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Sonic hedgehog delivered by an adeno-associated virus protects dopaminergic neurones against 6-OHDA toxicity in the rat

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Summary. Direct intracerebral administration of sonic hedgehog (SHH) reduces 6-OHDA and MPTP toxicity to nigral dopaminergic cells in rats and primates. To determine whether transfection of the DNA sequence for SHH using viral vectors also protects against 6-OHDA toxicity, a type 2 adeno-associated virus (AAV) incorporating 600 base pairs of N-terminal SHH DNA was generated to induce SHH expression in rat striatum.

AAV-SHH was injected into the striatum, 3 weeks prior to the initiation of an unilateral partial 6-OHDA nigro-striatal lesion. Animals receiving 4×10^7 viral particles of AAV-SHH showed a reduction in (+)-amphetamine induced ipsilateral turning over 4 weeks, when compared to animals receiving vehicle or a LacZ encoding vector. Following vehicle or AAV-LacZ administration, 6-OHDA caused a marked loss of striatal dopamine content and nigral tyrosine hydroxylase (TH) immunopositive cells. Following treatment with 4×10^7 viral particles of AAV-SHH the loss of striatal dopamine content was reduced and there was marked preservation of nigral dopaminergic cells. However, administration of 4×10^8 particles of AAV-SHH did not cause a significant change in (+)-amphetamine-induced rotation, striatal dopamine levels or the number of nigral TH immunoreactive cells following 6-OHDA lesioning compared to vehicle or AAV-LacZ treated animals.

The results show that SHH delivered via a viral vector can protect dopaminergic neurons against 6-OHDA toxicity and suggest that this could be developed into a novel treatment for PD. However, the effects maybe dose limited due to uncoupling of hedgehog receptor signalling at higher levels of SHH expression.

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Introduction

Parkinson's disease (PD) is due to the primary degeneration of nigro-striatal dopaminergic neurones and the subsequent reduction in caudate-putamen dopamine content (Carlsson et al., 1957; Ehringer and Hornykiewicz, 1960; Marsden, 1990). Symptomatic relief of motor abnormalities is achieved using dopamine replacement therapy in the form of L-DOPA or dopamine agonist drugs. However, there is no effective therapy that stops or slows disease progression or reverses pathological change.

A neurorestorative treatment for PD may be possible through the use of neurotrophic factors, such as brain derived neurotrophic factor (BDNF) and glial cell lines-derived neurotrophic factor (GDNF) which can reverse degeneration of the dopaminergic nigro-striatal pathway to varying degrees. However, neurotrophic factor based approaches are limited by their relatively short duration of effect (Sauer et al., 1995; Gash et al., 1996) and by the need for multiple intracerebral administrations (Sauer et al., 1995; Shults et al., 1995, 1996; Winkler et al., 1996; Gill et al., 2003). For example, intranigral administration of GDNF to rats on alternate days resulted in complete protection against 6-OHDA toxicity, whereas a single GDNF application was only partially effective (Sauer et al., 1995). In early stage PD, the chronic intraputaminal infusion of GDNF led to a lowering of L-DOPA dosage and decreased motor disability while no effects were observed on acute intraventricular administration (Gill et al., 2003).

Viral vectors containing the DNA for neurotrophic factor production provide a more sustained means of delivering protein to the basal ganglia in PD (Latchman et al., 2000). Adeno-associated viruses (AAVs) are highly suitable vectors since they infect neurones, achieving long lasting expression of genes of up to 4.7 kbp (Lo et al., 1999; Chamberlin et al., 1998). AAVs have minimal inflammatory properties and are non-pathogenic in man, since almost all viral gene sequences are deleted (Muzyczka, 1992; Ozawa et al., 2000). Transfection of the gene sequence for tyrosine hydroxylase (TH) into the striatum of unilaterally 6-OHDA lesioned rats using an AAV vector led to the sustained expression of TH and a reduction in apomorphine-induced rotation (Kaplitt et al., 1994). Similarly, AAVs can be used to transfect the DNA for BDNF and GDNF in 6-OHDA lesioned rats (Klein et al., 1999; Mandel et al., 1997; Bjorklund et al., 2000). Expression of BDNF in the substantia nigra of rats 6 months following administration of recombinant AAV reduced (+)-amphetamine-induced rotational behaviour following 6-OHDA lesioning although there was no reduction in dopaminergic cell death (Klein et al., 1999). In contrast, sustained expression of GDNF following AAV transfection prevented both nigral cell death and reduced behavioural deficits in 6-OHDA lesioned rats (Mandel et al., 1997; Kirik et al., 2000; Wang et al., 2002). However, the effect was site dependent. Thus, prior nigral GDNF-AAV transfection alone did not protect against striatal 6-OHDA toxicity whereas striatal or striatal and

nigral GDNF transfection completely protected the nigro-striatal pathway and prevented the onset of rotational behaviour (Kirik et al., 2000). Transfection with GDNF-AAV 4 weeks following the initiation of a striatal 6-OHDA lesion in rats also counteracted nigral cell death and produced a sustained reduction in apomorphine-induced rotation (Wang et al., 2002). These experiments all serve to show the utility of an AAV derived viral vector approach towards protection of the nigrostriatal pathway against toxin insult in rodents.

Sonic hedgehog (SHH) is another trophic protein able to stimulate the growth of dopaminergic neurons. SHH supports the survival of TH positive neurons in E14 rat mesencephalic explants following serum withdrawal (Miao et al., 1997). Importantly, SHH was active at low nanomolar concentrations and was more effective against the toxic actions of MPP^+ than equivalent concentrations of GDNF or BDNF (Miao et al., 1997). Neuroprotective effects of SHH towards dopaminergic neurons are also apparent *in vivo.* SHH administered into the striatum prevented 6-OHDA induced dopaminergic cell death and reduced (+)-amphetamine-induced rotation when administered before and after striatal toxin treatment (Tsuboi et al., 2002). In a previous study, we found that supranigral administration of a low concentration of SHH in MPTP treated common marmosets had a moderate ability to reverse motor disability and that it was more potent than GDNF (Dass et al., 2002; Costa et al., 2001). This suggests that sustained administration of the SHH protein through the use of viral vectors might induce significant preservation of the nigro-striatal pathway.

The aim of the present study was to assess the ability of AAV expressed SHH to protect the nigro-striatal pathway against 6-OHDA toxicity in the rat. A striatal injection of an AAV encoding and expressing human N-terminal SHH was used prior to 6-OHDA treatment, to investigate its ability to prevent the appearance of motor deficits, striatal dopamine deficiency and nigral cell death. The results suggest that SHH delivered as an AAV is capable of preventing nigral degeneration and that AAV-SHH treatment may be a viable neurotrophic approach to the treatment of PD.

Materials and methods

Male Sprague Dawley rats (200–225 g at the start of the study, Charles River USA) were housed at the Animal Services Unit, Biogen Inc. (USA), on a 12 h light/dark cycle in pairs or triplets, with free access to food and water. Experimental approval was obtained following review by the Biogen Institutional Animal Care and Use Committee (IACUC). All animals were weighed on a weekly basis.

Viral vector construction

Adeno-associated viruses were constructed expressing either SHH or LacZ. The constructs encoding the N-terminal human SHH gene or the LacZ DNA sequence were prepared to concentrations of either 2×10^{10} or 2×10^{11} genome copies per 1 ml in 0.1 M phosphate buffered saline (PBS) vehicle for intrastriatal injection.

AAV-LacZ and AAV-SHH adeno-associated viral vectors were generated by triple transfection into HEK 293 cells (American Type Culture Collection) cells. The AAV expression plasmid (the cis-plasmid) pAAV-CMV-LacZ was described previously (Fisher et al., 1996). The expression plasmid pAAV-CMV-SHH was made by insertion of the 660 base pair soluble secreted human SHH gene downstream of the CMV promoter in a psub201-derived AAV plasmid lacking rep and

cap. In this plasmid, the 5′ to 3′ organization was AAV ITR, CMV promoter, SHH transgene, SV40 polyadenylation signal, AAV ITR. The SHH gene encoded only the extracellular domain so that it would not be associated with the cell membrane. Virus was produced by triple transfection of pAAV-CMV-LacZ or pAAV CMV-SHH plus the rep and cap encoding plasmid pTrans-600 trans (Zhang et al., 2000) and the adenovirus helper function encoding plasmid pAdF6 (Zhang et al., 2000) into HEK 293 cells as described previously (Xiao et al., 1988). HEK 293 cells were harvested 48 hours after transfection and frozen. Thawed cells were lysed by sonication. The lysate was treated with RNase A and DNase I followed by deoxycholic acid treatment. AAV was purified by three sequential rounds of caesium chloride gradient ultracentrifugation and desalted as described previously (Fisher et al., 1996). The AAV genome copy concentration was determined by real time quantitative PCR. Plasmids pAAV-CMV-LacZ, pTrans-600 trans and pAdF6 were a gift from Drs. G.-P. Gao and J. M. Wilson (University of Pennsylvania).

Surgery

Four groups of rats were initially anaesthetized using sodium methohexital ($60\,mg \cdot kg^{-1}$, i.p.) and maintained during the surgical procedure with isoflurane. Using a Kopf stereotaxic frame, animals were injected with 2 µl of PBS (n = 13), 4×10^7 gc AAV-LacZ (n = 8), 4×10^7 AAV-SHH (n = 9), or 4×10^8 AAV-SHH (n = 9) into the striatum at the co-ordinates +0.7 mm AP, +2.8 mm L of bregma, and 5.0 mm ventral of the dura, with the tooth bar set to −3.3 mm according to the atlas of Paxinos and Watson (1986). This resulted in an AAV-LacZ dose of 4×10^8 gc and AAV-SHH doses of 4×10^7 and 4×10^8 gc. Injections were performed over 4 minutes with the needle left *in situ* for a further 10 minutes before withdrawal to prevent reflux. Some animals were killed 4 weeks following AAV injection to examine viral vector expression at the predicted peak time point of SHH expression and to establish the presence of SHH protein at the time of 6-OHDA lesioning (Davidson et al., 2000).

Three weeks following AAV injection, a partial 6-OHDA lesion of the striatum was undertaken in the remaining animals (Sauer et al., 1994). 6-OHDA was injected at the same co-ordinates as those used for the injection of the AAV vectors. 6-OHDA (16.5 µg 6-OHDA hydrochloride in 2 µl of 0.2% ascorbic acid in 0.9% NaCl, Sigma, Poole, UK) was injected over 4 minutes with the needle withdrawn 1 minute later. The scalp incision was closed using wound clips and animals were allowed to recover on a thermal blanket set to 37°C.

RNA Isolation and reverse transcription

RNA from striata transfected with AAV-SHH, AAV-LacZ, or untreated striatal tissue, was isolated using a Promega SV Total RNA Isolation system kit and the absorbance of 1 µl of the purified RNA (in 100 µl H_2O) was measured at a wavelength of 260 nm. The concentration of RNA in solution was then calculated using the formula: absorbance units $\times\, 71/40 =$ µg of RNA.

To create a cDNA library from the purified RNA, 14 µl of RNA was heated at 70°C for 3 minutes with 2 mM dNTPs (dATP, dCTP, dGTP, dTTP) and 4 µg of two random primers $d(T)_{18}$ and $d(N)_{10}$. This mixture was placed on ice after heating and 4 µl of AMV reaction buffer (250 mM Tris-HCl, 250 mM KCl, 50 mM $MgCl_2$, 50 mM dithiothreitol, 2.5 mM spermidine) and 1 µl of ribonuclease inhibitor were added to the solution. From this, 2 µl was removed and placed in a separate Eppendorf tube to act as a no-reverse transcription control. A volume of 2 µl of AMV reverse transcriptase (Promega) was added to the RNA sample, and this (and the control reaction) was heated sequentially at 37°C for 10 mins, 42°C for 30 mins, and 52°C for 20 mins. The reaction was terminated by heating at 80°C for 10 minutes, and the products were diluted 10 fold in RNAse free water and stored at −70°C.

Primer design

PCR primers for rat β-actin of 20 bp length (*Forward*: TCA TGA AGT GTG ACG TTG ACA TCC GT, *Reverse*: CCT AGA AGC ATT TGC GGT GCA CGA TG), were purchased from King's College London Molecular Biology Unit and amplified a sequence of 286 bp length. Primers for SHH of 20 bp in length were also designed with reference to the published sequence

of human and rat SHH (available on Pubmed-BLAST) and checked against the sequence of the AAV-SHH *(Forward*: TCG GGA AGA GGA GGC ACC CC, *Reverse*: CTC TGA GTG GTG GCC ATC TT). The design of the primers was based on the sequences of human (and hence virally encoded) SHH that shared little homology with rat SHH DNA. Products of primers were expected to be 320 bps long. Primers were diluted to a working concentration of 66 ng/μl.

Polymerase chain reaction

PCR using primers for β-actin or human SHH was performed to amplify the sample cDNA. Reagents were added on ice into ABgene 200 μl thin walled PCR tubes. The final reaction mixture consisted of 1.5 mM $MgCl_2$ 50 mM KCl, 10 mM TRIS-HCl, 0.1% Triton-X, 4 μM dNTPs, 66 ng (1 μl) of forward and reverse primer, 2.5 units of *Taq* DNA polymerase (Promega) and 2–4 μl of cDNA sample and were made up to volumes of 20–40 μl with PCR quality water. Striata derived cDNA from the animals injected with AAV-SHH, AAV-LacZ, or PBS, were used along with 2×10^5 gc of AAV-SHH as a source of DNA to amplify transfected human SHH or rat β-actin. To act as a further control, 4 μl samples from reactions in which reverse transcriptase were omitted from the preparation of cDNA, were also amplified using primers for SHH-320 bp fragment. The reaction tubes were then placed into a thermal light-cycler which was programmed to heat to 95°C for 40 s, 56°C for 30 s, and 74°C for 40 s, through 45 repetitions. After the cycles were completed, the reaction was left at 74°C for 5 mins before being cooled to 21°C. Agarose gels (1.5%) containing ethidium bromide (0.5 μM) were used to isolate individual products from the PCR reaction and these were visualised on a UV digitiser.

cDNA Isolation and sequencing

Bands of material amplified from AAV-SHH-transfected striata or from pure AAV-SHH virus were visualised under UV light, excised from the gel using a sterile blade and isolated and purified from the agarose gel. A 1 μl aliquot of the purified DNA was amplified by PCR under standard conditions, as before with SHH 320 bp fragment primers, and submitted for sequence analysis to King's College London Molecular Biology Unit. Results were used to search for similar DNA sequences using a standard BLAST search, and homology comparisons between the attained sequences were performed using BLAST 2 BLAST.

Behavioural analysis

Animals were tested weekly over a period of 4 weeks for (+)-amphetamine induced turning behaviour following initiation of the 6-OHDA lesions. A period of 10 minutes was allowed for the animals to acclimatise to the Columbus Instruments rotometer apparatus, prior to intraperitoneal injection of (+)-amphetamine sulphate ($5\ mg \cdot kg^{-1}$, i.p. Sigma). Net ipsiversive rotations were observed for 90 minutes following (+)-amphetamine challenge.

One week following the final challenge with (+)-amphetamine, the animals were killed by inhalation of CO_2 followed by decapitation. Striatal tissue was dissected away from the brain, frozen on dry ice and stored at −70°C. The midbrains, cut at the level of the optic chiasm, were placed in 4% paraformaldehyde for 1 week following which they were transferred into a 20% sucrose/1% PBS solution for 3 days for cryoprotection. Following equilibration (when the brains sank in sucrose solution), the brains were frozen and stored at −70°C.

HPLC

Striatal tissue samples were homogenized with in 0.2 M perchloric acid/100 mM EDTA (1:10 w/v ratio) and centrifuged for 15 minutes at 14,000 g at 4°C to precipitate lipids and large proteins away from the cytosol. The supernatant was further purified by filtration through a 0.22 μm Costar Spin-X tube for 5 minutes at 5000 g at 4°C. An aliquot (20 μl) of each sample was injected using an ESA 580 pump (195–200 PSI), onto a 150 × 3 mm RP-C18 HPLC column (ESA) from an ESA 540 auto sampler, which kept the samples at 4°C. The samples, along with a series of prepared standards for calibration (DA, DOPAC and HVA at 0.1 ng, 0.5 ng, 1 ng, 10 ng and 100 ng/ml of 0.2 M perchloric

acid/100 mM EDTA), were analysed for DA, DOPAC, and HVA content using a Coulochem 5200A electrochemical detector (350 mV, 500 nA) and processed using an ESA501 data station.

Immunohistochemistry

Nigral sections (60 μm) at the level of the third nerve were cut on a cryostat (Leica Instruments) and examined for the presence of TH-immunoreactivity (-ir) as described previously (Dass et al., 2002). Free-floating sections were incubated in primary monoclonal mouse anti-rat antibody in 0.1 M PBS-Triton X-100 (Chemicon Ltd) at 1:600 dilution overnight at room temperature. Sections were then incubated in the avidin-biotin peroxidase complex using the appropriate rabbit or mouse ABC Vectastain kit (Vector Laboratories). Immunoreactivity was observed using 3,3-diaminobenzidine (DAB, Sigma, Poole, UK) as the chromagen. Five sections per animal, taken at the level of the 3rd nerve, were analysed for quantification of TH positive cells using a Zeiss microscope.

Statistical analysis

All data was analysed initially using a two way ANOVA. For weight and rotational data, the effect of treatment and time was subsequently analysed using a non-parametric Kruskal Wallis test, followed by Dunn's multiple comparisons between all groups. Dopamine levels, dopamine turnover and cell counts were analysed for the effect of PBS, AAV-LacZ, or AAV-SHH between groups and between treated and untreated hemispheres using a one way analysis of variance, followed by a Tukey test between groups and between sides with a group using a paired two tailed t-test. If there were no significant differences ($P > 0.05$) between the 6-OHDA-viral particle-treated and the untreated hemispheres, then administration of the viral particle prevented/and or reversed the 6-OHDA induced degeneration.

Results

RNA Purification yield

After purification, the RNA concentration in samples of striatal transfected with AAV-SHH was $36.9\,\mu g \cdot ml^{-1}$ and $28.4\,\mu g \cdot ml^{-1}$ with AAV-LacZ. The intact untreated striatum had an RNA content of $45.4\,\mu g \cdot ml^{-1}$.

Identification of SHH expression in AAV-SHH transfected tissue

β-actin was strongly amplified by PCR of cDNA samples obtained from striatal tissue transduced with either AAV-SHH or AAV-LacZ, and from striatal tissue of normal rats (Fig. 1, lanes 1–3 respectively). In samples prepared in the absence of reverse transcriptase, AAV-SHH or AAV-LacZ transfected striatal tissue, PCR using 320 bp SHH primers produced small diffuse bands at approximately 250 bp, 157 bp and 66 bp (lanes 4–6). Striatal cDNA transduced with AAV-SHH (lane 7), exhibited bands of identical mobility to those observed when reverse transcriptase was omitted. However, an additional band was observed with a mobility that coincided approximately with the 325 bp marker. The cDNA samples from striatal tissue transduced with AAV-LacZ or untreated striata (lanes 8 and 9), produced similar bands to those seen in the absence of reverse transcriptase, but there were no additional bands in the region of the 325 bp marker. When amplified with SHH primers, the viral SHH DNA produced a band at approximately 325 bp that was of identical mobility to that obtained from AAV-SHH transduced striatal tissue. Faint and diffuse bands were also noted at 157 bp and 66 bp.

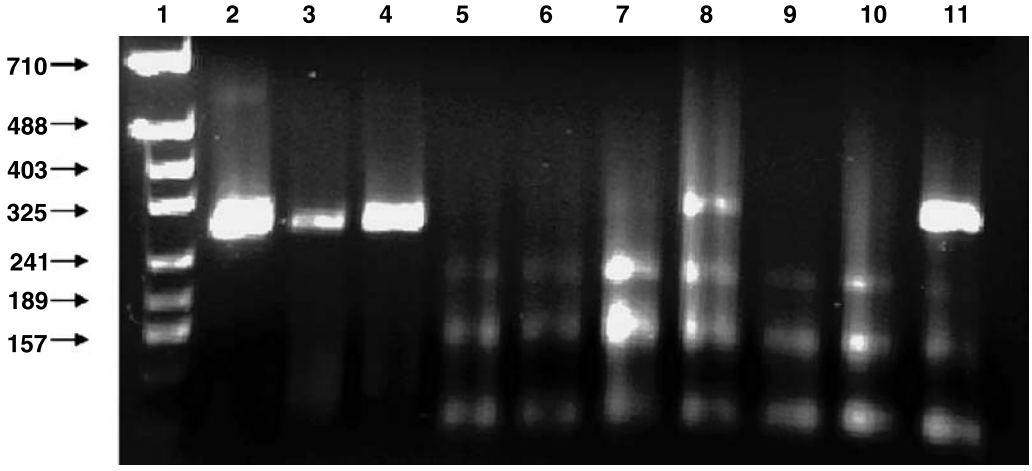

Fig. 1. PCR Amplification of AAV-SHH transduced striatal cDNA, controls with reverse transcriptase omitted, or 2×10^5 gc of AAV-SHH DNA using forward and reverse 320 bp fragment primers for human SHH or βactin. Lane 1: DNA ladder from pBluescript Sk+ plasmid digested with HPA II, giving bands at 710, 488, 403, 325, 241, 189, 157, 145, 109, and 66 bps. Lane 2: AAV-SHH transduced striatal cDNA, amplified with primers for β-actin. Lane 3: AAV-LacZ transduced striatal cDNA, amplified with primers for β-actin. Lane 4: cDNA from untreated striata, amplified with primers for β-actin. Lane 5: cDNA prepared in absence reverse transcriptase from AAV-SHH transduced striatal tissue, amplified using forward and reverse 320 bp fragment primers for human SHH. Lane 6: cDNA prepared in absence reverse transcriptase from AAV-LacZ transfected striatal tissue, amplified using forward and reverse 320 bp fragment primers for human SHH. Lane 7: cDNA prepared in absence reverse transcriptase from untreated striatal tissue, amplified using forward and reverse 320 bp fragment primers for human SHH. Lane 8: AAV-SHH transduced striatal cDNA, amplified with primers for human SHH. Lane 9: AAV-LacZ transduced striatal cDNA, amplified with primers for human SHH. Lane 10: cDNA from untreated striata, amplified with primers for human SHH. Lane 11: Viral AAV-SHH DNA (2×10^5 gc) amplified with primers for human SHH

cDNA Isolation and sequencing

Amplification of the purified cDNA from AAV-SHH transfected striata (lane 3) or from the SHH encoding viral vector (lane 2 and 4), resulted in single bands of approximately 320 bp being visualised on the gel (Fig. 1). A 314 bp sequence was identified for cDNA from AAV-SHH transduced striata, and a 315 bp length of DNA was identified from SHH encoding viral vector. When analysed using a BLAST 2 BLAST search, the two sequences for AAV-SHH transduced cDNA and virus PCR products were found to be 100% identical from bp 94 to 292. In addition, when entered into a standard BLAST search the AAV-SHH transduced cDNA sequence was 97% identical to human SHH (score 345) but only 88% identical to rat SHH (score 188).

Weight changes

All groups of animals significantly gained weight over the period of study following 6-OHDA lesions ($P<0.01$). The injection of PBS, AAV-LacZ or AAV-SHH was without effect on body weight or on the rate of weight gain.

Rotational behaviour

PBS treated rats exhibited marked (+)-amphetamine induced rotational behaviour during the 4 weeks following striatal 6-OHDA lesioning. Over time however, rotational behaviour tended to decline in the PBS treated group although this did not reach statistical significance (Fig. 2). Similarly, AAV-LacZ treated animals also exhibited (+)-amphetamine induced rotational behaviour during the 4 weeks following striatal 6-OHDA lesioning and again this declined over time.

One week after 6-OHDA lesioning, the 4×10^7 gc AAV-SHH group exhibited a significantly lower rotational behaviour when compared to both the PBS and AAV-LacZ treated rats ($P < 0.05$). The decrease in rotational behaviour was maintained for the remainder of the experiment. At 2, 3, and 4 weeks following 6-OHDA administration, the (+)-amphetamine induced rotational behaviour in animals receiving 4×10^7 gc AAV-SHH viral particle was virtually absent ($P < 0.01$), although increased investigative and rearing behaviour was noted. Compared to rotational behaviour in the first week after 6-OHDA administration, rotations within the 4×10^7 AAV-SHH group were significantly lowered in subsequent weeks ($P < 0.05$) (Fig. 2).

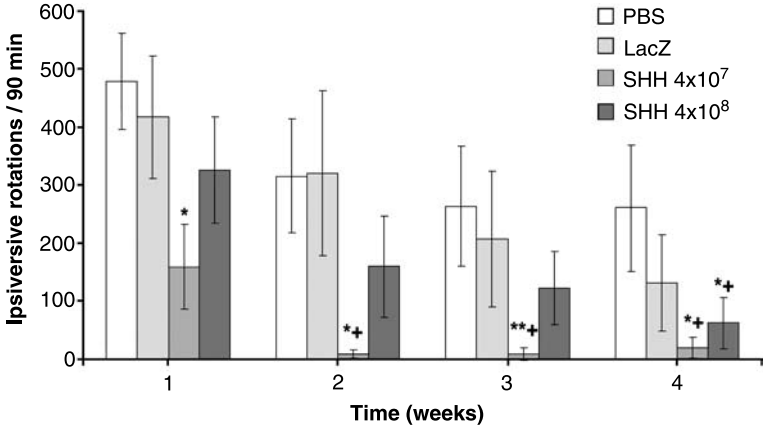

Fig. 2. Net amphetamine induced rotations (±S.E.M.) in PBS or AAV – treated groups following 6-OHDA lesion induction. Net amphetamine induced rotations over 4 weeks, following 6-OHDA lesion induction for PBS group (n = 13), LacZ 4×10^8 (n = 8), and SHH at 4×10^8 (n = 9) and 4×10^7 gc (n = 6). Two-way ANOVA indicated significant differences due to drug and time. Kruskal Wallis test followed by Dunn's multiple comparisons was used to compare all data for each variable. *Week 1*: 4×10^7 AAV-SHH group is significantly different from PBS and LacZ ($P < 0.05 = \star$). No other differences are observed between groups. *Week 2*: 4×10^7 AAV-SHH group is significantly different from all other groups ($P < 0.05 = \star$) and no other differences are observed between groups. *Week 3*: 4×10^7 AAV-SHH group is highly significantly different from all other groups ($P < 0.01 = \star\star$) and no other differences are observed between groups. *Week 4*: 4×10^7 and 4×10^8 AAV-SHH groups are significantly lower than PBS group ($P < 0.05 = \star$). No other differences are observed between groups. 4×10^7 AAV-SHH group is significantly lowered at weeks 2, 3, and 4, compared to rotational behaviour at week 1 ($P < 0.05 = +$). 4×10^8 AAV-SHH group is significantly lowered at week 4 compared to rotational behaviour at week 1. No other differences were found within groups

Animals treated with 4×10^8 AAV-SHH viral vector exhibited no significant change in rotational response to (+)-amphetamine challenge compared to PBS treated animals 1, 2, and 3 weeks after 6-OHDA lesioning. However, 4 weeks after 6-OHDA lesioning, the administration of 4×10^8 gc AAV-SHH in rats caused a significant reduction in (+)-amphetamine induced rotational behaviour compared to PBS treated animals ($P < 0.05$) (Fig. 2).

Striatal dopamine levels

In a preliminary set of experiments in unlesioned rats, neither AAV-SHH titres had any effects on striatal dopamine levels when compared to the control side (data not shown).

Dopamine levels were decreased by 85% in the striatal tissue ipsilateral to the injection of 6-OHDA in PBS treated animals compared to the contralateral intact hemisphere ($P < 0.05$) (Fig. 3a). Rats treated with AAV-LacZ also had significantly decreased levels of dopamine in the 6-OHDA-injected striatum ($P < 0.05$). Animals treated with 4×10^7 AAV-SHH viral particles showed no significant reduction in dopamine content in the ipsilateral striatum compared to the contralateral striatum. Indeed, 4×10^7 AAV-SHH treated rats had significantly higher levels of dopamine in the lesioned hemisphere when compared to the PBS, AAV-LacZ, or 4×10^8 AAV-SHH treated hemispheres ($P < 0.05$) (Fig. 3a).

In animals treated with 4×10^8 AAV-SHH, dopamine levels in the 6-OHDA lesioned striatum appeared to be somewhat higher than those found in the striatum of PBS or AAV-LacZ treated animals 4 weeks after the lesion, but these differences did not reach statistical significance.

Striatal dopamine turnover

In PBS treated animals, the 6-OHDA lesioned striatum showed a significant increase in dopamine turnover, as assessed by the DOPAC + HVA to DA ratio, when compared to the untreated hemisphere ($P < 0.05$) (Fig. 3b). Animals treated with AAV-LacZ tended to have increased dopamine turnover in the lesioned striatum relative to the contralateral intact striatum, although this was not statistically significant ($P = 0.11$). Rats treated with 4×10^7 gc AAV-SHH had lower dopamine turnover in both the 6-OHDA lesioned striatum and the contralateral intact striatum; there was also no difference in dopamine turnover between the lesioned and intact sides in this group. Administration of 4×10^8 gc SHH had no effect on dopamine turnover.

Tyrosine hydroxylase immunoreactivity in the substantia nigra

Injection of 6-OHDA into the striatum induced a 45% loss of TH positive cells in the ipsilateral SNpc of PBS treated rats when compared to the intact side ($P < 0.05$). Treatment with AAV-LacZ did not alter the loss of nigral TH positive cells produced by 6-OHDA lesioning. In animals treated with 4×10^7 AV-SHH, the 6-OHDA-induced reduction of nigral TH positive neurones was significantly prevented compared to rats treated with either PBS or AAV-LacZ.

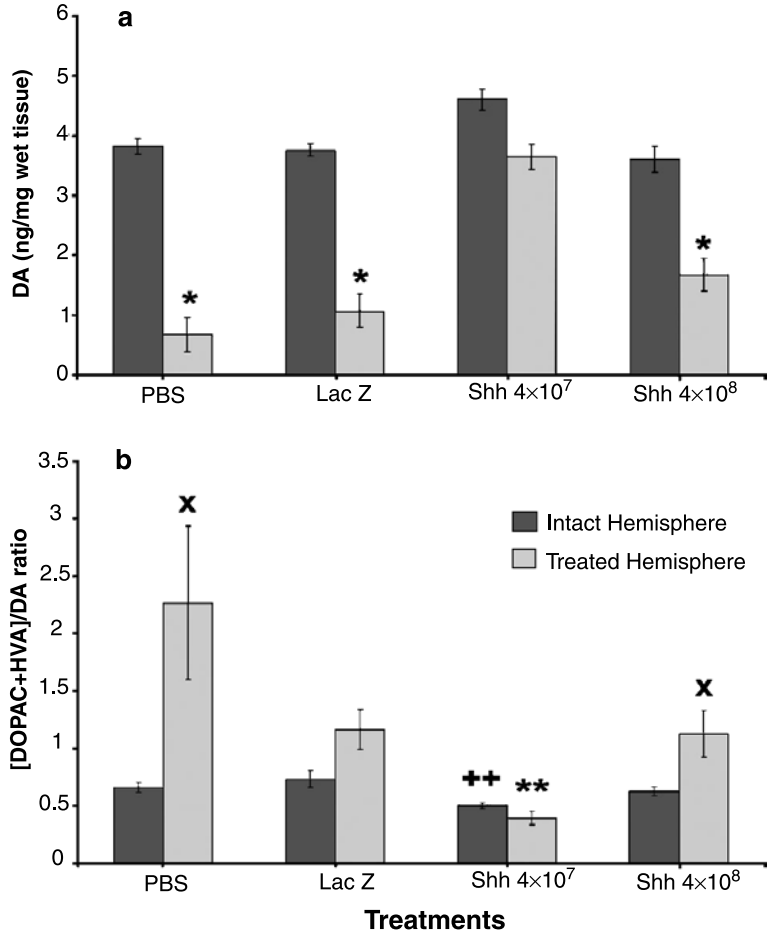

Fig. 3. Striatal dopamine levels (±S.E.M.) in AAV treated animals following 6-OHDA lesioning. Mean dopamine levels (ng/mg of wet weight tissue ±S.E.M.) from the striata of treated and untreated sides of PBS (n = 13), LacZ 4×10^8 gc (n = 8), Shh at 4×10^7 (n = 6) and 4×10^8 gc (n = 9). Two-way ANOVA indicated significant differences due to side but not drug treatment. Animals treated with 4×10^7 AAV-SHH exhibited no significant changes in dopamine when compared to the control side. All other groups displayed significant decreases in dopamine (* = $P < 0.05$) (Paired two tailed t-test) (**a**). Ratio of dopamine metabolites to dopamine in striata of PBS or AAV-treated groups. Ratio of dopamine metabolites to dopamine content ([DOPAC + HVA]/dopamine) in the treated striatum (±S.E.M.) for PBS (n = 13), LacZ 4×10^8 gc (n = 8), SHH 4×10^7 (n = 6), and SHH 4×10^8 gc (n = 9) groups. Significant differences are observed between groups for treatment and side (two-way ANOVA) (**b**). AAV-SHH 4×10^7 is significantly different from all other groups on the treated side ($P < 0.01 = **$) and also significantly different on the untreated side compared to the LacZ group ($P < 0.01 = ++$) No other differences were observed between groups. Between sides, PBS and AAV-SHH 4×10^8 groups have significant differences between sides ($P < 0.05 = \times$). No other differences were observed (Paired two tailed t-test)

The number of TH positive cells in the ipsilateral substantia nigra following the administration of 4×10^7 AAV-SHH was not different from that found in the intact contralateral hemisphere. However, following administration of 4×10^8

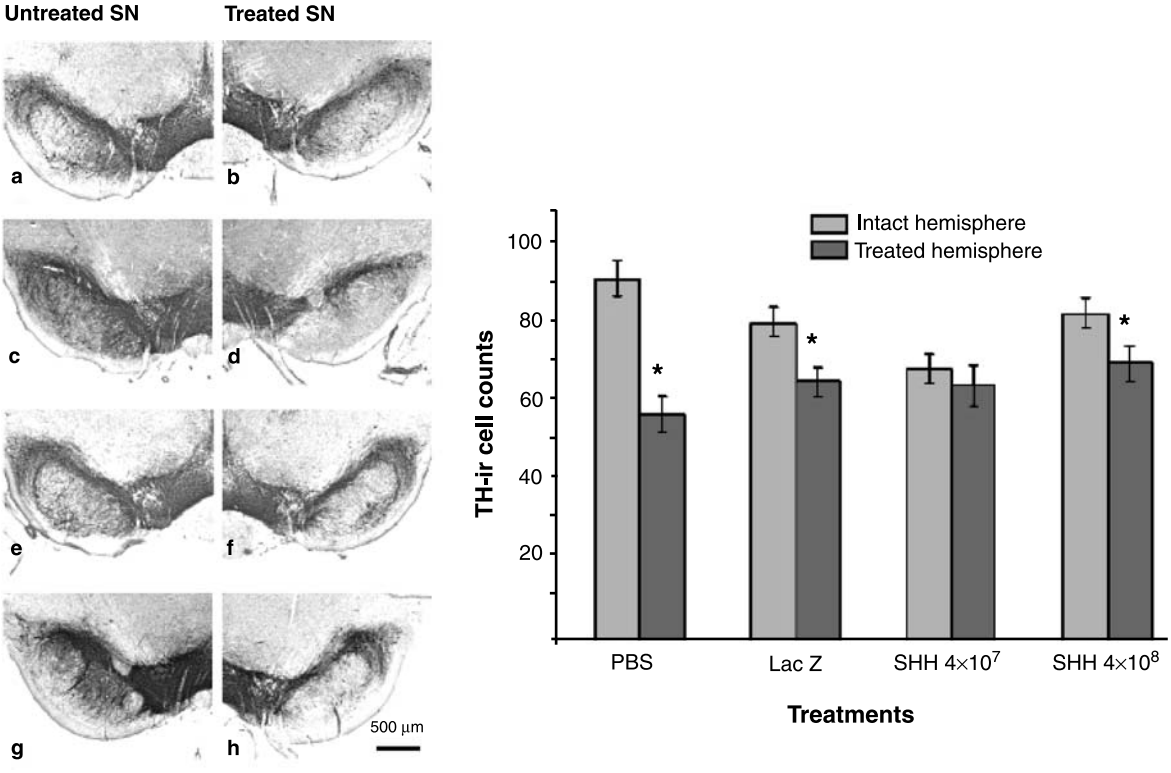

Fig. 4. Tyrosine hydroxylase immunoreactive cells at the level of the third nerve. Sections from the side of 6-OHDA administration are shown on the right, whilst the contralateral sides are shown on the left panels. Sections from animals treated with PBS – (**a**, **b**), AAV-LacZ 4×10^8 gc – (**c**, **d**), AAV-SHH 4×10^7 gc – (**e**, **f**), and AAV-SHH 4×10^8 gc – (**g**, **h**), are shown at 2.5× magnification. The right panel shows TH positive cell counts in the substantia nigra pars compacta following PBS, LacZ or AAV Treatments. TH positive cell counts in the substantia nigra pars compacta (±S.E.M.) of treated and untreated sides of two PBS groups (n = 13), LacZ 4×10^8 gc (n = 8), SHH at 4×10^8 (n = 9) and 4×10^7 gc (n = 6). Five sections per animal, taken from the level of the third nerve, were analysed Two-way ANOVA indicated significant changes due to side but not treatment (**b**). Animals treated with 4×10^7 gc of AAV-SHH exhibited no significant changes in TH+ve cells when compared to the control side. All other groups displayed significant decreases in TH positive cells ($\star = P < 0.05$ Paired two tailed t-test)

AAV-SHH the number of TH-ir cells was significantly smaller than the untreated substantia nigra (Fig. 4a, b).

Discussion

These data presented indicate that SHH delivered via an AAV vector may be capable of maintaining nigro-striatal dopaminergic function following a neurotoxic insult with 6-OHDA in the rat. Expression of the transfected human SHH mRNA was detected, indicating that the behavioural observations were attributable to treatment with AAV-SHH prior to 6-OHDA administration. Rotational behaviour induced by (+)-amphetamine administration was markedly reduced by pre-treatment with AAV-SHH and the striatal dopamine content was almost

entirely preserved in these animals. TH positive cell counts at the nigral level also showed a preservation of dopaminergic cells following treatment with AAV-SHH in 6-OHDA lesioned animals.

Using RT-PCR, expression of human SHH was confirmed in striatum of rats injected with AAV-SHH. The PCR product from amplification of pure viral DNA migrating to 325 bases mirrored the migration of the AAV-SHH cDNA product 325 bp band, suggesting that the two bands may be the same DNA sequence. Confirmation of the sequence proved the presence of human SHH cDNA, although the first 100 bases were not clearly resolved due to the low amount of PCR product recovered prior to analysis of the DNA sequence.

Previously, GDNF was shown to be effective in protecting dopamine containing cells from toxic insults as it completely prevented the appearance of (+)-amphetamine induced rotations in 6-OHDA treated rats (Shults et al., 1996; Bilang-Bleuel et al., 1997). However, experiments involving long-term treatment with GDNF in 6-OHDA treated rats, MPTP-treated monkeys and patients with PD have been complicated by the occurrence of side effects including weight loss and paresthesia (Zhang et al., 1997; Hoane et al., 1999; Kordower et al., 1999; Iravani et al., 2001; Nutt et al., 2003). In contrast, no adverse effects of AAV-SHH treatment were observed during the course of this study. No weight loss was observed, and in a previous study of SHH protein administration to MPTP-treated common marmosets, administration of SHH resulted in a slight weight gain (Dass et al., 2002).

Whether SHH is acting in a truly neuroprotective manner requires consideration. In the first week following 6-OHDA lesioning, rotational behaviour in response to (+)-amphetamine was present in AAV-SHH treated animals indicating that motor imbalance reflecting altered dopaminergic transmission between the two striatal hemispheres had developed. However, circling behaviour was reduced if not abolished in the subsequent weeks, suggesting that SHH acted to restore dopaminergic function rather than to protect against 6-OHDA toxicity. Optimal protection of dopamine neurons by GDNF from neurotoxic insult occurs when it is administered 6 hours before 6-OHDA lesioning (Kearns et al., 1997). However, in the case of AAV-SHH administration, protection against or prevention of a decline in striatal dopamine levels likely did not occur in the first week after 6-OHDA treatment, as administration of (+)-amphetamine elicited circling behaviour indicative of a decrease in the dopamine content of the ipsilateral hemisphere.

The increase in the turnover of striatal dopamine in the PBS treated group was expected as remaining dopamine neurones attempt to compensate for the partial loss of neurones by accelerating the synthesis of dopamine, indicated by increased levels of dopamine metabolites, DOPAC and HVA (Hefti et al., 1980, 1985). In animals treated with 4×10^7 gc AAV-SHH, however, dopamine turnover in the 6-OHDA lesioned striata was not different from that seen in the normal striatum. Therefore, the normalization of the dopamine turn over is likely due to a protective or restorative effect of 4×10^7 gc AAV-SHH.

The number of TH positive cells present in the SN in PBS and AAV-LacZ-treated animals indicated that only moderate damage had occurred at the nigral level following the intra-striatal administration of 6-OHDA. In these

partially-lesioned animals a loss of 20–30% TH positive cells was observed in the 6-OHDA treated side compared to the untreated hemisphere. A similar loss was also present in animals treated with 4×10^8 AAV-SHH, indicating that this dose of vector was unable to reduce nigral cell death. However, in animals treated with 4×10^7 gc AAV-SHH, the TH positive cell counts on the 6-OHDA treated side were virtually identical to those on the untreated side, showing a marked reversal of the 6-OHDA toxicity initiated in the striatum. However, it is not quite clear by what mechanism the effects of SHH on the nigrostriatal system is brought about. Recent evidence suggests that SHH acts in a restorative rather than a neuroprotective manner. It has been shown that intrastriatal administration of SHH robustly increased patched transcript leading to proliferation and differentiation of progenitor cells in the subventricular zone into new neurones (Charytoniuk et al., 2002). Furthermore, the evidence suggests that activation of the SHH pathway is a prerequisite for progenitor maintenance of stem cell niches in the adult brain (Machold et al., 2003).

The greater effectiveness of the 4×10^7 gc AAV-SHH dose over the 4×10^8 dose supports the previously suggested hypothesis of SHH possessing a bell shaped dose response curve (Marigo et al., 1996; Stone et al., 1996; Dass et al., 2002; Tsuboi et al., 2002; Charytoniuk et al., 2002). The molecular basis for this results from the ability of SHH to induce the expression of part of its receptor complex, patched-1. This protein acts to bind SHH and, if over-expressed, can quench SHH signalling, therefore providing an auto inhibitory loop to the SHH signalling system (Stone et al., 1996). In accord with this, it has been shown that overexpression of patched *in vivo* induced by adenovirus mediated over-expression of SHH results in dampening of the SHH pathway (Bergstein et al., 2002). Previous studies at using striatal malonate injections have indicated a loss of effectiveness of SHH when greater amounts of SHH protein or AAV-SHH are used (T. Engber, unpublished data). Questions arising about AAV-induced inflammatory reactions have also been investigated previously, but the concentrations used in this study were towards the lower end of the immune response spectrum (Davidson et al., 2000). Therefore, the loss of effectiveness of the AAV-SHH when used at a higher dose is unlikely to be due to a large immune response to the vector.

Overall, the behavioural results shown indicate that SHH delivered via a viral vector may be a promising strategy for treatment in PD. Following treatment with AAV-SHH, both behaviour and neurochemistry were improved whilst no adverse effects were observed. The behavioural effects were notably delayed in their appearance, suggesting that SHH acted in a restorative manner rather than protecting dopamine neurons from the toxicity of 6-OHDA.

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