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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.

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 tantalizing 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 [21,23]. 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 [5,30].

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 behavioral deficits induced by the lesion [1,3,14,25]. Similar studies using adeno-associated viruses also report sparing of dopamine neurones from the toxin [6,13,22,24,31]. AAV delivery of the dopamine synthetic genes tyrosine hydroxylase (TH), aromatic amino-decarboxylase (AADC) and GTP cyclohydrolase 1 (GCH1) has also been successful [2,7,19,32]. 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, 20], and in primates [21,23,29]. And in a therapy aimed at restoring dopamine function following a 6-OHDA lesion, a lentivirus containing the gene for was able to reduce functional recovery in rats [4].

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 (MIECMV). The construction and purification of this vector have been described previously. [8,10] Gerdes et al. [8], 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 – 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 2.5 mm² 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 – 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, characterizing the in vivo dynamics of RAd36 transduction and transgene expression, was carried out in normal rats [8]. 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 fetal 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

2.1. Experimental animals

Adult, female Sprague–Dawley rats were used for all experiments. All rats weighed 200–250 g 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.

2.2. 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 1.4-kb fragment from the major-immediate-early, murine

cytomegalovirus promoter (MIECMV) [12,16]. 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 4×10^{10} IU/ml [9].

2.2.1. 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 carried out using the co-ordinates of Paxinos and Watson [11]. Viral vectors, neurotoxins and vehicle control solutions, were delivered via a 30-gauge cannula connected to a 10- μ l Hamilton syringe in a microdrive pump set to deliver at 1 μ l/min. Injections of both 6-OHDA and viral vectors were 3 μ l in volume delivered over 3 min with 3 min 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), unilaterally into the median forebrain bundle. The toxin was used at a concentration of 3 μ g/ μ l (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 -2.3 mm below the interaural line.

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

2.2.3. Rotation tests—Lesion-induced rotation tests under the influence of the dopamine agonist methamphetamine 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 modeled after the design of Ungerstedt and Arbuthnot [30]. Rotation test scores were accumulated over a 90-min testing session following an intraperitoneal injection of methamphetamine hydrochloride (dissolved in 0.9% sterile saline) at a dose of 2.5 mg/kg of body weight. Rotations were expressed as total, net rotation scores (ipsilateral minus contra lateral) 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.

2.2.4. Histology—On completion of the experiment, animals were terminally anaesthetized by intraperitoneal injection of 1 mg/kg sodium pentobarbitone, then perfused transcardially with 100 ml of phosphate buffered saline (PBS) at pH 7.4, followed by 250 ml of 4% paraformaldehyde in PBS over a 5-min period. The brains were then removed from the skull and post-fixed by immersion in the same fixative solution for 4 h, then transferred to 25% sucrose in PBS. After equilibration in the sucrose solution, coronal sections were cut on a freezing stage, sledge microtome at a thickness of 60 μ m into 0.1 M TRIS-buffered saline, pH 7.4 (TBS) and stored at +4 °C prior to staining. All stains were carried out on a 1 in 6 series of sections. One series 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 5 min immersion (with agitation) in an ascending series of alcohols (70%, 95% and 100% ethanol), then 30 min immersion in a 50/50 mixture of chloroform and ethanol. Slides were re-equilibrated to water via 5 min immersion (with agitation) in 95% and 70% ethanol then distilled water. Staining was carried out by 5 min immersion in cresyl fast violet solution (5% in 0.1 M sodium acetate buffer, pH 3.5). Differentiation the stain and dehydration was carried out 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-min immersion in 3% hydrogen peroxide/10% methanol in distilled water, followed by washing and re-equilibration in TBS. After a 1-h preincubation 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 h at +4 °C. A known positive control, and a negative control in which the primary antibody was omitted, were also run. After thorough washing, a biotinylated anti-rabbit, secondary antibody (Dako, 1:200) in 1% normal goat serum in TBS was applied for 3 h. The sections were then washed for 30 min before application of 10% streptavidin–biotin–horseradish peroxidase solution (Dako, UK) in TBS for 90 min, followed by thorough washing and equilibration to 0.05 M Tris non-saline (TNS) solution at pH 7.4. The horseradish peroxidase label was revealed by a 10-min incubation in a 0.5% solution of diaminobenzidine tetra hydrochloride (Sigma-Aldrich, UK) in TNS containing 0.3 μ l/ml of hydrogen peroxide. Sections were finally mounted on gelatine-coated microscope slides dehydrated in an ascending series of alcohols, cleared and cover-slipped using DPX mountant.

2.3. 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 and in brains where there were several brain structures containing stained cells, sub-counts were carried out in each structure and the volume of staining estimated from these. Cell counts were carried out on a Leica DMRB microscope (Leica Microsystems UK) using a 10 $\times$ 10 eyepiece graticule and a $\times$ 20 objective.

2.4. 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

3.1. 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 3 μ l of RAd36, using the protocol described above. The titre of RAd36 used for each group was as follows: group 1, 1×10^4 IU/ μ l; group 2, 1×10^5 IU/ μ l; group 3, 1×10^6 IU/ μ l. All rats were sacrificed 4 weeks post-injection of the virus.

3.2. 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 postlesion. Rats were then

divided into three matched groups according to rotation scores. Twenty weeks later all rats received bilateral, striatal injections of 3 μ l of the LacZ adenoviral vector RAd36 at a concentration of $1 \times 10^6/\mu$ l. Group 1 was sacrificed and the brains were perfusion fixed, 3 days post-injection; group 2 at 1-week post-injection; and group 3 at 4 weeks post-injection.

4. Results

4.1. Experiment 1: In vivo, dose–response characteristics of RAd36

Fig. 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. While there were small amounts of staining of the corpus callosum in the brains of a number of animals in groups 1 (10^4 IU/ μ l) and 2 (10^5 IU/ μ l) (3 out of six brains in each group), only in group 3 (10^6 IU/ μ l) was extensive staining seen (in 4 out of 6 brains). Analysis of the volumes of β -galactosidase staining in the corpus callosum (see Fig. 2A) demonstrated that there was a significant increase in the levels of corpus callosum staining from the 10^4 IU/ μ l dose to the 10^5 IU/ μ l 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 or 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 (Fig. 2B), the ratios of the total striatal volumes of the injected versus the non-injected sides were 0.97 ± 0.02 , 1.02 ± 0.02 and 1.00 ± 0.01 in the 3×10^4 IU dose, 3×10^5 IU dose and 3×10^6 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 ± 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.

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

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 Figs. 3B, D, F and H).

In the β -galactosidase immunostained sections however, there was a clear asymmetry of staining in the two injected hemispheres (see Fig. 3A, C, E and G). On the intact side of the brain β -galactosidase immunoreactivity was confined almost exclusively to the striatum, in a volume centered around the site of injection. In contrast, on the dopamine-depleted side of the

brain, many brains had staining in other structures, principally in the corpus callosum overlying the injection site. Analysis of β -galactosidase-positive cell numbers (Fig. 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 (Fig. 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 Figs. 4B and C). Although there were lower overall numbers of β -galactosidase cells in the lesioned striatum (9244 ± 1298) than in the unlesioned striatum ($11,691 \pm 1467$) this difference was not significant. However, in the corpus callosum there were significantly larger numbers of cells seen on the lesioned side ($10,768 \pm 1571$) 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 indicates 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 (Fig. 4B). However, the numbers of cells counted in the corpus callosum do not change significantly during the time course of the experiment (Fig. 4C). As in experiment 1, no cells expressing the transgene were seen in areas afferent to the site of injection.

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 [25]. 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 [1,3,14].

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, particularly 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

[13,22]. In spite of the supposed immune privilege of 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 [6,7,24,31].

The inflammation observed following injection of adenoviral vectors was shown to be due to an immune response to viral proteins synthesized by infected cells [32]. 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 [19]. These vectors not only attenuated the host immune response but had the added advantage of a larger carrying capacity for introduced transgenes [2,18]. 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 [20]. 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 [29].

Adenoviral vectors have been used successfully deliver glial-derived neurotrophic factor (GDNF) in rat models of Parkinson's disease and to ameliorate the effects of 6-OHDA lesions [4,8,10,12,16,23], 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 [9] but there are no reports of direct comparisons of vector dynamics in the normal versus the hemiparkinsonian brain as in the present work.

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. who described a similar phenomenon where viruses with reduced or absent binding ability were seen to diffuse further in the brain than normal virus [29]. This phenomenon has also been noted for other substances notably following injection of heparin into rat brain [17].

The levels of LacZ expression observed in experiment 2 were generally lower than those seen in experiment 1 using a 3×10^6 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 [15,29]. 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 Fig. 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 this experiment is two to three logarithms lower than the doses used by other 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 (Fig. 5). 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 [28]. In another study gliosis was at a peak in the first week postlesion but had declined to control levels after 4 months [27]. In studies where 6-OHDA was injected directly into the striatum there were increased numbers of astrocytes [28] and microglia [11] 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 [26]. 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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Fig. 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 3×10^4 IU (A and B), 3×10^5 IU (C and D) and 3×10^6 IU (E and 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 (D–F) 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 1.0 mm.

Fig. 2.

Experiment 1: In vivo dose-response characteristics of RAd36 at 3×10^4 (group 1), 3×10^5 (group 2) and 3×10^6 (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. (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.

Fig. 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 sacrificed 1 week post-injection of 3×10^6 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 2 mm.

Fig. 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 β -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.

Fig. 5. High power ($\times 200$) photomicrographs of β -galactosidase expressing cells following injection of RAd36 into rat brain. (A) 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 also cell profiles show glial cell profiles.