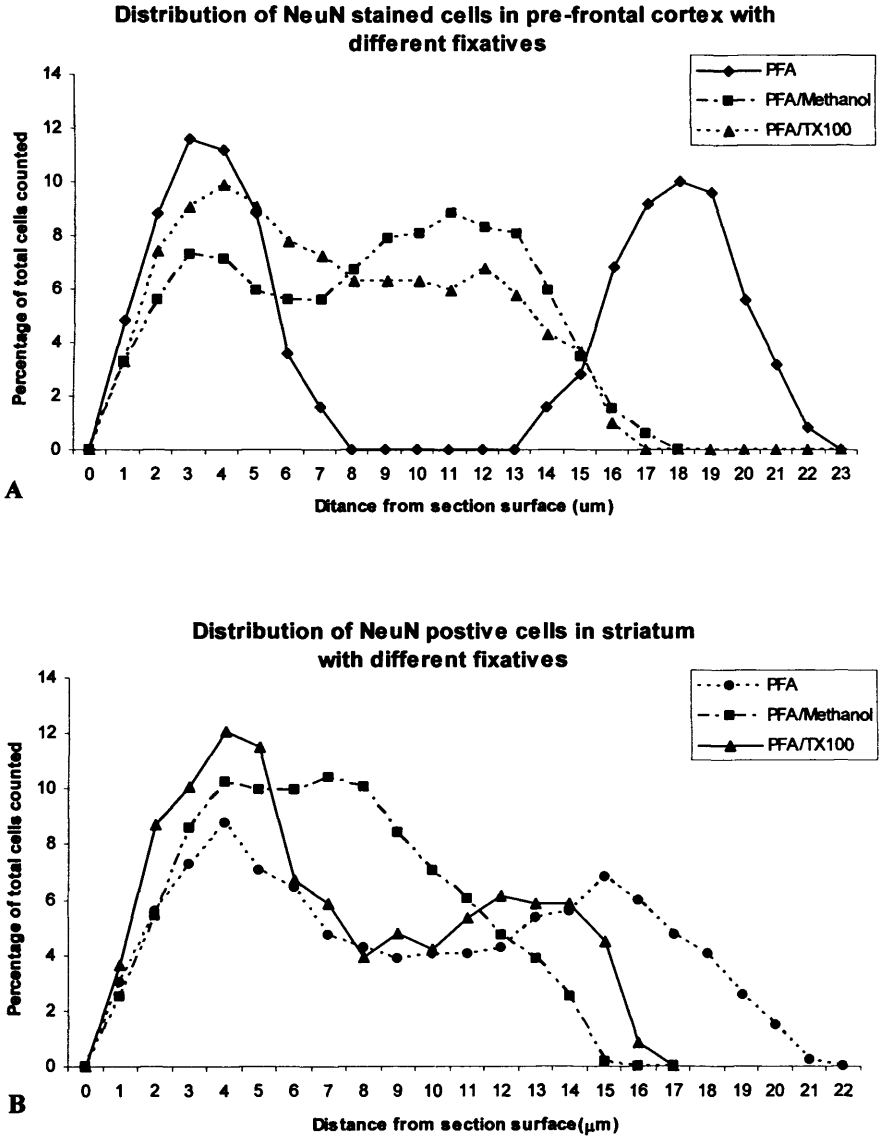

Figure 8. A. Plot of the number of NeuN positive cells in prefrontal cortex, counted at increasing distance from the surface, in sections from brains perfused with different fixatives (experiment 4). Note that cells in sections from the PFA fixed brains show the familiar two-peaked distribution However in sections from the brains fixed using methanol or TX100 containing fixatives the two-peak profile is dramatically reduced indicating successful staining of cells in the central thickness of the section. **B.** Plot of the number of NeuN positive cells in corpus striatum, counted at increasing distance from the surfac in sections from brains perfused with different fixatives (experiment 4). Note that cells in the PFA and PFA/TX100 fixed brains have a slight two-peaked distribution though this is not as pronounced as that seen on the prefrontal cortex.

Experiment 5. Modification of the fixative to affect cell membrane permeability

Rationale

Following experiment 4, it was hypothesised that the addition of membrane disrupting agents to the fixation process might preserve the cell membranes in a state which would allow full penetration of IHC staining.

Method

The brains from 3 normal rats were perfusion fixed using modifications of the standard paraformaldehyde fixative.

Condition 1)
Standard fixative 0.1M PBS/ 4% PFA

Condition 2)
Standard fixative containing 0.2% v/v triton X-100 (PFA/TX-100)

Condition 3)
0.1M PBS/ 4% PFA diluted with 20% v/v methanol (PFA/methanol).

Perfusion was carried out using the standard method and after removal from the skull, brains were post-fixed in the fixative used for perfusion for 4 hours. After completion of fixation, all brains were transferred to 25% sucrose in PBS until they sank. Sections were cut at 60μm thickness and stained immuno-histochemically using the NeuN antibody and the standard protocol. To assess the distribution of cells within the section thickness, counts of positively stained cells in the corpus striatum and pre-frontal cortex were carried out using a fixed-area counting frame on 5 sections per brain. The total number of stained cells seen at different depths from the surface of the section was noted.

Results

On examination of the sections it became clear that some sections did contain NeuN positive cells in the central thickness of the sections. Figures 8 shows the distribution through the section thickness of NeuN positive cells in sections through pre-frontal cortex or the corpus striatum for each condition expressed as the percentage of total cell counted. In the prefrontal cortex (PFC), sections from both PFA/methanol and the PFA/TX100 fixed brains contained NeuN positive cells throughout the section thickness whilst sections from the PFA-only fixed brain did not (figure 8A). Both methanol and TX100 were effective in permeabilising the cell membranes in the PFC. However, in the striatum, only the PFA/ methanol fixed brain showed full thickness staining in the striatum. Sections from the PFA/TX100 fixed brain had a similar distribution of stained cells to that seen in the PFA-only fixed brain (figure 8B).

Discussion

Addition of the membrane disrupting agents methanol or triton X-100 to the fixative solutions increased the depth of penetration of NeuN staining in both the cortex and striatum. The differences seen in the extent of penetration between these two structures is not clear. It is possible that the combination of perfusion followed by a period of post-fixation produces different degrees of fixation in different regions of the brain. The standard fixation protocol involves 5 minutes of perfusion of the brain followed by its removal from the skull and a further 4 hours immersion in the fixative (post-fixation). Replenishment of both para-formaldehyde and TX100 during the post-fixation period relies on diffusion of these substances from the surface of the brain. In the pre-frontal cortex, close to the surface of the brain, replenishment of these substances would be quicker than in the much deeper striatal tissue. This might explain why, in the pre-frontal cortex there is greater effectiveness of the TX100 in the PFA/TX100 treated brains (better access of TX100 in the cortex than in the striatum produces better permeabilization of the membranes), but a larger two layer effect in the PFA only brains (better access of PFA in the cortex than in the striatum increases the impermeability of the membranes).

Because cells were counted in equivalent volumes of tissue, we can compare counts between brains (figure 9) (notwith-standing possible differences in tissue distortion produced by the different methods of fixation). In the striatum, there were significantly more cells in the PFA/methanol treated sections compared to the PFA-only treated sections ($F(2,49)=8.69$; $p<0.001$). In the pre-frontal cortex, there were significantly greater numbers of cells in both the PFA/methanol and the PFA/TX100 when compared to the PFA only group ($F(2,52)=24.10$; $p<0.001$). In fact, the methanol treated striata contained 27% more stained cells than the PFA only fixed brain, providing us with an estimate of the number of striatal cells that are unstained in the central thickness of 60μm sections using the standard method of fixation. In the prefrontal cortex this difference was even greater and sections from brains prepared with either of the modified fixatives contained at least 2.5 times the number of stained cells than those from PFA-only treated brains.

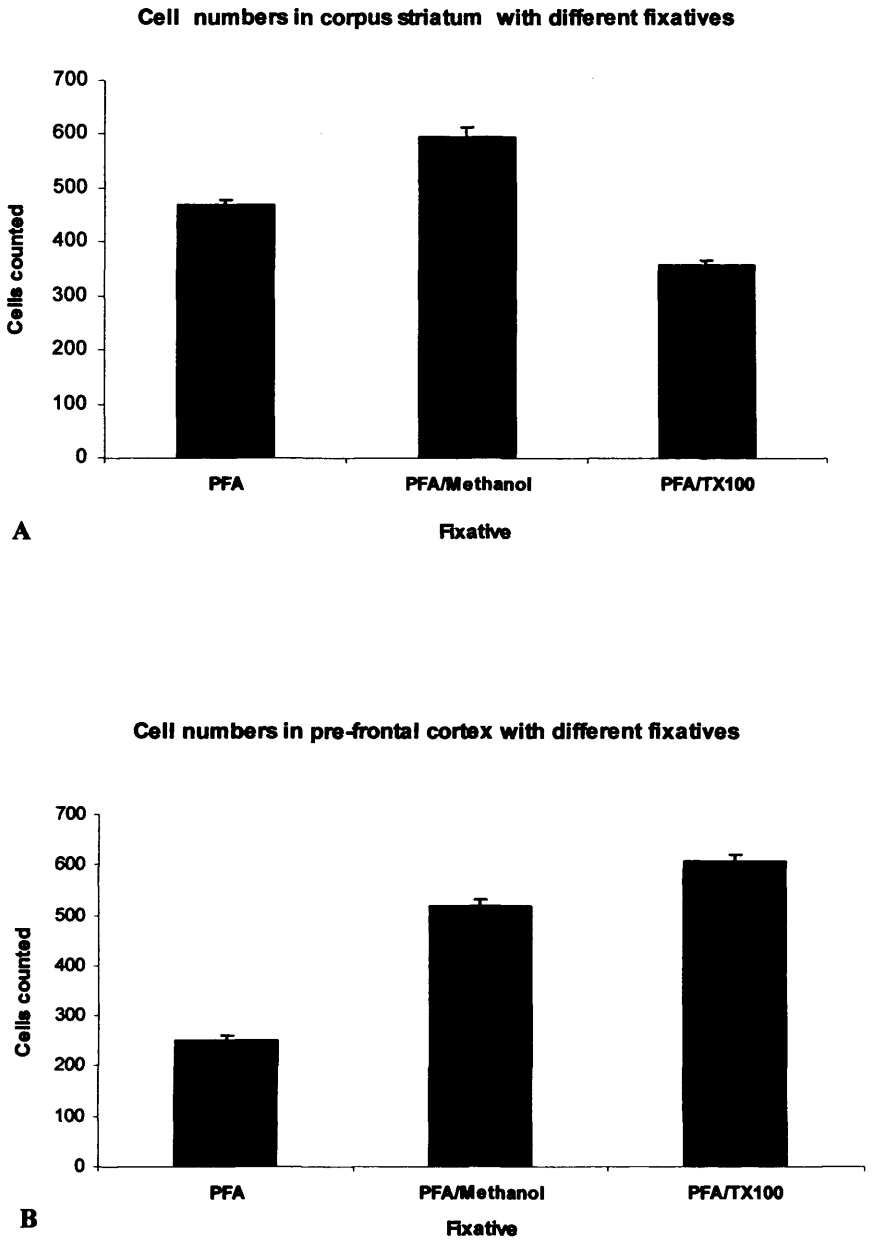

Figure 9. A. Experiment 5. The number of NeuN stained cells counted in an equivalent volume of striatum (A) and pre-frontal cortex (B) in sections from brain fixed using different modifications to the perfusate. PFA/Methanol fixed sections contain significantly more cells than the PFA fixed brain Error bars are SEMs.

Experiment 6:
Investigating the cause of membrane impermeability

Rationale

We hypothesise that the barrier to the penetration of IHC staining in thick sections of brain tissue is the cell membrane itself and that the observed impermeability is caused by the fixation of the tissue. Thus, the standard fixation protocol preserves membranes in such a way that they are impermeable to one or more elements of the IHC protocol and only cells which have had their membranes physically cut during sectioning are stained, producing the characteristic two layer staining seen in thick sections. However, the standard 4% paraformaldehyde based fixative is also used for fixation of in vitro specimens for immunohistochemical staining (typically mono-layers of cells in 24-well plates) and excellent staining is obtained on these specimens with a wide range of antibodies. If our hypothesis is

true we need to explain why the same fixative used in tissue culture does not produce the impermeability to staining seen in perfusion-fixed brain tissue.

One obvious difference is the duration of exposure to the fixative. In the standard protocol, perfused brain tissue is exposed to fixative for a minimum of 245 minutes; 5 minutes of perfusion (under pressure), plus 4 hours of post-fixation. In fact exposure is almost certainly longer than this, as it will take several hours after removal of the brain from fixative for the fixative to diffuse out of the brain. Even then PFA will persist at low concentration until (and even after) the brain is sectioned. In contrast, in tissue culture specimens, exposure to the fixative is just 10 minutes followed by several washes in PBS that remove all traces of fixative. It seems likely then that it is not exposure to PFA that is the cause of impermeability, but rather, over-exposure to PFA in perfusion-fixed tissue. To test this hypothesis we looked at the effect of reducing the concentration of paraformaldehyde in the perfusate.

Method

Three concentrations of fixative, 1%, 2% and 4% paraformaldehyde were prepared using a stock solution of 20% PFA diluted in PBS. For each fixative 3 rats were perfused, processed and the brain cut using the standard protocol (table 1). Sections were stained immunohistochemically for NeuN and histochemically using Cresyl violet. Cell counts were carried on the NeuN stained sections through the depth of the section in the striatum of five sections from each brain and the munber of stained cells were recorded at each level.

Results

There were differences in the density of staining of cresyl violet between the three conditions with a general decrease in the intensity of staining with decreasing concentration of paraformaldehyde (not shown). However, at high power the cell morphology appeared normal in all sections. Counts on NeuN sections showed that as previously, the brain fixed using the standard protocol (4% PFA) had the two-layer staining artefact. In sections from the 1% PFA and 2% PFA fixed brains this artefact was not present and we observed staining through the full thickness of the section (figure 10).

Discussion

The effect of paraformaldehyde concentration on the penetration of NeuN staining is clearly demonstrated. Sections from brains perfused with 1% and 2%, but not 4% paraformaldehyde produce full thickness staining. From these data we can conclude that the impermeability of cell membranes to the immunohistochemical method is caused by overexposure to paraformaldehyde fixation.

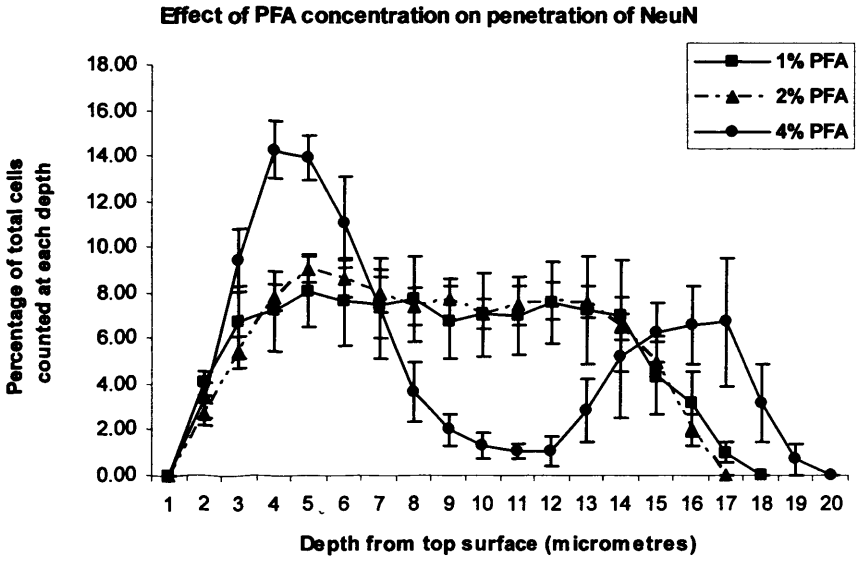

Figure 10. The effect of PFA concentration on the penetration of NeuN staining (experiment 6). The plot shows the percentage of the total number of NeuN stained cells counted at 1 micrometer intervals with increasing depth from the top surface of the section. Cell counts were carried out on 5 sections through the dorsolateral cortex from 3 rat brains for each condition. Note that whilst cell counts are distributed throughout the thickness of the section in the 1% PFA and 2% PFA conditions in the 4%PFA condition we see the biphasic plot characteristic of the two layer staining. Error bars are % corrected SEMs

General Discussion

In the Journal of Anatomy in 1996, Mayhew and Gunderson wrote; "From now on there can be no excuse for applying the biased counting methods of yesteryear. Their continued use, despite the availability of unbiased alternatives, should be seen as paying homage to history rather than advancing science." (Mayhew & Gundersen 1996) Whilst many might consider this a harsh position, several journals, including Neurobiology of Aging and The Journal of Comparative Neurology have nevertheless stipulated that all quantification of neuroanatomical data should be carried out using stereological techniques unless there are good reasons not to do so. In 2000, the Journal of Chemical Neuroanatomy published an issue dedicated to stereology in which stereology was described as the "gold standard" for quantitative anatomy (Kordower 2000) and in which it was stated "...an experiment can only be considered valid if the data have been collected in a reliable manner, according with the best current methodological standards, utilizing a reliable and unbiased method," strongly suggesting that stereological methods should be used wherever possible (Steinbusch et al. 2000).

Other workers have opposed the notion that stereology is optimal for, or indeed applicable to, many of the counting tasks that confront the neuroanatomist (Guillery & Herrup 1997; Hedreen 1998) and have argued for a less rigid approach when considering which methods of quantification are appropriate for neuroanatomical analyses. Notwithstanding, stereology has become generally established in the neurosciences as the method of choice for histological counting. In the present paper we investigate a problem associated with immunohistochemical staining, which makes the accurate estimation of cell numbers in tissue sections problematic and has a particular bearing on the use of stereological counting using the optical dissector.

In the present paper we have described the problem of full thickness immunohistochemical staining in thick sections of brain tissue; a problem that renders all methods of cell counting invalid and poses a particular problem for the application of unbiased stereological methods. We have demonstrated this using the simple method of focusing through the thickness of sections using a high magnification microscope objective and either plotting the numbers of cells counted at each depth or recording the depths at which stained cells are observed. We have investigated the nature of this artefact and concluded that it is not the thickness of the section itself which is the barrier to penetration, but elements within the section, most likely the plasma membranes of the cells themselves.

There are several strands of evidence in support of this conclusion. Firstly, in sections stained for the marker gene β-galactosidase, blood vessel walls expressing the gene exhibited two layer staining, despite the fact that all the solutions used had full access to the vascular endothelium via the lumen of the blood vessel. Secondly, full thickness staining of sections is observed when using non-immunohistochemical methods such as cresyl violet or haematoxylin and eosin. In comparison to the antibodies used in immunohistochemistry, dye molecules are relatively small and therefore more likely to overcome barriers to penetration. Also, prior to staining with histological methods, sections are routinely de-lipidised using solvents such as chloroform and methanol which disrupt the structure of cell membranes. Thirdly, we have shown that full thickness staining can be obtained by modifying the standard 4% paraformaldehyde to include membrane disrupting agents such as methanol or triton X-100. We have also investigated the cause of the membrane impermeability and have shown that the level of exposure to paraformaldehyde fixation is a factor in the two layer staining artefact. Reduction of the paraformaldehyde concentration from 4% to 1% or 2% inhibited the formation of the barrier to penetration and gave full thickness staining with immunohistochemical stains.

The nature of the barrier to immunohistochemical penetration is unknown. 4% paraformaldehyde should more properly be called 4% formaldehyde as the paraformaldehyde polymer (in theory at least) dissociates to the monomeric formaldehyde at low concentrations. Formaldehyde fixes tissue by cross-linking a wide range of tissue components including, proteins and lipids, both of which are present in cell membranes. Aldehydes are also known to be progressive fixatives such that the degree of cross-linking attained is proportional to the fixation time. The widespread use of 4% paraformaldehyde arose in the 1980s when immunohistochemical methods became available. 4% paraformaldehyde was introduced as a purified version of 10% formalin which contains methanol and other impurities, which were thought might adversely affect the antigenicity of tissue sections (Bancroft & Stevens 1990). Paraformaldehyde is difficult to dissolve in water and may persist in polymeric form in solution. This may or may not be an issue in the current work but experiments to

determine the stability and reproducibility of 4% paraformaldehyde solutions would be informative in this regard.

The problem of full thickness staining is not widely discussed in the scientific literature, though several other workers have commented on the problem in work centred on other investigations. Van de Berg et al analysed sections of rat brain, stained immunohistochemically for parvalbumin, by counting cells at five different depths in the section thickness. They reported that the central thickness (33%) of the section contained no stained cells. They went on to use an optical dissector method of counting using only the stained portions of the section as if these were two thinner stained sections within the thickness of the section itself (Van de Berg et al. 2003). A similar approach was adopted by Calhoun et al. to count synaptophysin immunoreactive boutons in rat hippocampus (Calhoun et al 1996) and by Jinno et al. investigating rat hippocampal GABAergic neurons (Jinno et al 1998) Kirik et al commented on two layered staining in sections of rats brain in which they were counting TH positive cell numbers in dopamine transplants and they too counted the stained portions using the optical dissector and made corrections to their calculations to account for this (Kirik et al. 2001).

Other workers have made detailed studies of the range of artefacts which are encountered during the cutting and staining of tissue sections. Gardella et al. looked at shrinkage of tissue sections in the z-axis during cutting and staining of either vibratome, cryostat or celloidin cut sections. (Gardella et al. 2003). They reported an aretefact similar to that reported here, namely that more cells were observed at the surfaces of the sections than in the central thickness, a problem which they attributed to greater compression of the section surfaces during dehydration. However, this is not the same artefact as described in the current paper. The stain used was thionin, a small molecular weight dye which was able to penetrate the sections easily and produce staining of cells in the full thickness of the section. Whilst we report a complete absence of staining in the central thickness of the section, they reported only differential distribution of stained cells in the z-axis. In a systematic investigation of tissue shrinkage artefacts, Dorph-Peterson et al. reported reduced cell counts in the central portion of thick sections stained using using the histochemical stain thionine. However, they concluded that the observed artefact was caused by differential shrinkage within the sections, such that the surfaces of the section were more compacted during processing (Dorph-Petersen et al. 2001).

In the modern neuroscience laboratory the failure to obtain full thickness staining with immuno-histochemical methods is a major problem Our results suggest that, using conventional methods of fixation and staining, immunohistochemically stained sections contain an unknown and variable portion of the section thickness in which there are no stained cells. Additionally, analysis of histological material increasingly depends on stereological methods and the optical dissector method of cell counting, which ought to be ideally suited to thick sections, cannot adequately be carried out on sections stained in this way. Our results suggest that the barrier to staining is the cell membrane itself (or some component of the extracellular matrix closely associated with the cell body) and this has further implications for the region of the section that is stained. We suggest that only those cells which have had their membranes or processes physically cut during sectioning are able to take up the immuno-histochemical staining. If this is so then the stained portions of the section may also contain intact cells that are unstained and even methods of counting that concentrate on the stained thickness of the section will underestimate true cell numbers. Whilst the problem of full thickness staining is primarily a histological issue, it is one that has special implications for stereological methods, because it would be desirable to use the optical dissector method in fully stained thick sections. Additionally, if the barrier to antibody penetration is associated with the cell membrane then even the most rigorous application of 3-dimensional counting will fail to produce an accurate estimate of cell numbers.

It is our experience that the two-layer artefact described herein is observed by many laboratories in the neuroscience field (personal communications with a large number of workers over many years). As a consequence, many laboratories that aspire to carry out unbiased stereological sampling on their material are unable to do so. The fixatives and perfusion protocol used by our laboratory are standard methods which are widely used in the neurosciences. If our conclusions are correct, then we would expect that any laboratory using such protocols is likely to experience two layer immuno-histochemical staining on thick sections whether they are aware of it or not. Some workers may be unaware of the two-layer effect because they use sections of less than 40μm thickness. In such sections unstained cells at the

centre of the section would be easy to overlook and whilst cells may be seen through the full thickness of the section one would expect to see fewer cells in the central portion. Alternatively, it is possible that some laboratories, assuming that the problem stems from the section thickness, have attempted to solve the problem by cutting thinner sections. Our results show that, depending on the size and shape of the cells being stained, cells could still be missed even in relatively thin sections using immunohistochemical protocols.

We conclude that modification of the widely used, paraformaldehyde-based, perfusion protocol will be necessary in order to achieve full thickness staining with immuno-histochemical methods. The task may not be an easy one. An ideal solution would be a modification of the fixative so that the same general perfusion protocols could be used and major modification of the staining methods would not be required. Any new protocol would ideally be as versatile as the present one in terms of the techniques that can be carried out on the tissue. In our laboratory, tissues preserved using the standard fixation protocol are stained immunohistochemically using more than 30 different antibodies as well as a number of histochemical stains. Whilst the addition of membrane disrupting agents and/or the reduction of the concentration of paraformaldehyde, are obvious modifications to try, there are pitfalls to consider. Membrane disrupting agents such as methanol also have fixative properties and have the potential to adversely affect the immunohistochemical detection of antigens within the tissue, particularly when using monoclonal antibodies which target a single tissue epitope. Reduction of the concentration of paraformaldehyde may also have the same effect, altering the antigenicity of compounds of interest so that they are undetectable by IHC. Clearly the chemical and anatomical preservation of our tissue must not be sacrificed for the sake of membrane permeability. We may therefore need to consider the use of compound fixatives, in which the concentration of PFA is reduced, and other fixative agents such as glutaraldehyde, picric acid or zinc chloride are added. We are currently investigating such options.

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Chapter 3

In Vivo Transgene Expression from an Adenoviral Vector is Altered Following a 6-OHDA Lesion of the Dopamine System

3.1 Introduction and supplementary information

3.2 Manuscript:
In Vivo Transgene Expression from an Adenoviral Vector is Altered Following a 6-OHDA Lesion of the Dopamine System.
E. M. Torres, C. Monville, P. R. Lowenstein, M. G. Castro, S. B. Dunnett.
(Accepted for publication in the Journal of Molecular Brain Research).

3.1 Introduction and supplementary information

Work investigating the potential use of viral vectors in-vivo is usually carried out in normal animals. Because of my previous experience working with Herpes simplex vectors, I was aware that the pathological state of the brain could influence the behaviour of injected viral vectors. Subsequent experiments (see paper 3) would rely on the direct injection of adenoviral vectors in a model of Parkinson's disease where the rat brain had been unilaterally depleted of dopamine by injection of 6-hydroxydopamine. It was decided therefore to investigate the behaviour of the adenoviral vectors to be used in these studies when injected into the dopamine-depleted striatum of the rat and to compare this to the non depleted situation.

All of the experiments contained in this paper, the subsequent analysis of the collected data and the writing of the manuscript itself was undertaken by the author of this thesis.

S.B. Dunnett, P. R. Lowenstein, M. G. Castro and C. Monville, were involved in the planning of experiments and provided help and advice throughout. C. Monville also assisted during surgery and in the rotational testing of the large numbers of animals involved.

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Research report

In vivo transgene expression from an adenoviral vector is altered following a 6-OHDA lesion of the dopamine system

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Abstract

We have investigated the in vivo dynamics of an adenovirus-based, LacZ expressing vector, RAd36, at different doses, when injected unilaterally into the corpus striatum of normal rats. We have further investigated the characteristics of this vector in the presence of a 6- OHDA lesion of the nigrostriatal pathway. The dopamine-depleting lesion had an effect on both the number and the distribution of cells transduced by the adenoviral vector. The lesioned side of the brain contained significantly greater numbers of β-galactosidase positive cells than the unlesioned side at 3 days, 1 week and 4 weeks post-injection and the distribution of transduced cells was altered by the presence of a dopamine lesion. We conclude that the increased levels of transgene expression seen in the lesioned hemisphere are due to a change in the diffusion characteristics of the injected vector in the lesioned hemisphere. These results indicate that, when investigating the use of virus- based vectors, ultimately for use in gene therapies in the CNS, the in vivo dynamics of the vector need to be assessed not only in the normal brain, but also in the pathological brain state such as animal models of target diseases.

Theme: Disorders of the nervous system
Topic: Degenerative disease: Parkinson's
Keywords: Adenovirus; Viral vectors; Animal model; Gene therapy; 6-OHDA; Parkinson's disease

1. Introduction

The use of viral vectors as tools for direct gene therapy in the central nervous system is a tantalising prospect for neurodegenerative conditions such as Parkinson's and Huntington's diseases. Highly efficient vectors have been developed over recent years, for the direct delivery of genes into the adult nervous system. These include Herpes simplex virus (HSV), adenovirus (AV), adeno-associated virus (AAV) and lentivirus (LV) derived systems [1,2]. Such technology allows the direct transfer of genetic material into post mitotic cells in the adult brain. In theory, it should be possible to deliver genes of interest such as growth factor genes, or the genes necessary for the synthesis of dopamine, directly into the Parkinsonian brain and in this way to halt or even reverse the deficits induced by the loss of dopamine in this condition [3,4].

Growth factors, such as glial derived neurotrophic factor (GDNF), delivered using viral vectors, have been shown to be effective in animal models of Parkinson's disease (PD). Several groups have demonstrated that pre-delivery of GDNF into rat brain using an adenoviral vector, prior to a 6-hydroxydopamine (6-OHDA) lesion can dramatically reduce the cell loss and/or behavioural deficits induced by the lesion [5-8]. Similar studies using adeno-associated viruses also report sparing of dopamine neurones from the toxin [9-13]. AAV delivery of the dopamine synthetic genes tyrosine hydroxylase (TH), aromatic amino-

decarboxylase (AADC) and GTP cyclohydrolase 1(GCH1) has also been successful [14-17]. Lentiviral vectors have been used for direct delivery of GDNF or the GDNF related protein, neublastin into the brain and have proved protective against the effects of a 6-OHDA lesion in rats [18,19], and in primates [20][1;2] And in a therapy aimed at restoring dopamine function following a 6-OHDA lesion, a lentivirus containing the gene for GDNF was able to enhance functional recovery in rats. [21]

The current work is based on a type 5 adenovirus-based vector called RAd36. The vector is a first generation adenoviral vector having the E1 and E3 regions of the genome deleted to render the virus replication defective. The vector contains the LacZ gene from Escherichia coli driven by the highly efficient, major-immediate-early promoter from the murine cytomegalovirus (MIEmCMV). The construction and purification of this vector have been described previously. [22-23] Gerdes et al. [22], injected RAd36 into the corpus striatum of normal rat brain at doses from 10^4 to 10^8 infectious units (IU). Injections in the 10^4 to 10^7 range transduced large numbers of cells in the striatum and adjacent corpus callosum, as shown by β-galactosidase immunohistochemistry. Only at the highest dose of 10^8 IU was there any noticeable adverse reaction to the virus and associated tissue damage. Optimal transgene expression was seen following injection of 10^7 IU. This resulted in a cross sectional area of transduced cells of $2.5mm^2$ at the site of injection. Although cell counts were not carried out at all doses, the authors reported a near 100% efficiency of transduction (i.e. nearly 1 cell transduced for every IU injected) at low doses (10^1 to 10^3 IU injected) and demonstrated that the mCMV promoter was three orders of magnitude more efficient when compared to the hCMV promoter.

The work described above, characterising the in-vivo dynamics of RAd36 transduction and transgene expression, was carried out in normal rats [22]. Our interest is to use adenoviral vectors to deliver transgenes in models of Parkinson's disease and in transplantation-based therapies for the purpose of improving the survival of foetal dopaminergic cell implants. In this regard there were two critical issues to be addressed prior to the use of this vector system in our animal models. Firstly, to establish the injection parameters which would allow us to obtain transgene expression within a sufficient volume of striatal tissue for therapeutic effect and to determine the optimal titre of virus to use to obtain maximal gene expression without adverse effects. Secondly, to investigate whether the in vivo properties of the vector might be affected by the 6-OHDA lesion used to induce Parkinsonism.

2. Materials and Methods

Experimental animals

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

Adenoviral vectors

The recombinant, LacZ containing adenoviral vector (RAd36) used in this work has been previously described. Briefly, the vector is a first generation, replication defective, E1, E3 deleted, type 5 adenovirus vector into which has been inserted the Escherichia coli LacZ gene. The inserted gene is driven by a 1.4kb fragment from the major-immediate-early, murine cytomegalovirus promoter (MIEmCMV) [24,25]. Vectors were grown using a complementing HEK-293 cell line (derived from human embryonic kidney) and purified by caesium chloride gradient centrifugation to a titre of 4 x 10^{10} IU/ml [26].

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 carried out using the co-ordinates of Paxinos and Watson. [27] Viral vectors, neurotoxins and vehicle control solutions were delivered via a 30-gauge cannula connected to a 10μl Hamilton syringe in a micro drive pump set to deliver at 1μl/minute. Injections of both 6-OHDA and viral vectors were 3μl in volume delivered over 3 minutes with 3 minutes allowed for diffusion of the injected solution into the brain before careful withdrawal of the cannula.

Unilateral Dopamine lesions were carried out by stereotaxic injection of 6-hydroxydopamine hydrobromide (Sigma-Aldrich, UK) into the median forebrain bundle. The toxin was used at a concentration of 3μg/μl (calculated as the free base weight) dissolved in a solution of 0.1% ascorbic acid in 0.9% sterile saline. The stereotaxic co-ordinates used for injection were as follows: A= -4.4, L= -1.0, V= -7.8, with the nose bar set at -2.3mm below the interaural line.

RAd36 injections.

The RAd36 stock solution (4 x 10^7 IU/μl) was diluted 40-4000 fold using 0.9% sterile, isotonic saline to working titres of 1 x 10^6 - 1 x 10^4 IU/μl The stereotaxic co-ordinates used were as follows: A= +0.6, L= ±3.0, V= -4.5, with the nose bar set -2.3mm below the interaural line.

Rotation tests.

Lesion induced rotation tests under the influence of the dopamine agonist meth-amphetamine were carried out 2 weeks 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 modelled after the design of Ungerstedt and Arbuthnot [68] Rotation test scores were accumulated over a 90-minute testing session following an intraperitoneal injection of methamphetamine hydrochloride (dissolved in 0.9% sterile saline) at a dose of 2.5mg/Kg of body weight. Rotations were expressed as total, net rotation scores (ipsilateral minus contralateral) over the session. Rats with a net rotation score of less than 600 turns per session were considered to have a poor lesion and were eliminated from the study.

Histology.

On completion of the experiment, animals were terminally anaesthetized by intraperitoneal injection of 1mg/Kg sodium pentobarbitone, then perfused transcardially with 100ml of phosphate buffered saline (PBS) at pH 7.4, followed by 250ml of 4% paraformaldehyde in PBS over a 5 minute period. The brains were then removed from the skull and post-fixed by immersion in the same fixative solution for 4 hours, 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 60μm into 0.1M 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 of sections from each brain was stained using the general neuronal stain cresyl-fast violet as follows. Sections were mounted onto gelatine coated microscope slides and allowed to dry at room temperature overnight. Slides were then dehydrated by five minutes immersion (with agitation) in an ascending series of alcohols; (70%, 95%, and 100% ethanol), then 30 minutes immersion in a 50/50 mixture of chloroform and ethanol. Slides were re-equilibrated to water via 5 minutes immersion (with agitation) in 95%, and 70% ethanol then distilled water. Staining was carried out by 5 minutes immersion in cresyl fast violet solution (5% in 0.1M sodium acetate buffer, pH3.5). Differentiation of the stain and dehydration was carried out simultaneously in an ascending series of alcohols (70%, 95%, and 100% ethanol) before clearing in xylene and cover slipping using DPX mounting medium.

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 TBS. Endogenous peroxidase enzyme activity was quenched using a 10 minute immersion in 3% hydrogen peroxide/ 10% methanol in distilled water, followed by washing and re-equilibration in TBS. After a 1 hour pre-incubation period in a solution of 3% normal goat serum/0.1% Triton X-100 in TBS, sections were incubated in a monoclonal β-galactosidase antibody (Promega UK) at a 1:10,000 dilution in 1% normal goat serum/0.1% Triton X-100 for 60 hours 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 anti-rabbit, secondary antibody (Dako 1:200) in 1% normal goat serum in TBS was applied for 3 hours. The sections were then washed for 30 minutes before application of 10% strepavidin-biotin-horseradish peroxidase solution (Dako UK) in TBS for 90 minutes, followed by thorough washing and equilibration to 0.05M Tris non-saline (TNS) solution at pH 7.4. The horseradish peroxidase label was revealed by a 10-minute incubation in a 0.5% solution of diaminobenzidine tetra hydrochloride (Sigma–Aldrich UK) in TNS containing 0.3ul/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.

Morphometry

In experiment 1, comparative levels of gene expression were carried out by measuring the area of β-galactosidase staining in each section and using these data to estimate the total volume of brain in which the marker gene was expressed. These measurements were carried out using a PC based image analysis system equipped with Scion-Image software. Measurements of total striatal volume on both the vector-injected and normal sides of the brain were taken in a similar fashion so that any gross tissue damage could be assessed by comparing the ratio of the striatal volumes on each side. In experiment 2, assessment of the extent of marker gene expression in the brain was carried out using the same image analysis system. The numbers of β-

galactosidase positive cells in each brain was estimated using a Leica DMRB microscope (Leica Microsystems UK) using a 10 x 10 eyepiece graticule and a x20 objective. In brains where were several brain structures contained stained cells, sub-counts were carried out in each structure, and the volume of staining estimated from these.

Statistical Analysis

ANOVA tests were carried out using the statistical package Genstat 5 (Version 3.2; Lawes Agricultural Trust, Rothamsted, UK). Two-tailed values of $p<0.05$ were considered significant.

3. Procedure

Experiment 1: In vivo, dose-response characteristics of RAd36 mediated gene expression.

In this dose-response experiment 3 groups of 6 normal rats each received a unilateral striatal injection of 3µl of RAd36, using the protocol described above. The titre of RAd36 used for each group was as follows. Group 1; 1 x 10^4 I.U./µl, Group 2; 1 x 10^5 I.U./µl, Group 3; 1 x 10^6 I.U./µl. All rats were sacrificed 4 weeks post-injection of the virus.

Experiment 2: The effects of a 6-OHDA lesion of the median forebrain bundle on RAd36 mediated gene expression.

Eighteen rats received a unilateral 6-OHDA lesion of the median forebrain bundle, followed by amphetamine-induced rotational testing at 2 weeks and 4 weeks post lesion. Rats were then divided into three matched groups according to rotation scores. Twenty weeks later all rats received bilateral, striatal injections of 3µl of the LacZ adenoviral vector RAd36 at a concentration of 1 x 10^6/µl. Group one was sacrificed and the brains were perfusion fixed, 3 days post injection, group two at 1-week post injection and group three at 4 weeks post injection.

4. Results

Experiment 1

Figure 1 shows representative sections from each group of rats stained for β-galactosidase and cresyl fast violet. On brain sections stained using cresyl fast violet there was no evidence of an inflammatory reaction in any of the vector-injected brains, at any of the doses used. The injected striata had a normal morphology and architecture, with no evidence of cell loss or tissue damage. In most brains a small scar at the site of the needle tract could be seen, containing unresolved blood pigments, but this was the only physical evidence of the injection seen in any animal.

There was a gradual increase in the levels of β-galactosidase staining in the striatum with increasing dose of the injected vector. A noticeable feature was that, as the volume of β-galactosidase expression in the striatum increased with the dose of vector, immunoreactivity was increasingly seen in structures outside the corpus striatum, specifically in the corpus callosum and/or the ependymal layer of the lateral ventricles. Whilst there were small amounts of staining of the corpus callosum in the brains of a number of animals in groups 1 (10^4 I.U./µl) and 2 (10^5 I.U./µl), (3 out of six brains in each group), only in group 3 (10^6 I.U./µl) was extensive staining seen (in 4 out of 6 brains). Analysis of the volumes of β-galactosidase staining in the corpus callosum (see Figure 2A) demonstrated that there was a significant increase in the levels of corpus callosum staining from the 10^4 I.U./µl dose to the 10^5 I.U./µl dose of vector ($F_{2,15} = 4.74$, $p<0.05$). All brains were examined for the presence of cells in areas of the brain afferent to the striatum into which injected virus might have been retrogradely transported, such as the neocortex or the substantia nigra pars compacta, but no immunoreactive cells were found in afferent areas.

To determine whether of not there was any gross loss of tissue in the striatum as a result of adenoviral vector injection, cresyl violet stained sections were used to compare the total volume of the corpus striatum on the injected versus the uninjected sides of the brain in each dose group (Figure 2B). The ratios of the total striatal volumes of the injected versus the non-injected sides were 0.97 $\pm$0.02, 1.02 $\pm$0.02, and 1.00 $\pm$0.01, in the 3x10^4 IU dose, 3x 10^5 IU dose and 3x 10^6 IU dose groups respectively. There were no significant differences between sides in any group and the mean ratio of the three groups combined was 1.00 $\pm$0.01. These data suggest that following injection of the RAd36 adenoviral vector, there are no gross cytotoxic effects at any of the doses used in this study.

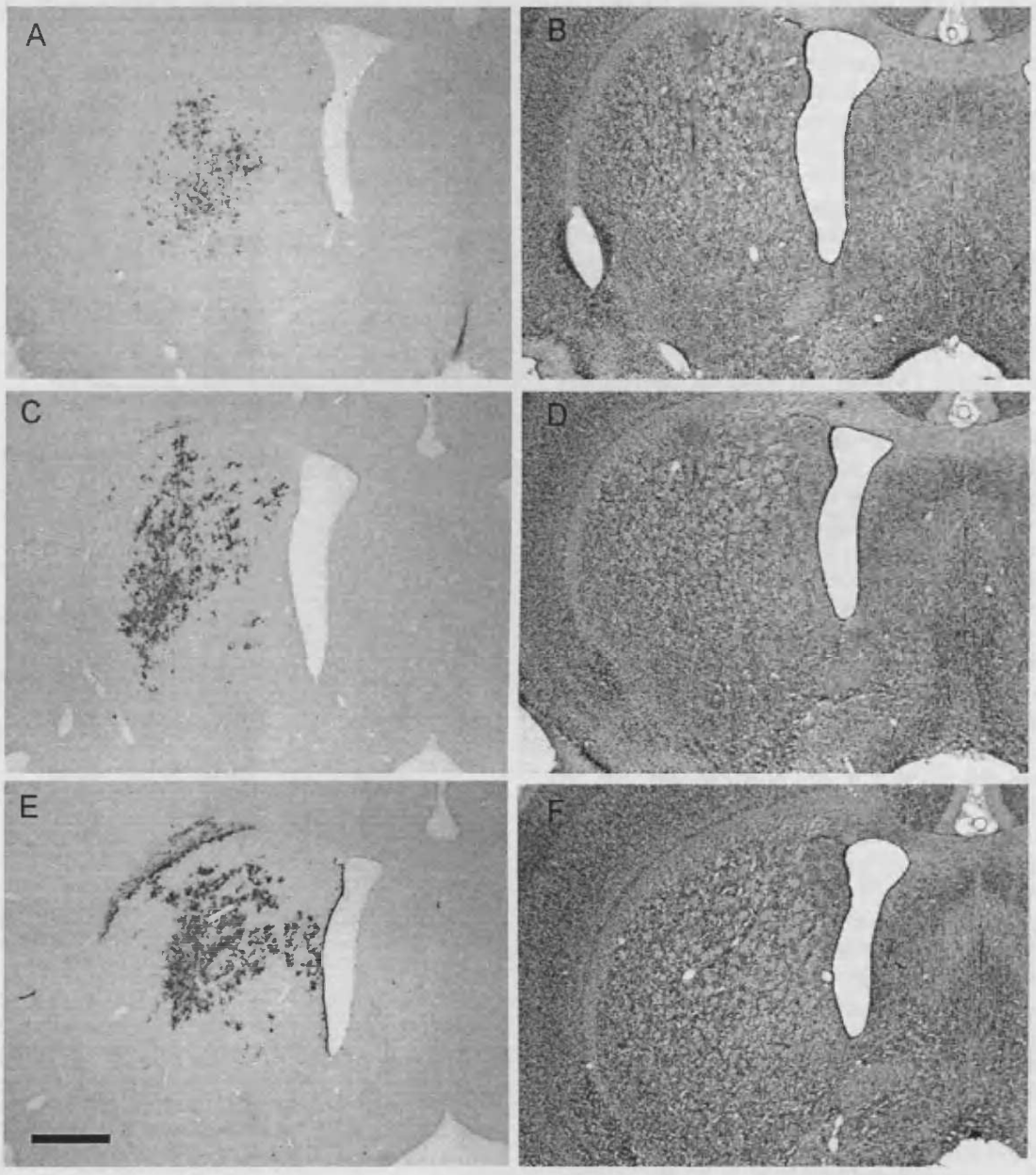


Figure 1. Experiment 1. In vivo, dose-response characteristics of RAd36. Photomicrographs of representative sections of brain stained for β-galactosidase immunohistochemistry (A,C,E) or cresyl violet (B,D,F) 4 weeks post injection of the adenoviral vector RAd36 at doses 3 x 10^4 IU, (A,B), $3x10^5$IU (C,D) and $3x10^6$IU (E,F). There is an increase in the volume of brain containing β-galactosidase immunoreactivity from the lowest dose group (A) to the middle dose group (C). There is a further increase in staining volume in the highest dose group (E) with the additional feature of a densely stained area in the corpus callosum in most of the animals in this group. Cresyl violet stained sections show normal striatal morphology in all dose groups. No evidence of inflammation or gross tissue damage was seen with this virus in any animal. Scale bar is 1.0mm

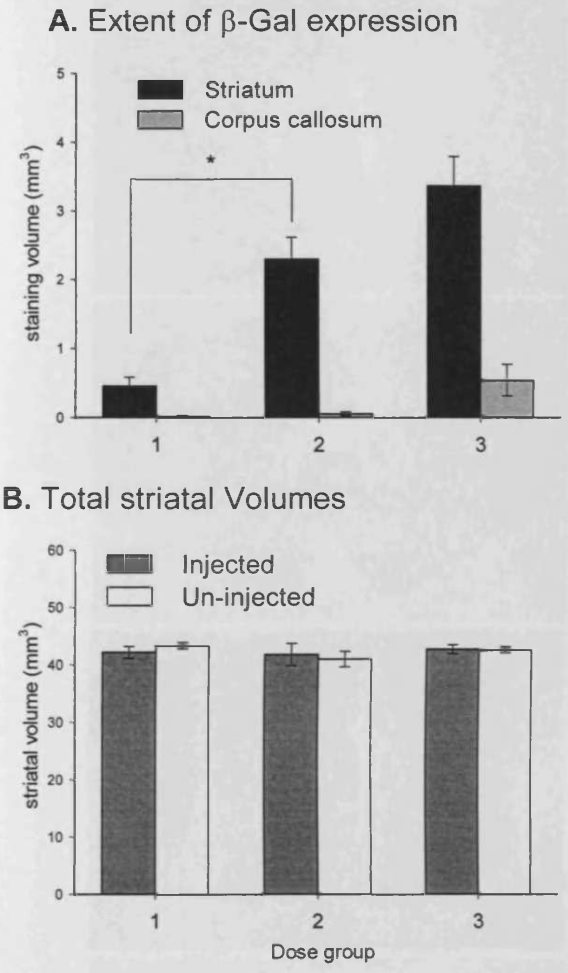


Figure 2. Experiment 1. In vivo, dose-response characteristics of RAd36 at $3x10^4$ (group 1), $3x10^5$ (group 2) and $3x10^6$ (group 3) IU doses. A) Volumes β-galactosidase immunoreactivity in both the striatum and the overlying corpus callosum, 4 weeks following injection of different doses of RAd36 adenoviral vector into the striatum. In the striatum, there was a significant increase in the volume of staining observed between dose 1 and dose 2. B) Plot of the total striatal volumes on the injected and non-injected sides of the brain in all groups. The injected and non-injected volumes do not differ significantly indicating that there is no gross loss of striatal tissue on the injected side. Error bars are SEMs

Experiment 2.

Examination of cresyl violet stained sections showed that there was little or no evidence of an inflammatory reaction in any of the adenoviral-vector injected brains at any time point. There was no evidence of striatal atrophy, or of ventricular enlargement. Small scars, similar to those seen in experiment 1, were the only evidence of a striatal injection (See Figure 3B, 3D, 3F and 3H).

In the β-galactosidase immuno-stained sections however, there was a clear asymmetry of staining in the two injected hemispheres. (See figures 3A, 3C, 3E, and 3G). On the intact side of the brain β-galactosidase immunoreactivity was confined almost exclusively to the striatum, in a volume centred around the site of injection. In contrast, on the dopamine depleted side of the brain, many sections had staining in other structures, principally in the corpus callosum overlying the injection site. Analysis of β-galactosidase positive cell numbers (Figure 4A) demonstrated that the 6-OHDA lesioned side of the brain contained significantly greater numbers of β-galactosidase positive cells than the unlesioned side at all of the time points observed ($F_{1,16} = 14.85$, $p<0.001$).

The differences in mean cell numbers seen in the lesioned and unlesioned sides of the brain were due in part to the distribution of stained cells in the two hemispheres. On the unlesioned side of the brain most staining was seen around the site of injection in the corpus striatum, similar to that seen in the medium dose group in experiment 1. On the lesioned side of the brain however, immunoreactivity often extended into the overlying white matter of the corpus callosum (Figure 3) similar to that seen in the high dose group in experiment 1. This is demonstrated by comparing the numbers of cells counted in the corpus callosum and striatum on each side of the brain (see Figures 4B and 4C). Although there were lower overall numbers of β-galactosidase cells in the lesioned striatum (9244 $\pm$1298) than in the unlesioned striatum (11691 $\pm$1467) this difference was not significant. However, in the corpus callosum there were significantly larger numbers of cells seen on the lesioned side (10768 +1571) compared to the unlesioned side (1457 +1035) of the brain. ($F_{1,16} = 37.11$, $p<0.001$).

There was a slight but significant decline in the numbers of transduced cells in both hemispheres between 3 days and 4 weeks post injection of the virus ($F_{2,16} = 4.87$, $p<0.05$). Analysis of these data indicate that the decrease in the number of positive cells with time is due to a fall in the number of cells counted in the striatum on both sides of the brain (Figure 4B). However, the numbers of cells counted in the corpus callosum do not change significantly during the time course of the experiment. (Figure 4C). As in experiment 1, no cells expressing the transgene were seen in areas afferent to the site of injection.

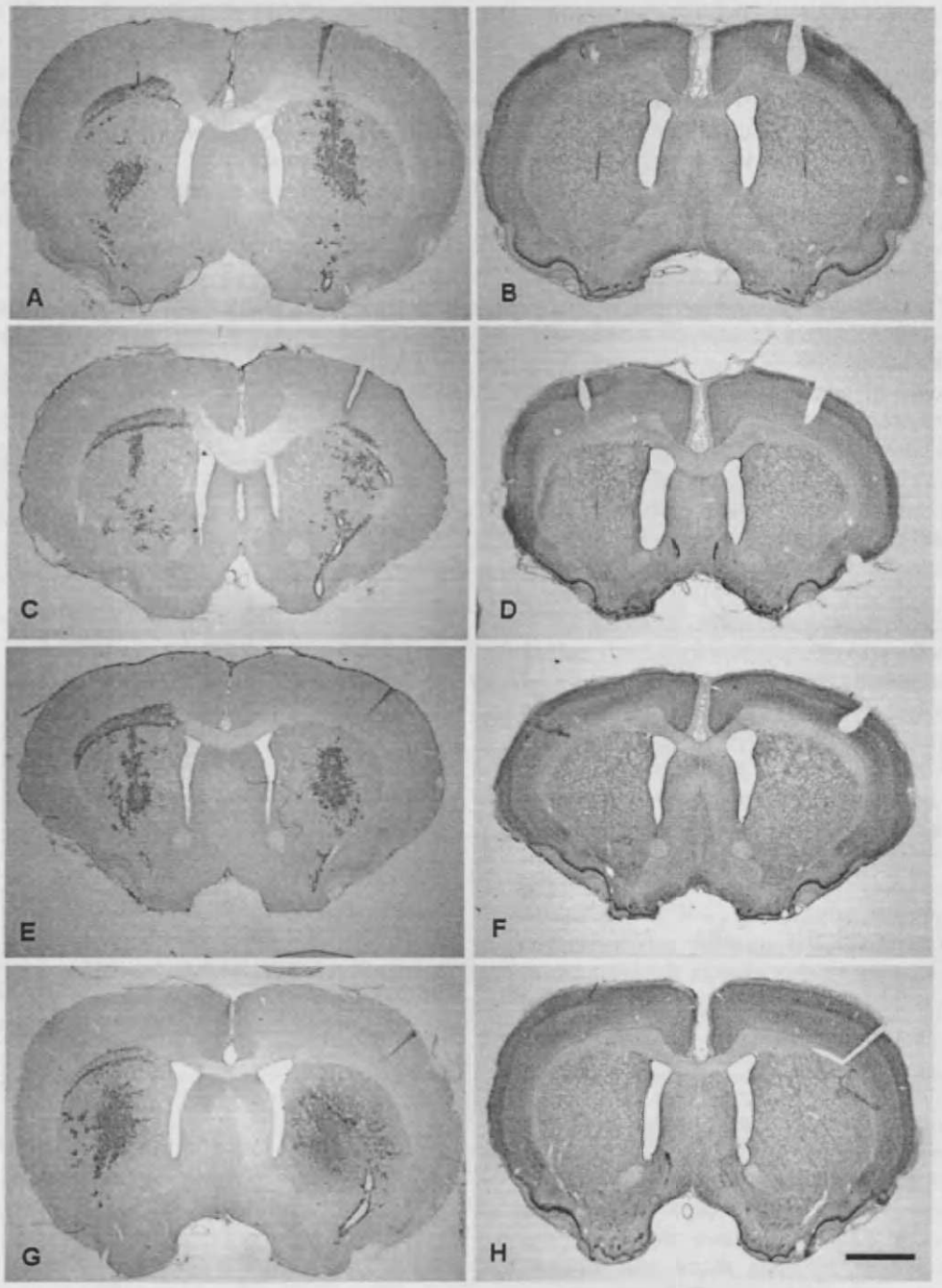
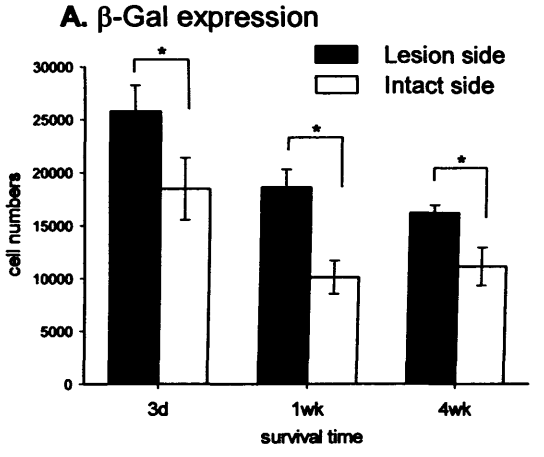
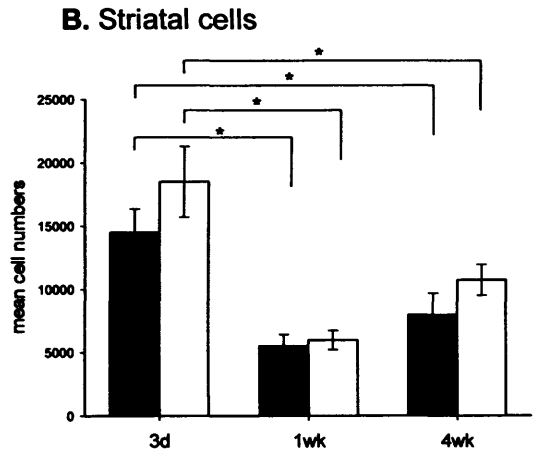
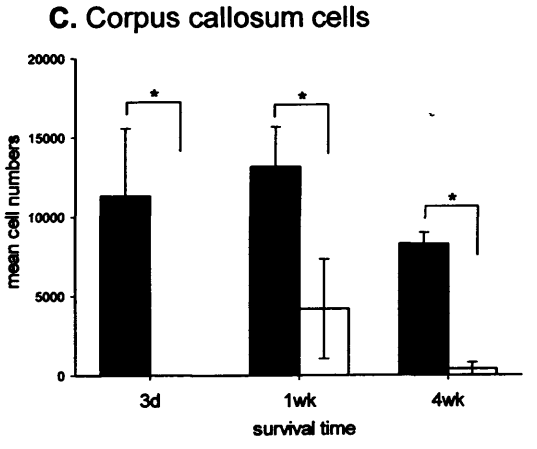


Figure 3. Representative sections from experiment 2. Each row (A-B; C-D; E-F; G-H) shows pairs of adjacent sections, from the brains of 4 different animals from the group sacrificed 1 week post-injection of $3x10^6$ IU of RAd36. In the left hand column are sections taken at the level of the injection site and stained for β-galactosidase (A, C, E, G) In the right hand column are adjacent sections stained with cresyl fast violet. In all pictures, the 6-OHDA lesioned side of the brain is on the left hand side of the image (note the orientation mark in the cortex on the unlesioned side of each section made at the time of sectioning). The β-galactosidase stained sections show clearly the differential distribution of gene expression in the two hemispheres. On the unlesioned side most expression is in the striatum. On the lesioned side, striatal expression appears slightly lower and there is an additional area of staining in the overlying corpus callosum. In the cresyl fast violet stained sections note the needle tracts in both hemispheres but the absence of inflammation, tissue damage or ventricular enlargement. Scale bar is 2mm.

Our results show that, following direct injection of Rad36 into the brain, both the number of cells expressing LacZ and the distribution of those cells in the brain are affected by the presence of a dopamine depleting, 6-OHDA lesion. Consequently, the calculation of the volumes and titres of virus injections and of the subsequent doses of therapeutic gene expression are likely to be possible only in tissue which accurately models the pathology of the disease concerned.







5. Discussion

The use of adenovirus gene transfer into the brain dates back to 1993 when Le Gal La Salle et al. reported that replication deficient adenoviral vectors were efficient vectors for the transduction of neural cells both in vitro and in vivo. They used a multiply deleted, replication deficient vector containing the LacZ gene under the control of the Raus sarcoma virus promoter (RSV) without apparent cytopathic effects. In vitro almost all cells in a culture derived from superior cervical ganglia were transduced and a similar efficiency was reported in vivo around sites of injection in rat hippocampus and substantia nigra with expression extending up to 2 months post injection [3]. In the same year, other workers using similar vectors reported efficient transduction in a number of brain areas following intracerebral injection in both rats and mice [4-6].

These so-called first generation adenoviral vectors were derived from either type 2 or type 5 wild-type adenoviruses and possessed only minimal gene deletions in the E1 region of the genome which were enough to render them replication deficient. However, it became apparent that despite earlier evidence to the contrary, such vectors, particular at high titres, were able to illicit a strong immune response following injection in vivo. This was seen particularly following injection into peripheral targets such as the lung and the liver and was associated with cytotoxicity in transduced cells followed by rapid down regulation of the transgenes [7;8].

Figure 4. Experiment 2. A) Time course of β-galactosidase expression in the lesioned and unlesioned hemispheres of the brain; total cell counts at 3 days, 1 week and 4 weeks post injection. The numbers of cells seen in the lesioned and intact sides of the brain were significantly different at all time points. B) Striatal cell counts. Time course of b-galactosidase expression in the lesioned and unlesioned striata; total cell counts at 3-days, 1-week and 4-weeks post injection. In the striatum β-galactosidase cell numbers were not significantly different on the lesioned and unlesioned sides at any time point. However 3 day cell counts on both sides of the brain were significantly higher than those at both the 1 week and 4 week time points. C) Time course of β-galactosidase expression in the corpus callosum in lesioned and unlesioned hemispheres of the brain. The numbers of cells seen in the lesioned and intact sides of the brain were significantly different at all time points. Error bars are SEMs

In spite of the supposed immune privilege the brain, inflammation was also reported following intracerebral injection of first generation viruses. As well as local activation of astrocytes and microglia there was also expression of major histocompatibility antigens T cell activation [9-12].

The inflammation observed following injection of adenoviral vectors was shown to be due to an immune response to viral proteins synthesised by infected cells [13]. In order to overcome this, second generation vectors were designed containing additional deletions of the E2 (primarily), and E3 or E4 regions of the genome [14]. These vectors not only attenuated the host immune response but had the added advantage of a larger carrying capacity for introduced transgenes [15;16]. More recently, so-called "gutless" adenoviral vectors have been developed which contain no viral coding sequences and display much reduced levels of immunogenicity in vivo [17]. Vectors of the type used in the present study have been assessed for their inflammatory properties. Thomas et al. injected a related vector RAd35 (having the same backbone as RAd36) into normal rats at a range of doses and studied inflammatory responses 3 and 30 days post injection using a range of markers. At doses in the range from 10^6 IU to 10^8 IU there was minimal activation of astrocytes and microglia and transgene expression remained stable over the 30 day study period. Above doses of 10^8 IU, cytotoxicity and chronic inflammation were observed and transgene expression became progressively down-regulated with increasing dose [18].

Adenoviral vectors have been used to successfully deliver glial derived neurotrophic factor (GDNF) in rat models of Parkinson's disease and to ameliorate the effects of 6-OHDA lesions, [2;19-23] and Horellou et al used an adenoviral vector containing the tyrosine hydroxylase gene (TH) to partially restore dopamine function to the hemiparkinsonian rat brain. Mild host responses to CNS injections of the adenoviral vectors in these models have been reported [24] but there are no reports of direct comparisons of vector dynamics in the normal versus the hemiparkinsonian brain as in the present work.

The data from experiment 1 indicate that at the doses used, the adenoviral vector RAd36 is an efficient, non-toxic vector capable of transducing large numbers of cells in the adult rat brain following intrastriatal injection. There was a dose response to an increasing titre of the injected virus and expression of the transgene product was evident at 4 weeks post-injection (the longest time point considered in the present study). An interesting phenomenon was the appearance of β-galactosidase expressing cells in structures other than the targeted striatum, in the high dose group. Why this should be is not certain, since we might expect the diffusion of injected virus to be similar in all groups. It seems likely that in the lower dose groups most viruses become bound close to the site of injection and in this way, diffusion away from the injection site is limited. In the high dose group, there may be saturation of virus-binding sites close to the injection site, which would allow the injected virus particle to diffuse further from the injection site and to transduce cells in a greater volume of the brain. This would concur with work done by Thomas et al. al. who described a similar phenomenon where viruses with reduced or absent binding ability were seen to diffuse further in the brain than normal virus [18]. This phenomenon has also been noted for other substances notably following injection of heparin into rat brain [25].

The levels of LacZ expression observed in experiment 2 were generally lower than those seen in experiment 1 using a 3 x 10^6 dose of virus. This is likely to be due merely to variability between experiments and does not detract from the relative differences seen between the lesioned and unlesioned sides of the brain in experiment 2. The data clearly demonstrate that, following bilateral, striatal injection of the adenoviral vector Rad36, levels of LacZ expression are greatest on the side of the brain which received a 6-OHDA lesion of the median forebrain bundle. The pattern, distribution and diffusion of recombinant adenovirus vectors and the expression of the transgenes can vary and is dependent on the dose, volume and speed at which the viruses are delivered within the brain parenchyma [18,[26]. All of these parameters were carefully standardized in the present experiment. Both the lesioned and unlesioned sides of the brain were injected in an identical fashion using the same stock of virus. The same titre of virus was used on each side and was delivered in the same volume of vehicle and using the same rate of injection. However, the distribution of marker gene expression observed was clearly different in the intact and 6-OHDA lesioned hemispheres of the brain and we can only conclude from the results shown in Figure 3 that the differences seen in the distribution of transgene expression are due to the presence of a 6-OHDA-induced lesion. When considering how one might limit gene expression to the striatum in the lesioned brain, it is possible that a reduction of the dose of virus injected into the lesioned striatum might prevent the extraneous expression of transgene products. However, the dose used in the current experiment was two to three logarithms lower than the doses used by other

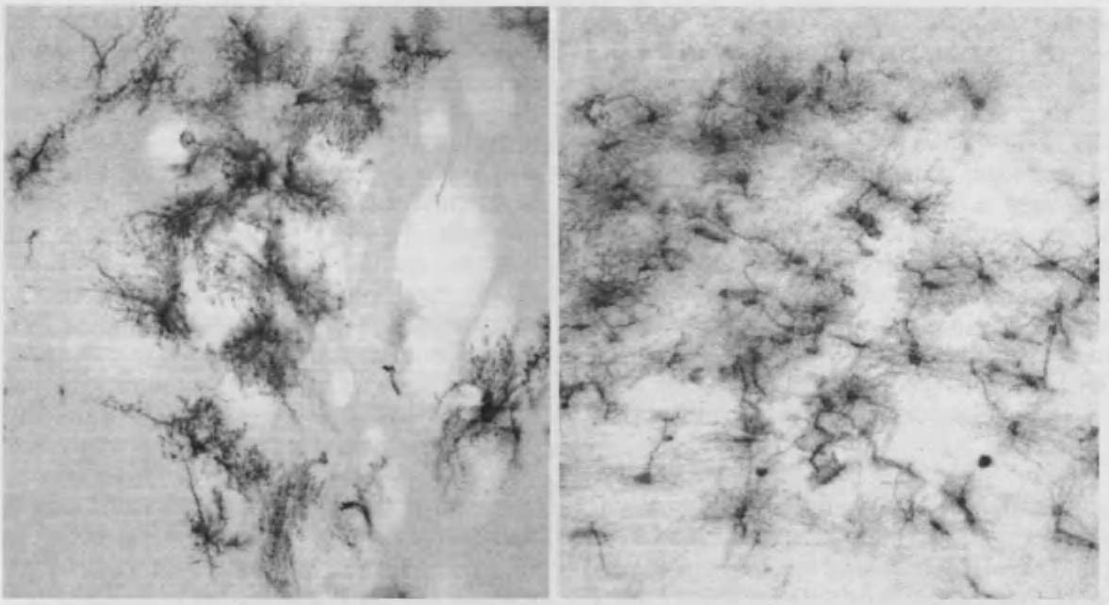


Figure 5. High power (x200) photomicrographs of β-galactosidase expressing cells following injection of RAd 36 into rat brain. A. In the striatum positively stained cells have multiple processes and have the appearance of glial (microglial or astrocytic) cells. Neuronal profiles were rarely seen. B. In the corpus callosum cells also have the appearance of glia.

groups in experimental gene therapy paradigms of Parkinson's, and we therefore conclude that lowering the viral dose further might be detrimental in providing the desired therapeutic benefit.

Presently, we can only hypothesize as to why a distal lesion of the median forebrain bundle and the subsequent removal of the dopamine innervation to the corpus striatum should affect the expression levels and distribution of an adenovirally delivered transgene. One possibility is that the withdrawal of dopamine terminals from the lesioned striatum has altered either the diffusion characteristics of the virus, or the distribution of virus binding sites in the striatum. This hypothesis is in line with the results from experiment 1 in which binding in the corpus callosum was seen only following injection of the highest titre of the virus and would also explain the results from experiment 2 in which the volumes of staining and cell numbers observed in the lesioned striatum were lower than those in the unlesioned hemisphere. Experimentally, the permeability of brain tissue is difficult to measure using anything other than the substance under investigation. Injection of tracers or dyes which may have very different diffusion characteristics is seldom informative. However, by using antibodies against a marker other than the vector transgene product (e.g. viral coat proteins) it should be possible to look at the brain minutes or hours after vector injection and determine whether the diffusion of the vector in the lesioned striatum is different to that in the intact brain.

Another possibility is that the 6-OHDA lesion has altered the population of cells in the lesioned striatum. At high magnification, the β-galactosidase expressing cells seen were glial in nature, in both the striatum and corpus callosum and on both sides of the brain. The dopamine-depleting 6-OHDA lesion of the median forebrain bundle has been shown to produce acute gliosis in the striatum as shown by increases in levels of the astrocyte marker GFAP. Stromberg et al. demonstrated gliosis 1 month post lesion which had down regulated by 7 months post-lesion [27]. In another study gliosis was at a peak in the first week post lesion but had declined to control levels after 4 months [28]. In studies where 6-OHDA was injected directly into the striatum there were increased numbers of astrocytes [28] and microglia [29] in the lesioned striatum. In contrast, another study looking at GFAP levels in the striatum 15 months after a 6-OHDA lesion found no long term gliosis [30]. We shall be investigating further the nature of the changes to the dopamine depleted striatum which might be the cause of the present observations. A systematic time course study of the inflammatory responses in the striatum following a median forebrain bundle will be carried out. Against this background RAd36 will be injected at different time points to see if the levels and distribution of transgene expression correlate with any of the inflammatory changes seen.

We conclude that, following viral vector delivery, the in-vivo dynamics of transgene

expression in the rat brain are altered in the presence of a 6-OHDA lesion of the median forebrain bundle. Removal of the dopaminergic innervation of the striatum prior to the injection of a LacZ containing adenoviral vector led to increased numbers of transduced cells and to a change in the distribution of those cells within the lesioned hemisphere. This finding has important implications for the use of viral vectors, both in animal models of brain disease and for any potential clinical applications. The differences seen in the lesioned and non-lesioned hemispheres in the present study would be likely to affect both the potency and the efficacy of any potentially functional transgene. Clearly, when investigating any gene therapy ultimately intended for therapeutic use it will be necessary to conduct pre-clinical studies in a host environment that mimics as closely as possible the pathology of the diseased brain. The diseased brain may be structurally different to the normal state lacking normal structural elements such as the afferent dopamine terminals in PD or containing additional structures such as scar tissue. There may be additional cell types or increased numbers of cells, notably those cell types involved in inflammatory processes. In addition, inflammation in the CNS is associated with leakage of the blood brain barrier and the diseased brain may also contain blood derived inflammatory cells, proteins and cytokines all of which may interact with injected vectors. We recommend that all future investigations into direct application of gene therapies in the CNS should take full account of these issues.

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Chapter 4

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

4.1 Introduction and supplementary information

4.2 Manuscript:

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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(Accepted for publication in Brain Research Bulletin)

4.1 Introduction and supplementary information

In 1999, using the marker of cell division bromo-deoxy-uridine, Sinclair et al demonstrated that virtually all of the surviving TH-positive neurons seen in transplants from E14 rat embryos had undergone their final cell division *in utero,* prior to excision from the embryo. In contrast, none of the neurons that had undergone cell division in the grafts post-implantation, expressed the dopaminergic phenotype. It was hypothesised that the numbers of dopamine cells in E14 grafts were low because many of the dopaminergic cells implanted had not yet differentiated. Thus, the dopamine "precursor" 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.

The aim of the present study was to investigate two questions. Firstly, could the provision of differentiation factors to dopamine grafts at the time of implantation improve the yield of dopamine cells in the graft? Secondly, were the adenoviral vectors used to deliver LacZ successfully to the rat brain in previous experiments capable of delivering therapeutic genes to dopamine grafts?

S.B. Dunnett, P. R. Lowenstein, M. G. Castro and C. Monville, were involved in the planning of experiments and provided help and advice throughout. C. Monville also assisted during surgery and in the rotational testing of the large numbers of animals involved.

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Research Article

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 neurones 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 3-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.

Key Words: Adenovirus; viral vectors; animal model; gene therapy; 6-OHDA; Parkinson's disease; dopamine; transplant; sonic hedgehog.

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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 neurones 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, most 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 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;43;74].

Neural Transplantation

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 behaviors caused by a unilateral lesion of the dopamine system and to ameliorate behavioral 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;31;61]. 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 [41;54] and others showing little benefit. More critically, in recent clinical trials some patients developed significant side effects, 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 [30], although advances are being made in developing animal models of the neurobiological basis of L-DOPA and graft associated dyskinesias [39;64], with optimism that the interaction between chronic L-DOPA treatment and dopamine rich grafts can be understood and subsequently managed to avoid this unwanted side effect.

Graft Survival

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 [37;42;50]. Poor dopamine cell survival and/or incomplete 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 [41], 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;62].

Aims

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 [28;67]. 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) [28].

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 cells 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

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 [33], RAd/LacZ [67] 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.

Experimental animals

Adult, female Sprague-Dawley rats were used, weighing 200-250g 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.

Adenoviral vectors

The construction and characterisation of the adenoviral vectors used in the work have been described previously [28;33;67]. 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 [63]. 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 cytomegalovirus-derived promoters of human origin or murine origin [67;70] (see table 1).

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 [52].

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 10μl Hamilton syringe in a microdrive pump set to deliver at 1μl/min. The toxin was used at a concentration of 3μg/μl (calculated as the free base weight) dissolved in a solution of 0.2mg/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.

Viral vector injections.

On completion of post-lesion rotational testing, rats were divided into groups, matched according to their rotation scores and then 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 = ±3.0, V = -4.5. All virus injections were 3µl in volume and carried out using the same parameters for injection as the 6-OHDA lesions.

Table 1. Summary of Adenovirus vectors used

Vector	Transgene	Promoter*	Final titre
RAd35	Lac Z	MIEhCMV/	1 x 10^7IU/µl
RAd Shh	Shh-N	MIE*m*CMV/	1 x 10^6IU/µl
RAdGDNF	GDNF	MIE*h*CMV/	1 x 10^7IU/µl

Promoters are derived from either murine (mCMV) or human cytomegalovirus (hCMV)

Ventral mesencephalic grafts.

VM grafting was carried out two 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 2µl of the cell suspension containing the cells from 1 VM, injected at the same coordinates used for the viral vector injections.

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

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 [68]. Following an intraperitoneal injection of methamphetamine hydrochloride (dissolved in 0.9% sterile saline) at a dose of 2.5mg/kg of body weight, rotation test scores were recorded and are reported as net scores (ipsilateral minus contralateral) over a 90-minute session. Only rats with a net rotation score of ≥600 turns per session were used in the experiment. Grafted rats were rotation tested 4 weeks and 6 weeks post-implantation using the same method.

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 3mm-thick coronal slice was cut at the level of the injection and the both intact and injected striata dissected out and collected into 0.5ml 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 13000 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 hours at room temperature. Samples of purified GDNF in serial dilutions (0-1000pg/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 hours 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 (490nm) was measured in an automated plate reading system within 1 h of the reaction.

Histopathology

On completion of behavioural testing, animals were terminally anaesthetized 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 minute period. The brains were then removed from the skull and post-fixed by immersion in the same fixative solution for 4 hr, 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 40μm into 0.1M 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 hr 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 hr. The sections were then washed for 30 min before application of 10% strepavidin-biotin-horseradish peroxidase solution (Dako) in TBS for 90 min, followed by thorough washing and equilibration to 0.05M 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 0.3ul/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.

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 a10 x 10 eyepiece graticule and a x20 objective on the same sections and corrected using the Abercrombie formula [1].

Statistical Analysis

ANOVA tests were carried out using the statistical package Genstat 5 (Version 3.2; Lawes Agricultural Trust, Rothamsted, UK). Two-tailed values of $p<0.05$ were considered significant.

3. Results

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 wk post-lesion to a net contralateral score, 4 and 6 wk 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 difference sbetween 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.

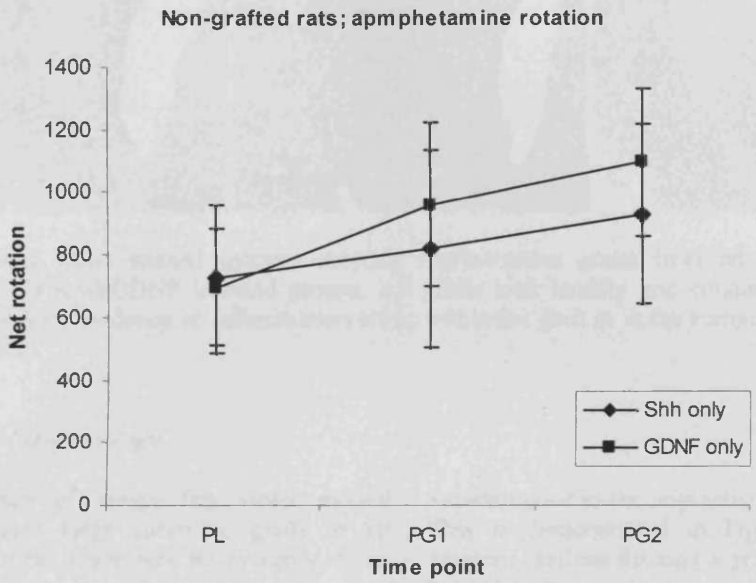

Figure 1. Amphetamine rotation in control animals with 6-OHDA lesions and striatal injections of either RAd/Shh or RAd/GDNF. Time points are 6-weeks (PL), and 8 weeks (PG1) and 10 weeks (PG2) post-lesion at time points corresponding to post graft testing in the graft groups. There is no recovery of amphetamine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

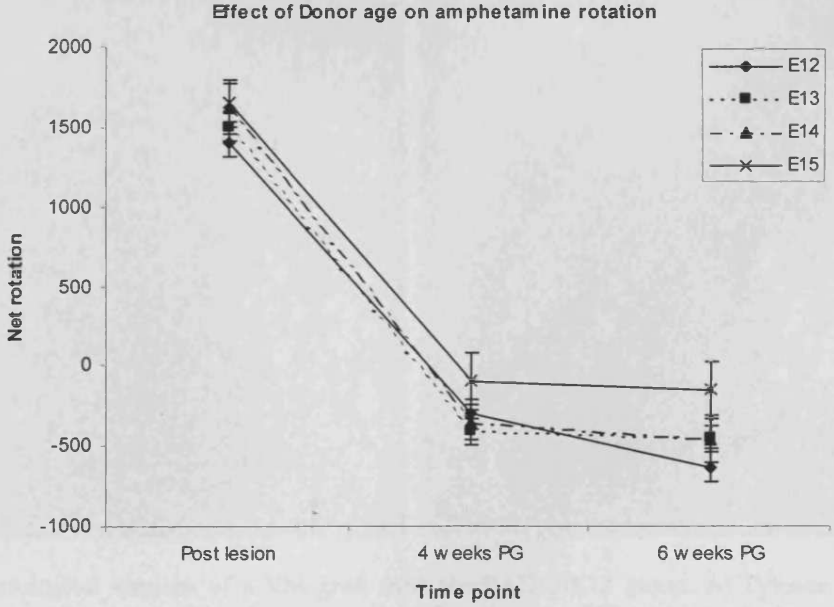

Figure 2. Post lesion and post graft amphetamine rotations by embryonic donor age. There was no effect of virus pre-treatment on post graft rotation. All graft groups showed an over-compensatory rotational response.

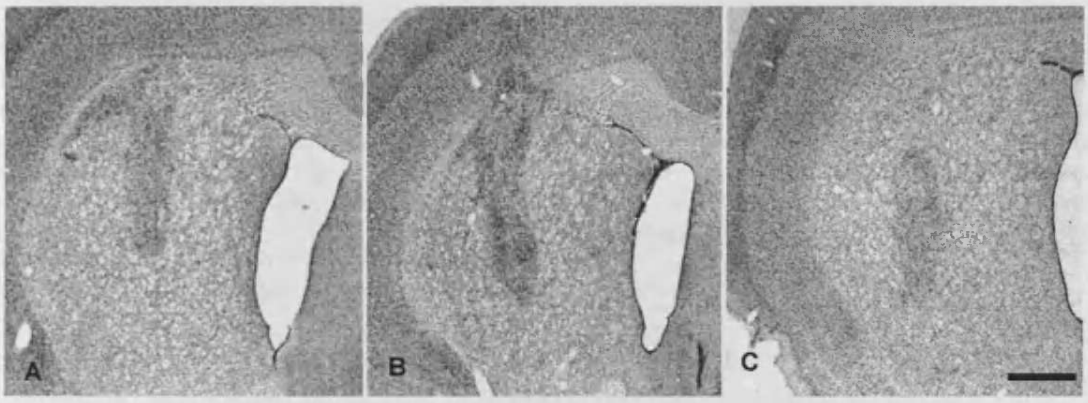

Figure 3. Cresyl violet stained sections showing representative grafts from rats in A) control, B) RAd/Shh and C) RAd/GDNF injected groups. All grafts look healthy and contain large numbers of neurons. There is no evidence of inflammation either within the graft or in the surrounding host striatum. Scale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

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 immuno-histochemically for TH and β-galactosidase respectively. LacZ transduced cells were seen in intimate contact with the VM grafts.

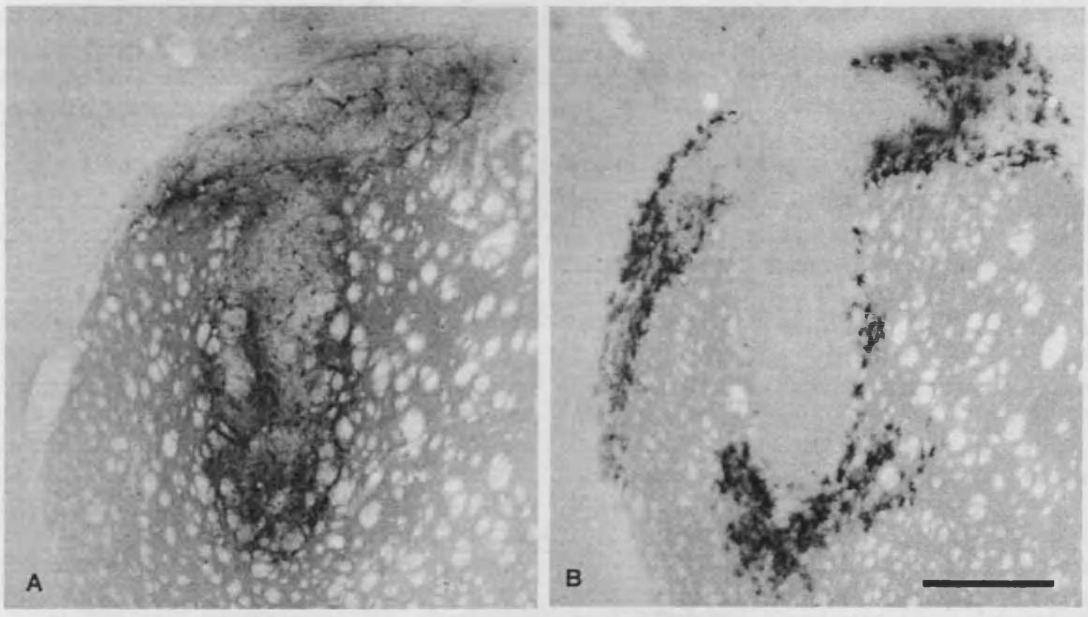

Figure 4. Histological sections of a VM graft from the RAD35/E12 group. A) Tyrosine hydroxylase immuno-staining shows a healthy dopaminergic graft with many TH positive neurons and displaying extensive outgrowth into the surrounding striatum. B) β-galactosidase immuno-staining on an adjacent section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

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. Figure 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 at this age. Similarly, figure 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.

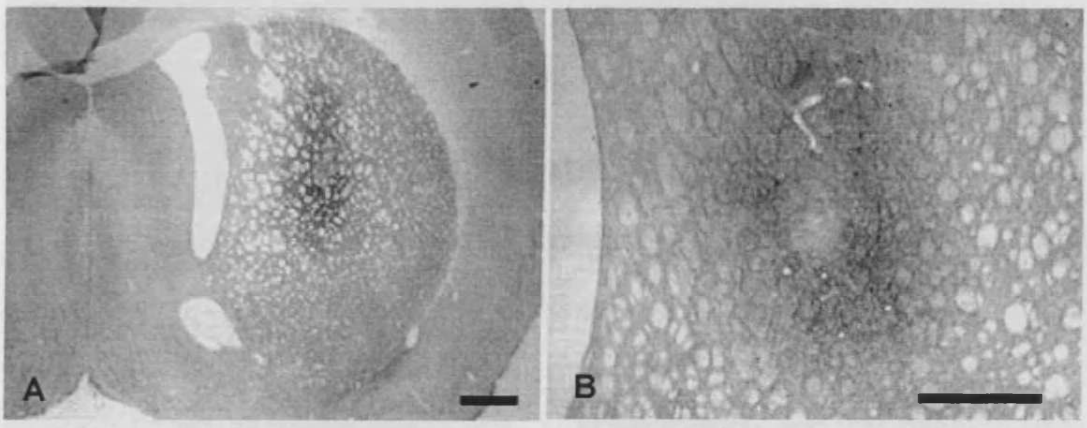

Figure 5. Expression of transgenes in the grafted striatum following viral vector injections. A. GDNF immunoreactivity around a small section of graft in a brain from a GDNF/Graft group. B. Shh expression surrounding a graft in a section from an animal in a Shh/Graft group. Shh staining shows immunoreactive 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 bars are 1mm.

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 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 figure 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 $\pm$35 pg/mg of tissue, over and above the background levels of GDNF detected in Rad35 injected and non-injected striata.

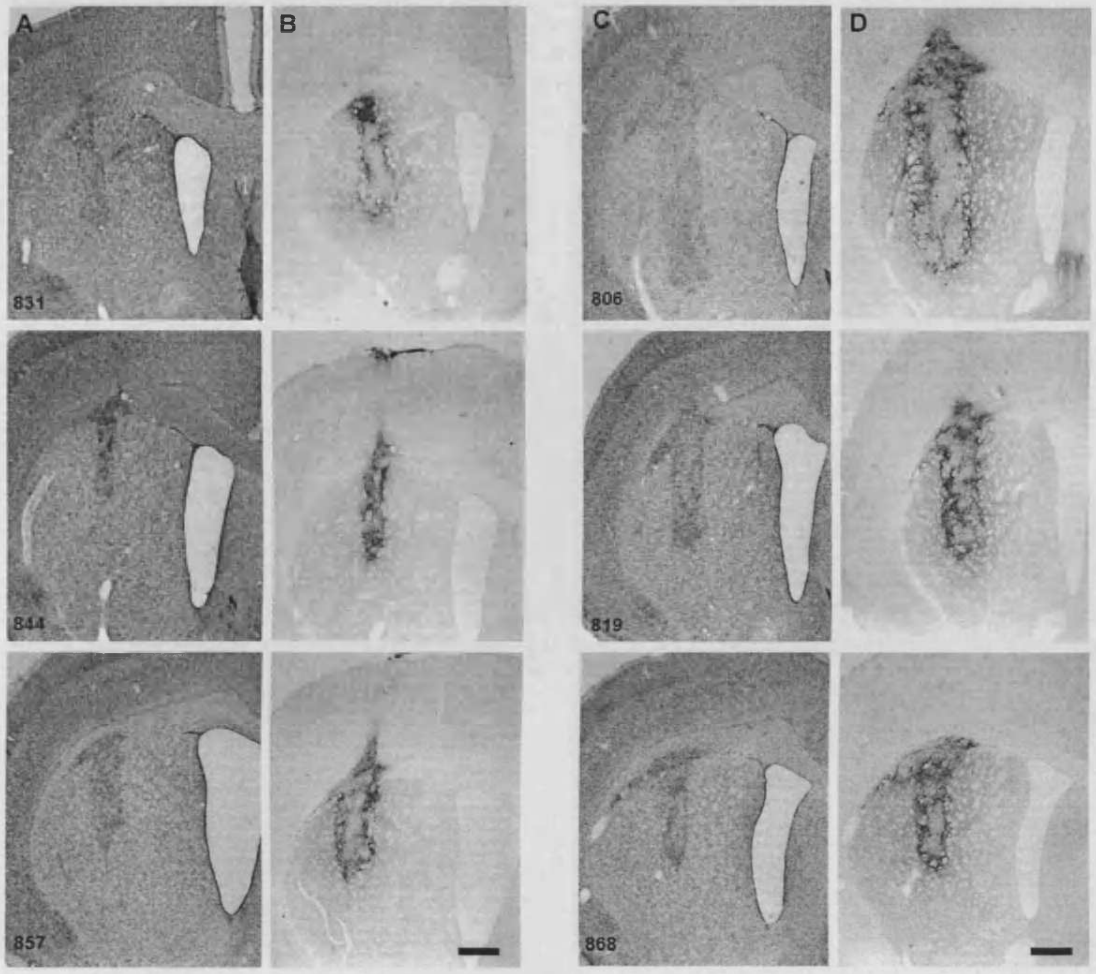

Figure 6. Representative sections of E12 donor age grafts from saline (columns A and B) and Shh (columns C and D) treated groups stained for cresyl violet (columns A and C) and in adjacent sections TH (columns B and D). Grafts treated with the Shh vector contained significantly greater numbers of cells that the saline treated group. Numbers are individual rat numbers. Scale bars are 1.0mm

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 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.

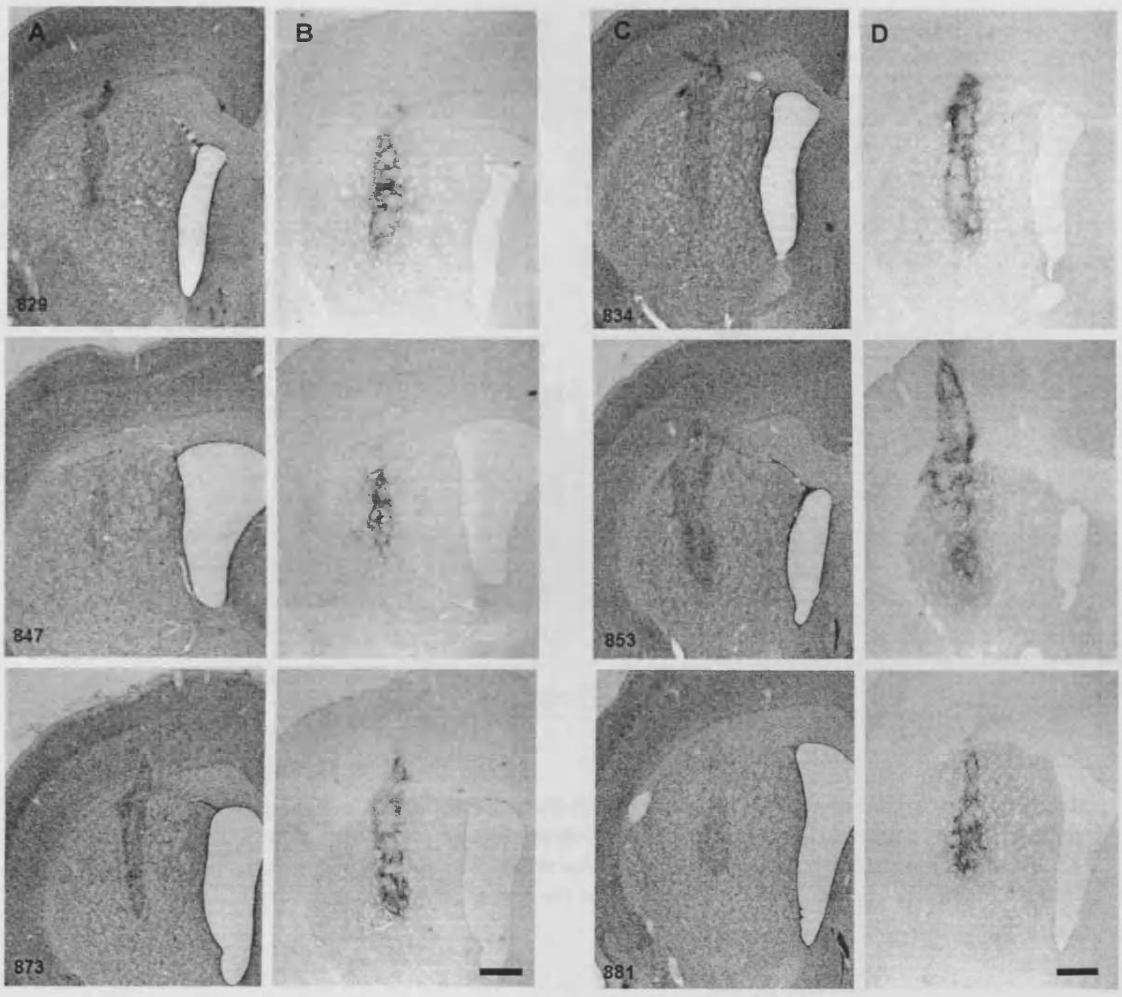

Figure 7. Representative sections showing E14 donor age grafts from saline (columns A and B) and GDNF (columns C and D) treated groups stained for cresyl violet (columns A and C) and adjacent sections for TH (columns B and D) from 3 rats in each group. Grafts treated with the GDNF vector contained significantly greater numbers of cells that the saline treated group. Numbers are individual rat numbers. Scale bars are 1.0mm

The use of the transcription factor Shh in the present study was influenced by work carried out in 1999 by Sinclair et al [60], in which it was shown that virtually all of the surviving TH-positive neurons in transplants from E14 rat embryos had undergone final cell 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 were low because the many of the dopaminergic cells implanted had not yet differentiated. Thus, the dopamine "precursor" 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 a factor (Shh) known to be important in dopaminergic differentiation to dopamine grafts using adenoviral vectors.

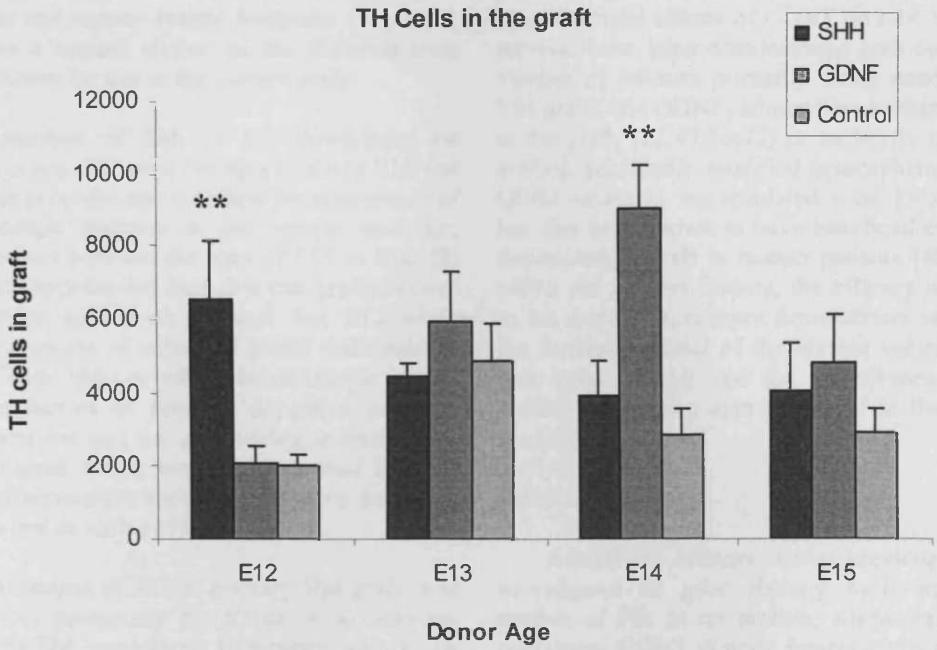

Figure 8. Plot of estimated mean TH cell numbers in the dopaminergic grafts in each group. There was no significant effect on cell numbers at any age group in the control group. However at the E12 donor age, Shh treated grafts contained significantly more cells than all other treatment groups and at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

Differentiation Factors

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. It is expressed in the notochord and floor plate of the developing midbrain as a 45kd protein that undergoes proteolytic cleavage into carboxy terminal (Shh-C) and amino-terminal (Shh-N) fragments [44]. The 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 [34]. 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 [35;53]. 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];[55].

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 neurones 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) [65] and 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. [71] 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 to E16 and thus both precedes and overlaps the appearance of dopaminergic neurons in the ventral midbrain, which occurs between the ages of E11 to 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 [49];[34;45].*

Application of Shh to primary VM grafts was 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 [73]. 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 also 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.

GDNF

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. Whilst 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.[40] 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 neurones.[5;26;27]. 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;46;69] adenovirus (AV) vectors [13;14;33;66] and LV vectors [4;11;29;36]. 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;47;56;72] or indirectly using co-grafted, genetically-modified neurospheres [51] or GDNF-secreting encapsulated cells [59]. GDNF has also been shown to have beneficial effects on dopaminergic graft in human patients [48]. 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.

Adenoviral Vectors

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;38]. In a strategy aimed at replacing dopamine synthesis in the depleted striatum, Horellou and colleagues used an adenoviral vector containing the TH gene to partially restore dopamine function in the hemiparkinsonian rat brain [32]. Only one study has reported the application of viral vectors to dopamine implants by pre-loading of the host striatum. Sanchez-Capelo et al delivered transforming growth factor beta (TGFβ1) to VM grafts by prior injection of an adenoviral vector into the striatum [58]. 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 when using this 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. In the current work pilot studies involving direct injection of the current vectors led us to choose two weeks post injection of the vectors as the time point best satisfying these criteria.

Conclusion

The improvement of E12 graft survival following treatment with RAd/Shh in the present study is an interesting result. Since at this donor age very few dopamine neurones 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 [60]. 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 development, 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. [7] 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 precise 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;33;57] 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 neurones; 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.

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Chapter 5

Improved survival of primary dopamine grafts in a rat model of Parkinson's disease by use of younger embryonic donor age tissue

5.1 Introduction and supplementary information

5.2 Manuscript:
Improved survival of primary dopamine grafts in a rat model of Parkinson's disease using young embryonic donor age tissue
E. M. Torres, C. Monville, M.A. Gates, V. Bagga, S. B. Dunnett. (2005)
(*To be submitted to Nature Neuroscience as a short report*)

5.1 Introduction and supplementary information

The work described in the previous chapter looked at the application of adenoviral vectors containing either Shh or GDNF to dopamine grafts derived from embryonic donors of different ages. The original experiment included an E11 embryonic age group, not reported in the final manuscript. This was because E11 derived grafts produced unexpectedly large grafts, which were unaffected by any of the virus treatments. (See figure S1). Control E11 grafts contained 5 times more cells than control E14 grafts and with DA cell survival rate of more than 30%, were larger than any primary, unmodified grafts reported previously. Because of the importance of this finding and because the E11 data added little to the observations made on the E12-E15 grafts it was decided not to include these data in the manuscript so that they could be investigated more fully. This chapter presents further work investigating the potential of E11 derived VM grafts.

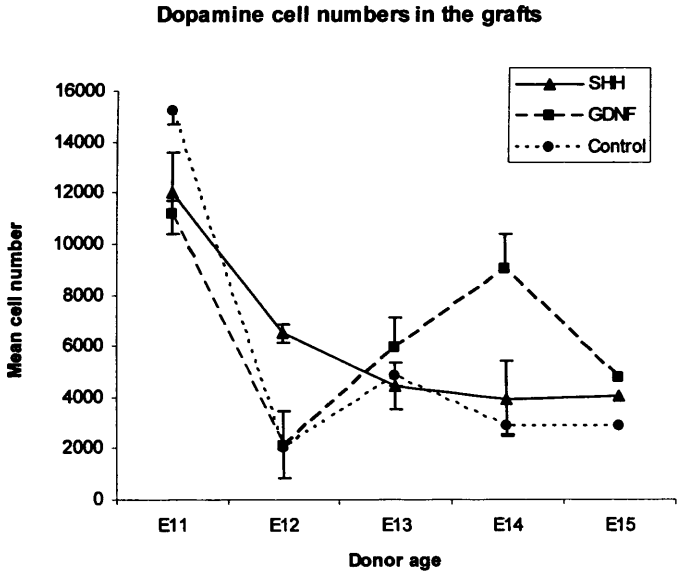

Figure S1. Effects of RAd viral vector applications on mean TH cell numbers in embryonic VM grafts derived from donors of different ages. The mean TH cell number in grafts of the E11 group was 12779 (SEM = 1229) Numbers of TH cells were significantly greater in E11control grafts when compared to those the standard E14/15 donor ages (t_{28}=2.53, $p<0.05$).

S.B. Dunnett, P. R. Lowenstein, M. G. Castro and C. Monville, were involved in the planning of these experiments and provided help and advice throughout. C. Monville also assisted during surgery and in the rotational testing of the large numbers of animals involved. The help of V. Bagga was invaluable in the staging and dissection of the very young embryos used in this study.

Research Report

Improved survival of primary dopamine grafts in a rat model of Parkinson's disease using young embryonic donor age tissue

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Abstract

The paradigm for dopamine replacement by transplantation of embryonic dopamine cells was established in the rat model of Parkinson's disease more then two decades ago. The initial protocols, which used solid implants of embryonic ventral mesencephalon, were superseded by the use of embryonic cell suspensions and such grafts were shown to survive well, innervate the host striatum, release dopamine and reverse some of the behavioural deficits caused by the Parkinsonian lesion. At that time, the optimal age of embryo used for donor tissues was determined as embryonic donor age E14 (post plug). This was arrived at chiefly from knowledge of the developing nervous system and corresponded to the age at which maximum dopamine cell neurogenesis was known to occur. Since then, the techniques for dissection of ventral mesencephalon from the donor embryos, preparation of cell suspensions and methods of implantation have lead to improved graft survival. However, the survival of primary graft tissue is still relatively poor and typically less than 10% of implanted dopamine neurons survive. Poor survival has been demonstrated in human grafts also and is one of the main factors limiting the use of dopamine grafts as a treatment for Parkinson's disease.

Using modern methods of graft preparation, we have readdressed the issue of the optimum donor age for dopamine grafts. In a rat model of Parkinson's disease, rats received unilateral 6-OHDA lesions of the median forebrain bundle followed by dopamine grafts derived from embryos of crown rump length 4mm, 6mm, 9mm, or 10.5mm. Grafts derived from 4mm embryos survived poorly, with less than 1% of the implanted dopamine cells surviving. Grafts derived from 9mm and 10.5mm embryos were similar to those seen in previous experiments with survival rates of 8% and 7% respectively. The best survival was seen in the group that received 6mm grafts, which were significantly larger than all other graft groups. Mean cell survival in the 6mm group was 36%, an extremely high rate for primary untreated VM cell grafts applied as a single placement and more than five-fold larger than the 10.5mm group. As VM tissues of this age contain few differentiated dopamine cells we conclude that the large numbers of dopamine cells seen in the 6mm grafts differentiate post-implantation and consider the in-vivo conditions which allow this differentiation to take place and the implication of this work for the future of dopamine grafting.

Key Words: Animal model; dopamine graft; donor age; 6-OHDA; Parkinson's disease.

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Introduction

One of the most promising therapies for the treatment of Parkinson's disease is the replacement of lost dopamine neurons by implantation of dopamine cells derived from embryonic ventral mesencephalon (VM). The technique was pioneered in animal models more than twenty years ago when solid pieces of embryonic substantia nigra were implanted into cortical cavities or into the ventricular spaces of Parkinsonian rats.[8] Such grafts were able to survive in the host brain and induce graft-derived behavioural recovery in simple tests of motor asymmetry. Subsequently, techniques for preparing the embryonic VM as a cell-suspension were developed, allowing implantation of the tissue directly into the de-afferented striatum. [19,44] In these studies functional recovery was shown to be dependent on the topographical placement of the graft tissue as well as the reinnervation of the host striatum by the implanted dopamine cells. The implanted cells were able to mature, send out axons into the host tissue, make appropriate synaptic connections and importantly, deliver dopamine to the target tissue.[16,20,10]

Embryonic dopamine tissue allografts into human subjects were first carried out in the mid 1980s,[28,31] and several clinical centres have succeeded in producing long-lasting functional recovery in Parkinson's patients following embryonic dopamine cell implants.[18,34] Not all clinical centres have the same success and the technique has both detractors and supporters. However, there is little doubt that, in a subset of patients, transplants derived from donor embryos of the correct age and implanted using a robust surgical protocol, have been able to provide considerable therapeutic benefits. In successful cases the implanted dopamine cells provide a continuous low dose of dopamine to the denervated striatum in a manner that eliminates the peak-trough regime of orally administered L-DOPA. Patients are able to reduce their L-DOPA intake dramatically to a fraction of the previous dosage and in some cases L-DOPA treatment can be stopped altogether. The associated side affects are much reduced or eliminated altogether. Long term studies of these patients have shown that transplantation therapy is long lasting and that it has the potential to extend treatment of PD patients well beyond the therapeutic window provided by L-DOPA.[29,39] Clinical effects take the form of 30-50%, improvements in motor tests of more time spent in the "on-phase" without debilitating motor symptoms and in many patients, a reduction in the doses of drugs needed to control their symptoms.[14,22,24,25,32,53]

Despite its success, transplantation therapy is not widely or routinely used as a therapy for PD because of the complexity of the ethical, logistical and quality control issues which surround the use of material derived from fetal donors.[4,7] Principal among these issues is the long-standing problem of the relatively poor survival of the implanted, dopaminergic neurons in the host brain. In animal models, although survival rates of up to 40% have been reported, in studies using techniques to enhance cell survival, typically only 5-10% of implanted embryonic dopamine neurons survive in the host brain.[13] Poor survival has also been confirmed in human patients,[27,30,35] and is considered a major factor in the incomplete recovery seen in many clinical trials.

When carrying out this study it became apparent that the precise estimation of embryonic donor age is a rather complex issue. Firstly, there is imprecision in the ageing of commercially acquired time-mates. Depending on the day of pairing, the dam and sire are together for 17 hours (overnight) or for 24 hours and the precise time of fertilization is unknown. As a result the developmental stage of embryos obtained in this way can vary considerably. Secondly, the donor age can be expressed either as post-coitus age where day zero is taken as the day of conception or post-plug age where day zero is taken as the day after mating when the vaginal plug is observed. Finally, two different rat embryo staging scales are used by workers in the field, one proposed by Ambrus et al [2] (in widespread use by American workers) and one proposed by Dunnett et al,[17] (principally used by European workers) modified from an earlier scale by Olson et al,[36] and Seiger et al.[45] Both scales estimate embryonic age from measurement of the crown rump length (CRL) of the embryos. The two scales agree well between the ages of E13 to E15, but disagree at ages younger than E13. On the Ambrus scale, a 6mm embryo would be aged E12 whereas on the Dunnett scale the size of an E12 embryo is given as 8mm. The latter scale does not give ages younger than E12 but extrapolation of the plot would make a 6mm embryo E11. In the present work, to avoid confusion, and to allow direct comparison with other experiments, CRL is used as the main index for comparison between groups.

Methods

Experimental animals

Forty, adult female Sprague-Dawley rats were used, weighing 200-250g 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.

Surgery

All surgery was performed under gaseous anesthesia (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. [37]

Unilateral Dopamine lesions

Dopamine lesions were carried out by injection of 6-Hydroxydopamine (hydrobromide, Sigma) unilaterally into the median forebrain bundle out using a 30-gauge cannula connected to a 10µl Hamilton syringe in a microdrive pump set to deliver at 1µl/minute. The toxin was used at a concentration of 3µg/µl (calculated as the free base weight) dissolved in a solution of 0.2mg/ml ascorbic acid in 0.9% sterile saline. The stereotaxic co-ordinates used for injection were: A= -4.4, L= -1.0, V= -7.8 (below dura), with the nose bar set at -2.3mm below the interaural line. Injections were carried out over 3 min. with 3 min. allowed for diffusion before careful withdrawal of the cannula.

Rotation

Lesion induced rotation tests under the influence of the dopamine agonist meth-amphetamine were carried out 2 weeks 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, following an intraperitoneal injection of methamphetamine hydrochloride (dissolved in 0.9% sterile saline) at a dose of 2.5mg/kg of body weight. Rotation test scores were accumulated over a 90-min. test session and are reported as net scores (ipsilateral minus contralateral) over the 90-minute session. Only rats with post lesion net rotation score of $\geq$600 turns per session were used in the experiment (n=32). Grafted rats were tested 4 weeks and 6 weeks post-implantation using the same method.

Dopaminergic grafts

Dopamine cell grafting was carried out 8-5-6 weeks post lesion following completion of rotational testing. The ventral mesencephalon was dissected from Sprague-Dawley rat embryos of a range of donor ages. The sizes of the embryos used and the estimated donor ages are given in table 1. VM grafts were prepared as a cell suspension according to a standard protocol.[17] Briefly, the ventral mesencephalon was dissected from each embryo. After washing in Hank's balanced salt solution (HBSS), the dissected pieces were placed in a solution of 0.1% % trypsin in 0.05% DNAase in Dulbecco's minimum eagle medium (DMEM) for thirty minutes then washed in two changes of DMEM containing 0.05% DNAase.

Dissociation of the dissected pieces was carried out in 200µl of DMEM containing 0.05% %DNAase by trituration with Gilson pippettors. Initial trituration was carried out with a 1ml (blue) pipette tip (15-20 triturations) then a finer 100µl (yellow) pipette tip was used (15-20 triturations) to achieve the final, single cell suspension. The suspension was then spun in a bench-top micro centrifuge at 2000rpm for 3 minutes. The supernatant was removed and the pellet of cells resuspended in DMEM containing 0.05% DNAase at a concentration of 1 VM per 2µl.

Grafted rats received 2µl of the cell suspension injected into the dopamine depleted striatum at stereotaxic coordinates: A = +0.6, L = $\pm$3.0, V = -4.5 (with nose bar set at –2.3mm) using a 30 gauge cannula attached to a 10µl Hamilton syringe, driven by an electronic syringe pump. After injection of the graft material, the cannula was left in place for 3 minutes to allow the diffusion of injected material, prior to careful removal of the cannula from the brain. Grafts in each group were derived from at least two cell suspensions prepared from two separate rat litters. Each injection consisted of 2µl of cell suspension containing the cells from 1 VM injected at the same coordinates as the viral vector injection.

Table 1 Details of VM graft suspensions.

CRL (mm)	Estimated Donor Age	Mean Cells/μl Implanted
4.0 (±0.25)	E10-10.5	34,000
6.0 (±0.25)	E11-11.5	220,500
9.0 (±0.5)	E12.5-13	336,500
10.5 (±0.5)	E13.5-14	422,000

On completion of behavioural testing, animals were terminally anaesthetized by intraperitoneal injection of 200mg/Kg sodium pentobarbitone, and then perfused transcardially with 100ml of phosphate buffered saline (PBS) at pH 7.4, followed by 250ml of 4% para-formaldehyde 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 hr, 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 40μm into 0.1M 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. 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 TBS. Endogenous peroxidase enzyme activity was quenched using a 10 minute immersion in 3% hydrogen peroxide/ 10% methanol in distilled water, followed by washing and re-equilibration in TBS. After a 1-hour 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 hours 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 hours. The sections were then washed for 30 minutes before application of 10% strepavidin-biotin-horseradish peroxidase solution (Dako) in TBS for 90 minutes, followed by thorough washing and equilibration to 0.05M Tris non-saline (TNS) solution at pH 7.4. The horseradish peroxidase label was revealed by 10 min incubation in a 0.5% solution of diaminobenzidine tetrahydrochloride (BDH) in TNS containing 0.3ul/ml of hydrogen peroxide. Sections were finally mounted on gelatine coated microscope slides dehydrated in an ascending series of alcohols, cleared, and coverslipped using DPX mountant.

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 calculated from cross-sectional areas measured on TH stained sections on a regular series (1 in 6) through the entire graft. Cell counts were carried out on a Leica DMRB microscope using a 10 x 10 eyepiece graticule and a x20 objective on the same sections and corrected using the Abercrombie formula.[1] Measurements of cell size were made using a x100 objective on the same system. Outgrowth from the graft was assessed by measurement of the density of staining around the graft using the Scion-image system. At high magnification the density of TH immuno-staining was measured on 3 sections per animal using the x40 objective. Using a measuring frame of 0.25mm^2 area, the density of staining was measured immediately adjacent to the graft and then at 0.5mm intervals at increasing distance from the graft edge both laterally to the corpus callosum and medially to the ventricle wall. Optical densities are expressed as a percentage of the density of staining in the intact striatum measured over averaged fields.

Results

Amphetamine rotations

The group of rats that received 4mm grafts showed no recovery from pre-graft levels on amphetamine rotation testing. 6mm, 9mm, and 10.5mm graft groups all showed decreased rotation levels 4 weeks post grafting and an over-compensatory rotation response by 6 weeks post grafting. Analysis of variance between mean rotations at different donor ages revealed significant differences in the levels of rotation seen between treatments at 4 weeks ($F_{3,26}$ = 9.93, $p<0.001$), and 6 weeks post grafting ($F_{3,26}$ = 23.43, $p<0.001$). Post-hoc t-tests revealed that at 4 weeks post grafting, 4mm group rotations differed significantly from 6mm (t_{13}.= 5.24, $p<0.001$); 9mm (t_{13}.= 6.85, $p<0.001$) and 10.5mm (t_{13}.= 5.24, $p<0.01$) rotations. At 6 weeks post grafting 4mm group rotations differed significantly from 6mm (t_{13}= 5.78, $p<0.001$) 9mm (t_{13}.= 6.50, $p<0.001$) and 10.5mm (t_{13}=5.80, $p<0.001$) rotations.

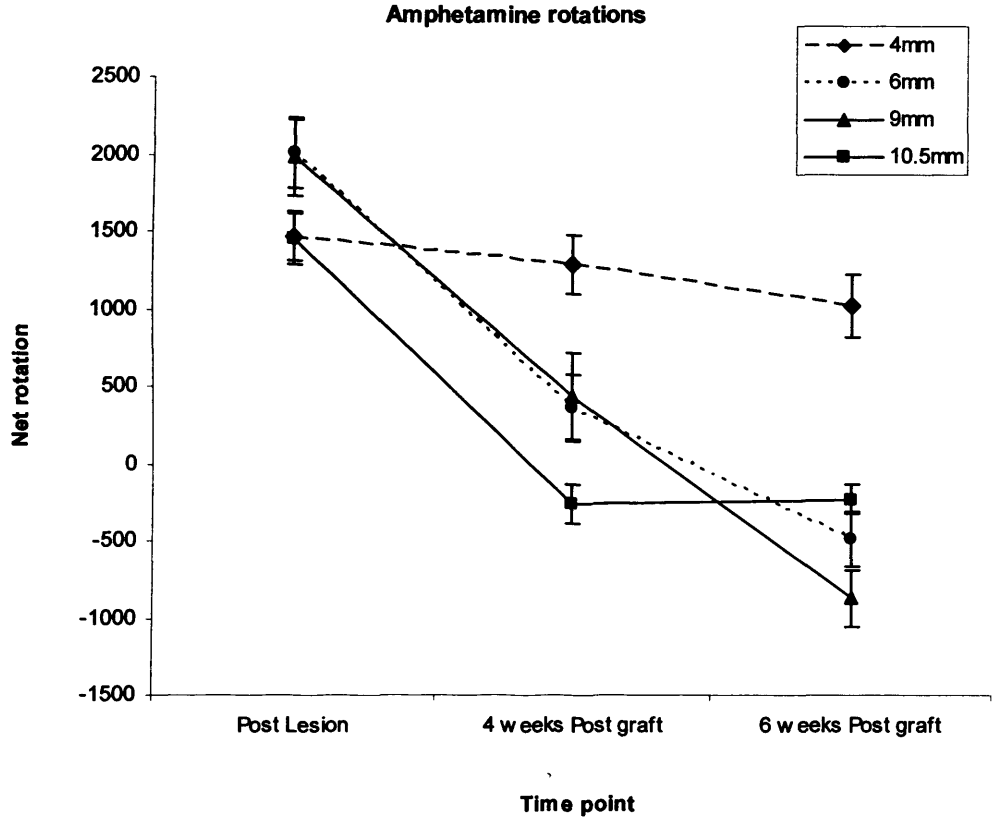

Figure 1. With the exception of the 4mm graft group all other groups produced a recovery of the lesion-induced behavioural deficit seen on amphetamine induced rotation. In the 6mm, 9mm and 10.5mm graft groups there was a change from the net ipsilateral rotation observed post-lesion, to a net contralateral rotation post-grafting, the classical over-compensatory graft response. At 4 weeks post grafting the net rotational response of the 10.5mm group was significantly greater than either the 6mm or 9mm graft groups though there were no differences in the rotational responses of the 6mm, 9mm and 10.5mm graft groups at 6 weeks post-grafting. This suggests that the maturation and integration of 6mm and 9mm grafts may occur more slowly than that of the E13.5 graft group.

Histology

Examination of cresyl violet stained sections showed the presence of grafts in the striatum in all graft groups. Grafts contained mixed populations of neuronal and non-neuronal cells and there was no evidence of an inflammatory reaction either within the graft or in the surrounding striatum. TH immunohistochemistry revealed large numbers of TH positive cells and extensive TH outgrowth from the grafts into the surrounding striatum (see figure 2). The 4mm grafts survived poorly with no surviving TH cells observed in several of the brains in this group. Grafts in the 6mm group were notably larger than those seen in any of the other groups occupying much of the striatum and had an almost circular profile, compared to the oval or tear-drop shaped grafts normally seen (see figure 2). In addition the rostro-caudal extent of the grafts was unusually large for single placement grafts extending in some cases more than 2mm and occupying a large portion of the striatal volume (see figure 3). The estimated mean graft volumes for each group were: 4mm: 0.051mm^3 (0.027); 6mm: 4.33 mm^3 (0.86); 9mm: 1.24 mm^3 (0.156), and 10.5mm: 0.618 mm^3 (0.221) (figures in brackets are SEMs). Analysis of variance confirmed that there were significant differences in graft sizes between donor age groups ($F_{3,25}$ = 13.08, $p<0.001$). 4mm graft volumes were significantly smaller than the standard 10.5mm graft volumes (t_{13} = -2.29, $p <0.05$), and 6mm graft volumes were significantly larger than 10.5mm graft volumes ($t_{12} = 3.42$, $p <0.01$).

The survival of dopamine cells in dopamine grafts is commonly expressed as a proportion of the total number of dopamine cells in the adult rat substantia nigra compacta. The most extensive review of survival rates used a figure of 35,000 cells in the intact adult substantia nigra,[13] and the following calculation are made using this estimate for direct comparison. The mean numbers of TH cells in grafts derived form each donor age is summarized in figure 4. Graft survival in the 10.5mm group was 6.9%, which is typical of unmodified grafts derived from donors of this age. The survival rate of 9mm grafts 10.7% and in 4mm grafts just 0.5%. The best survival rate was seen in the 6mm graft group and was 35.6%, a five-fold greater than the 10.5mm grafts and an extremely high survival rate for primary untreated VM cell grafts. Analysis of variance indicated significant differences between groups ($F_{3,26}$ = 13.17, $p<0.001$).

Correlation of rotations and cell numbers.

In previous experiments carried out by our group and in other laboratories, the numbers of surviving TH cells in the graft do not correlate well with post graft rotations. In the present study the much wider range of graft sizes presented an opportunity for readdressing this correlation. To allow for the influence of the hypersensitivity of the lesioned striatum, post-graft rotation scores were corrected by subtracting the pre-grafting amphetamine score for each animal. Figure 5 shows the plot of TH cell numbers against the post graft rotational change. There is a good correlation between these two factors ($r = 0.841$)and the plot suggests that maximal over-compensatory rotation is achieved when there are 4500-5000 cells in the graft but any further increase in cell numbers in the graft does not increase overall rotation further.

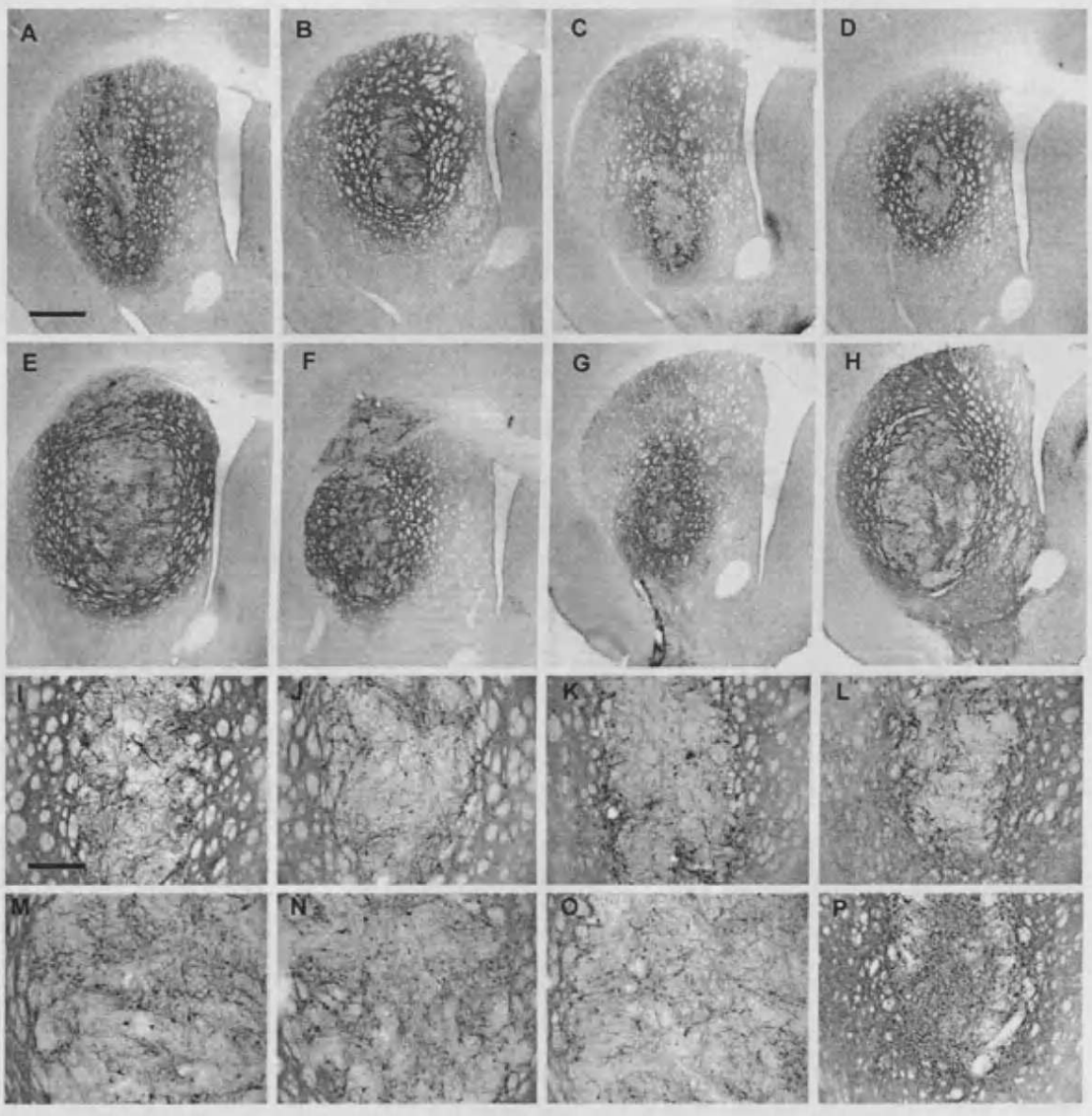

Figure 2: Morphology of dopamine grafts from 6mm and 9mm donor embryos (TH stained sections).
A-D; Low power images of representative 9mm grafts from 4 different animals. Grafts are typical, and similar to those seen from 10.5mm donors (not shown) consisting of a mass of tissue containing many TH positive cells distributed in patches within and around the periphery of the graft. Dense TH staining around the graft is dopamine re-innervation of the host striatum. E-H; Low power images of representative 6mm grafts from 4 different animals. Very large grafts contain many TH positive cells with an apparently more even distribution within the graft, and dense innervation of the striatum. Scale bar is 1mm. Higher magnification of E13 grafts showing cell morphology and distribution in 9mm (I-L) and 6mm grafts (M-P). Scale bar is 500μm.

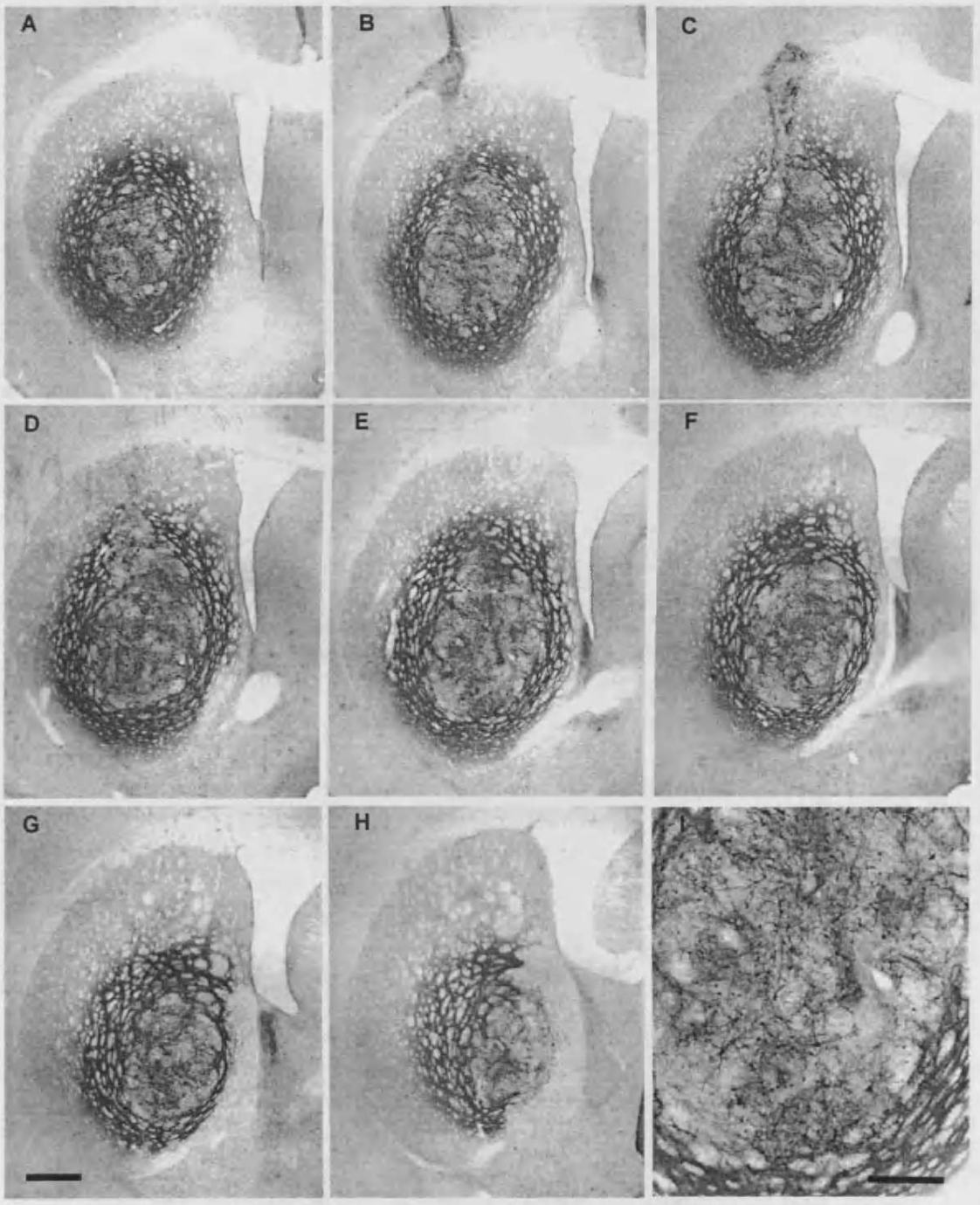

Figure 3: A 1 in 6 series through a dopamine graft from the 6mm donor group, stained for tyrosine hydroxylase. The rat selected is the median animal in this group based on graft volume. A-H; Medium power images showing the size and position of the graft in the host striatum and the dense halo of re-innervation around the graft in each section. Each section is 40μm thick and the graft extends more than 1.9mm in a rostro-caudal direction. Scale bar is 1mm. I. Higher power figure showing both TH stained cells and patches of TH innervation within the body of the graft. Scale bar is 500μm.

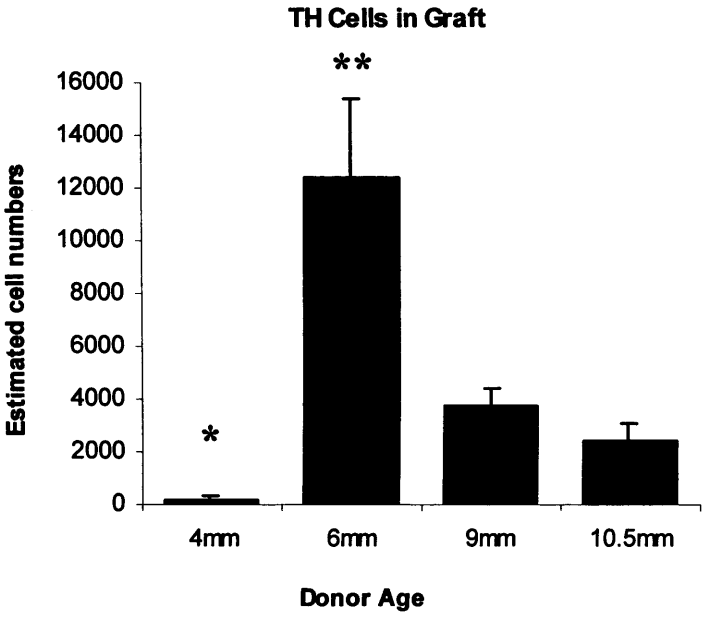

Figure 4. Mean TH cell numbers in grafts derived from 4mm, 6mm, 9mm and 10.5mm donor embryos. 6mm graft cell numbers were significantly greater than those seen in 10.5mm grafts ($t_{13} = 3.552$, $p < 0.01$). The numbers of cells seen in 4mm grafts were significantly lower than in E14 grafts ($t_{14} = 3.190$, $p < 0.01$).

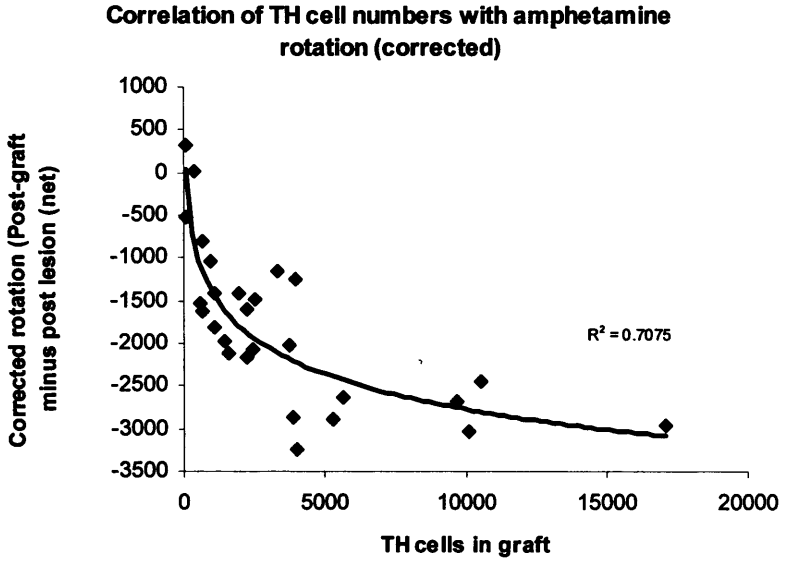

Figure 5. Correlation of post-graft amphetamine rotations with TH cell numbers in the graft with line of best fit (logarithmic). Net rotation score are corrected by subtraction of the pre-graft rotation score for each animal.

Discussion

The poor survival of DA neurons in nigral grafts has required the use of multiple donors per recipient in clinical trials of PD cell transplantation, greatly reducing the feasibility of this approach as a therapy for PD. Despite a great deal of research effort over the last two decades, the goal of one embryo per patient for Parkinson's disease transplantation has not yet been achieved. If the survival of implanted dopamine cells could be routinely boosted from the present levels of 5-10% up to 30%, or more, then it is conceivable that this target might be reached and one of the main obstacles to the use of human embryonic tissue for PD therapy would be overcome. Improvement of graft survival has been widely investigated using a variety of different approaches, and recently summarized in reviews by Brundin et al, [13] and Sortwell et al.[47] The two areas of research that have received most attention and have yielded the most promising results have been improvements in the dissection and preparation of the embryonic tissue, and the addition of trophic factors to the tissue prior to implantation.

Changes in the techniques of preparation of DA grafts have seen a progressive move towards more physiological and nutritional media during handling the donor tissue. Changes in the methods of dissociation, in particular changes in the enzymes used in this process have also led to improved graft survival and such changes have much improved the reliability and reproducibility of cell suspensions.[5,12,23] Changes in the techniques used for implantation have also had beneficial, and for a given amount of implanted tissue, graft survival is higher when tissue is implanted in multiple sites in the striatum, rather than as a single injection or using glass micropipettes in place of metal cannulae to reduce trauma.[6,33]

In recent years, remarkably little work has been carried out regarding the optimal age of embryos for use as VM donor tissue on the widespread assumption that this issue has been satisfactorily resolved. We have carried out a literature search of papers published using embryonic dopamine-rich grafts in rat models of Parkinson's disease. A total of 210 papers were reviewed, from 1976 to 2004 (see table 2). No single study addressed the optimal age of embryo for dopamine grafts directly. Over the 28 years of study, the donor ages used for dopamine grafts ranged from E13 to E20 with a corresponding CRL range of 8-34mm. (see figure 6). Because of the differences in the way methods are reported in each paper it is difficult to compare between papers. Most workers did not specify whether the embryonic ages used were calculated form the day of mating or from the day of vaginal plugging. A more accurate comparison was possible in the minority of papers (64) in which the CRL was reported (see fig. 7).

The first papers detailing embryonic dopamine grafts into rat brain were published between 1976 and 1979, using embryos aged E16-17 (15-20mm CRL) as solid pieces of tissue implanted into cortical cavities.[8,38,48] Between 1980 and 1985, E16-17 was the predominant age used, and at that time, these embryos were considered to be "relatively young" in comparison to those used previously in other graft studies[9]. The use of E16-17 donors was based on what was then known about embryonic development and the appearance of dopamine cells in the VM, from pioneering studies using solid grafts in an intraocular transplant paradigm, undertaken by Seiger and Olson.[36,45]

The use of cell suspensions was pioneered by the group led by Bjorklund et al and was first published in 1980, using E16-17 donor age tissue. The use of solid tissue grafts continued until 1985,[40,49] though by this time most workers were using cell suspensions, which, unlike solid grafts, could be implanted directly into the striatum. Donor embryos younger than E16 were used by some workers at this time: in 1983 Schmidt et al used E13 VM tissue,[44] and in 1985, Arbuthnott et al used E14.[3] However, the mean embryonic age used remained above E16 until 1985. Five years later in 1990, the mean donor age used had fallen to E14 where it has remained until the present day. We have been unable to find any papers using donor embryos younger than E13 (8mm CRL). Mean CRL values from the 64 papers in which these data were reported, range from 10-34mm and are shown in figure 7.

In the present study, the survival rate of 36% seen in the 6mm (E11) graft group is considerably higher than any previously reported work using primary, untreated VM cell grafts. Indeed the figure compares favourably to the best survival rates of grafts treated with combinations of factors to enhance dopamine cell survival. Several factors have been used successfully to improve on the generally reported figure of 5-10% of primary grafts. GDNF has been used to increase survival rates to 15-30%;[42,43,51,54] and other workers have used the related factor neurturin to produce 20% survival.[41] Survival rates of 34% and 18% have been reported in graft treated with bFGF[52]and GDF-5,[51] respectively.

Table 2. A chronology of dopamine grafting in rat models of PD

Year	Papers	Donor Age	Notable landmarks
1976-1979	3	E16-17	First dopamine grafts (solid)
1980	2	E16-18	First use of cell suspension grafts
1981-82	2	E15-17	First use of E13 donor age tissues
1983	2	E15-17	
1984	2	E16-17	
1985	5	E14-18	E14 donor age used/Last use of solid grafts
1986	7	E14-18	
1987	3	E13-14	Second use of E13 donor age tissues
1988-89	11	E14-20	
1990-93	36	E13-17	
1994	18	E13-20	Last use of E20 donor age
1995-96	32	E13-17	Mean donor age falls below E15
1997	9	E13-14	
1998-1999	34	E13-16	
2000-01	14	E13-17	Last use of E17 donor age
2002-2004	19	E13-14	Mean donor age falls below E14

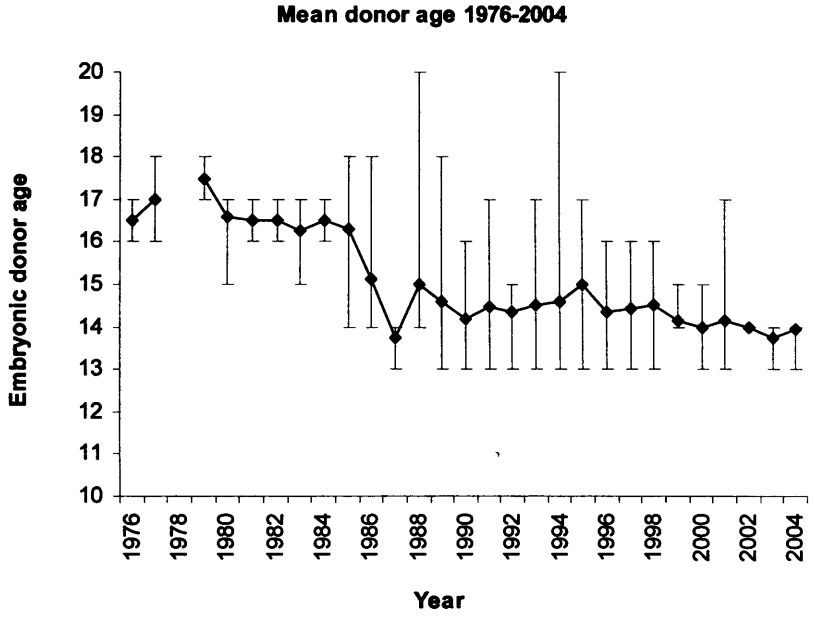

Figure 6. Mean embryonic age of donor tissues used in rat models of PD. Figures are derived from 210 papers published from 1976 to 2004. Error bars denote the maximum and minimum embryonic donor ages used in each year.

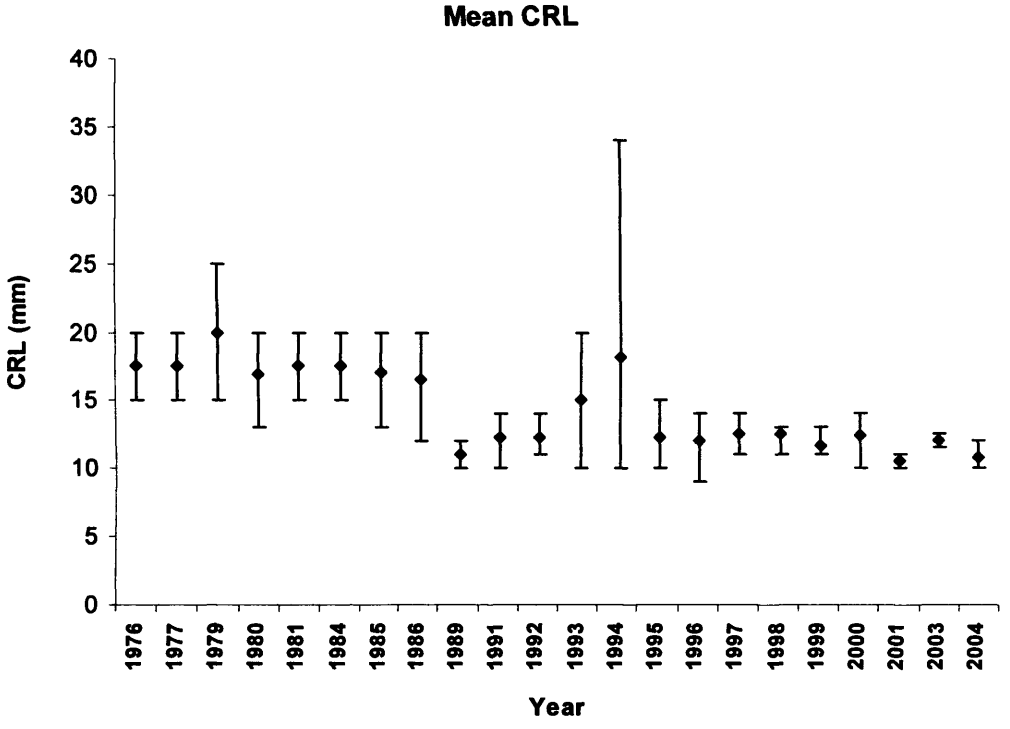

Figure 7. Mean CRL (mm) of donor embryos used in rat models of PD. Figures are derived from 64 papers published in the period 1976 to 2004. Error bars denote the maximum and minimum CRLs used in each year.

During embryonic development, dopaminergic neurons are reliant on factors produced in the underlying floor plate. Hynes et al used E9 explants of neural plate to induce dopamine neurons in the dorsal midbrain ectopically. One of the main factors involved was sonic hedgehog (Shh) and blocking of Shh using antibodies was shown to prevent DA cell development.[26] Cells in the ventral mesencephalic floor plate are important in the period of development when dopamine neurons finish proliferation and differentiate into their final phenotype. The involvement of floor plate cells was further demonstrated by Cohen et al. using the floor plate marker FP4 and by blocking of the floor plate cells transcription factor HNF-3β.[15] Farkas et al used E12 (post-plug) VM and showed that a transforming growth factor TGF-β significantly increased the numbers of TH positive cells in culture and that the presence of both Shh and TGF-β were required for DA cell differentiation in vitro.[21] Fibroblast growth factor 8 (FGF8) has been shown to work in conjunction with Shh in the establishment of the topographical distribution of the developing substantia nigra.[11]

It has been hypothesized that the majority of the dopaminergic cells seen in standard E14 grafts are cells that had differentiated prior to excision from the embryo, and that cells which have not yet done so are unable to complete their development into the dopaminergic phenotype.[46] The adult rat substantia nigra and ventral tegmental area contain 30,000-40,000 dopamine neurons and it is known that in the developing embryo 50% of these have undergone their final division by the age of E14. This means that that in an E14 graft there is still a substantial loss of the implanted, differentiated cells and on average only 2000-3000 of a potential 20,000+ differentiated dopamine cells survive. It has been shown previously that the trophic factor glial-derived neurotrophic factor GDNF can improve E14 dopamine graft survival and it seems likely that the mechanism involved is the protection of differentiated dopamine cells during the implantation process. In a previous experiment we demonstrated that adenoviral-vector delivered GDNF improved the survival of E14 and E15 dopamine grafts, but was ineffective in younger aged grafts. In the same experiment, adenoviral

delivery of Shh improved the survival of E12 grafts but not grafts from E13, E14 or E15 embryos. We concluded from these data that GDNF is able to improve the survival of dopamine cells, provided that they have differentiated prior to implantation, via a protective mechanism.

Grafts derived from 6mm donor embryos have few differentiated cells at the time of excision. Most of the surviving cells seen in these grafts must therefore have completed their differentiation post-implantation. The precise mechanism involved in the transformation of dopamine precursor cells into mature differentiated dopamine cells within the grafts is unknown, but is likely to involve many of the same factors and the same cells involved in the normal development of the embryonic VM. We hypothesize that the VM dissected from the 6mm embryo may contain cells derived from the neural floor plate which in normal development produce differentiation factors and may continue to do so in the dopamine implant. Cohen et al demonstrated that in cultures derived from E12 VM dopamine cells were observed in clusters, intimately associated with clusters of cells derived from the floor plate demonstrated using the marker FP4.[15] In a study aimed primarily at investigating the effects of lowered oxygen on VM cells in vitro, E12 VM cells expanded and cultured over a 5-day period gave high yields of dopamine cells (18% of total neuronal numbers) even in normal culture conditions, The presence of the dopaminergic differentiation factor FGF8 was demonstrated within the cultures indicating that there was an association in vitro, between dopamine cell precursors and accessory cells capable of producing the factors necessary for their proliferation and differentiation into mature dopamine neurones.[50] Studies are in progress to investigate this association in VM grafts, to determine whether floor plate cells are present in 6mm grafts, and if so, what factors are produced by these cells.

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Chapter 6

Amphetamine Induced Rotation in the Assessment of Lesions and Grafts in the Unilateral Rat Model of Parkinson's Disease

6.1 Introduction and supplementary information

6.2 **Amphetamine Induced Rotation in the Assessment of Lesions and Grafts in the Unilateral Rat Model of Parkinson's Disease**
E. M. Torres, S. B. Dunnett.
(To be submitted to European Neuropsychopharmacology)

6.1 Introduction and supplementary information

Amphetamine induced rotation is an important tool for the assessment of lesion and graft survival in the unilateral model of PD. The work in this chapter arose from the observation that the temporal rotational response to amphetamine seen in grafted rats was different to that seen in lesioned animals. Not only was the direction of rotation reversed, but the temporal distribution of rotation rates within the 90-minute testing session was also altered. Lesioned rats displayed initially low rates of turning which rose to plateau rate 30-40 minutes after injection. Grafted rats on the other hand had an initial peak of responding which fell to a plateau after 20-minutes. The early peak of rotation had been noted by several other workers but had not been investigated in any detail and had been explained principally as the superimposition of stereotypical responses and amphetamine induced rotations.

Because of the extreme sensitivity of the lesioned striatum amphetamine rotation can only confirm the presence of a graft and tells us little about the size of the graft itself. The current work was carried out to investigate amphetamine rotation further in the hope of obtaining better indices of graft survival in the living animal.

Research report

Amphetamine Induced Rotation in the Assessment of Lesions and Grafts in the Unilateral Rat Model of Parkinson's Disease

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Abstract

Amphetamine induced rotation is widely used as an index of lesion deficits and of graft-derived recovery in the unilateral rat model of Parkinson's disease (PD). In the present study, we have analysed the time course and profile of the rotational response of both lesioned rats and rats with lesions and dopamine grafts. In lesioned rats, following an intraperitoneal injection of 2.5mg/kg methamphetamine, the net (ipsilateral) rotation exhibited a typical dose response, with minimal rates of rotation in the first 10 minutes post injection, rising slowly to a maximum after 20-30 minutes and then remaining at that level for the duration of the session (90 min). Grafted rats however, exhibited a peak of (contralateral) rotation in the first 10 minutes following injection, which then fell to a minimum after 30 minutes and remained steady for the remainder of the session. The initial peak in rotation does not occur following a control injection of physiological saline, and is therefore not a conditioned response. Following an injection of a lower dose of amphetamine (1.25mg/kg) the amplitude of the initial contralateral response increases, falls more quickly and then rises to a second peak of rotation at the end of the session. The pattern of rotation seen in grafted rats is dependent on the presence of a graft but does not correlate to graft size or to the amplitude of the overall contralateral response. It is hypothesised that the initial peak of rotation seen in grafted rats is due to the release of dopamine from the graft at low levels of amphetamine in the brain and that the subsequent fall in the rate of rotation is due to activation of the intact side of the brain as the levels of amphetamine in the brain increase. The rate of responding in the first 10 minutes following amphetamine injection correlates well with total rotation scores and might be used as an alternative index for identifying the presence of a graft requiring much shorter testing times.

Key words: Animal model; dopamine graft; 6-OHDA; Parkinson's disease, amphetamine; rotation.

Introduction

One of the most widely used rat models of Parkinson's disease (PD) is the unilateral 6-hydroxydopamine (6-OHDA) lesion of the nigrostriatal bundle which results in almost complete (>97%) dopamine depletion in the striatum on one side of the brain and causes a range of motor deficits.[4,17,2,7,25,31] In our laboratory this model is used to assess the efficacy of embryonic dopamine transplants for the treatment of PD. The main index used to assess the efficacy of both 6-OHDA lesions and dopamine grafts is rotational behaviour induced by the administration of the dopamine active drug amphetamine. In rats that have received a unilateral lesion of the dopamine system, intraperitoneal administration of 2.5mg/kg of amphetamine causes a strong ipsilateral rotation of 6-20 turns a minute over a 90 min testing session. Implants of embryonic dopamine cells derived from E14 ventral mesencephalon (VM) are able to ameliorate the amphetamine-induced rotational deficit producing a reduction in the amplitude of rotation and classically, an over-compensatory response where the direction of rotation shifts from an ipsilateral to a contralateral at rates of up to 25 turns a minute.[3,13]

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We have observed that in rats that have received a 6-OHDA lesion followed by an embryonic dopamine graft, the contralateral rotational response to amphetamine initiates very quickly after injection of the drug, and then falls to a lower level after 10-20 min. This is in contrast to the response seen in lesion-only rats where the initial response is low, rising to a peak of rotation 20-30 min after injection. The present paper seeks to address two questions. Firstly, what is the nature of the difference in the amphetamine response in lesion-only and lesion-grafted rats? Secondly, is the current use of total rotations over 90 min the best parameter for the assessment of lesions and grafts or can more detailed investigation of the response yield other useful indices of lesion and graft function?

General Methods

Experimental animals

The present study presents a detailed analysis of rotational behaviour in a large number of rats which were used in a series of experiments investigating methods of improving dopamine graft survival. Adult, female Sprague-Dawley rats were used, weighing 200-250g 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.

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.[27]

Unilateral Dopamine lesions

Dopamine 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 10µl Hamilton syringe in a microdrive pump set to deliver at 1µl/min. The toxin was used at a concentration of 3µg/µl (calculated as the free base weight) dissolved in a solution of 0.2mg/ml ascorbic acid in 0.9% sterile saline. The stereotaxic co-ordinates used for injection were; A= -4.4 (behind bregma), L= -1.0 (from midline), V= -7.8 (below dura), with the nose bar set at -2.3mm 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.

Rotation

Drug-induced rotation was assessed using a bank of 8 automated rotometer bowls modelled after the design of Ungersted and Arbuthnott.[32] Rats were injected i.p. with 2.5mg/kg of methamphetamine hydrochloride (Sigma chemicals UK) and placed immediately into the apparatus. Total turns in each direction were measured in 10 min bins over 90 minutes and rotation scores expressed as the net ipsilateral minus contralateral rotations, Rotational testing was carried out 2 weeks and 4 weeks post lesion to obtain an estimate of the extent of dopamine depletion in each animal. Only rats with a net rotation score of $\geq$600 turns per session were used in the experiment. Grafted rats were tested 4 weeks and 6 weeks post-grafting using the same protocol. In additional experiments rats were also tested following i.p. injection of 1.25mg of amphetamine or sterile saline in test sessions up to 120 minutes.

Dopamine grafts

On completion of post-lesion rotational testing rats were divided into matched groups according to their rotation scores and then received unilateral dopamine graft into the lesioned striatum. Dopamine grafts were prepared from same-strain rat embryos aged E12-E14 (post-plug) using a standard protocol.[12] Briefly, the VM was dissected from each embryo. After washing in Hank's balanced salt solution (HBSS), the dissected pieces were placed in a solution of 0.1% trypsin (Worthington Biochem corp. NJ USA) in 0.05% DNAase (Sigma chemicals UK) in HBSS for 20 min then washed in two changes of 0.05% DNAase in HBSS. Dissociation of the dissected pieces was carried out by trituration using a Gilson pippettor. Initial trituration was carried out with a 1ml (blue) pipette tip (15-20 triturations) then a finer 100µl (yellow) pipette tip was used (15-20 triturations) to achieve the final, single cell suspension. The suspension was then spun in a bench-top micro centrifuge at 2000rpm for 3 minutes. The supernatant was removed and the pellet of cells resuspended in 0.05% DNAase in HBSS at a concentration of 1 VM per 2µl. Grafted rats received 2µl of the cell suspension injected into the dopamine depleted striatum at stereotaxic coordinates: A=+0.6, L= $\pm$3.0, V= -4.5 using a 30 gauge 10µl Hamilton syringe. After injection of the graft material, the cannula was left in place for 3 min prior to careful removal of the cannula from the brain.

Histopathology

Animals were terminally anaesthetized by i.p. injection of 200mg/kg sodium pento-

barbitone, and then perfused transcardially with 100ml of phosphate buffered saline (PBS) pH 7.4, followed by 250ml of 4% paraformaldehyde in PBS over 5 min. 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 40μm into 0.1M 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 nissl stain; cresyl fast violet. A second series was stained immunohistochemically for tyrosine hydroxylase, using 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 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 hr 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. Known positive controls, and negative controls, in which the primary antibody was omitted, were also run. After thorough washing, a biotinylated, rat-adsorbed anti-mouse, secondary antibody (Vector UK) a a dilution of 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% strepavidin-biotin horseradish peroxidase solution (Dako UK) in TBS for 90 min, followed by thorough washing and equilibration to 0.05M Tris non-saline (TNS) solution at pH 7.4. The horseradish peroxidase label was revealed by 10 min incubation in a 0.5% solution of diaminobenzidine tetrahydrochloride (BDH) in TNS containing 0.3μl/ml of hydrogen peroxide. Sections were finally mounted on gelatine coated microscope slides and dehydrated in an ascending series of alcohols, cleared, and coverslipped using DPX mountant.

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 calculated from cross-sectional areas measured on TH stained sections on a regular series (1 in 6) through the entire graft. Cell counts were carried out on a Leica DMRB microscope using a 10 x 10 eyepiece graticule and a x20 objective on the same sections and corrected using the Abercrombie formula.[1] Measurements of cell size were made using a x100 objective on the same system.

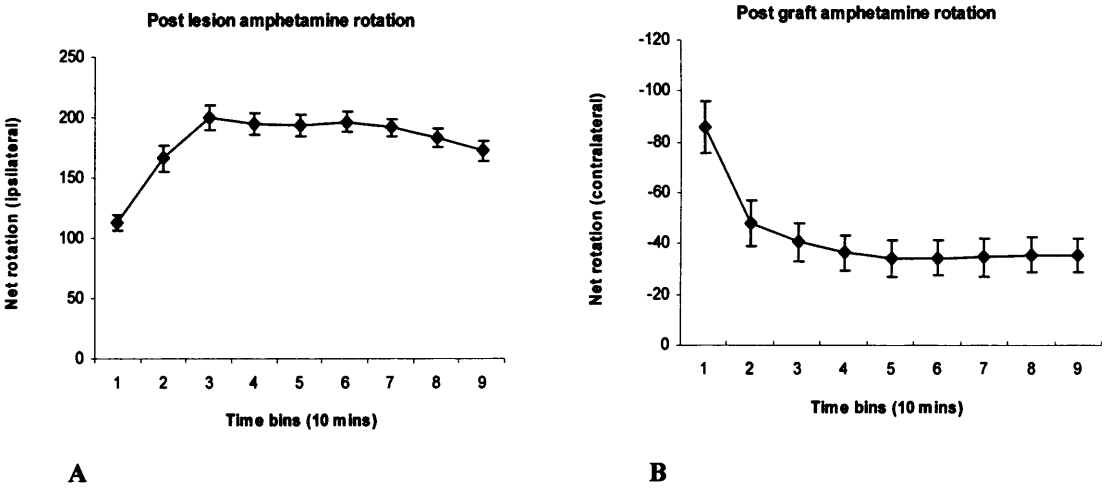

Figure 1 Amphetamine induced rotational response in lesioned and grafted rats. A) Ipsilateral response of lesioned rats following injection of 2.5mg/kg methamphetamine. B) Contralateral response (Y-axis is negative) of the same rats 6 weeks post grafting at the same dose.

Experiment 1

Fifty-eight rats that had received unilateral 6-OHDA lesions of the median forebrain bundle and grafts of embryonic ventral mesencephalon were tested for methamphetamine induced rotation using the standard dose (2.5mg/kg) for 90 min at time points of 4 weeks post lesion and 6 weeks post grafting.

Results

Figure 1 shows plots derived from the mean rates of turning (net) in 10-min time bins over the 90 min session. In lesioned rats there was a net ipsilateral rotational response. In the first 10-minute time bin, net rotation was at a minimum, at approximately 10 turns per min. The rate of rotation rose to a maximum of 20 turns per min, 30 min after injection and stayed at that level for 30-40 min before falling slightly in the last 20 min. In grafted rats the net response was in the contralateral direction. All rats in the group had a surviving graft (see below) and each exhibited not only significant reduction of methamphetamine induced rotation but also the classical "over compensatory" response described above. Net contralateral rotations peaked in the first time bin at approximately 9 turns per minute and then fell over the next 20-30 min to a stable level of around 4 turns/ min.

To determine whether the early peak in graft rotation was related to the magnitude of graft function, the data from the 58 rats were re-categorized according their total overcompensatory response. Rats were divided into 4 groups [0-199, 200-399, 400-699 or 700+ net rotations] and the mean rotation in each time bin over the 90 minute session was plotted for each group. Analysis showed that the initial peak of rotation was seen in all four groups, suggesting that the same mechanism was responsible, independent of the overall level of overcompensation (Figure 2). These data also suggested that the initial peak of rotation might be proportional to the overall over compensatory response.

A plot was made of the net rotations recorded in these initial peaks against the total over compensatory response for each group (figure 3). The correlation of peak of rotation with overall response was high. This indicates that the rotations observed in the first initial peak, before the levels of rotation fall to baseline, are a good index of the total rotations over 90 min. As in previous work, there was no relationship between TH cell numbers and overcompensation of rotation. (ANOVA; $F_{3,43} = 1.93$, $p=0.136$, n.s.).

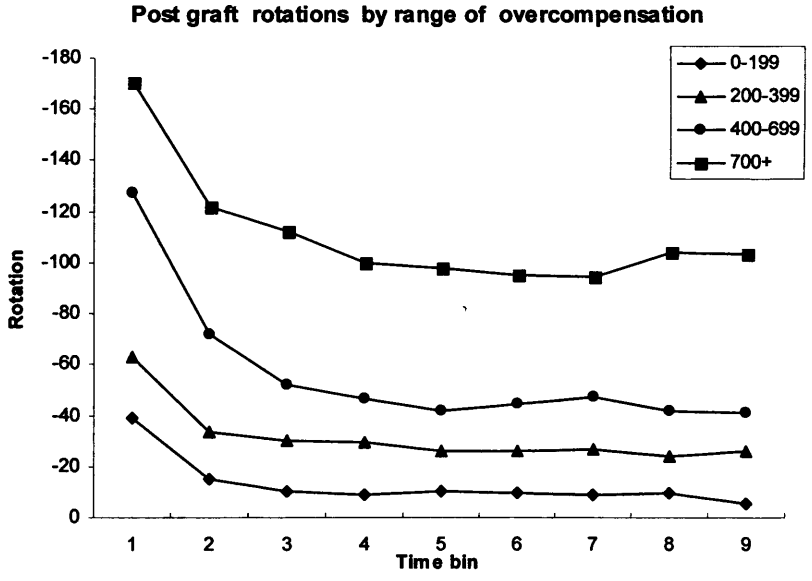

Figure 2. Net rotation following amphetamine administration after animals were ranked, and then divided into 4 groups according to their total net response. The peak of rotation in the early time bins is seen in all groups irrespective of the overall response. (Error bars are not shown because the plot allocates animals to subgroups a posteriori based on the ranges of mean scores).

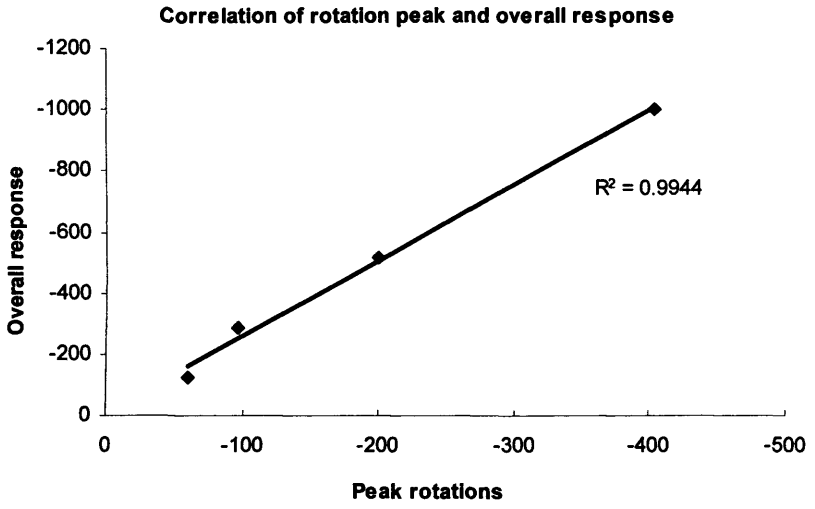

Figure 3 Correlation of the initial peak in rotation following amphetamine injection with the overall rotational response. ($r = 1$ $p < 0.05$)

Experiment 2

Other workers have observed that animals can develop a conditioned response to repeated administration of amphetamine.[2,7] To investigate this possibility in the current work, 8 lesioned and grafted rats were given an injection of 0.3ml of physiological saline i.p. and placed in the rotometer apparatus for 20 min prior to administration of a second injection of 2.5mg/kg of amphetamine.

Results

Figure 4 shows a plot of net rotations in each 10 min time bin in experiment 2. Only low levels of rotation were observed following the saline injection (average net rotation of 1 turn per min over 20 min) but subsequent injection of amphetamine resulted in high levels of rotation (average net rotation of 174 turns per min) in the following 10 min time bin, clearly demonstrating that the initial peak of rotation is caused by the drug and is not a conditioned effect of the injection per se.

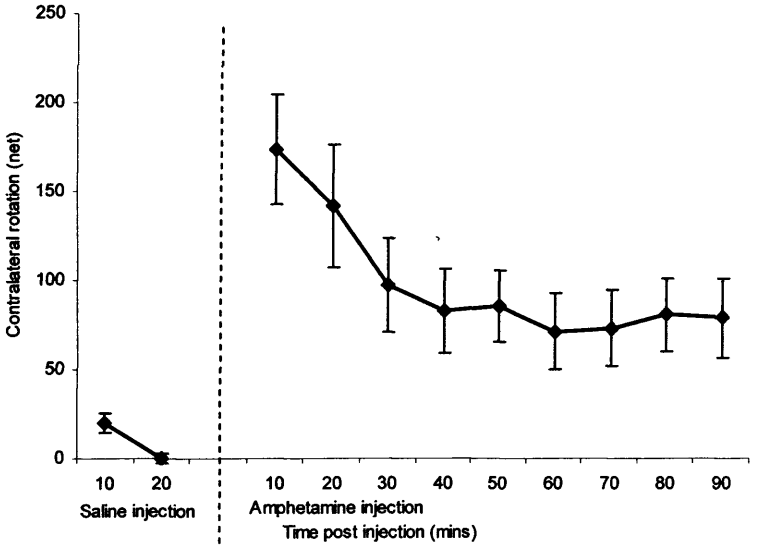

Figure 4. Experiment 2. The effect of a saline injection on grafted rats. Rats were injected with physiological saline instead of amphetamine and tested in the rotometer apparatus for 20 minutes prior to injection of amphetamine. Saline injection did not produce the initial peak of rotation seen with amphetamine and demonstrates that the peak is not a conditioned response.

Experiment 3

Rationale

The rotational response to a lower dose of amphetamine was tested using 8 lesioned and grafted rats which had previously been tested using the standard dose of amphetamine (mean response at 2.5mg/kg of amphetamine was 854) 1 week later rats were injected with a lower dose (1.25mg/kg) and rotation was monitored for 120 minutes post injection.

Results

Figure 5 shows a comparison of the temporal response following administration of the two doses of amphetamine. The 2.5mg/kg dose produced a characteristic response curve where the highest response (204 turns in 10 minutes) was recorded in the first time bin with the rotation rate falling to between 75-100 rotations in subsequent time bins. The overall response to 1.25mg/kg of amphetamine was almost 3-fold higher than that seen at the standard dose, with a mean of 2487 net rotations. The low dose response was higher than the standard response at all time points, with a higher rate of turning in the first bin (246 turns), falling to 150 turns in the third time bin then rising steadily to reach a peak of 252 turns at the end of 90 minutes. Rates of rotation fell in the last 3 time bins, presumably as the dose of amphetamine in the brain fell with time.

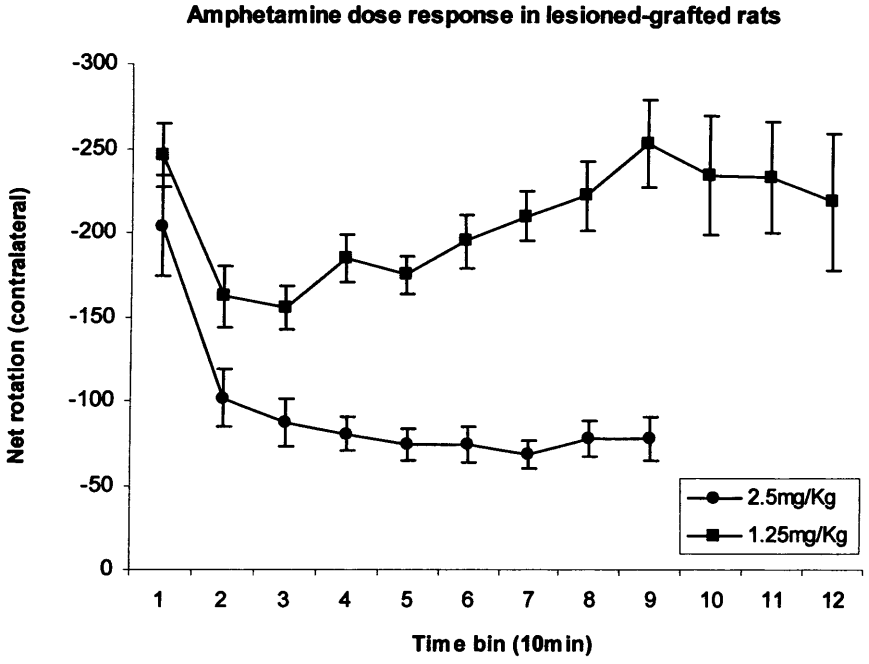

Figure 5. Net rotational response of lesioned-grafted animals to different doses of amphetamine (1.25mg/kg and 2.5mg/kg). The lower dose of amphetamine produces higher rates of rotation at all time points. The net rotation reflects differential activation of the intact (normal) and grafted (supersensitive) striata and this is clearly greater at the lower amphetamine dose. Error bars are SEMs.

Discussion

A unilateral lesion of the dopamine system results in asymmetry of both the levels of dopamine in the brain and the sensitivity of the brain's response to it. In the unilateral rat model of PD, a 6-OHDA lesion of the median forebrain bundle (MFB), results in ipsilateral loss of dopamine in the corpus striatum and nucleus accumbens as well as in areas outside the striatum such as the olfactory tubercles, septum and pre-frontal cortex. In the striatum and nucleus accumbens there is up-regulation of both the number and sensitivity of dopamine receptors and both of these brain areas play a part in the rotational response. The asymmetrical basis of the amphetamine-induced rotation is thought to be due to differences in dopamine physiology between the intact and lesioned striata. The nucleus accumbens on the other hand is thought to be involved in the initiation and amplitude of the response[25] and disruption of accumbens function, either by lesioning or receptor blockade, eliminates the rotational response. [23,24]

Amphetamine induced rotation is not the direct result of asymmetrical striatal and accumbens activity affecting motor output and the precise pathways have yet to be identified.[16,28,31] There is crossover of the motor output from the striatum, the right side controlling the left limbs and the left side controlling the right limbs. If rotation were a purely motor phenomenon, then a unilateral lesioned rat should rotate contralaterally rather than ipsilaterally which it does not. Rather, the nature of the asymmetry is thought to be due to positional preference in bilateral space. Hence a lesioned rat neglects contralateral (lesion affected) space and has a positional preference in response to the ipsilateral side and rotation is directed accordingly. In grafted rats, the activation of the supersensitive receptors in the lesioned striatum by graft-derived dopamine is proportionally greater than the normal activation of the intact striatum and results in a positional preference for the contralateral side and subsequent rotation in that direction.[15,31]

The situation is somewhat different in rats that have received a unilateral lesion induced by striatal, rather than MFB administration of 6-OHDA. These animals have higher net rates of amphetamine-induced rotation than MFB lesioned rats.[9] The reason for this has not yet been fully explained, but is thought to be due to the relative sparing of nucleus accumbens dopamine innervation in such animals.[11] Thus, whilst striatally-lesioned rats have a similar striatal asymmetry to MFB lesioned rats, their rotational response is higher because the accumbens is intact on both sides of the brain and overall levels of motor activity are higher. If this is correct, then the amplitude of the rotational response can be seen as a product of the accumbens "drive" and the striatal asymmetry and net rotation scores might be affected by changes in either brain region.[24,25] This view is supported by work showing that lesions to the nucleus accumbens in unilaterally Parkinsonian rats have the effect of decreasing net rotation,[22,29] whereas grafts of DA rich tissue into the nucleus accumbens have the effect of increasing rotation rates.[5]

Amphetamine is a presynaptic (indirect) dopamine agonist that causes increased dopamine release and a reduction in dopamine re-uptake, resulting in enhanced dopamine availability at the post-synaptic receptors. In a rat with a unilateral 6-OHDA lesion of the MFB, the striatum and accumbens (as well as septal, and cortical areas) on the ipsilateral side of the brain are almost completely (>95%) de-afferented of dopamine. In such animals, the amphetamine response is therefore a result of unilateral activation of the dopamine system on the intact side with little or no activation of the lesioned side of the brain. From this perspective, the characteristics of the rotation seen in figure 1A can be regarded as the response of the intact striatum to amphetamine. The gradual increase in the rate of rotation seen in the first 30 minutes (bins 1-3) reflects increasing levels of dopamine in the brain and/or increasing activation of dopamine receptors with time. The pharmaco-kinetics of DA release following amphetamine have been demonstrated by in vivo micro dialysis and show a peak of dopamine release 40 minutes after injection of 2.5mg/kg of amphetamine,[26] corresponding to the temporal response observed in the lesioned animals in this study.

The situation in the lesioned-grafted rats is more complex, as amphetamine stimulates dopamine release both in the intact striatum and from the graft located in the lesioned striatum. Activation of the intact side will have the effect of driving the rotational response in an ipsilateral direction whilst activation of the graft on the lesioned side of the brain will tend to drive the response contralaterally. Thus, at any given time point, the rotation seen in lesioned-grafted animals, will result from differences in the amplitude of the dopamine response on the two sides of the brain. The net overcompensation of rotation seen in lesioned-grafted animals is believed to be due to a (subnormal) dopamine release acting on hypersensitive receptors in the lesioned striatum, so that the resultant level of post-synaptic activation is higher than normal. Thus, release of dopamine from the graft, even at low levels, induces a greater response on the ipsilateral side of the brain than the normal

dopamine release on the contralateral side and overall rotation towards the contralateral side.

The peak of net rotation seen in the first time bin is interesting and though it has been previously described, it has not been fully investigated or explained. Rioux et al reported three types of temporal response to amphetamine (5mg/kg) in lesioned-grafted rats, all of which showed a peak in rotation in the first 5-10 min post injection. The differences in responses between types were attributed to different sizes of graft and differences in their location within the host striatum.[30] The present results exhibit the same time course, but suggest that the phenomenon is not explainable in terms of graft sizes as the same pattern of rotation was seen irrespective of net overcompensation and was not correlated to graft size. Bi-phasic response of grafted rats has also been reported by Herman et al.[19]

We can infer from the response curve of lesion-only rats that it takes some time for the intraperitoneally administered amphetamine to elicit peak concentrations of dopamine in the brain. In lesioned and grafted rats the initial peak of responding may be due to differential effects of low levels of dopamine in the intact and lesioned striata. The blood brain barrier in the grafted striatum is disrupted in the first week following implantation but has been shown using infusions of the vital dye Evans blue to have reformed after 12 days.[6] We can assume therefore that the dynamics of drug access to both striata are similar. Immediately after injection, small amounts of dopamine in the intact striatum would induce a correspondingly small drive towards ipsilateral turning as seen in the early time bins in lesioned animals. However, on the grafted side of the brain, small amounts of dopamine released by the graft will have a large effect on the supersensitive receptors there and induce a larger drive towards contralateral turning. The net result therefore, is a high rate of contralateral rotation in the first ten minutes after injection, when the dopamine levels in the brain are low. In later time bins, as the concentration of amphetamine in the brain rises the response of the intact striatum increases accordingly until the imbalance between the ipsilateral and contra-lateral drive decreases to a minimum resulting in a gradual decrease in net contralateral rotation (see figure 6).

This hypothesis is consistent with the experimental results in the present paper. Firstly, the peak response is seen in all grafted animals regardless of the overall amplitude of the over compensatory response, due to the hypersensitivity of the lesioned side of the brain at low drug levels. Secondly, 1.25mg/kg of amphetamine induces a greater rotational response than 2.5mg/kg (1800 vs. 845 rotations over 90 minutes, see figure 5), because at the lower dose, the influence of the intact side is reduced whilst the response of the lesioned-grafted side of the brain remains high due to the influence of supersensitive receptors. Additionally, the low dose of amphetamine exhibits a second peak of rotation, as the response of the intact side starts to wane, eventually reaching a level equivalent to that seen in the first time bin. The rate of rotation subsequently falls as the dose of amphetamine in the brain becomes limiting even for the supersensitive lesioned-grafted side of the brain. Cragg et al (2000) demonstrated that the dynamics of dopamine release from VM grafts are different to those of the intact striatum. Whilst the levels of dopamine released by the graft were significantly lower than those on the intact side, levels of dopamine re-uptake were reduced and the extra-cellular lifespan of dopamine greatly extended.[8] The extended period of action of dopamine on the grafted side of the brain may help to explain this second peak of rotation in grafted animals.

A further possible explanation of the two-peak amphetamine response needs to be considered, namely the possibility that stereotypical responses to high doses of amphetamine might competing with the turning response. Amphetamine, particularly at high doses, has been shown to induce stereotypy, causing rats to carry out repetitive limb and oro-facial behaviours to the exclusion of general locomotor activity and this type of responding has been shown to be dopamine dependent.[9,10,21] In a recent paper, Gentry et al measured both stereotypy and general motor activity in normal rats following injections of amphetamine at dose of 0.3, 1.0 and 3.0mg/kg.[18] They showed that at the highest dose of amphetamine stereotypical behaviour progressively inhibited locomotor activity, causing a reduction of high levels of activity seen 30 minutes post injection to a minimum level at 40-100 minutes post injection. At later time points (100-400 min post-injection), stereotypical responses fell and levels of activity rose once more (though not to the initial high levels seen), resulting in a biphasic response similar to the response shown here in the lesioned-grafted animals at the 1.25mg/kg dose. However, they observed no stereotypy at 0.3mg/kg dose or at the 1.0mg/kg dose; the closest to the 1.25mg/kg used in the present study. There are a number of differences between these results and the present work. The time courses of the bi-phasic response observed are different, though this may be accounted for by differences in the dose used.

The smaller size of the second peak of

responding seen by Gentry is interesting and was also observed by Herman et al previously.[20] This is consistent with falling levels of amphetamine over the period of observation and is different from the matched peaks seen in the present study. In the present study the first and second peaks of rotation are of equal amplitude, which is consistent with the hypothesis that net rotation is the result of the interaction between the intact and grafted hemispheres and that the two peaks represent the net response at equivalent drug levels in the brain, the first peak as levels rise and the second as levels fall. It should also be noted that no stereotypic response have been observed in lesion only animals, though this could be due to the lack of activation of hypersensitive receptors on the intact side of the brain, which in the main, contributes to the response.

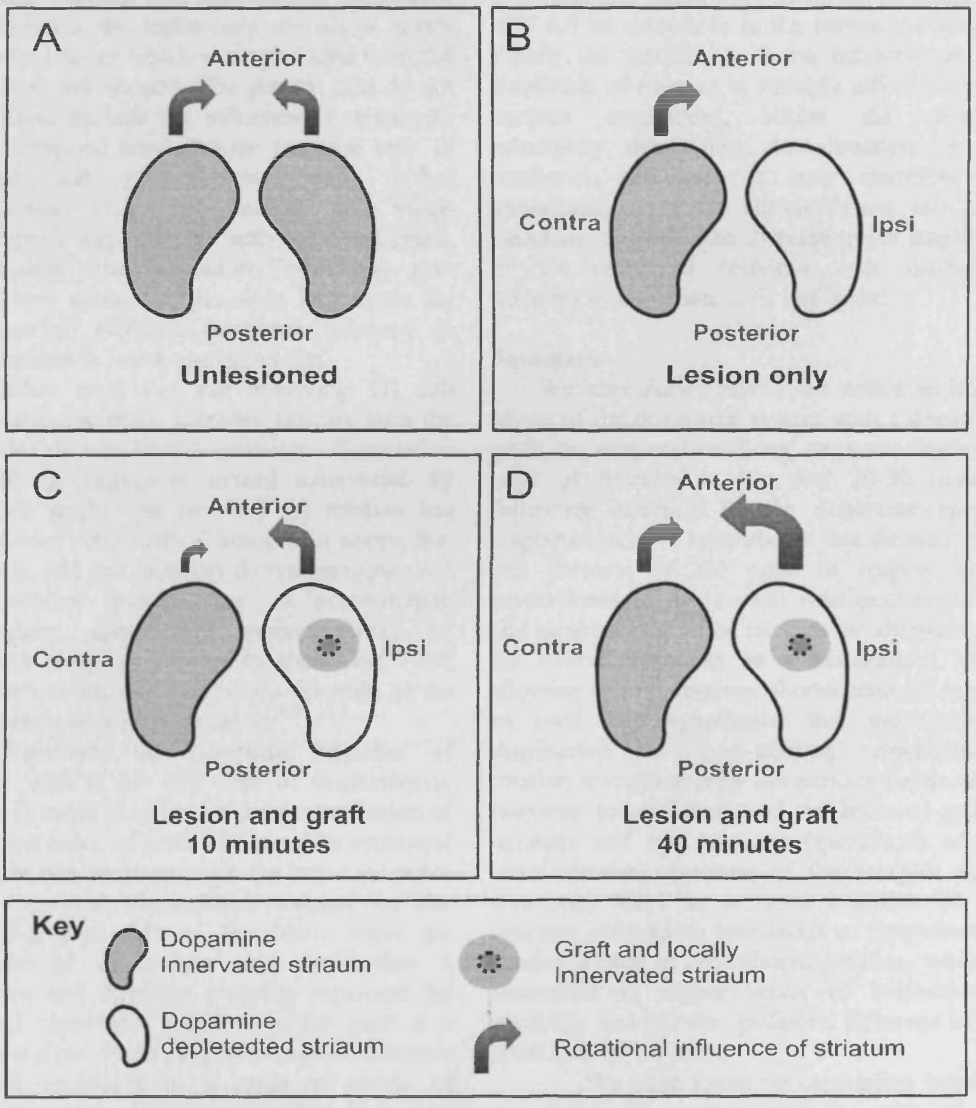

Figure 6. Hypothesised influence of the ipsilateral and contralateral striata during amphetamine-induced rotation. A. In a normal rat the influence of both striata is approximately equal and net rotation is zero. B. In the lesioned rat, the dopamine depleted striatum has no influence. The intact striatum induces ipsilateral influence and the net rotation is in the ipsilateral direction. C. In lesioned and grafted rat 10 minutes post injection, striatal levels of amphetamine and dopamine release are low. The intact striatum induces minimal ipsilateral influence. Receptor super sensitivity in the lesioned striatum induces a large contralateral influence and contralateral rotation is maximal. D. In lesioned and grafted rat 40 minutes post injection, striatal levels of amphetamine and dopamine release are high on both sides. The intact striatum induces an ipsilateral influence on rotation and the supersensitive lesioned striatum induces a contralateral influence only slightly larger than the intact side. As a result overall contralateral rotation is lower than in C.

Previous workers have reported a two-peak response to the direct dopamine agonist apomorphine, in lesion-only rats in which the trough of rotation was associated with overt stereotypical behaviours such as strong postural asymmetry, licking, scratching and biting behaviours.[25,27] In the present study, no overt stereotypical behaviours were observed under the influence of amphetamine (though no formal investigation was undertaken) and the bi-phasic rotational response was seen only in the grafted rats and not in the lesion-only animals or in rats with poor lesions which were excluded from the study (data not shown). The present data do not allow us to exclude the influence of stereotypy on the temporal amphetamine response seen in lesioned and grafted rats, and further experiments will be needed in which stereotypical responses are actively investigated. Nevertheless, the interactive hypothesis presented here remains a plausible hypothesis for the observed biphasic rotational response to amphetamine in lesioned-grafted rats.

Neither graft size nor surviving TH cell numbers in the graft, correlate directly with the recovery of ipsilateral rotation. Correlation between the degree of striatal innervation by dopamine grafts and recovery on rotation has been shown previously,[14] however it seems that generally, TH cell numbers do not correlate well with rotation scores, due to post-synaptic mechanisms, specifically hypersensitivity, of dopamine receptors induced by the lesion,[20] and other factors such as the specific location of the grafted cells within the striatum.[20,30]

However, the two-peak response of rotation seen at the low dose of amphetamine (figure 3) holds promise for the determination of an in vivo index of graft size based on rotational data. The two peaks seen on the low dose curve likely represent the rotation induced by the lesioned-grafted side of the brain when the influence of the intact side approaches a minimum and therefore probably represent the maximal observable response of the graft at a particular dose. Future experiments will compare maximal responses at a range of doses of amphetamine to determine whether such data might be used as an index of graft size.

As the rotation induced by the lesioned-grafted side is caused by dopamine release from the graft, we might expect the magnitude of these peaks to be proportional to the size of the graft. However, we have not been able to find a correlation between the size of the dopamine graft and any of the indices of rotation studied. There are a number of reasons why this is the case. Rotation is also influenced by the levels of super-sensitivity in the lesioned striatum, which vary from rat to rat. Additionally, the relatively small volume and the single placement of the graft means that any long term changes in super sensitivity induced by the graft will be confined to a relatively small volume of the striatum and may not be detectable in the current paradigms. Finally, as mentioned in the introduction, the amplitude of rotation is strongly affected by the nucleus accumbens, whilst the striatum principally determines the direction of the rotational response. It may therefore be unrealistic, using the current lesion and graft paradigm, to expect an increase in the amplitude of the rotational response with increasing numbers of dopamine cells implanted

Summary

We have shown that in rats with a unilateral lesion of the dopamine system with a dopamine graft, the temporal rotational response displays a peak of rotation in the first 20-30 minutes following injection of the dopamine agonist amphetamine. We have shown that the amplitude and duration of the peak of responding is proportional to the overall rotational response, and might therefore be used as an alternative to the overall response as a behavioural index allowing testing sessions shorter than 90 min to be used. We hypothesise that the temporal distribution of post-grafting amphetamine rotation is explicable by competition between the response (contralateral) of the lesioned-grafted striatum and the response (ipsilateral) of the intact striatum. Because of the receptor super sensitivity there is a rapid response of the lesioned striatum to low levels of amphetamine causing a peak of contralateral rotation, which is attenuated as higher levels of amphetamine gradually increase the ipsilateral influence of the intact side of the brain.

We have found no correlation between any index of rotation and the size of the dopamine graft. This is likely to be due to a number of factors, such as the relatively local effects of a single graft placement, and variability in placement of grafted tissue within the supersensitive striatum.

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Chapter 7

General Discussion

This chapter provides a brief summary of the work presented in this thesis, over and above the detailed discussions included in the individual manuscripts.

General Discussion

Whilst the primary concern of this thesis has been the improvement of dopamine graft survival, other directly related issues have arisen during the course of work and an effort has been made to address these. Chapters dealing with the failure of full thickness immuno-histochemical staining, and the investigation of post-graft amphetamine rotation have been included in this thesis as important themes in their own right, and because they have directly influenced the investigations of dopamine cell transplantation efficacy.

The failure of full thickness immuno-histochemical staining.

The problem of two layer staining in immunohistochemically stained sections is one which we have been aware of in our laboratory for many years and it is clear from talking to collaborators and colleagues that they too experience the same phenomenon. It is an important issue because, whilst our standard immunohistochemical protocols give excellent staining with a wide range of antibodies, the presence of the two-layer staining artefact means that each section contains within it a population of unstained cells that we are unable to visualize and quantify. As a result, any measurements taken from such sections will be biased, and cell counts will inevitably underestimate true cell numbers.

A particular problem is that the two-layer staining also prevents use of stereological counting methods, which are considered to be superior to the manual counting and correction methods used previously. It has been suggested by some workers that stereological counting could be carried out in two-layer sections by treating each stained layer as a separate, thin sub-section and adjusting the data accordingly. Whilst it is true that such a method of counting is technically possible, the validity of results obtained in this way relies on the assumption that the failure of staining in the central thickness is due to a failure of the immuno-staining to penetrate far enough into the section. Implicit in this assumption is that all the cells within the stained portions of the section have been visualized. The present results demonstrate that this is not the case and that even the strictest stereological sampling method will fail to generate unbiased data, or to estimate accurately cell numbers in two-layer stained sections.

In Chapter 2, none of the modifications to the immuno-histochemical protocol was able to produce staining in the central thickness of the section. The failure of staining was not due to inadequate exposure to either antibodies, the streptavidin-biotin complex or the DAB colour reaction. A key observation was that the endothelium of vascular elements, transduced with a LacZ-expressing adenovirus also displayed the two layer staining artefact, despite direct access of all the solutions used in the staining process to these cells, via the lumen of the blood vessel. The conclusion was therefore, that the barrier to staining was not the thickness of the tissue sections per se, but some component either within the tissue, or of the cells themselves, preventing penetration of one or more reagents involved in the immunohistochemical protocol.

Subsequent investigations showed that the addition of membrane-disrupting agents such as detergents or alcohols to the fixative were able to overcome the barrier to full thickness staining and implicated the lipid rich cell membrane itself (or some component of the connective tissue intimately associated with this) as the barrier to staining. Thus, immunohistochemistry was only able to stain cells near the surface whose membranes had been cut during sectioning. This hypothesis explains the two layer staining seen in blood vessels, and the failure of staining to extend beyond blood vessels into the surrounding tissue. This also explains the differential depth of penetration of staining seen when using different antibodies as this will depend on the size and complexity of the cell type being stained.

An important finding was that the barrier to penetration can be affected by altering the concentration of paraformaldehyde fixative used to preserve the tissue. The observation that dilution of the standard 4% PFA to 2% or 1% was able to remove the barrier to penetration suggests that overexposure to PFA fixation itself is responsible for formation of the barrier. Thus, in the future, modification of the fixative to achieve full thickness staining should be possible using reduced concentrations of PFA and the inclusion of other fixatives such as methanol, ethanol or picric acid. The challenge will be to preserve the epitopes involved in antibody binding across a wide range of stains whilst at the same time retaining the simplicity and versatility of the current perfusion/fixation protocols.

Amphetamine induced rotation and assessment of lesion and graft survival.

Chapter 6 dealt with the use of amphetamine-induced rotation in the assessment of lesion and graft survival in the unilateral model of PD. This work arose from the observation that the temporal rotational response to amphetamine seen in grafted rats (with a prior 6-OHDA lesion) was different to that seen in lesioned animals. Not only was the direction of rotation reversed (as expected), but the temporal distribution of rates of responding within the 90-minute testing session was also altered. Following an injection of amphetamine, lesioned rats display initially low rates of turning which rise to plateau rate 30-40 minutes after injection. Grafted rats on the other hand have an initial peak of responding which falls to a plateau after 20-minutes.

The early peak of amphetamine rotation exhibited by grafted rats had been noted by other workers but had not been investigated in any detail or adequately explained. The best explanation was that the initial peak of rotation is due to the hypersensitive response of the dopamine denervated striatum to dopamine released from the graft, and that the initial response is potentiated by the development of stereotypical behaviours as the levels of amphetamine in the brain rise.

The present work presents an alternative hypothesis based on the interaction of the intact and grafted striata to differing levels of amphetamine throughout the testing period. (See chapter 6 figure 6.) In the current hypothesis the rotational bias at any time point is the result of the difference between the influence of the intact side of the brain (ipsilateral) and that of the lesioned or lesioned/grafted grafted side of the brain (contralateral). The hypothesis readily explains the responses of both lesioned and lesioned/grafted rats to the different levels of amphetamine in the brain at different time points in the testing session. The issue of stereotypy cannot be excluded, although the observation that the profile of responding at lower doses of amphetamine exhibits the same peak-trough profile, argues against this. In addition, no overt stereotypical behaviours have been observed, but future work will need to include direct recording of any stereotypic responses. There was good correlation between the amplitude of the initial peak of responding and the total overall response in grafted animals, and it may be that in future work this might be preferred as an index of graft induced rotation allowing the use of shorter testing sessions to assess lesion and graft efficacy. Poor correlation of

graft size with indices of rotation is in line with experiments reported by other workers. Single placement grafts are unable to attenuate the hyper-sensitivity of the lesioned striatum as a whole especially over the relatively short time scale of 4-6 weeks used in the current work. Multiple graft placements, longer time scales, and better grafts may be able to reverse hyper-sensitivity and improve the correlation between graft size and rotation. It seems logical that the amount of dopamine produced by the graft (and therefore graft size) should influence the magnitude of the amphetamine response and be discernable using appropriate testing conditions Further experiments are being planned to see if differences in the rotational responses to different doses of amphetamine might be used as an in vivo measure of graft size in individual animals.

The effect of the 6-OHDA lesion on viral vector transduction.

In chapter 3 it was shown how a 6-OHDA lesion affected the distribution of transgene expression following injection of an adenoviral vector. In the presence of a dopamine-depleting lesion of the MFB, gene expression was more widespread, and transduced large numbers of cells in areas outside the striatum. It was concluded that the differences seen were probably due to differences in the diffusion characteristics of the lesioned striatum, either because of reduced numbers of viral-vector binding sites, or because of changes in the permeability of the extracellular space.

Although the observed results are likely to be specific to the viral vector and the lesion model used, they illustrate an important general point: that testing of vectors in the normal brain is of only limited value and that pre-clinical testing needs to be carried out using models which match as closely as possible the disease being studied. Investigation of the *in vivo* dynamics of transgene expression must consider the levels and locations of gene expression as well as the temporal profile of expression, all of which may be affected by the disease pathology. If a treatment requires expression of a functional gene (e.g. GDNF) in the striatum, it is important that the expression of GDNF in other (afferent or efferent) areas are investigated, together with the manner in which these and other characteristics of gene expression might be altered by the lesion used.

Improvement of dopamine graft survival.

Dopamine cell transplantation continues to be a realistic prospect as a therapy for late stage Parkinson's disease when conventional L-DOPA based treatment starts to fail. In the limited numbers of clinical trials that have been carried out, some patients have derived great benefit from dopamine implants. In the best cases, patients achieve significantly improved neurological scores, both on and off L-DOPA, and are able to reduce their daily drug doses, thereby enjoying a much-improved quality of life. Additionally, long term imaging of transplant patients and post-mortem studies have shown that dopamine grafts are long lasting and therefore have the potential to continue to provide benefit for the life of the patient.

Despite the obvious benefits, dopamine implantation is not routinely used as a therapy for PD for a number of important reasons. Firstly, implanted embryonic dopamine cells do not survive well in the adult brain either in animal models or in human clinical trials. Typical survival rates are 5-10%, and evidence suggests that dopamine cell survival in human patients is no better. Thus, one of the principal reasons for the limited numbers of trials carried out so far has been the large amounts of embryonic tissue required (5-6 embryos per patient, per side of the brain) for optimal therapy, greatly exacerbating the ethical and logistical problems in procuring donor tissue.

Secondly, recent changes in both UK and EU regulations require that, beyond clinical trials, any routine therapy will have to make use of "good manufacturing practice" and quality control during the preparation of materials to be implanted, which will be especially difficult to achieve when using primary tissue donors.

Thirdly, not all patients in clinical trials have shown significant benefit and the reasons for the variability of response amongst patients are not fully understood. Whilst some of the variability may arise from the variable nature of the tissues implanted, or differences in the surgical protocols used, it is likely that much of the variation also arises from differences in the patient population and all of these possibilities will require further investigation before any transplantation of any type of cell into PD patients can proceed.

Finally, in two recent high profile clinical trials, significant side effects were observed in a subset of patients following dopamine cell implants. Most serious among these were graft-induced dyskinesias, where patients demonstrated uncontrollable movements even

in the absence of L-DOPA treatment. Whilst the mechanisms underlying such side effects are likely to be elucidated in the near future, these results have nonetheless been a setback to PD transplantation and will need to be resolved before clinical trials can be resumed.

This thesis has primarily addressed the first issue, that of poor graft survival and the limited availability of embryonic donor tissue. The loss of 90-95% of the dopaminergic neurons implanted means not only that several donors are required for optimal human transplants, but also that, in both animal and human grafts, most of the cells in the mature graft are not dopamine cells. In the rat model of PD, a typical dopaminergic graft is cylindrical, or teardrop shaped and the dopamine cells within the graft mostly occupy positions near the surface of the graft (see Chapter 1 figure 1). This means that much of the space occupied by the graft is made up of non-dopaminergic cells that contribute little or nothing towards functional recovery and may be detrimental to the functioning of the host striatum, effectively inducing a space-occupying lesion. Thus, the issue of graft survival is not merely one of increasing dopamine cell yield or decreasing the requirement for donor tissue. Increased dopamine cell survival will mean that for a given number of mature dopamine cells, grafts may be smaller, occupying less space in the host striatum, and able to more efficiently re-innervate the host striatum without impairing striatal function. It is likely therefore, that the full potential of dopamine grafts in terms of functional recovery have not yet been fully realized, and whilst the future of transplantation therapy for PD most likely lies with the grafting stem cells, primary cell transplantation still has an important part to play as proof of principle and for studies defining optimization and mechanisms of action of DA grafts. Until the maximum functional efficacy of primary transplants is determined, the full potential of dopamine cell transplantation will not have been realized and the benchmark for stem cell therapies has therefore yet to be adequately established.

The present work has contributed two exciting developments in this regard. Firstly, the improvement of graft survival using differentiation factors is a mechanism not previously described. Delivery of the differentiation factor Shh using an adenoviral vector was able to dramatically increase dopamine cell yield in E12 dopamine grafts, not only when compared to untreated E12 controls but also when compared to the supposed "optimal" E14 grafts. Other workers have shown effects at other ages and Shh is known

to persist in the developing brain beyond the age of E14. Thus the effect of Shh on dopamine neurons is dose dependent, and future experiments looking at dose responsiveness of Shh treatment may well yield greater benefits or demonstrate effects of Shh on grafts of other donor ages. The application of differentiation factors to DA grafts provides an alternative strategy for enhancing the survival of dopamine grafts and, depending on the age of the donor tissue used, such factors have the potential to enhance dopamine cell yield when used alone, or when combined with neurotrophic or other factors already proven to enhance graft survival.

The second and potentially more exciting development is the discovery that young donor age tissue, derived from donors 1-2 days younger than any used previously, produce dopamine cell yields many-fold greater than standard E14 derived grafts. This is exciting because the survival rate of more than 30% described here, if reproducible in other species, may already be sufficient to achieve the goal of 1 embryonic donor per patient. For human transplants, this would depend on identification of the equivalent donor age embryos in humans and an investigation of whether or not tissue from such embryos exhibits the same capacity for DA cell generation as the 6mm rat embryos. Rat embryos of this size are at stage 14-15 on the Carnegie scale, which in the human embryo is approximately 35-36 days post-ovulation (C35-36). This is a relatively young age and terminations at this age would likely be medical rather than surgical. Future experiments will compare 6mm and 10 mm rat embryos in vitro and will generate data that allow us to assess the potential of C35-36 should such tissue become available in the future.

Because E11 VM contains no mature dopamine neurons, the survival of E11 grafts must therefore involve development of DA precursors, post-implantation, into fully differentiated neurons via mechanisms as yet undetermined. At this early stage in the investigations the relative contributions of cell proliferation, differentiation and maturation processes is not known, although all are likely to be involved. Once determined, such processes will tell us much about the development of grafted dopamine cells in the host brain, the nature of the signals required, and the identity of the cells responsible for guiding the development of the dopaminergic phenotype. Subsequently, there is potential to manipulate these mechanisms to further increase DA cell yields from E11 grafts or those derived from other donor ages. For example, if dopamine cell

precursors are shown to undergo a period of proliferation after implantation, this would provide an opportunity either *in vivo* or *in vitro* to expand the numbers of cells prior to differentiation. Alternatively, boosting levels of differentiation factors or application of anti-oxidants or growth factors might be used to further improve E11 graft survival. It is reasonable therefore to assume that the current high levels of survival seen in untreated grafts can be boosted further and, if equally applicable to human embryonic tissue, might provide enough tissue from a single embryo for the treatment of one or more patients.

The functional capacity of young donor grafts is unknown, potentially exciting quantity. That the grafts have the ability to reverse the amphetamine rotational deficit has already been shown, and TH immunohistochemistry has demonstrated widespread innervation of the surrounding host striatum. However, the dopaminergic phenotype of the TH positive cells has yet to be proven and although young grafts contain many more TH positive cells than conventional grafts it may be that not all of these are dopaminergic. On the other hand, the amelioration of lesion deficits observed so far was achieved using sub-optimal, single-placement grafts of large volume. Recovery of function should be better achieved using multiple graft placements, possibly implanting less tissue, (to avoid potential space-occupying lesion effects) and attempting to reinnervate the greatest possible volume of dopamine-depleted striatum using the least amount of implanted tissue. Because of the morphology of standard dopamine grafts, in which the majority of implanted cells are non-dopaminergic, this optimum has never been achieved and the potential of young donor age grafts is high in this regard.

The work with young donor age tissue presented in the current thesis is only the start of our investigations. The potential appears great, and future experiments will hopefully yield more important discoveries that contribute to the future of dopamine cell replacement for Parkinson's disease.

Appendix 1

Gene Therapy applications for Parkinson's disease

E. M. Torres, C. Monville, S. B. Dunnett

A1.1 Introduction and supplementary information

A1.2 Manuscript:

Gene Therapy applications for Parkinson's disease

E. M. Torres, C. Monville, S. B. Dunnett
(Invited review for Publication in "Gene Therapy for Neurological Disorders." Edited by P. R. Lowenstein and M. G. Castro. Published by Karger, (Switzerland)

A1.1 Introduction and supplementary information

The following manuscript was written as an invited review chapter for publication in the book "Gene Therapy for Neurological Disorders" Edited by P. R. Lowenstein and M. G. Castro Published by Karger, (Switzerland). The sections covering the use of HSV vectors and Lentiviral vectors were contributed by Dr. C. Monville, the remainder of the manuscript was the work of E. M. Torres. Professor S. B. Dunnett provided guidance direction and editorial input.

Review

Gene Therapy applications for Parkinson's disease

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Introduction

The degeneration of nigrostriatal dopamine neurons in the human brain is known to be the primary pathology of Parkinson's disease (PD) and underlies the profound motor syndrome associated with the disease, involving bradykinesia, rigidity and tremor. The discovery in the 1950s that administration of the dopamine precursor L-3, 4-dihydroxyphenylanaline (L-DOPA) could reverse a similar dopamine-denervation syndrome in experimental animals (Carlsson et al. 1957), rapidly led to the clinical application of L-DOPA. When administered orally, together with the peripheral decarboxylase inhibitor carbidopa, L-DOPA proved an effective therapy for this previously untreatable condition. Subsequent innovations have increased the potency and efficacy of L-DOPA therapy, such as the development of slow releasing forms of the drug, as well as use of other dopamine agonists such as bromocriptine. Since the mid nineteen seventies, countless thousands of Parkinson's sufferers have benefited from the treatment and to this day, L-DOPA remains the mainstay of treatment for PD.

However, despite the obvious benefits, pharmacological treatment of Parkinson's patients has serious limitations. It is not curative and the underlying disease progresses inexorably with time. In the early stages of the disease, a proportion of the degenerating dopamine neurons still survive and these contain the enzymes necessary to synthesize dopamine from L-DOPA once it arrives at the target area. In addition, there are intrinsic mechanisms which compensate for the progressive decrease in dopamine levels (Zigmond et al. 1990). There is up-regulation of the numbers of striatal dopamine receptors and an increase in dopamine receptor sensitivity. There is also down-

regulation of dopamine degradation in nerve terminals. Together, these compensatory mechanisms have the overall effect of increasing the efficacy of smaller and smaller amounts of endogenous dopamine as the levels decrease with time. However, as the disease progresses, patients need increasing and more frequent doses of L-DOPA to obtain therapeutic benefit and the ameliorative properties of the drug become complicated by the development of debilitating side effects, mainly in the form of drug-induced dyskinesias and on-off fluctuations in drug response (Marsden & Parkes 1976). These typically develop after 5-10 years of drug therapy and arise from the interaction of the increasing drug load, in particular the peak and trough nature of drug levels in the blood, with the increased dopamine-sensitivity of the brain. Continuous intravenous delivery of low dose L-DOPA has been shown to dramatically reduce drug induced dyskinesias, but is in itself not a practical therapy for a predominantly elderly out-patient population. (Schuh & Bennett, Jr. 1993). Inevitably, patients reach an end-stage of the disease when the side effects of therapy come to outweigh the benefits and drug treatment becomes increasingly problematic. Consequently, there has for a long time been the need for an alternative to L-DOPA for these end stage PD sufferers.

PD presents a tantalising target for gene therapy mainly because the principal pathology arises from the degeneration of a single group of cells the consequences of which are manifested in a circumscribed and well characterised neural circuitry. Although the aetiology of the disease is unknown, the root cause of the clinical pathology is known to be the death of dopamine containing cells in the substantia nigra pars compacta (SNc) and the subsequent loss of dopamine innervation to the principal target area, the striatum. The failure of the dopaminergic input to the striatum results in an up-regulation of the gamma-amino-butyric acid (GABA) output from this nucleus to the globus pallidus pars externa (GPe). As a consequence, there is down-regulation of the GABA output of the GPe to both subthalamic nucleus (STN) and the substantia nigra pars reticulata (SNr), resulting in disinhibition of both these nuclei. The resulting up-regulation the glutamatergic output of the STN causes over excitation of efferent areas including the SNr and globus pallidus pars interna (GPi). It is the up-regulation of these two nuclei in particular which is thought to account for the characteristic symptoms of PD, in particular tremor, rigidity and bradykinesia (Albin et al. 1989). (See PD affected areas in Fig. 1)

PD can be modeled in animals by lesioning of the dopamine cells of the SNc. This is done principally using dopamine cell specific toxins such as 6-hydroxydopamine (6-

OHDA), or 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP). Total bilateral destruction of the dopamine system in rats is seriously debilitating on the recipient animal and the animals need intensive care simply to remain alive (Zigmond & Stricker 1973). A more common approach therefore, is a hemi-Parkinsonian model in which 6-OHDA in injected unilaterally, either into the ascending nigrostriatal nerve bundle or into the striatal terminal field. The resulting dopamine asymmetry in the brain produces a corresponding motor asymmetry and a measurable rotational behaviour in response to amphetamines or the dopamine agonist apomorphine (Ungerstedt 1971b; Ungerstedt 1971a). The magnitude of the rotational response is sensitive to therapeutic strategies and is widely used as the main index of functional recovery in this model. The unilateral 6-OHDA model has also been used in primates as has the peripheral injection of MPTP to produce a bilateral, partial dopamine lesion. Chronic peripheral administration of MPTP at low doses over extended periods of time can also produce an animal model in monkeys that is progressive and has many of the hallmarks of the idiopathic disease (Hantraye et al. 1992). Conversely, intracarotid administration can be used to produce a unilateral lesion similar to the 6-OHDA lesion in rats, without the need for surgical intervention in the brain (Bankiewicz et al. 1986).

Whilst L-DOPA therapy in patients is targeted at dopamine replacement in the striatum, treatments aimed at correcting some of the downstream consequences of dopamine depletion have also enjoyed some success. Based on the direct and indirect pathways of the basal ganglia circuitry described above, lesions have been used to ablate downstream targets in order to "correct" the up-regulated outputs of the striatum in GPi and STN (Bergman et al. 1990). Such experiments led to the introduction of pallidotomy and subthalamotomy as experimental surgical treatments for PD (Laitinen et al. 1992). This approach has not been without its problems however and in some patients there have been severe side effects. Except in a small number of patients who have a drug-resistant tremor, this treatment has now fallen out of favour (Okun & Vitek 2004). More promising has been a technique known as deep brain stimulation in which high-frequency stimulating electrodes implanted to the sensorimotor region of either the GPi or STN of Parkinson's patients are able to inhibit the up-regulated output of these nuclei and to modulate most of the motor symptoms of the disease (Limousin et al. 1995). Patients also gain better control of "on" time and are typically able to reduce their dopaminergic medication. (Benabid 2003; Betchen & Kaplitt 2003; Bronte-Stewart 2003).

One of the most studied alternative therapies and one that still holds considerable promise, has been neural transplantation. Dopaminergic cells, extracted from developing embryos, are implanted ectopically into the dopamine-depleted striatum. The implants are able to form dopamine cells in the host brain which send out axons into the surrounding tissue. They make appropriate synaptic connections and most importantly, deliver dopamine to the target tissue. Originally developed in animal models in the 1980s and 1990s, such transplants have since been carried out in humans in a number of centres around the world (Dunnett et al. 2001; Olanow et al. 1997). There have been successes as well as failures and the technique has had as many detractors as supporters. However, there is little doubt that, in a definable subset of patients, transplants derived from donor embryos of the correct age and implanted using a robust surgical protocol, are able to provide considerable therapeutic benefits. In successful cases the implanted dopamine cells provide a continuous low dose of dopamine to the denervated striatum in a manner that eliminates the peak-trough regime of orally administered L-DOPA. Patients are able to reduce their L-DOPA intake dramatically to a fraction of the previous dosage and in some cases L-DOPA treatment can be stopped altogether. The associated side affects are much reduced or eliminated altogether. Long term studies of these patients have shown that transplantation therapy is long lasting and that it has the potential to extend treatment of PD patients well beyond the therapeutic window provided by L-DOPA (Lindvall & Hagell 2001; Peschanski 2001). However, despite its success, transplantation therapy is not widely or routinely used as a therapy for PD. The reasons for this are less to do with efficacy of the treatment itself and more to do with the complexity of the ethical, logistical and quality control problems which surround the use of fetal donor tissues (Barker 2002; Bjorklund et al. 2003).

Targets for Parkinson's disease gene therapy

The disrupted circuitry described above, defines the regions of the brain at which novel treatments for PD, including gene therapies, might be targeted. Broadly speaking, gene therapies can be applied in one of two ways: either by direct (*in vivo*) injection of transgenes into the brain or blood stream using a suitable gene vector, or indirectly (*ex vivo*), by packaging transgenes into accessory cells for subsequent implantation into the affected areas. In the case of PD, there are a number of potential direct approaches (see Fig 1). Dopamine replacement in the striatum, a direct correlate of L-DOPA therapy is an obvious one. A number of research groups have succeeded in transducing striatal

cells with one or more of the genes involved in dopamine synthesis and have reported beneficial effects in animal models. The three principal genes thought to be required are tyrosine hydroxylase (TH), L-aromatic amino acid decarboxylase (AADC) (the first two enzymes involved in the conversion of tyrosine to dopamine) and GTP cyclohydrolase 1 (GTPCH1) the enzyme which synthesises an important TH cofactor, tetrahydrobiopterin (BH_4). A further factor involved in the packaging and transport of dopamine, vesicular monoamine transporter (VMAT), may also be required (Kang et al. 2001b). Transduction of TH alone will probably be insufficient for de-novo dopamine synthesis *in vivo*, although gene therapy using an adeno-associated virus (AAV) vector containing only the AADC gene, in conjunction with peripherally administered L-DOPA has been shown to partially reverse 6-OHDA induced deficits in rats (Leff et al. 1999), (Sanchez-Pernaute et al. 2001).

However, novel synthesis of L-DOPA *in vivo* will almost certainly require transduction of two or more of the TH, AADC and GTPCH1 transgenes. Some promising work has already been carried out in this regard. In a rat model of PD, Mandel and colleagues (1999) showed that injection into rat striatum of AAV vectors containing genes for both TH and GTPCH1 led to demonstrable L-DOPA production *in vivo* over long periods of time, and was able to ameliorate the deficits produced by the 6-OHDA lesions. (Mandel et al. 1999). Kirik et al used a TH-containing AAV vector in combination with BH_4 in a partial lesion model to show good recovery on a range of behavioural tests (Kirik et al. 2002a). Transduction of TH and AADC using a Herpes simplex (HSV)-based vector was carried out by Sun et al who succeeded in partially reversing apomorphine induced rotation and showed up to 10,000 TH/AADC positive cells in the striatum seven months after treatment. (Sun et al. 2003c)

Transduction of TH, AADC and GTPCH1 in combination will likely be the optimal approach for dopamine replacement therapy, and several methods of gene delivery have already been investigated. AAV vectors have been used to introduce genes for TH AADC and GTPCH1 into rat (Shen et al. 2000) and monkey (Muramatsu et al. 2002) striatum. resulting in amelioration of lesion induced deficits. Azzouz and colleagues used a "tricistronic" lentivirus (LV) vector in a rat model with comparable results (Azzouz et al. 2002d). See also (Bankiewicz et al. 2000).

An alternative to the dopamine replacement strategy is the provision of neurotrophic factors to the Parkinsonian brain in an attempt to halt or reverse dopamine cell degeneration. Glial cell line derived neurotrophic factor (GDNF) is a prime

candidate for a PD gene therapy. Direct administration of GDNF protein has been shown to have potent ameliorative and reparative effects in both rodent and primate models of PD (Bjorklund et al. 1997; Gash et al. 1996b; Gash et al. 1998b; Gash et al. 1998a; Gash et al. 1996a) and a report of efficacy in a pilot clinical study has attracted particular interest (Gill et al. 2003). In animal models using gene therapy, GDNF has been reported to protect dopamine cells against the effects of a dopamine lesion when delivered using AAV (McGrath et al. 2002; Wang et al. 1996) adenovirus (AV) vectors (Choi-Lundberg et al. 1998; Connor 2001) and LV vectors (Kordower et al. 2000a; Bilang-Bleuel et al. 1997; Gerin 2002; Chen et al. 2003b). GDNF has also been used in combination with the anti-apoptotic Bcl-2 delivered using an HSV vector and found to be effective in protecting against the effects of 6-OHDA. (Natsume et al. 2001c). Given the progressive nature of PD, it is likely that any growth factor therapy will need to be administered continuously over a long period of time in order to sustain dopamine neuron survival and function in the long term. Moreover, since GDNF does not cross the blood-brain barrier and GDNF receptors are widely distributed throughout the body, delivery will need to be directly into the brain. Because the primary cellular pathology of PD is confined mainly to the dopaminergic neurons of the substantia nigra, supplying therapeutic genes to these neurons by injection of viral vectors into localised sites in the forebrain is the most obvious strategy.

An innovative strategy by Luo and colleagues targeted the deep brain areas at which electrical stimulation has been directed (Luo et al. 2002). They used AAV vectors to introduce the GABA-synthetic enzyme glutamic acid decarboxylase (GAD) into the subthalamic nucleus in an attempt to inhibit the output of this region. Following success in animal models, the go-ahead has now been given for a clinical trial using this approach in patients. (Luo et al. 2002)

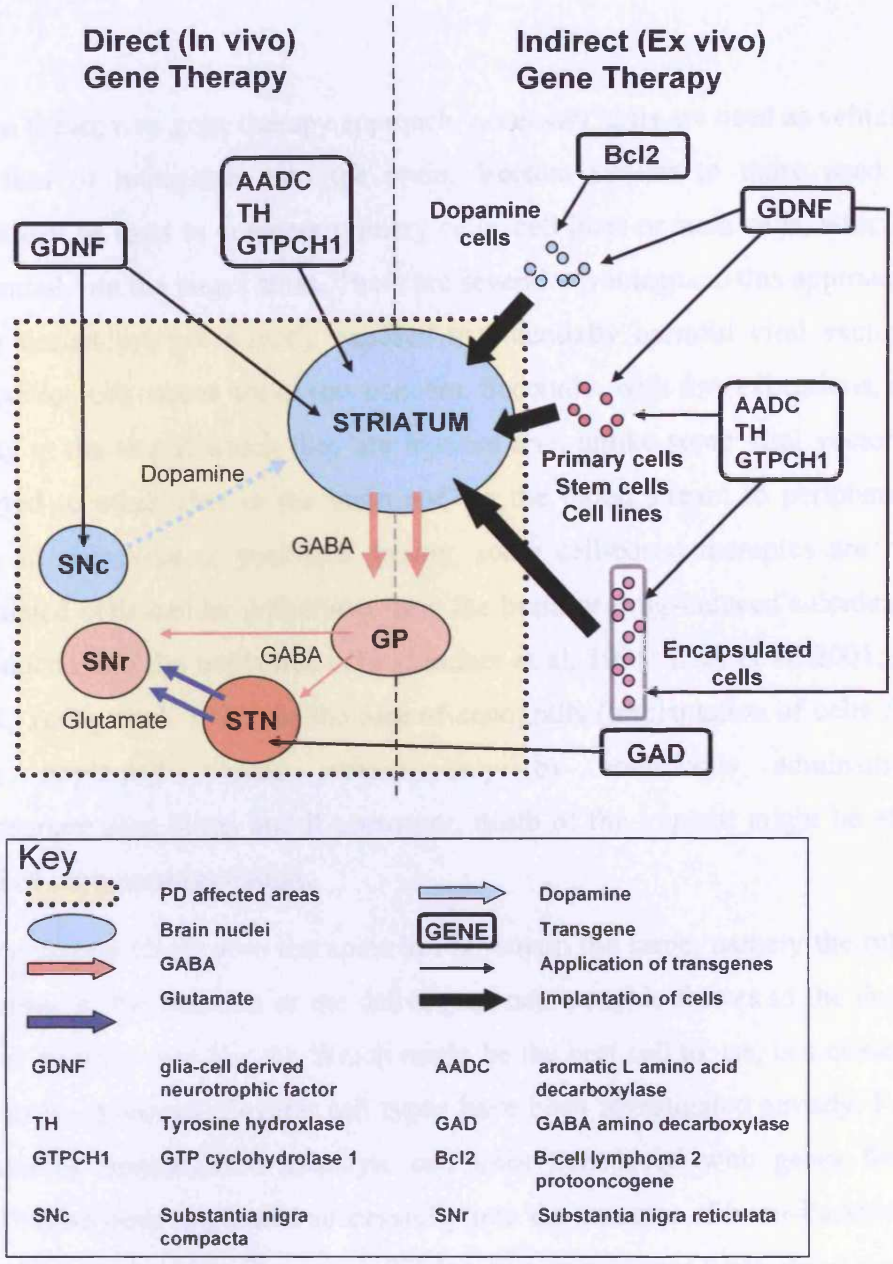

Figure 1 PD affected brain areas and targets for gene therapeutic approaches. PD affected areas are shown in the yellow shaded box. There is a degeneration of the nigrostriatal dopamine pathway (dashed blue arrows) and upregulation of GABAergic output form the striatum to the external segment of the globus pallidus (double orange arrows). This causes down-regulation of GABAergic output from from this nucleus (thin orange arrows) and in turn, up-regulated glutamatergic output from the STN to the SNr and Gpi (pink arrows). Strategies for CNS gene therapy may be 'direct' (left), where transgenes are injected directly into the host brain; or 'indirect' (right), where transgenes are introduced into accessory cells before implantation into the host brain. The diagram shows how these therapies might be applied to PD. Genes of interest might be targeted at a number of affected areas. Direct application of neurotrophic genes such as GDNF to either the striatum or SNc might be used to rescue the degenerating dopamine system. Alternatively, dopamine synthesis might be established in the striatum by the introduction of genes for TH, AADC and GTPCH1. Another direct approach might be to down-regulate the overactive STN by the introduction of a gene for GAD to enable synthesis of GABA in this nucleus. Indirect approaches fall into two main categories. Modification of primary cells and modification of cell lines. The survival of primary dopamine grafts might be enhanced by the introduction of GDNF or of anti-apoptotic genes such as Bcl2. GDNF and the dopamine synthetic enzymes TH, AADC and GTPCH1 might be introduced into primary cells such as host astrocytes, stem cell lines or cells contained within polymer capsules prior to implantation into the host striatum.

In the *ex vivo* gene therapy approach, accessory cells are used as vehicles for the introduction of transgenes into the brain. Vectors similar to those used in direct therapies can be used to engineer primary cells, cell lines or stem cells, which can then be implanted into the target areas. There are several advantages to this approach. Firstly, the host tissues are not directly exposed to potentially harmful viral vectors and so safety and toxicity issues are of less concern. Secondly, with few exceptions, implanted cells stay at the site at which they are injected and, unlike some viral vectors, are not transported to other sites in the brain nor via the blood stream to peripheral tissues. Thirdly, in the event of problems arising, some cell-based therapies are reversible. Encapsulated cells can be withdrawn from the brain or drug-induced suicide genes can be introduced into the implanted cells (Lindner et al. 1995; Date et al. 2001; Shingo et al. 2002; Tseng et al. 1997). In the case of xenografts (implantation of cells from other species) implanted tissues survive only by continuous administration of immunosuppressant drugs and if necessary, death of the implant might be effected by removal of immunosuppression.

The targets for *ex vivo* therapies in PD remain the same, namely the replacement of dopamine in the striatum or the delivery of neurotrophic factors to the degenerating dopamine neurons (see Fig. 1). Which might be the best cell to use, is a question which has yet to be answered. Several cell types have been investigated already. Primary rat fibroblasts or immortalised astrocyte cell lines transduced with genes for TH and GTPCH1 have been implanted successfully into the striatum of hemi-Parkinsonian rats as. (Bencsics et al. 1996; Chen et al. 2003a). The same genes have also been shown to have functional effects when delivered using rat bone marrow stromal cells (Schwarz et al. 2001). *Ex vivo* GDNF protection against 6-OHDA lesions has also been demonstrated using adrenal chromaffin cells, (Espejo et al. 2001) astrocytes, (Cunningham & Su 2002) and neural stem cells (Akerud et al. 2001).

A final PD gene therapy strategy could be the enhancement of dopamine cell transplantation. One of the major and long-standing problems in the use of foetal transplants has been the relatively poor survival of implanted dopamine neurons. In animal models, although survival rates of 20% and even 40% have been reported, typically only 5-10% of implanted embryonic dopamine neurons survive in the host brain. Put another way, more than 90% of the implanted dopamine cells (or cells which

have the potential to become dopamine cells) die, or never develop a dopaminergic phonotype following implantation into the host brain (Brundin et al. 2000). Poor survival has also been confirmed in human patients (Kordower et al. 1997; Lindvall et al. 1994; Olanow et al. 1996) and is considered a major factor in the incomplete recovery seen in many clinical trials. In experimental models, the problem of poor survival can be simply overcome by increasing the amount of donor tissue used, since the degree of functional recovery has been shown to correlate with the number of dopaminergic cells surviving implantation in these models (Dunnett et al. 1988). However, in the clinic, the necessity for donor tissue from multiple embryos is problematic. Up to six embryos per patient, per side of the brain are now considered to be necessary for optimal therapy (Lindvall 2000; Hagell & Brundin 2001). The logistical difficulty of obtaining a sufficient quantity of fresh tissue has proved extremely problematic, such that over the fifteen years that this therapy has been actively explored, transplantation therapy for PD has been carried out in only a few centres around the world and to date only a few hundred patients have received implants.

A great deal of effort has been put into finding alternative sources of dopamine cells for implantation. Embryonic dopamine cells from pigs have been investigated (Armstrong et al. 2002; Jacoby et al. 2002). However cross species transplant studies still have problems of immune rejection and the theoretical possibility that contaminating strains of virus might be able to cross the species barrier have led to significant regulatory and safety concerns. Expansion of precursor cells derived from embryonic primary tissue to increase the number of dopamine cells has also received much attention. Fetal progenitor cells can be expanded indefinitely in the form of neurospheres which can then be differentiated into neurons and glia. However such cells have resisted attempts to encourage their differentiation down the dopaminergic lineage, despite more than a decade of research (Lindvall 2003). More recently, embryonic stem (ES) cells have been considered. These are derived from the blastula stage of the developing embryo and in theory have the potential to form all cell types of the body. In spite of some promising preliminary reports in mouse models, using mouse ES cells (Kim et al. 2002) (Bjorklund et al. 2002), we are still a long way from achieving reliable control of the steps required to generate dopamine neurons from ES cells and for the cells to retain differentiation following transplantation in vivo. Nevertheless there

remains widespread optimism that these problems are solvable, and the ES cell remains an exciting prospect for the future (Kawasaki et al. 2002; Morizane et al. 2002).

In theory, if the survival of implanted dopamine cells could be routinely boosted from 10% up to 50% or more, then an optimal therapy might still be achieved using a single embryo per patient, and the practical problems surrounding the limited availability of primary fetal tissue for PD therapy would be much reduced. To this end, several centres are exploring a variety of different approaches to reduce the processes involved in cell death and promote neuronal survival in tissues destined for transplantation (Brundin et al. 2000).

Viral vectors for Parkinson's disease gene therapy

Whilst it might be argued that the potential targets for PD gene therapy have largely been established, the optimal means of delivery of therapeutic genes into the brain is still far from decided and is the subject of vigorous research effort. The issue is complex, as evidenced by the wide range of vector systems which are currently being investigated. In the race to find the best vector for gene delivery for the central nervous system, the front-runners have already been mentioned; AAV, AV, LV and HSV-based vectors appear to be the main contenders. Each vector system has benefits and drawbacks and we shall not go into a detailed comparison here (For review see (Fawcett et al. 2001)). Ultimately, in the race for the perfect CNS gene therapy vector, there may be more than one winner. It is likely for example, that the optimal vector for *in vivo* delivery of one growth factor to the striatum may not be the one best suited for in *ex vivo* delivery of the same or another factor to embryonic dopamine cells. Efficient delivery of the genes for dopamine synthesis may well be best achieved using a third vector.

There are a number of issues to be considered when choosing the best vector for delivery of a particular transgene. Common to all is the issue of toxicity. The vector must not be cytotoxic itself, nor cause cell death by initiating a cycle of virus replication in host cells. As a consequence, all virus-based vector systems have genes deleted to reduce cytotoxicity and render them replication-deficient. In addition the vector, and the process used to produce it, must be non-immunogenic such that no potentially damaging

immune response is induced in the host. The use of *ex vivo* gene therapy is one way of avoiding this.

The tropism of viral vectors is also an issue. For direct application, the vector must be able to infect non-dividing cells and transduce them successfully. Transduction relies both on successful delivery of the transgene to the cells of interest and on successful activation of the gene promoter, which may be cell specific. For example, genes driven by the glial-fibrillary-acidic-protein (GFAP) will express only in glial cells, whilst a neuron-specific-enolase promoter will only be switched on in neuronal cells. Control of promoter expression is more problematic. Viral gene promoters are prone to switching off after a short period of time. On the other hand promoters which drive gene expression indefinitely may have unforeseen consequences and many think that for gene therapy in humans, some form of controllable expression may well be required. The use of the gene promoters linked to tetracycline responsive elements has already shown some promise (Vogel et al. 2004; Kafri et al. 2000b; Chtarto et al. 2003).

Hands on experience with viral vectors

In our laboratory, we are investigating alternative approaches to PD gene therapy using a variety of viral vector systems. Firstly, a GDNF delivery, dopamine cell protection approach using HSV-based vectors, in collaboration with Cantab Pharmaceuticals (now Xenova, Cambridge UK) and with the group of Dr. Stacey Efstathiou at the University of Cambridge. Secondly a dopamine replacement strategy using a tricistronic LV vector containing gene inserts for TH, AADC and GTPCH1, produced by Oxford Biomedica (Oxford, UK). Thirdly, a strategy for increasing the survival of dopamine cell transplants using AV vectors containing genes for GDNF and Sonic hedgehog (Shh), in collaboration with P.R. Lowenstein and M. G. Castro of the Gene Therapeutics Research Institute, Cedars-Sinai Medical Centre (University of California, Los Angeles,USA)

All of our studies are carried out using a unilateral 6-OHDA lesion model of PD in the rat. Dopamine lesions are carried out using 6-OHDA.(Hydrobromide) at a free base concentration of 3.5mg/ml. All surgery is conducted under isoflurane anaesthesia in a Kopf stereotaxic frame, with the nose bar set 2.3mm below the interaural line, The toxin is administered either as a single injection into the median forebrain bundle

(MFB) (A = -4.4, L = -0.1, V = -7.8) or using the 4-site striatal injection paradigm based on that described by Kirik et al (A= +1.3, L= -2.6; A= +0.4, L= -3.0; A= -0.4, L= -4.2; A= -1.3, L= -4.6; all injections at V= -5.0) (Kirik et al. 1998). MFB injection of the toxin results in an acute lesion in which nigrostriatal dopamine function is disrupted immediately and dopamine cell death occurs within 1-2 days following injection, to produce a near-complete (>95%) unilateral lesion . The striatal injection paradigm induces dopamine cell death over 1-3 weeks and is better suited to treatment strategies aimed at cell protection.

Dopamine grafts are prepared from the dissected ventral mesencephalic (VM) of embryos aged E14 (14 days after formation of the post-copulatory vaginal plug). Dissociation of the tissue is carried out using trypsin and mechanical triturating to produce a single cell suspension. (Fricker et al. 1996). When grafting, each rat receives an amount of tissue equivalent to one VM injected into the dopamine depleted striatum either as a single injection (A= +0.6, L= -3.0, V = -4.5) or as four injections at the same coordinates used for the striatal 6-OHDA administration.

Behavioural assessment of the lesions and grafts is undertaken by screening all animals for drug-induced rotation in a bank of rotometer bowls (Ungerstedt 1971a) over 90 min following intraperitoneal (i.p.) injection of 2.5 mg/kg methamphetamine i.p. and over 60 min after a subcutaneous (s.c.) injection of 0.05 mg/kg apomorphine. Anatomically, the lesions, grafts and vector injections are assessed by TH immunohistochemistry (illustrated in Fig. 2).

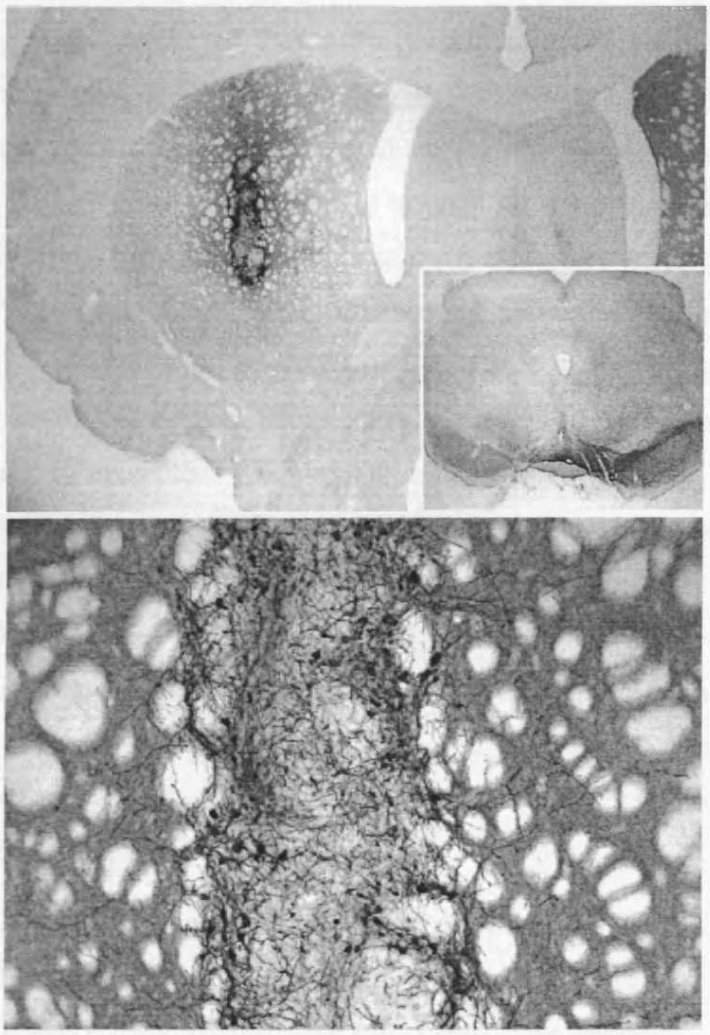

Figure 2. Typical appearance of dopamine grafts in rat model of Parkinson's disease **A**. Appearance of an E14 derived graft implanted into the dopamine depleted striatum, as visualised by TH immunohistochemistry. The graft contains many TH positive cells which re-innervate the surrounding striatum (dark halo around the graft). **B**. Higher power photograph of the graft showing TH cell morphology and TH processes within and radiating from the graft. *Inset.* TH staining of the substantia nigra showing loss of dopamine cells unilaterally on the side of the lesion injection.

GDNF gene therapy for Parkinson's disease using HSV Vectors

The progressive nature of Parkinson disease offers an opportunity for gene therapy aimed at slowing or blocking the degenerative process. As a candidate vector for gene delivery, vectors derived from disabled Herpes simplex viruses (HSV) are potentially very attractive. HSV has a natural tropism for mammalian neurons. It has a large genome into which large or multiple transgenes can be inserted (Latchman & Coffin 2001b). More than 84 viral genes are encoded, approximately half of which are essential for viral replication in a permissive tissue culture environment (Fink et al. 2003). In the generation of gene therapy vectors, nonessential genes may be deleted, to allow the insertion of exogenous genetic material and vector toxicity can be reduced, and safety improved, by the simultaneous deletion of several immediate-early (IE) genes (Hobbs & DeLuca 1999; Krisky et al. 1998b; Samaniego et al. 1997; Wu et al. 1996). Transgene sequences can be introduced into the defective vector genomes by homologous recombination, and it is possible using appropriate complementing cell lines to prepare high titre vector stocks that are free from contaminating replication-competent viruses and that have normal targeting characteristics without the ability to replicate or reactivate *in vivo* (Ozuer et al. 2002).

Safety is clearly a critical consideration in the development of any gene therapy designed to treat human disease. Whilst many other vectors have yet to reach clinical trials, the safety of HSV vectors in humans has already been examined. In two separate trials, replication-competent HSV vectors were injected into the brain as a potential treatment for glioblastoma (Yeung & Tufaro 2000; Markert et al. 2001) and no significant vector-related adverse effects were reported.

Another factor in favour of the use of HSV is that its genome does not integrate into the mammalian genome but remains as an episomal element within the nucleus of the infected cell. The use of other viral vectors which insert into the host genome has potential problems. Two studies have shown insertional mutagenesis could lead to leukemia (Cavazzana-Calvo et al. 2000; Check 2002). Further, viral vectors that integrate into chromosomes have been shown to be more likely to insert into active genes than into non-coding regions (Nakai et al. 2003). Thus, the non-integrating nature of HSV may well add to it's suitability as a gene therapy candidate vector.

Table 1 Summary of HSV-based vectors used in the current work

Vector *	Backbone	Transgene	Source
DH1J4	-gH/Multiple IE deletions	GDNF	Cantab
DH1J1	-gH/Multiple IE deletions	LacZ	Cantab
gHLAPGDNF	-gH/-Tk/-LAP	GDNF	U.C.
gHLAPLacZ	-gH/-Tk/-LAP	LacZ	U.C.
CS1	-gH/-Tk/-LAP	LacZ	U.C.

* All HSV transgenes are driven by the LAT promoter. Tk = thymidine kinase; LAP = latency associated promoter. U.C. = University of Cambridge

We have undertaken an investigation of the potential of different HSV vectors to deliver GDNF in a rat model of PD. The vectors involved in the work are summarised in table 1. Viral vectors were obtained from two different collaborators and the construction of the vectors reflects the different approaches of each collaborating group. The vectors prefaced DH were obtained from Cantab Pharmaceuticals of Cambridge. The vectors prefaced gH and CS were obtained from the group led by Stacey Efstathiou, based in the Department of Virology of Cambridge University. Deletion of the essential glycoprotein gH, which is important in viral entry and cell-to-cell spreading (Forrester et al. 1992), restricts replication to a single cycle in both vectors. The endogenous latency-associated transcript (LAT) regions are also deleted to reduce the possibility of intra-genomic recombinations with wild type viruses. Transgene expression is driven by a 3.3 kb fragment of the LAT promoter (LAP) important in long term transgene expression in the PNS (Lachmann & Efstathiou 1997; Marshall et al. 2000). The LAP/transgene cassette is inserted into either the gH/Tk locus (DH vectors) or between the IRL sites separating the unique-long and unique-short regions of the genome (g/CS vectors). The main difference between the two vectors is the extent of viral gene deletion undertaken. As well as the gH and LAT deletions the gH/tk are deleted in the thymidine kinase (tk) locus, a non essential gene which is a known neuro-virulence determinant (Efstathiou et al. 1989). Tk negative viruses are attenuated and reactivate poorly in animal models. The DH1J-based vectors have multiple deletions of the ICP4, ICP22, ICP47 and ICP27 immediate early genes and as a consequence have very limited IE gene expression which in theory should greatly decrease their cytotoxicity.

Previous studies have suggested that, when injected into the striatum, HSV is taken up by terminals and retrogradely transported to the cell bodies of afferent neurons

(Latchman & Coffin 2001a) including the critical DA neuronal populations in the SN. In an initial study, (Monville et al. 2004) we compared the ability of GDNF to protect nigrostriatal neurons against the effects of a 6-OHDA lesion when delivered using either the DH1J4 or gHLAPGDNF constructs. DH1J1 and gHLAPLacZ were used as the respective controls. Striatal injection of both of the HSV-GDNF constructs was effective in protecting against a subsequent 6-OHDA lesion as measured by the number of surviving DA neurons in the substantia nigra (Fig 3). However, only ghLAPGDNF constructs (the least deleted) were able to alleviate the behavioural deficits caused by the lesion, and then only partially.

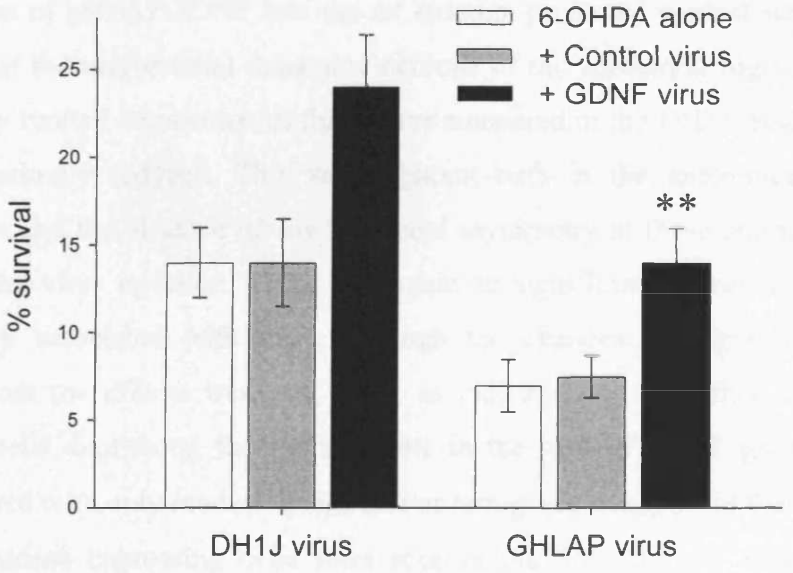

Figure 3. Protection of dopaminergic neurons by a GDNF containing virus following a 6-OHDA lesion. Only the in the GHLAP virus (right) did GDNF produce significantly higher survival then untreated controls.

An unexpected and as yet unexplained result was that injection of the DH1J1 and DH1J4 vectors was associated with severe cytotoxicity and tissue loss at the site of injection. Whilst the data showed that the modest recovery of function on amphetamine rotation was specific to the GDNF vector, the inhibition of apomorphine-induced rotation was comparable with both GDNF and control viruses. This was probably due to death or dysfunction of striatal neurons wherein the receptor super-sensitivity responsible for this form of rotation lies. (Barker & Dunnett 1994). Although the anatomical observation of partial neuroprotection is in line with the hypothesis that

GDNF could be neuroprotective, the cytotoxicity associated with the virus needed further investigation.

The second batch HSV-GDNF vector from the University of Cambridge (See table 1) was less deleted than the Cantab vector lacking only the gH, tk and LAP genes. Previous studies using similar vectors containing the LacZ gene under the control of the LAP, have shown that following delivery in the striatum, β-galactosidase expression was detected within anatomically related CNS regions distal to the site of injection (Scarpini et al. 2001c). In a second experiment we evaluated the ability of this alternative source of HSV-GDNF to protect nigral dopamine neurons against 6-OHDA toxicity using both anatomical and behavioural criteria. As with the dH1J4 vector, injection of gHLAPGDNF into the rat striatum protected against subsequent 6-OHDA lesion of the nigrostriatal dopamine neurons of the substantia nigra. However, despite the very limited attenuation of this vector compared to the DH1J-based vectors, toxicity was markedly reduced. This was apparent both in the anatomical integrity of the striatum and the absence of any rotational asymmetry in these animals associated with the initial virus injection. There was again no significant improvement in behavioural recovery associated with this. Although the changes in nigral cell survival were significant the effects were not large, as indicated by the rather limited numbers of nigral cells expressing the marker gene in the gHLAPLACZ group, which may be associated with only modest uptake and/or retrograde transport of the virus, Only few β-galactosidase expressing cells were seen in the SNc and the number of cells seen dropped off dramatically from 3 days to 4 weeks post injection. The reduction of transgene expression with time is due not to the death of infected cells, but has been shown to be the result of silencing of the non-integrating viral of the LAP promoter (Scarpini et al. 2001b).

In theory, the more heavily deleted (DH) viruses should cause less inflammation and tissue damage, and transduce greater numbers of host cells than the gh/tk deleted vectors. Indeed that is the primary purpose of extensive gene deletion. It was something of a surprise therefore to observe that when injected into the hemi-Parkinsonian rat brain, the more deleted vector caused the most inflammation and damage. Our suspicions fell on the method of preparation and purification of the virus. The conventional method of HSV production (used by the university) was to harvest the virus from the supernatant of the complementing cells, concentrate this by

centrifugation and then to purify the virus using an ultracentrifuge-based liquid (Ficoll) gradient method. By contrast, the dH1J vectors were prepared using a novel method, devised with a mind to possible future commercial scaling of virus preparation. The virus was harvested by lysing of the complementing cells in a high molarity sodium sulphate solution, which was then filtered to remove cell debris. Purification was by means of an affinity column with heparin as the binding agent. We hypothesized that the Cantab method might be co-purifying substances which were immunogenic in the rat brain such as proteins derived form the complementing cell line or large numbers of undeveloped or dead virus particles.

In a third experiment we compared the *in vivo* toxicity, uptake and retrograde transport of a common LacZ vector, prepared using the two different preparation protocols. The CS1 virus was chosen for comparison, as this virus was capable of being grown on the different cell lines used by the two groups. Two batches of CS1 virus were prepared according to either the salt-harvest/affinity (CS1c) or the supernatant-harvest/centrifugation (CS1v) methods. When injected into the normal rat striatum the CS1c preparation was found to be significantly more toxic than the CSv preparation using a variety of measures. CS1c produced greater inflammation, more blood vessel enlargement and fewer transduced cells at all time points. indicating that the protocol for viral preparation was indeed the source of the toxicity seen (See Fig. 4 and 5).

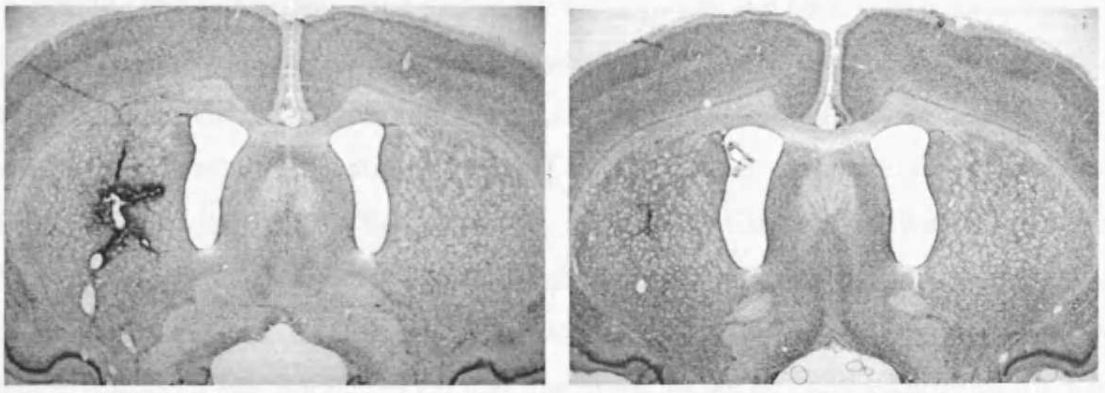

Figure 4. Comparison of the inflammation seen following injection of CS1 prepared using two different protocols. Cresyl fast violet stained sections of rat brain 4 weeks after striatal injection of 3 x 10^6 IU of HSV-LacZ virus (CS1). A. CS1c prepared using the commercial method. There is residual inflammatory infiltrate around the injection site and in the overlying corpus callosum with enlargement of blood vessels and some perivascular cuffing. Despite this there is no obvious distension of the adjacent lateral ventricle that would indicate extensive tissue loss in the striatum. B. CS1v prepared using the conventional method. A small focus of inflammation at the site of injection is all that remains. There is no evidence of a widespread inflammatory process or of striatal tissue loss.

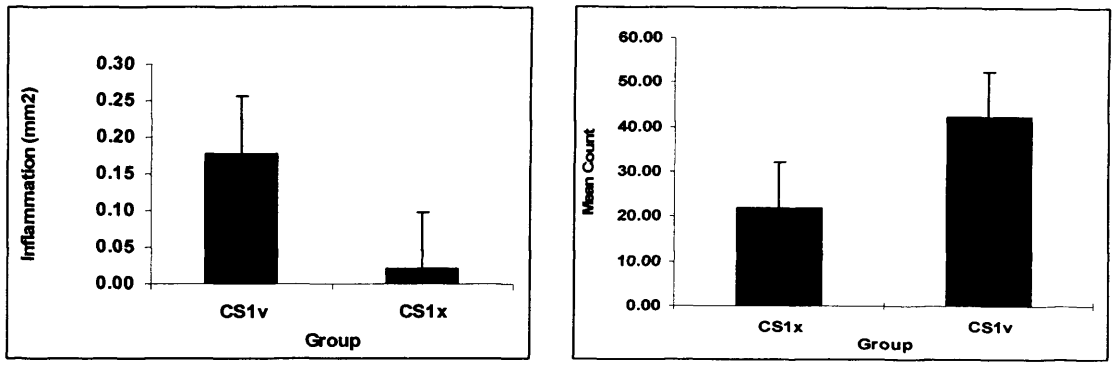

Figure 5. Comparison of the inflammation seen following injection of CS1 prepared using two different protocols. A. CS1 prepared using the commercial method (CS1c) produced more inflammation than CS1 prepared using the conventional method (CS1v). B. By contrast there were fewer β-galactosidase positive cells in the CS1c injected than the CS1v injected brains.

In our experiments with HSV-based vectors we were able to detect β-galactosidase expression, at least at short time points, but were not able to detect GDNF expression using immunochemistry or ELISA. In the meantime we have seen a significant effect of GDNF on dopamine cell survival in one of the two experiments, suggesting that GDNF was expressed at similar levels to that seen with β-galactosidase. Moreover, Natsume and colleagues have shown that after rats were injected with an HSV-GDNF vector, the substantia nigra exhibited a diffuse GDNF immunoreactivity around the injection site and that GDNF was released from the transduced cells (Natsume et al. 2001b). Another possibility is that GDNF expression was not sustained long enough for us to detect it with the methods used. In our studies, high levels of β-galactosidase expression were seen after 3 days. In a previous study, using similar HSV-based vectors, it was demonstrated that maximum levels of expression from the LAT promoter were seen two weeks post injection into rat brain. (Scarpini et al. 2001a). As a compromise, we elected to inject the virus one week before the 6-OHDA lesion in the

hope of producing the maximum protection possible. HSV gene expression typically became silent 3-4 weeks post injection. This was sufficient to yield partial, but not complete, protection of TH neurons. The paradigm used involved a four site striatal lesion but just a single striatal injection of virus and this may have been a limiting factor in the extent of GDNF protection seen.

In conclusion, experiments with both of the HSV vector types testedhave hinted at the potential od these vectors for PD but both have problems which need to be overcome. The LAT promoter, being non-integrating and of viral origin is being silenced in the mammalian brain and the evaluation of the multiply-deleted DH1J based vectors has been hampered by the processes used to produce and purify them. The finished preparation appears to contain pyrogenic substances that elicit a powerful immune response in rat brain resulting in severe inflammation, cytotoxicity and tissue loss. Injection of DH1J4 into the Parkinsonian brain produced an encouraging (if limited) recovery of function, despite this. Modification of the methods of virus preparation to reduce the *in vivo* toxicity is necessary and only then can the full potential of these vectors be properly investigated.

Ex vivo application of viral vectors

The cell replacement strategy in PD has been based on the observation that restoration of dopamine in the striatum by neural grafts can lead to substantial and long-lasting functional recovery. Extensive studies in animal models have lent support to this approach and the limited data available from human transplants have been encouraging. However, the poor survival of the grafted cells (typically 5-10% of the implanted dopamine neurons) remains an issue. Much research has focussed on strategies to improve the yield of dopamine cells, principally so that the number of embryos required per treatment might be reduced. The application of factors to the dopamine grafts using gene therapy is one such strategy and we have examined the potential of the vectors being tested in our laboratory to deliver transgenes to embryonic ventral mesencephalon cells.

Suspensions of cells prepared from the ventral mesencephalon (VM) of embryos of E14 gestational age were exposed to HSV or recombinant adenoviral (RAd) vectors, each containing the LacZ transgene for 1-3 hours at a range of molarities of infection

(MOI, expressed as infectious units per cell). Excess vector was removed by repeated spinning and washing of the cells. The infected cells were then resuspended in culture medium and plated onto cover slips coated with poly-lysine and allowed to differentiate. After 1 week of incubation, the cultured cells were fixed using 4% PFA and processed for β-galactosidase immunohistochemistry. This infection protocol was seen to be efficient even at the shortest time point of 1 hour, an observation that is compatible with the requirement for the excised embryonic dopamine cells need to be implanted as quickly as possible to maintain viability.

The HSV and RAd vectors produced very different results. Expression of β-galactosidase in cultures infected with HSV vectors was seen exclusively in neurons (Fig. 6). The HSV used in this experiment was DH1J1, with the transgene under LAP promoter control. This promoter is neuron-specific so that even if the vector had infected non-neuronal cells in the culture gene expression would be limited to neurons. Interestingly, despite the toxicity seen *in vivo* with this vector there was no obvious toxicity seen *in vitro* even at highest molarities of infection used (250 infectious units per cell) adding weight to the hypothesis that the toxicity seen *in vivo* with this vector was due to an immune response of the host to some component of the injected virus preparation.

Cultures infected with RAd vectors presented a very different picture (Fig. 7). Most β-galactosidase expression was seen in cells with an astrocytic profile and only occasionally were immunoreactive neurons seen. This mirrors the *in vivo* appearance of these vectors in which they appear to have a primarily glial tropism. This is potentially problematic for the use of RAd vectors to deliver differentiation factors, depending on whether the transgenes concerned are expressed or have their action within the transduced cell. Whilst diffusible factors such as GDNF and Shh should work equally well in transduced neurons or glia, other genes which might require expression within the target neurones (such as those encoding transcription factors) are likely to be ineffective.

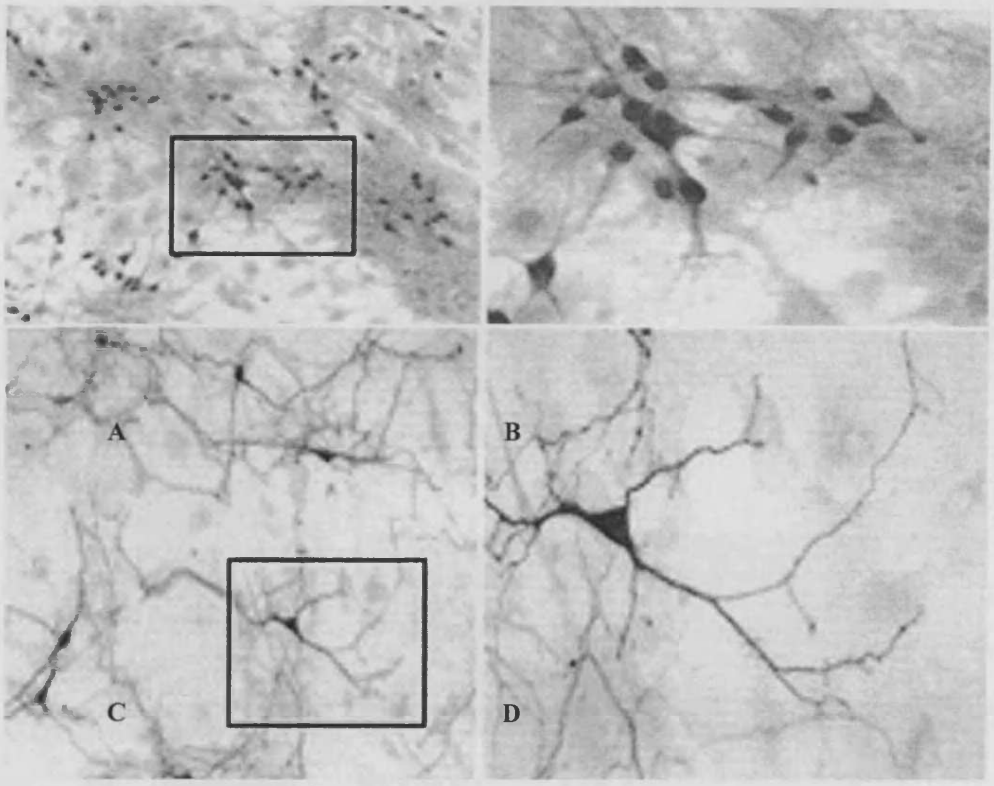

Figure 6 HSV *in vitro*. Photomicrographs of E14VM cultures 1 week post infection with HSV-LacZ. A,B. Immunostaining for β-galactosidase reveals mumerous positive cells with a neuronal profile. C,D. TH immunohistochemistry (bottom panel) shows well differentiated dopamine neurons in all cultures. Scale bars = ...

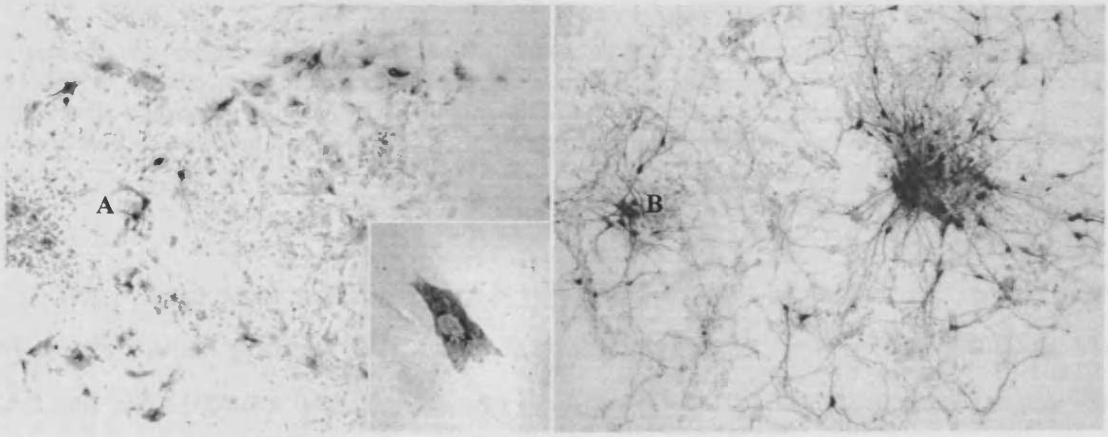

Figure 7 RAd *in vitro*. Photomicrographs of E14Vm cultures 1 week post infection with RAd-LacZ. A. Immunostaining for β-galactosidase reveals mumerous positive cells with a glial profile (inset). B.All cultures contain clumps of healthy well differentiated TH positive cells.

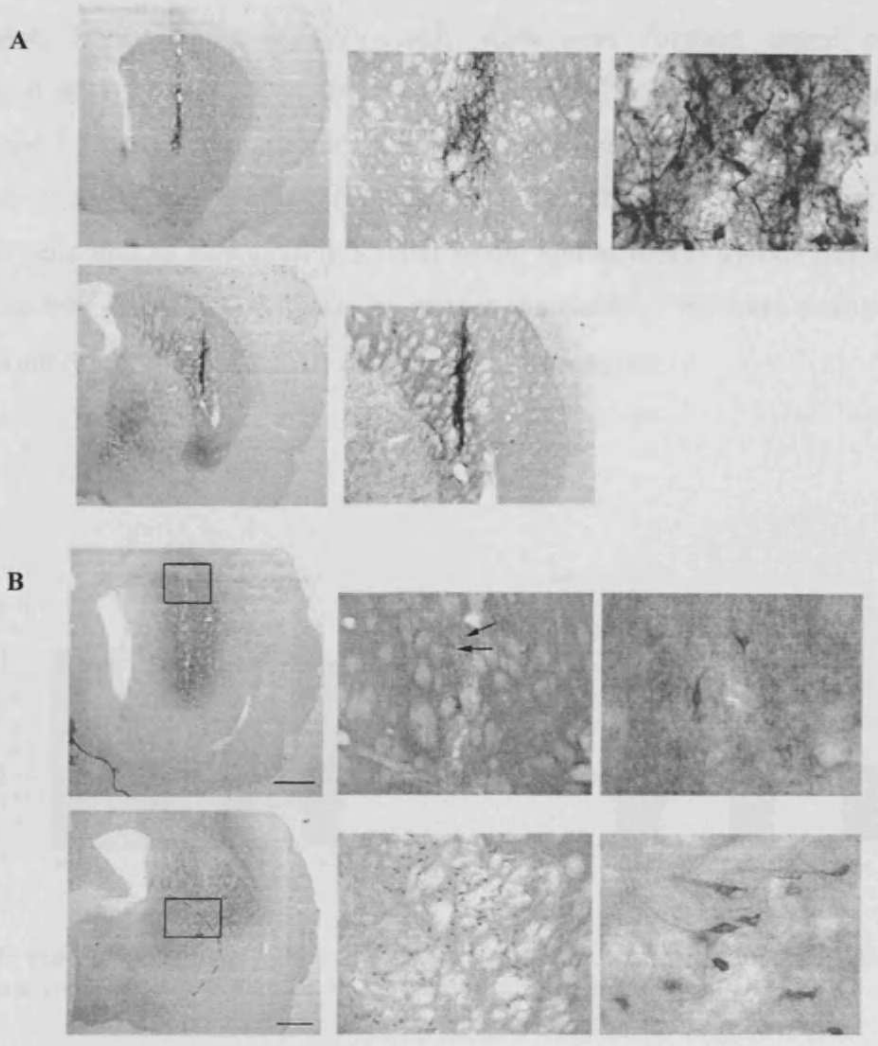

Figure 8. *In vivo* appearance of vector modified dopamine grafts A. TH staining of grafts treated with HSV-GDNF. Whilst small, the grafts contained many TH positive cells B. GDNF expression from grafted cells transduced with HSV/GDNF and implanted into rat striatum. Expression was seen within the graft and surrounding tissue as well as in surrounding areas such as globus pallidus. Scale bar is 700μm

The *in vitro* work described here is typical of the sort of studies carried out with any vector system prior to their use in *in vivo* experiments. In experiments using the RAd and HSV vectors we were able to determine information regarding toxicity the efficiency of infection, levels of transgene expression and the tropism of the vectors and these dat were influential in the manner in which the vectors were deployed in vivo. Following the *in vitro* observations we have explored the potential of *ex vivo* application of HSV-GDNF vectors to dopaminergic cells prior to transplantation in the rat hemi-Parkinsonian model. Rats with a unilateral lesion of the median forebrain bundle were allocated to matched groups according to their post-lesion amphetamine rotation scores. Rats then received grafts of a standard E14Vm cell suspension treated as follows: No

treatment; HSV-GDNF; or HSV-LacZ. Rats were rotation tested post-graft and sacrificed at a number of time points post-lesion for postmortem analysis. In rats sacrificed 17 weeks after surgery TH immunochemistry revealed grafted cells in the striatum 5 days after grafting (Fig. 8). We also observed GDNF expression in the grafted cells and in surrounding striatal tissue and adjacent globus pallidus, probably due to GDNF secretion and uptake by cells in the vicinity. We were unable to detect any expression of either β-galactosidase or GDNF expression.

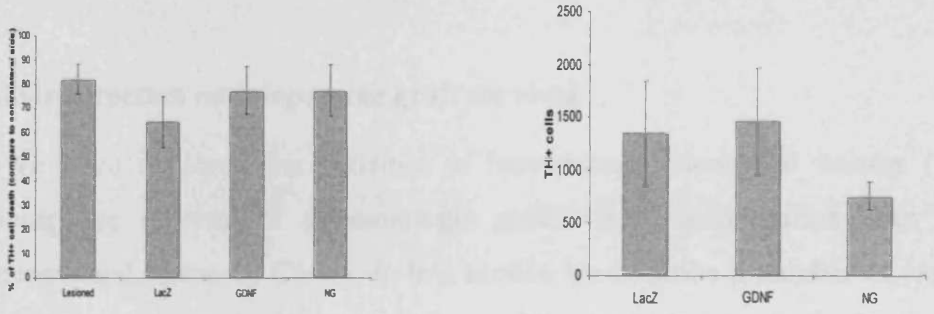

Figure 9: Postmortem analysis following *ex vivo* treatment of dopamine grafts with HSV vectors. A. TH positive cells count in the SNc. B. TH positive cells count in the graft.

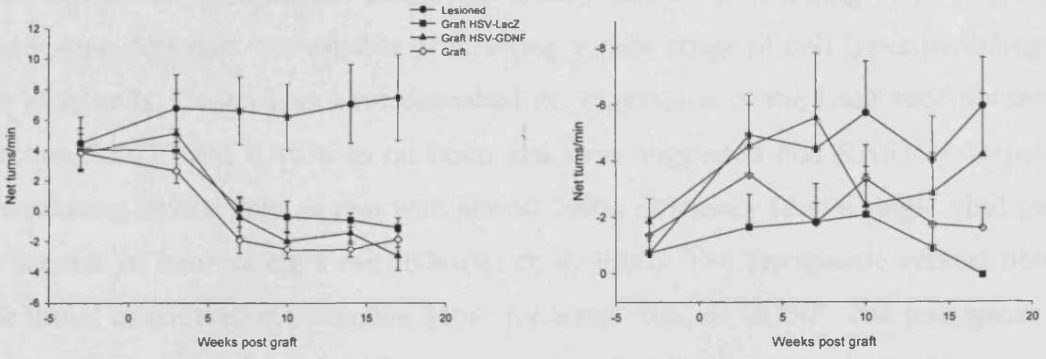

Figure 10: Drug-induced rotations. Amphetamine (left). Apomorphine (right)

There was no rescue of dopamine neurons in the SNc following HSV-GDNF treatment in the striatum (Fig. 9A). and counts of TH positive cells in the dopamine grafts revealed no significant differences between groups (Fig. 9B). There was amelioration of drug induced motor asymmetries induced by amphetamine (Fig. 10A) and apomorphine (Fig. 10B) in all graft groups. The improvement obtained with apomorphine was delayed in onset and weaker than that seen with amphetamine. However, no significant differences were observed between the HSV-GDNF, HSV-LacZ and un-treated graft groups. It is likely that the levels of GDNF produced by the implanted cells were too low in these experiments or that the timing of GDNF expression was delayed, issues which are discussed in more detail below.

Adenoviral vectors and dopamine graft survival

We have explored the potential of recombinant adenoviral vectors (RAd) for improving the survival of dopaminergic grafts, in a collaboration with Pedro R. Lowenstein and Maria G. Castro. In this section we describe a number of experiments which illustrate the methods used and the problems which arise in the investigation of new gene therapy vectors

The RAds used in the work have been described previously. They are first generation vectors in which deletions or substitutions have been made in the E1 and E3 regions of the viral genome. The vectors are produced using a *trans*-complementing cell line established from human embryonic kidney cells. The resulting virus particles are replication deficient, but capable of infecting a wide range of cell types including non-dividing cells. Co-workers have described the expression of the LacZ recombinant AV vectors RAd35 and RAd36 in rat brain and have suggested that RAd36 is capable of transducing striatal cells *in vivo* with almost 100% efficiency (that a single viral particle is capable of transducing a cell) (Gerdes et al. 2000). The therapeutic vectors obtained for initial consideration contained genes for either Shh, or GDNF. The transgenes were driven by cytomegalovirus-derived promoters of either human or murine origin (see Table 2).

Table 2

Summary of Adenovirus vectors used in these experiments

Vector	Backbone	Transgene	Promoter*
RAd35	TypeV/-E1,-E3	Lac Z	MIE*h*CMV/
RAd36	Type V/-E1,-E3	Lac Z	MIE*m*CMV/
RAd Shh	Type V/ -E1,-E3	Shh	MIE*m*CMV/
RAdGDNF	Type V/ -E1,-E3	GDNF	MIE*h*CMV/

* ***m*** = promoter derived from murine CMV, h = promoter derived from human CMV

Methods of application of viral vectors to dopamine transplants

Our work using HSV and LV is aimed at treating the Parkinsonian deficit caused by a dopamine-depleting lesion and focused on direct injection of the vector into the host brain. By contrast the application of viral vectors to dopamine transplants is not so straightforward and a number of different routes of administration are possible.

- Firstly, as above, the vectors might be applied directly to the embryonic dopamine cell suspension embryo prior to implantation into the host brain.

- Secondly, accessory cells such as astrocytes could be transduced for co-grafting together with embryonic dopamine cells, either as a mixed suspension or in separate grafts, distinct in location and/or in time from the dopaminergic grafts.

- Thirdly, as in the HSV and LV GDNF experiments, the vectors could be injected directly into the host striatum. This could be done prior to implantation of the dopaminergic graft so that the transgene product would be expressed in the region of the host brain into which the graft is to be placed.

- A final route of application might be the vectors to be injected into the donor embryos *in utero*, prior to excision of the ventral mesencephalic tissue used for grafting.

(See figure 11):

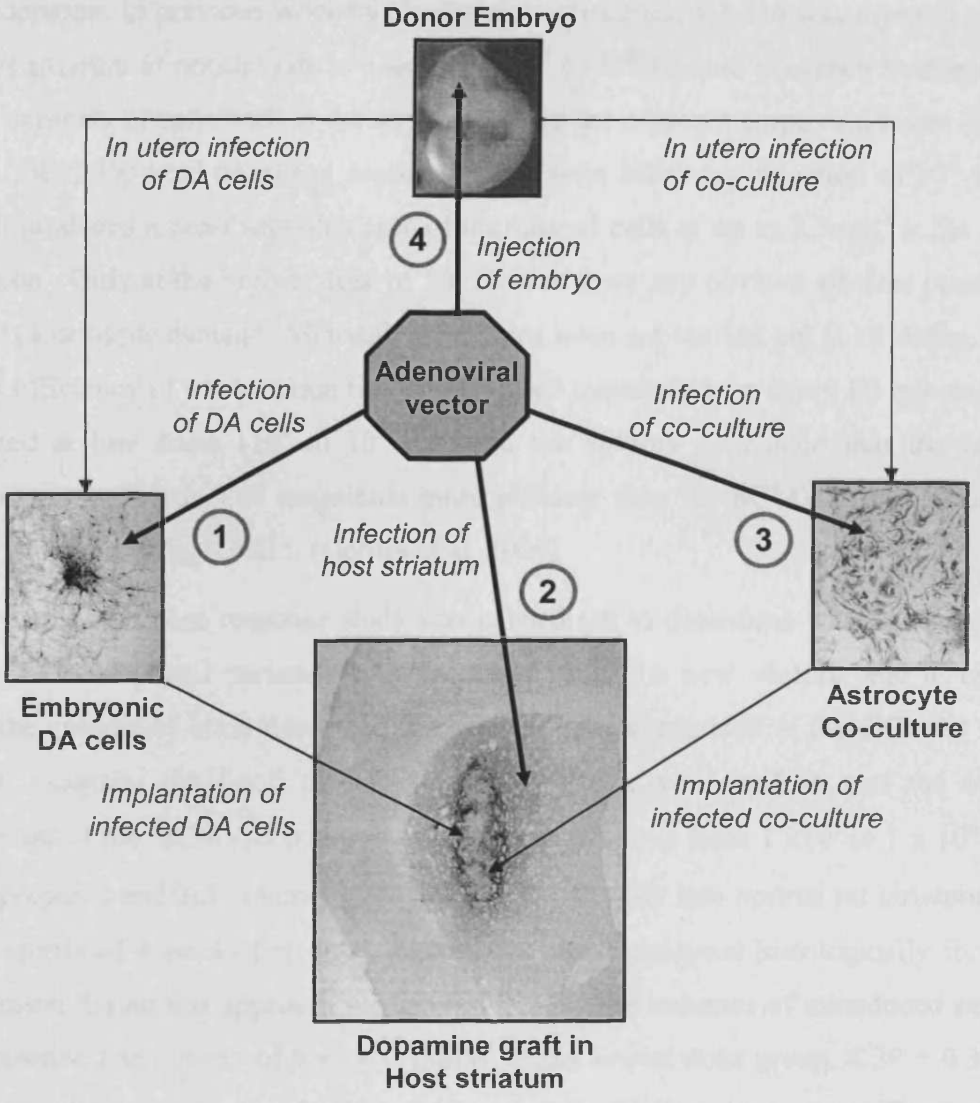

Figure 11. Diagram to show potential routes of application of AV vectors to dopamine grafts. **1.** Transduction of primary dopamine neuron suspensions with neurotrophic genes or genes important in DA cell differentiation. **2.** Delivery of the same transgenes to the host striatum prior to grafting. **3.** Transduction of co-cultures of cells (e.g. astrocytes) for co-grafting with dopamine grafts. **4.** Delivery of transgenes to the donor embryo in-utero prior to excision of graft tissue.

In vivo dynamics of adenoviral vectors in the hemi-Parkinsonian brain

The RAd36 vector contains a LacZ gene driven by the highly efficient, major-immediate-early promoter from the murine cytomegalovirus (MIEmCMV). The construction and purification of the vector has been described previously (Thomas et al. 2000). Our initial assessment involved the direct injection of this vector into the normal

rat striatum, to provide a direct comparison of these vectors with the others under consideration. In previous work by Gerdes and colleagues, RAd36 was injected into the corpus striatum of normal rats at doses from 10^4 to 10^8 IU, and was seen to transduce a large numbers of cells both in the striatum and in the adjacent corpus callosum (Gerdes et al. 2000). Optimal transgene expression was seen following injection of 10^6-10^7 IU, which produced a cross sectional area of transduced cells of up to 2.5mm^2 at the site of injection . Only at the highest dose of 10^8 IU was there any obvious adverse reaction to the virus or tissue damage. Although cell counts were not carried out at all doses, a near 100% efficiency of transduction (i.e. nearly 1 cell transduced for every IU injected) was reported at low doses (10^1 to 10^3 IU), and the authors concluded that the mCMV promoter was 3 orders of magnitude more efficient than the hCMV promoter used in other vectors such as RAd35. (Gerdes et al. 2000)

An *in vivo* dose response study was carried out to determine what, in our hands, would be the optimal parameters for injection using the new vectors, and to observe both the volume of brain tissue and the level of gene expression at the different doses. As an example, the LacZ containing RAd36 vector was used to test the *in vivo* properties of the mCMV-containing vectors. Log dilutions from 1 x10^4 to 1 x 10^6 IU/μl were prepared and 3μl volumes were injected unilaterally into normal rat striatum. Rats were sacrificed 4 weeks post-injection and the brains analysed histologically for LacZ expression. Using this approach we determined that the volumes of transduced striatum per injection had a mean of 0.45 $\pm$ 0.13mm^3 in the lowest dose group, 2.29 $\pm$ 0.32mm^3 in the middle dose group and 3.36 $\pm$ 0.43mm^3 in the highest dose group. There was no evidence of an inflammatory reaction nor of any difference in striatal volume on the injected and uninjected sides, indicating that there was no significant cell loss due to the vector injection (figure 12).

At the highest dose used, some transduced cells were also seen outside the injected striatum notably in the corpus callosum. This was probably due to the spread of viral particles beyond the site of injection following local saturation of virus binding. Expression of transgenes outside the target area may or may not be desirable, depending on the therapeutic target. However it is crucial to know where transgenes are expressed when considering the parameters of the therapy itself. In our experiments, the RAds appeared not to be retrogradely transported away from the striatum after injection (or at least do not transduce cells in these areas) as no transgene expression was observed in

afferent or efferent areas such as substantia nigra, neocortex or globus pallidus. This is not true of other vectors notably HSV, where retrograde transport is common, and where account needs to be taken of the possible effects of cell transduction in these areas.

One cannot assume of course, that the levels of infection, the dynamics of dispersal, and levels of gene expression will all be the same in pathological as in a normal brain. Since the aim of our work was to use AV vectors to deliver transgenes in models of PD and transplantation-based therapies, we also investigated whether or not the presence of the 6-OHDA lesion might affect the *in vivo* properties of the vector. In a simple experiment, RAd36 virus was injected bilaterally into the striata of rats that had previously received a unilateral 6-OHDA lesion of the median forebrain bundle, the classic hemi-Parkinsonian lesion. Rats were then sacrificed at 3 days, 1 week or 4 weeks post injection to determine the distribution of gene expression in the brain (Torres et al. 2004)

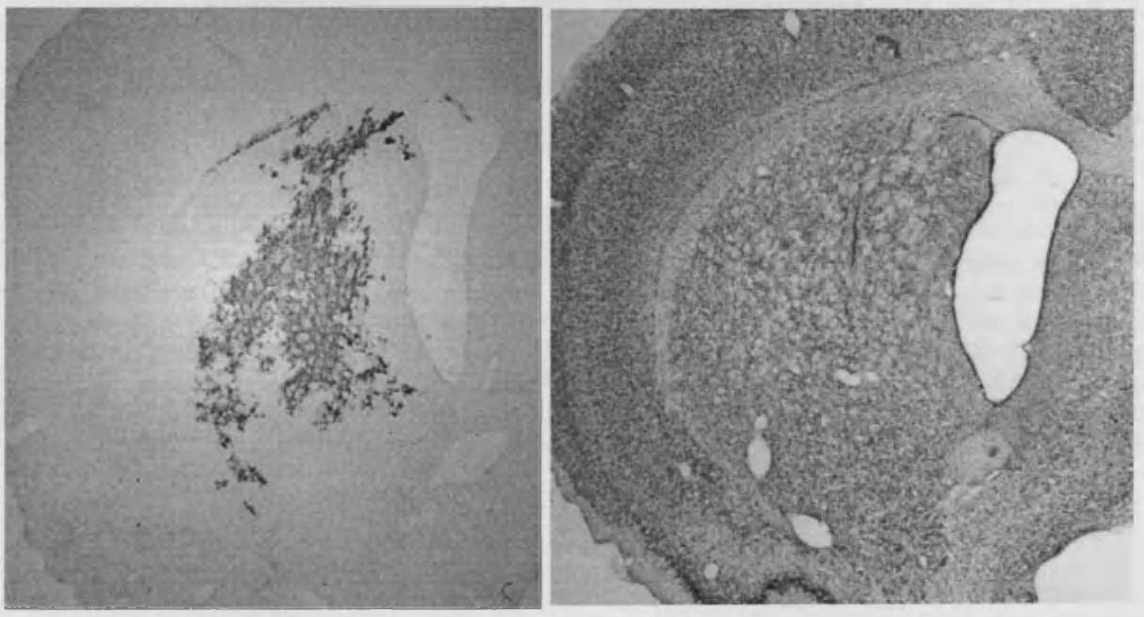

Figure 12. Histological appearance of virus injected into rat brain 4 weeks post-injection of the 3 x 10^5 IU of RAd36 virus. Photographs show the injected striatum from a single animal. A. β–Galactosidase immunohistochemistry shows large numbers of transduced cells in the striatum around the injection site and extending to the overlying corpus callosum. B. In the cresyl violet stain, the only indication of the injection is a small scar marking the site of the injection cannula. There is no indication of an inflammatory reaction. The regular spacing of the pale staining fibre bundles and the lack of tissue shrinkage indicates that little or no striatal cell loss has occurred.

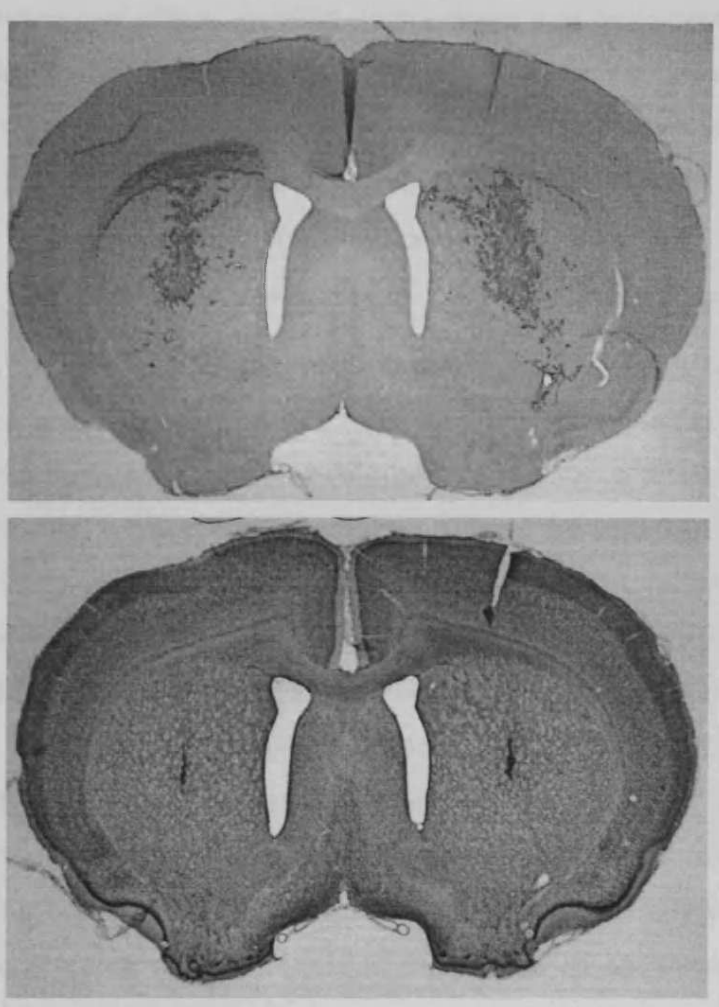

Figure 13. Histological appearance of the hemiparkinsonian rat brain following bilateral injection of the LacZ containing AV vector. RAd36. A. Section stained for β-galactosidase showing transgene expression in the intact hemisphere (right) similar to that seen following injection into in normal animals. β-galactosidase staining is seen mostly around the injection site in the striatum. However, in the dopamine depleted hemisphere (left) there an additional region of β-galactosidase staining is seen in the overlying corpus callosum and levels of staining in the striatum are generally lower. B. Cresyl violet stained section showing that striatal morphology appears normal 1 week post injection of the vector, apart from minor needle damage. There is little evidence of any inflammatory reaction (Reproduced from Torres et al 2004, with permission).

As in the normal rat brain, there was little evidence of an inflammatory reaction in any of the AV-vector injected brains at any of the time points examined (Fig. 13). Nor was there any evidence of striatal atrophy, or of ventricular enlargement. Small scars, similar to those seen following injection of vehicle solutions alone were the only evidence of a striatal injection seen in the β-galactosidase immuno-stained sections. However, evident in most brains, was an asymmetry of staining in the two injected hemispheres. On the unlesioned side of the brain, β-galactosidase immunoreactivity was

confined almost exclusively to the striatum in a volume of brain centred around the site of injection. In contrast, on the 6-OHDA side of the brain, many cases had staining in other structures, principally in the corpus callosum overlying the injection site (Fig 13). Analysis of β-galactosidase positive cell numbers demonstrated that the 6-OHDA lesioned side of the brain contained significantly greater numbers of β-galactosidase positive cells than the unlesioned side at all of the time points observed.

There were 15% fewer β-galactosidase seen cells in the lesioned striatum than in the unlesioned striatum, although this difference was not statistically significant. By contrast, there were significantly more cells seen in the corpus callosum on the lesioned side, by nearly 40%, compared to the numbers seen on the unlesioned side of the brain. The results clearly indicated that the presence of a 6-OHDA lesion affected both the distribution of transduced cells and the levels of transgene expression, although the reason why the hemi-Parkinsonian lesion affects the *in vivo* dynamics of the injected vector is not known. Altered diffusion characteristics of the de-afferented striatum is one possibility, reduced numbers of vector bindings sites due to removal of the dopamine terminals is another; experiments are underway to investigate this phenomenon further. Whatever the outcome, potential gene therapy vectors will need to be tested in appropriate animal models of the disease as an essential component of determining clinical utility.

As previously, no cells expressing the transgene were seen in brain nuclei – either cortex, nigra or thalamus – that project to the striatal site of injection. This indicates either that the injected vector was not retrograde transported from the injection site or that it was unable to transduce affected cells after transport. The significance of this observation is that, once the parameters of injection are optimized, the volume of brain infected can be limited to the site of injection, avoiding unwanted expression of transgenes in afferent areas.

The application of adenoviral vectors to dopaminergic grafts

In 1999 Sinclair and colleagues reported differences in the differentiation of dopamine in nigral grafts compared to the time course of development in the normal embryo, using the marker for dividing cells bromo-deoxyuridine (BrdU) injected into developing embryos and dopamine grafts (Sinclair et al. 1999). The peak of dopamine

cell neurogenesis was found to be between embryonic ages E13 and E15, in agreement with previous work done by Altman et. Al. using tritiated thymidine (Altman & Bayer 1981). They then studied dopaminergic implants derived from these embryos and determined the proportions of BrdU and TH positive cells present in the grafts. Surviving dopamine neurons in transplants from E14 rat embryos consisted almost exclusively of neurons which had undergone their final cell division prior to excision from the embryo. It was concluded that the numbers of dopamine cells in Parkinsonian grafts were low, not just because dopamine cells had failed to survive implantation. Rather, that many of the implanted cells which would have subsequently differentiated into dopaminergic neurons *in situ* failed to do so after transplantation, suggesting that some critical developmental signals are absent in the adult host brain. Based on this hypothesis, we have reasoned that one possible strategy to improve the yield of dopamine neurons in VM grafts would be to treat the VM graft tissue suspensions prior to implantation with the factors required for dopamine differentiation.

In choosing which factors might be effective, developmental studies have suggested a number of candidates which are implicated in the development and/or differentiation of dopaminergic neurons. Glial cell derived neurotrophic factor (GDNF) has been used both as a factor to prevent DA cell degeneration in lesion models, and had been shown to promote the growth of DA *in vitro* and in dopaminergic implants and is known to be involved in the normal development of the nigrostriatal dopamine system.[references] Whilst not a known differentiation factor this was considered to be a useful positive control which could be used to test the efficacy of the vector and promoter systems. Alternatively, the differentiation factor Sonic Hedgehog (Shh), is involved in the early neuronal specification and has been implicated directly in the development of substantia nigra (Hynes & Rosenthal 1999; Ye et al. 1998). The work described here investigates the application of these two vectors to improve the survival of dopaminergic grafts.

The experiment we describe here was designed to determine whether improved survival of dopamine grafts could be achieved by transducing the host striatum with factors that might improve the survival of dopaminergic grafts. RAd vectors containing either Shh or GDNF were used. All rats first received a 6-OHDA lesion of the median forebrain bundle. After behavioural assessment of the lesion, rats were allocated to balanced groups according to their amphetamine rotation scores. Prior to grafting, the

ipsilateral striatum was injected with either the Shh, GDNF or LacZ vectors. A control group received an injection of saline. Two weeks post injection of the vectors (allowing time for the transgenes to switch on and any post surgical trauma to subside), rats received a standard E14 VM graft into the same striatum. To allow for possible effects of Shh or GDNF expression on the lesioned DA system, control groups without a dopamine graft were also included (Fig. 14).

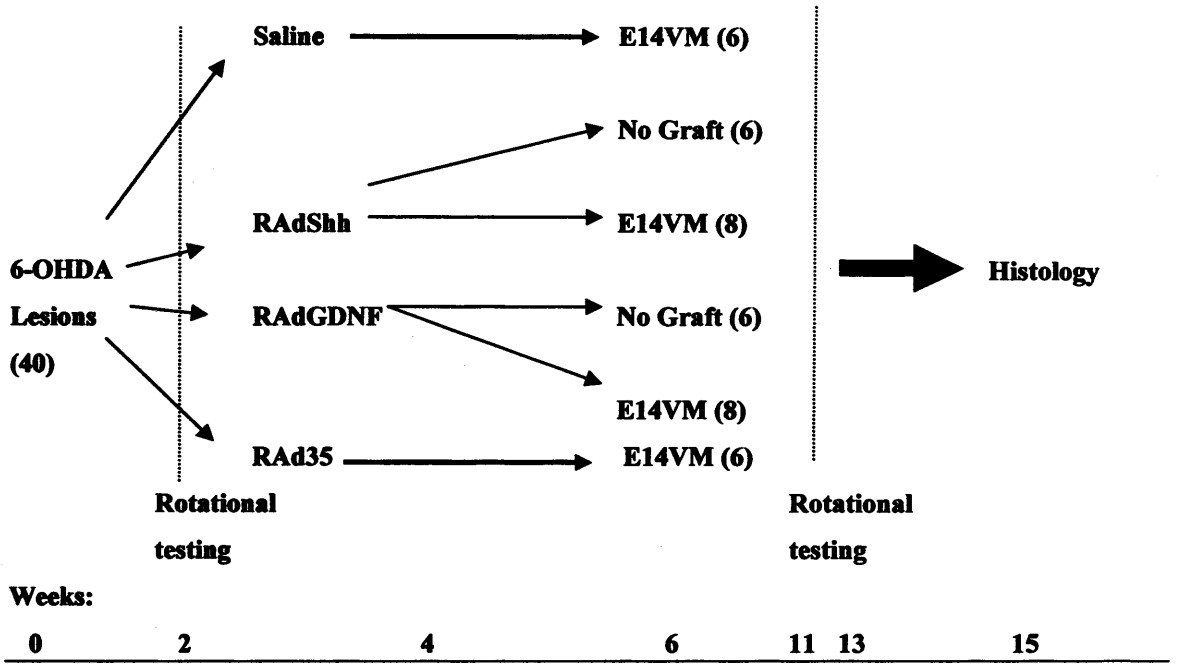

Figure 14. A schematic plan of the experiment investigating striatal delivery of AV vectors to dopamine transplants.

All graft groups showed a reduction in post-lesion amphetamine rotation and showed the over compensatory rotation in the contralateral direction which are associated with good grafts (Fig. 15). Though there were no differences in the graft induced rotational response between graft groups. Conversely, non-grafted groups showed no amelioration of the rotational deficit, whether or not they had received injections of RAD/Shh or RAD/GDNF alone, indicating that neither vector was able to effect the rescue of intrinsic dopamine cells or their terminals from the effects of the lesion.

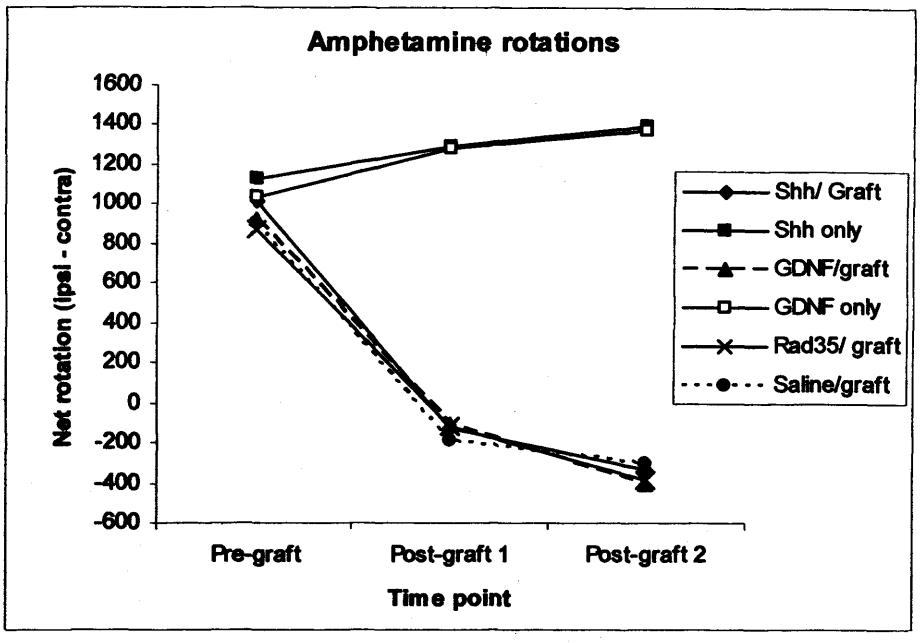

Figure 15. Rotation in rats with unilateral nigrostriatal lesions, following intrastriatal transfection with Shh or GDNF, with or without VM grafts. All grafted groups showed good recovery from the lesion induced rotational deficit, showing over the over-compensation (rotation in the opposite direction rather than dropping to zero) which is typical of good grafts. However there were no significant differences in the graft induced recovery between Shh treated and GDNF treated groups, whether grafted or not.

Post-mortem, histological examination of the brains revealed healthy grafts containing many TH positive cells in all graft groups. At the time of writing, *in vivo* levels of Shh and GDNF have yet to be determined but the protein product from all three vectors injected was easily detectable using immunohistochemistry (Figs. 16-18) Fig. 16 shows the distribution of transgene expression surrounding a dopamine graft in an animal from the RAd35-graft group. Image analysis of the grafts revealed no significant differences between graft volume or TH cell numbers between groups.

Our results show that RAd vectors cad be used to deliver transgene products to dopaminergic grafts using the method of prior transduction of the host striatum and that the transduced striatum is a permissive environment for dopamine transplantation. The diffusible factors Shh and GDNF were detectable immunohistochemically in the striatum surrounding the grafts 13 weeks following vector injection indicating that the window of possible therapy is extensive. However, there was no detectable effect of any virus treatment on graft survival. A number of possible explanations could be proposed. Firstly, the levels of GDNF and Shh produced by transduction may have been

insufficient. Secondly, the levels of variability in graft size within groups was large. Thirdly, the age of embryo from which dopaminergic cells were obtained may be too old. Certainly the differentiation factor Shh is likely to have its effect at a younger age of dopamine cell development. Experiments are now underway to address the levels of transgene product expression and the effects of this method of treatment on grafts of different embryonic ages.

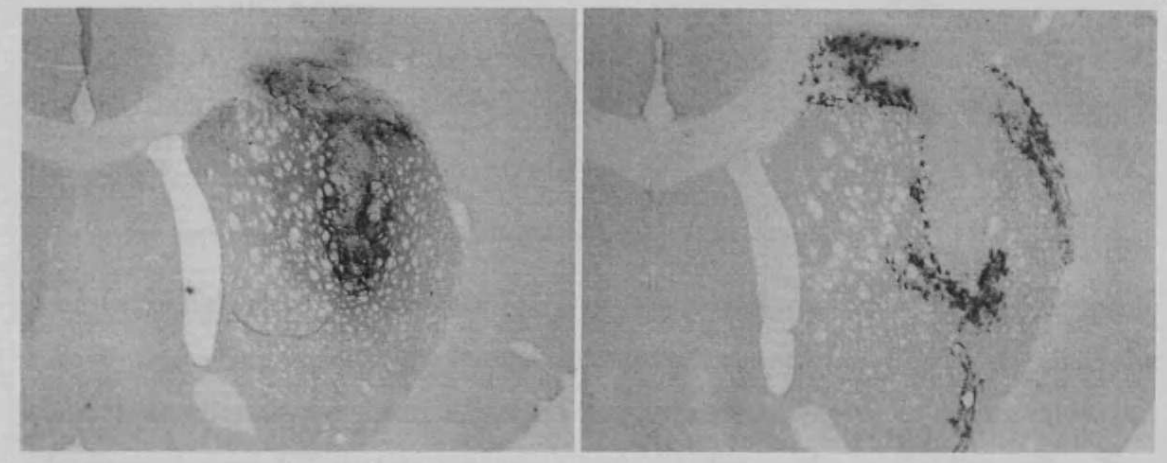

Figure 16. Adjacent sections of rat brain from the Rad35 graft group stained for TH and β-galactosidase. A. TH stained section typical of the grafts seen in all groups showing numerous positive cells with processes extending within the graft and into the surrounding striatum. B. β-galactosidase stained section, in which the graft is seen as a large, unstained volume mainly in the dorsal striatum and overlying corpus callosum. β-galactosidase staining can be clearly seen in both striatum and corpus callosum in close proximity with the graft. In the RAd/SHH and RAd/GDNF graft groups such an arrangement bring the grafted tissue into intimate contact with potentially beneficial transgene products.

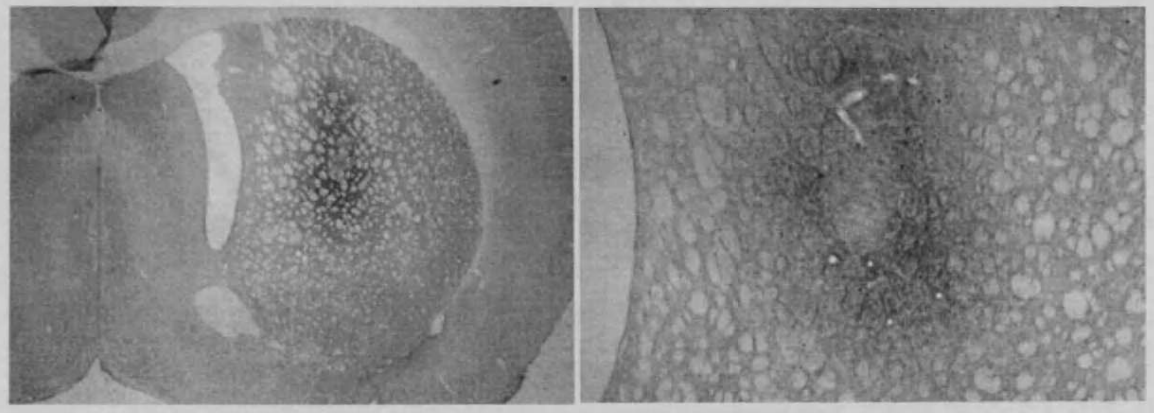

Figure 17. A. GDNF expression in the striatum following RAd/GDNF injections. Dense immunoreactivity surrounds a small section of graft in a brain from the GDNF/Graft group. B. Shh expression surrounding a graft in a section from an animal in the Shh/Graft group. Unlike GDNF, Shh staining shows immunoreactive cell bodies as well as diffuse staining of the striatal parenchyma.

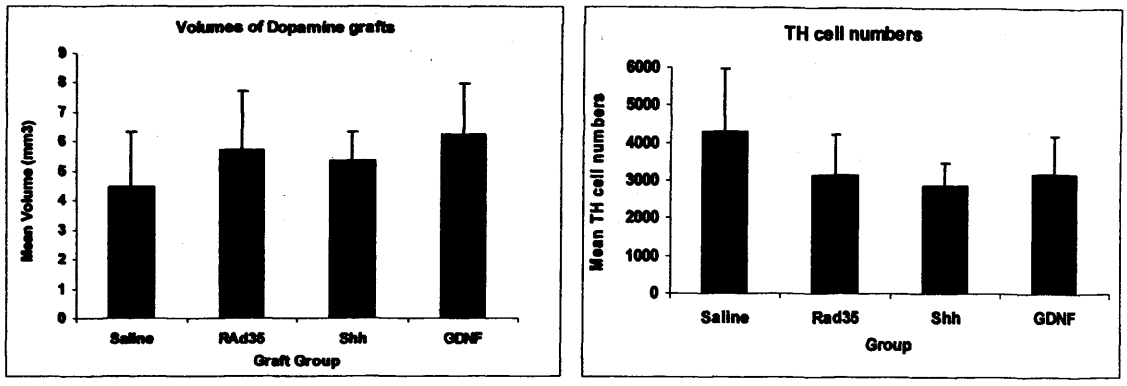

Figure 18. There were no significant differences in mean graft volumes (left) and TH cell numbers (right) between groups.

Dopamine replacement using Lentiviral vectors

Lentiviral (LV) vectors are derived from a group of highly pathogenic retroviruses, which includes the human immunodeficiency virus HIV. They share the useful properties of the commonly used oncoretroviral vectors, with the additional advantage that the LV vectors can infect both dividing and non-dividing cells. They have a large cloning capacity, at least 9kb, and are stably integrated into the genome of the target cells, properties which are favourable for long-term expression of transgenes in the nervous system (for review see (Bjorklund et al. 2000b). Notwithstanding, there are safety concerns regarding the use of lentiviral vectors, particularly those which are HIV derived. Chief among these is the possibility of reversion to replication competent forms (Castro et al. 2001). However, in this regard LV vectors are little different to any other potential viral vector and in theory, can be attenuated to obtain replication-defective, non-pathogenic vectors. HIV-1-based lentiviral vectors have been used with success for gene transfer into the primate central nervous system (CNS). Kordower and colleagues (Kordower et al. 2000b) recently showed that a lentiviral vector encoding GDNF could protect TH-positive neurons and preserve dopaminergic terminals in MPTP and aged monkeys a multiply-attenuated, self-inactivating GDNF containing LV vector was successfully used to protect against the effects of a 6-OHDA lesion in a mouse model of Parkinson's disease (Bensadoun et al. 2000b).

Because of the concerns surrounding the use of HIV-based vectors, systems which are non-pathogenic in humans, such as those based on equine or feline LV, are particularly attractive candidates for clinical use. (Poeschla et al. 1998; Mitrophanous et

al. 1999; Johnston et al. 1999; Curran et al. 2000). Our laboratory has been collaborating with Oxford Biomedica (UK) in the evaluation of an equine LV-based vector for possible use in the treatment of PD. The vector is derived from equine infectious anaemia virus (EIAV), which is non pathogenic in humans. The vector is multiply attenuated and self-inactivating. Azzouz and colleagues (2002d) have used a tricistronic self-inactivating EIAV vector expressing TH, AADC GTPCH1 in a single transcription unit to achieve functional improvement in the rat hemi-Parkinsonian model. They showed significant reduction the number of turns of apomorphine-induced motor asymmetry with (Azzouz et al. 2002c). More recently they have demonstrated neuroprotection in a rat model of PD using an EIAV/GDNF vector (Azzouz et al. 2004b).

Following the demonstration of efficacy of the tricistronic dopamine replacement vector (EIAV/DA) in ameliorating drug-induced rotation, we attempted to investigate the effects of the vector using a wider range of behavioural tests for parkinsonian deficits. Rats received a unilateral 6-OHDA lesion of the median forebrain bundle and were tested post-lesion using amphetamine and apomorphine induced rotation. Only animals showing at least 7 net turns/min with amphetamine and 3 net turns/min with apomorphine were included in the study (38 in total). Rats were divided into 6 matched groups based on their rotation scores as follows: A lesion only group, two EIAV/DA groups receiving different doses of LV (EIAV/DA1; 12 x 10^6 transforming units (TU) and EIAV/DA2; 24 x 10^6 TU), two matched control vector groups (EIAV/LacZ1;12 x 10^6 TU and EIAV/ LacZ2;24 x 10^6 TU) and a positive control group which received a standard E14 VM dopamine graft.

All rats underwent rotational testing at 3 week interval post surgery. They were also tested on a range of motor tests including the staircase (paw-reaching), rotarod, and cylinder tests. The grafted group was the only group of animals to show an amelioration of rotational deficit (Fig. 19). EIAV/DA had no apparent effect on amphetamine-induced rotations, neither were we able to reproduce the effect on apomorphine rotations obtained by Azzouz and colleagues. As a consequence there were no significant effects of the EIAV/DA on measures of spontaneous motor activity (data not shown).

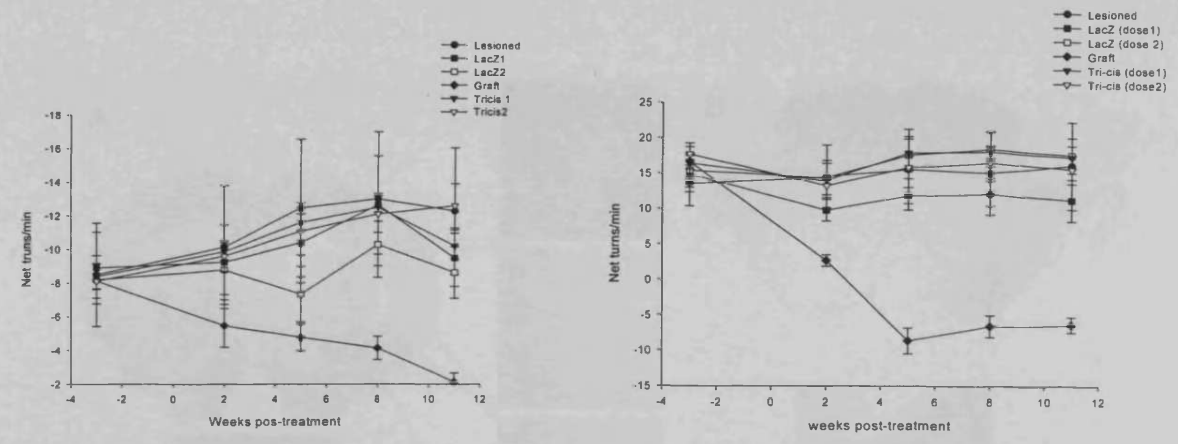

Figure 19: Drug-induced rotations. A. Apomorphine-induced rotations. B. Amphetamine-induced rotations.

The lack of efficacy of the EIAV/DA was puzzling. Post-mortem analysis of brain sections stained for the neuronal marker NeuN revealed no obvious damage or toxicity induced by the vectors (Fig. 20). β-galactosidase histochemistry showed that there was successful transduction by the EIAV/ vectors of cells in the striatum, the cortex and the substantia nigra pars reticulata (SNr), indicating transport of either the protein or virus in both retrograde and anterograde directions (Fig. 21). TH and AADC histochemistry was weaker than that seen for β-galactosidase (Fig. 22) and unlike β-galactosidase, expression of TH and AADC in the cortex or the SNr was not seen.

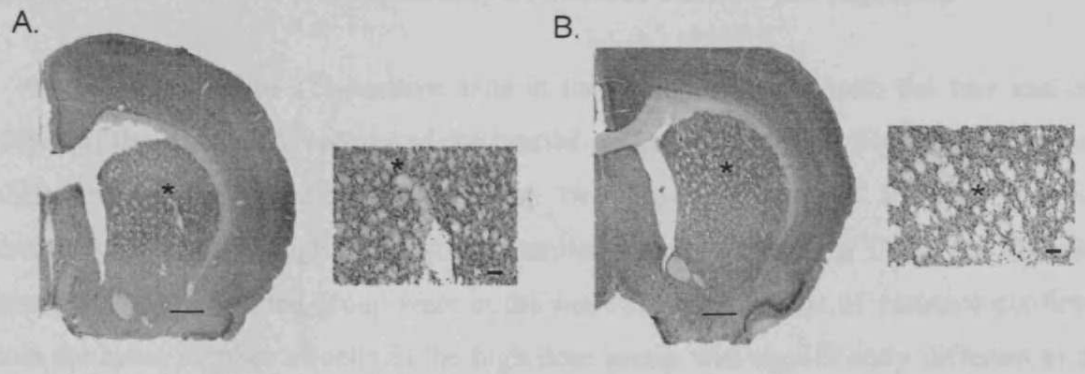

Figure 20: NeuN histochemistry. A. Rat injected with EIAV/DA (high dose) shows minimal neuronal depletion at the level of the needle track. B. The same animal at a more posterior level did not show any neuronal depletion or toxicity, scale bar = 1 mm. The star show a higher magnification x10, scale bar = 100 μm.

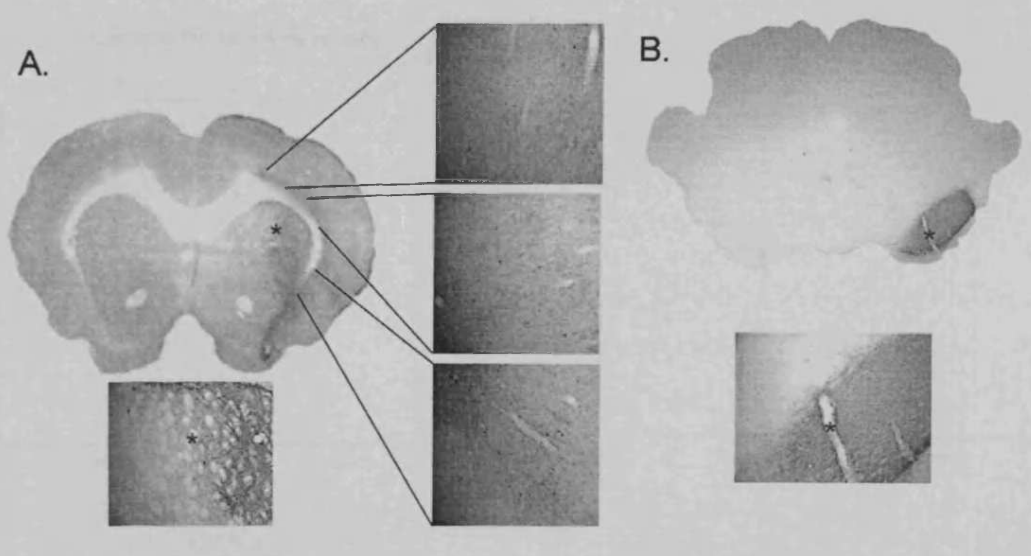

Figure 21: β-galactosidase immunohistochemistry following EIAV/LacZ. A. Expression in striatum and cortex. . B. Expression in SN pars reticulata.

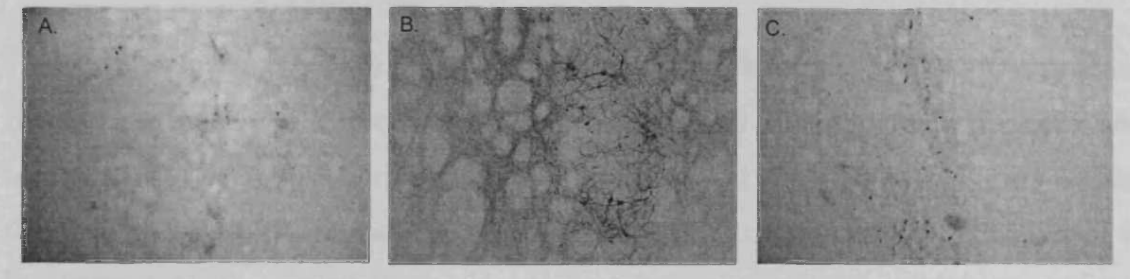

Figure 22: A. TH histochemistry in rat injected with EIAV/DA (high dose). B. TH histochemistry in rat grafted with E14VM. C. AADC histochemistry in rat injected with EIAV/DA (high dose).

The numbers of TH-positive cells in the groups injected with the low and high doses of the EIAV/DA vector and the grafted group are shown in Fig. 23. There was a difference in cell numbers between the two doses of vectors. Perhaps the most encouraging result though was that the number of cells expressing TH in the high dose group and in the grafted group were in the same range. Analysis of variance confirmed that the mean number of cells in the high dose group was significantly different to that in the low dose group ($F_{2,14}$ = 19.98, $P<0.001$) but not statistically different to the number of grafted cells. The numbers of AADC-positive cells seen were slightly more than the TH cell numbers. Once again there was a significant difference in cell numbers between the low and the high dose groups ($F_{1,10}$ = 23.36, $P<0.001$)

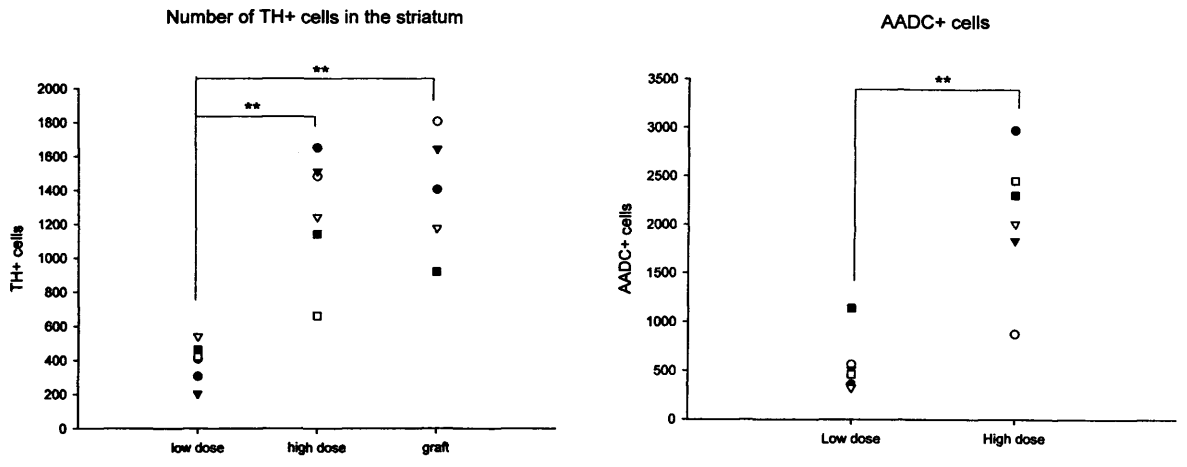

Figure 23: A. Counts of TH+ cells in the striatum. B. Counts of AADC+ cells in the striatum. The headings need to align in the different panels. Labelling A, B.

In conclusion, we have been able to demonstrate that EIAV vectors can produce high levels of β-Gal expression in the striatum and in retrograde (Cortex) and anterograde (SNr) targets. Expression of genes of interest (TH and AADC) was weaker than had been reported previously, and these were not expressed at levels sufficient to be detected by the protein assays used. As a result, transgene expression was not associated with any significant improvement in behavioral recovery. This may be due to a weak expression of both enzymes required for the production of DA in the striatum await HPLC analysis of tissue samples which will tell us more about the levels of DA produced by the vector in the striatum and might explain the reasons why no improvement was observed. These issues are developed in further detail in the general discussion at the end of this chapter.

General Discussion

The studies carried out in our laboratory on the application of viral vector gene therapy in PD models have looked at three different viral vectors using three different therapeutic strategies and serve to illustrate both the nature of such investigations and the difficulty of the issues involved in converging on a successful therapeutic strategy.

The HSV-based viral vectors were intended to deliver the neurotrophic factor GDNF to the hemi-Parkinsonian brain in order to protect against the effects of a subsequent 6-OHDA lesion. With one of the vectors used, a protective effect of the HSV/GDNF vector was demonstrated on the numbers of dopamine cells in the SNc, together with an associated amelioration of drug-induced turning behaviour. However, the effects were variable and of relatively limited magnitude. Two major issues arise out of these experiments. Firstly, we were unable adequately to evaluate the multiply-deleted dH1J type vectors due to the presence of toxicity, which was shown to be unrelated to viral gene expression. Secondly, the LAT promoter used in the HSV constructs appears to be silenced after just a few weeks *in vivo* making it unsuitable for long term expression and sustained delivery of GDNF (Monville et al. 2004).

The inflammation and damage caused by dH1J-HSV vectors in our studies is not typical of that seen by other workers. The method used in the production and purification of these vectors was shown to induce a level of toxicity *in vivo*. One theory is that the method of salt harvest used produces virus stock containing large numbers (possibly a majority) of dead or fragmented virus particles and that, unlike the gradient purification used for the gh/tk deleted vectors these are probably not removed by affinity column purification. The presence of virus-bound cellular components from the complementing cells in the preparation is also a possibility.

Despite the obvious advantages of HSV vectors in terms of tropism, transgene capacity, and the potential to establish latency, immune responses and toxicity are serious concerns that could limit the use of these vectors to treat chronic neurodegenerative disorders in humans (Lowenstein et al. 1994; Laquerre et al. 1999). HSV toxicity has been linked to the host shut-off functions of some viral gene products and to the direct toxicity of others. Although recent vector modifications have made HSV vectors less toxic (Glorioso et al. 1994; Krisky et al. 1998a), the potential of the present generation of HSV vectors may be limited for gene therapy (Samaniego et al.

1998). Our results confirm that, under some still poorly understood conditions of vector construction, virus toxicity can be reduced into the range where therapeutic application may be acceptable. However, of greater concern, the present data also indicate that the reduction of toxicity is associated with reduction of transduction levels, which may impose an absolute limit to the feasibility of using HSV vectors for efficient gene transfer. Nevertheless, it is worth emphasizing the fact that the potential of the most deleted HSV vector may not have been seen, masked as it was by the methods of production and purification used.

The observed silencing of the LAT promoters raises the issue of which promoter will be the best to use for Parkinson's gene therapy. The processes regulating the establishment of and reactivation from latency in HSV are not well understood. Wild type infections of HSV-1 can result in the establishment of latency in neurons in which the LAT regions of the genome maintain long term expression. It seems logical then to suppose that in an HSV-based vector, transgenes inserted into the region of the virus from which the LATs are expressed, and driven using LAT-derived promoters might effect long term expression also. It is currently unknown which viral genes are involved in the establishment of a latent infection, but it is known that *de novo* viral protein synthesis is not required. Latency is related to the expression of the LAT regions of the genome, which are expressed from a promoter that is highly active in neurons. LAT expression prevents the lytic replication cycle by down-regulation of genes associated with lytic infection. Reactivation of the latent virus can be induced by different stimuli, such as stress and UV irradiation (Kootstra & Verma 2003). Thus, it is possible that the inflammation caused by direct injection of HSV into the brain could be overriding the establishment of latency and be directing the virus down the lytic pathway, though this has yet to be demonstrated. The functions of the LATs remain unknown, although several putative roles have been suggested. These include: efficient establishment of latency (Perng et al. 2000b; Thompson & Sawtell 1997), effective reactivation from latency (Perng et al. 1994; Block et al. 1993; Perng et al. 1996a; Drolet et al. 1999; Perng et al. 1999; Loutsch et al. 1999; Perng et al. 1996c), and prevention of apoptosis in infected neurons (Perng et al. 2000a). However, it is clear that the LAT genes are not an absolute requirement for establishment, maintenance, or reactivation from latency (Ho & Mocarski 1989; Javier et al. 1988; Sedarati et al. 1989; Steiner et al. 1989).

The lack of a promoter that supports long-term expression in forebrain neurons has limited the utility of the HSV based system so far. A number of viral promoters that support long-term expression in other systems do not similarly support significant levels of long-term expression from HSV-1 vectors (Zhang et al. 2000). At 1 month after gene transfer into either the midbrain or striatum, HSV-1 plasmid vectors containing either the HSV-1 immediate early (IE) 4/5 promoter or the cytomegalovirus (CMV) IE promoter support expression in only a small percentage of the number of cells observed at 4 days (During et al. 1994b; Song et al. 1997a; Fraefel et al. 1996). The Maloney murine leukaemia virus long-terminal repeat (LTR) promoter supports expression in retrovirus vectors; however, in recombinant HSV-1 vectors, this promoter supports only limited long-term expression in primary sensory neurons, and only low levels of long-term expression in forebrain neurons (Dobson et al. 1990). A number of neuron-specific cellular promoters have also been examined, and the results are similar to those obtained using viral promoters. An HSV-1 plasmid vector that contains the neurofilament heavy subunit (NF-H) promoter supports high-level expression at 4 days, but expression is absent at 1 month (Wang et al. 1999b). In contrast to the results with viral and neuronal-specific promoters, two promoters that are active only in specific types of neurons support significant levels of long-term expression from HSV-1 plasmid vectors (Kaplitt et al. 1994a; Song et al. 1997b; Wang et al. 1999a; Jin et al. 1996b). One is a vector that contains the preproenkephalin promoter and supports expression for 2 months in both the amygdala and the ventromedial hypothalamus (Kaplitt et al. 1994b). Secondly, vectors that contain either a 6.8- or a 9-kb fragment of the TH promoter can support expression for 8-10 weeks in catecholaminergic neurons of the substantia nigra pars compacta and locus coeruleus (Jin et al. 1996a; Song et al. 1997c). In a very recent study, Sun and colleagues (Sun et al. 2003d), have developed a helper virus-free packaging system and a promoter that supports long-term expression in forebrain neurons by adding an upstream enhancer from the TH promoter to the NF-H promoter. They have shown, in the rodent hemiparkinsonian model, that this vector could support high-level expression of TH and AADC transgenes for up to 7 months and could improve the functional behaviour in lesioned rats.

For trophic factors such as GDNF, short term expression may be sufficient to provide neuroprotection against acute insults (e.g. trauma, stroke, graft survival at time of implantation), including acute injection of neurotoxins such as 6-OHDA. Other

studies have shown that neuroprotection could be effected in the CNS by transient HSV-mediated expression of appropriate transgenes (During et al. 1994a; Natsume et al. 2001a; Sun et al. 2003b). By contrast, protection against a chronic lesion or slowly progressive neurodegenerative disease is likely to require more long-term stability and, for clinical safety, regulated expression. So, despite their potential advantages, HSV-based vectors are still problematic for therapeutic application both in terms of levels and duration of expression and of toxicity.

Our work to evaluate the potential of recombinant AV-based vectors to deliver differentiation and trophic factors to embryonic dopamine grafts used an approach aimed at improving the yield of dopamine cells within the grafts. An important issue arising from these studies was the "tropism" of the adenoviral vectors used. Whilst the RAds were capable of transducing cells in rat striatum and of expressing a number of different transgenes, almost all the transduced cells seen were glial. *In vitro*, the transduction efficiency was low and the expression seen was similarly, predominantly glial. Adenoviral vectors are known to be able to infect a wide range of cell types in the CNS (Davidson & Bohn 1997) including neurons and the restriction of gene expression to glia in our studies is probably due to preferred activation of the CMV promoters in these cells. Retrograde transport has been reported with adenoviral vectors using and Rous sarcoma virus (RSV) promoter. (Perng et al. 1996b) The lack of expression in neurons presents a problem for the application of these vectors directly to embryonic dopamine cells, particularly for the delivery of transcription factors which will require transduction of the target cells. On the other hand, for diffusable factors such as GDNF and Shh, glial transduction may be sufficient for the delivery of these factors to developing dopamine cells in a neural transplant and a recent study by collaborators successfully used an RAd/CMV/Shh construct to protect against the effects of a 6-OHDA lesion (Hurtado-Lorenzo et al. 2004)

Initial experiments demonstrated clearly that the *in vivo* dynamics of AV vectors were affected by the presence of a 6-OHDA lesion, transducing greater numbers of cells than in the unlesioned brain and causing gene expression in areas outside the targeted area (Torres et al. 2004). This is an important finding, which may have implications for all directly administered gene therapies. The uptake and diffusion of any type of injected vector is likely to be affected by a number of different parameters. The density of the brain nucleus injected, the local topography of neurons and glia, nerve terminals

and axon bundles, blood vessels and ventricles, will all affect the distribution of the vectors post-injection. That the local environment can be altered by the disease process is evident. A 6-OHDA lesion of the median forebrain bundle not only removes dopamine axons and terminals from the striatum but also induces striatal up-regulation of astrocytes and microglia for up to 4 months post-lesion. (Pasinetti et al. 1999; Rataboul et al. 1989; Sheng et al. 1993; Stromberg et al. 1986). Whether or not this results in the striatum being more or less permissive to virus particle diffusion, or whether there are more or fewer virus binding sites available to virus particles is not known. Whatever the physical or chemical causes of altered transgene expression, it is clear that the knowledge to be gained from injections of viral vectors into normal brain is limited and pre-clinical evaluation will need to be carried out in animal models which mimic as closely as possible the pathological conditions of the disease under study.

The experiment in which host striatum was transduced with RAds prior to implantation of a dopamine graft, whilst demonstrating no detectable functional effects, was nonetheless highly informative. Transgene products from all three of the vectors used were detectable in the host striatum, surrounding and in intimate contact with the dopamine grafts. Following injection of the GDNF and Shh containing vectors, these diffuseable factors were also detected immunohistochemically within the graft tissue. As yet we can only hypothesise on the failure of effect on dopamine cell survival in these grafts. It is possible that the age of the donor tissue used (E14) was too old. Certainly we might expect the maximal effect of Shh to occur at younger ages (Briscoe & Ericson 2001; Goetz et al. 2002; Matsuura et al. 2001). GDNF on the other hand has previously been shown to improve the survival of dopamine grafts derived from E14 embryos (Apostolides et al. 1998; Brundin et al. 2000; Helt et al. 2001; Yurek & Fletcher-Turner 1999). It may be that the dose of GDNF needed is higher than that delivered by the RAd/GDNF.

When considering the appropriate dosage for trophic factors it is worth considering that too much GDNF may have a detrimental effect. Georgievska and colleagues used a lentiviral vector to deliver GDNF to the rat striatum 4 weeks prior to a unilateral 6-OHDA lesion (Georgievska et al. 2002d). GDNF expression in the striatum was seen up to 9 months after injection and was successful in protecting the dopamine neurons of the SNc from the effects of the lesion. However in another experiment, long term GDNF expression was found to be associated with a down-regulation of TH

expression in the striatum, and aberrant sprouting of nerve terminals in areas afferent to the striatum. (Georgievska et al. 2002a) It is perhaps not surprising that unregulated over-expression of GDNF in the brain should have effects beyond those initially intended. Nevertheless it highlights the fact that dosage and longevity of expression of transgenes in the brain is an important issue and one which will need addressing for any potential gene therapy.

Transport of the injected vectors or their transgene product away from the site of injection into areas of the brain unaffected by the disease is another issue for consideration. It is our experience that HSV-based vectors are retrogradely transported from the site of injection to afferent areas. Following striatal injection of HSV, the highest levels of transduced cells are seen in the cortex and SNc. Gene therapy using these or any other retrogradely transported vector will need to consider the effects of transgene expression in areas projecting to the site of injection as well as in the target area itself. Conversely, because of their glial tropism, the RAd vectors studied avoid the problem of retrograde effects. Whilst it is possible that these virus particles may still be taken up into nerve terminals and transported back to the projecting nuclei, it seems that the CMV promoter used in these constructs is inactive in such circumstances and we found no evidence of transgene expression in any area of the brain outside the immediate site of injection using RAd vectors. The suitability of the CMV promoters used in the AV vectors for long term expression *in vivo* has yet to be assessed. We have observed expression for up to 3 months with no sign of shutting down of the promoter elements. The present promoters are probably adequate for pre-clinical studies but, once again, regulatable promoters will need to be considered for human applications.

Adenovirus-based and HSV-based viral vectors were among the first considered for CNS gene therapy and it is clear that many issues pertaining to their use remain unresolved. It is likely that more recently developed vectors, such as those derived from LV or AAV, may present fewer issues of toxicity and stable expression, and in the long term may prove more tractable. Certainly, these vectors are already at a more advanced stage of development than their predecessors (Finkelstein et al. 2001). This view has been given support from several recent studies of their utility *in vivo* (Kirik et al. 2000; Kirik et al. 2002b; Palfi et al. 2002c; Wang et al. 2002; Palfi et al. 2002b; Kirik et al. 2002c; Bjorklund et al. 2000a).

Our experiments with the tricistronic lentiviral vector demonstrated that the transduction efficiency of these vectors is high, as shown previously, and detectable levels of all three transgene products were seen. The TH, AADC and GTPCH1 transgenes used are considered by some to be the minimal set necessary for replacing dopamine-production by neurons (Kang 1998; Kang et al. 2001a; Azzouz et al. 2002a). However, unlike previous studies using a similar vector (Azzouz et al. 2002), no functional efficacy was demonstrated despite detectable expression of the transgenes. The precise reasons for this difference are unclear. One hypothesis is that the levels of striatal dopamine produced in our study were not sufficient to reduce the behavioural supersensitivity in these animals, a necessary requirement for functional recovery on the apomorphine-induced rotation test. In previous work, (Azzouz et al. 2002b) the number of cells expressing the transgenes was between two and three times that seen in our study (5000 ± 700 vs 2076 ± 289 AADC-positive cells and 4800 ± 400 vs 1281 ± 145 TH-positive cells), although in a transplant of primary dopaminergic VM neurons these numbers would be more than sufficient to reverse a similar rotation asymmetry (Brundin et al. 1999). The tricistronic vector relies on IRES sequences to produce expression from three transgenes using a single promoter. However, gene expression from an IRES sequence is lower than that seen from the initial promoter and reduces with each IRES in the sequence (Metz et al. 1998). Thus, it may be that the sequence of transgenes is important for controlling the levels of dopamine synthesis and that the Azzouz construct (AADC/TH/GTPCH1) is simply more potent than the one used in the current work (TH/AADC/GTPCH1).

Whatever the problems with the tricistronic vector, lentiviruses may yet have great potential for PD gene therapy. They have a number of advantages in being able to integrate into non-dividing cells, with a large carrying capacity and the ability for stable integration into the genome of the target cells. In the current generation of vectors, the particles are pseudotyped with the G envelope protein of the vesicular stomatitis virus (VSV-G) which gives the vector the capacity to infect a broad range of tissues, including the nervous system, and is probably responsible for their high affinity for fully differentiated neurons within the CNS (Blomer et al. 1997c; Miyoshi et al. 1998; Naldini et al. 1996a; Naldini et al. 1996c). The level of expression in the brain may be further increased by the introduction of the woodchuck promoter regulatory element (WPRE) into the vector construct (Deglon et al. 2000a; Zufferey et al. 1998). The VSV-

G pseudotyped LV vectors are highly efficient in transducing cells in both striatum and substantia nigra, and in both sites the majority of the transduced cells are neurons (Blomer et al. 1997b; Deglon et al. 2000b; Kordower et al. 1999; Naldini et al. 1996b; Azzouz et al. 2004a). Moreover, in the striatum, stable expression of reporter genes has been observed for up to 6 months without any signs of toxicity or adverse inflammatory reaction in the host tissue (Blomer et al. 1997a).

There have been a number of encouraging studies using LV vectors containing a GDNF transgene (Sun et al. 2003; Palfi et al. 2002; Ostenfeld et al. 2002; Kordower et al. 2000; Georgievska et al. 2002; Bensadoun et al. 2000; Azzouz et al. 2002). However, there are important issues related to the level and site of expression of the transgene. It has been shown, for example, that GDNF can have a negative effect when sustained expression levels are high, resulting in down regulation of the expression of TH (Rosenblad et al. 2003; Georgievska et al. 2002b). So, as with other vector systems, the development of vector constructs in which transgene expression is switcheable may be necessary for clinical application. For such purposes, regulatable promoters such as the tetracycline-based systems have been explored with some success (Kafri et al. 2000a; Kafri 2001; Regulier et al. 2002; Regulier et al. 2003). The potential of LV has been further demonstrated in the CNS by their use in the creation of models of neurodegenerative disease. A lentivirus expressing mutated human huntingtin protein with extended glutamine repeats was introduced into rat striatal neurons to produce a model of Huntington's disease pathology (de Almeida et al. 2002). In another study a lentivirus expressing human wild-type or mutated human α-synuclein has been used to transduce rat nigral dopamine neurons as a model of PD (Lauwers et al. 2003; Lo et al. 2002).

The brief assessment, outlined in this chapter of the potential of gene therapy for the treatment of Parkinson's disease has been very much from the perspective of the neuroscientist rather than that of the virologist. The development of the optimal vector or vectors lies very firmly in the hands of the latter. From the position of the former, it would be fair to say that of the three vectors types under study in our laboratory, LV currently looks to be the most promising for clinical purposes though there remain issues of safety yet to be addressed, particularly the use of HIV derived vectors in humans. Another issue, common to all vector systems, is the regulation and control of transgene expression, and those responsible for authorising the use of gene therapies for

clinical use are more than likely going to insist on switchable gene promoters. The strength of adenoviruses resides in them being non-toxic and having a proven ability to express transgenes at high levels and for long periods in the CNS. The tropism seen in the current work is almost certainly promoter related, and a new generation of "gutless" adenoviruses, containing no viral DNA coding sequences at all, holds great hope for the future for these vectors. As for HSV based vectors, their affinity for the mammalian nervous system remains a major point in their favour as does their ability to establish latent infection within the host. The problems of promoter shutdown and processing toxicity which we have experienced need to be conquered but these vectors remain firmly in contention for gene therapy purposes.

Ultimately, the choice of the vector system is will depend on the genes to be delivered and the treatment strategy to be employed. Indeed, the choice of strategy may turn out to be just as difficult. For example, controlled delivery of GDNF as a preventative therapy to halt the degeneration of the dopamine system (if achievable) would seem to be most desirable therapy. However, clinical intervention will need to be early on in the disease process, when patients are still responsive to conventional L-DOPA therapy and the ethical and regulatory issues surrounding the use of invasive brain surgery on such patients is likely to be a considerable hurdle. Conversely, the dopamine replacement strategy, whilst very much an "end stage" reparative approach and therefore seemingly the second choice therapy, may have an easier route into the clinic precisely because the balance between risk and clinical need is easier to assess.

It is still early days for Parkinson's gene therapy and much has been learnt already. Few of the problems arising seem insurmountable and as our knowledge increases then a future therapy draws ever closer. However, for the moment at least, gene therapy for Parkinson's disease remains a tantalising prospect.

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