Recombinant adeno-associated viral vector (rAAV) delivery of GDNF provides protection against 6-OHDA lesion in the common marmoset monkey (*Callithrix jacchus)*

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Abstract

Glial cell line derived neurotrophic factor (GDNF) has shown potential as a treatment for Parkinson's disease. Recombinant adeno-associated viral vectors expressing the GDNF protein (rAAV-GDNF) have been used in rodent models of Parkinson's disease to promote functional regeneration after 6-OHDA lesions of the nigrostriatal system. The goal of the present study was to assess the anatomical and functional efficacy of rAAV-GDNF in the common marmoset monkey (*Callithrix jacchus)*. rAAV-GDNF was injected into the striatum and substantia nigra four weeks prior to a unilateral 6-OHDA lesion of the nigrostriatal bundle. Forty percent of the dopamine cells in the lesioned substantia nigra of the rAAV-GDNF treated monkeys survived, compared with 21% in the untreated monkeys. Fine dopaminergic fibres were observed microscopically in the injected striatum of rAAV-GDNF treated monkeys, suggesting that rAAV-GDNF treatment was able to prevent, at least in part, loss of dopaminergic innervation of the striatum. Protection of dopamine cells and striatal fibre innervation was associated with amelioration of the lesion-induced behavioural deficits. rAAV-GDNF treated monkeys showed partial or complete protection not only in the amphetamine and apomorphine rotation but also in head position and the parkinsonian disability rating scale. Therefore, our study provides evidence for the behavioural and anatomical efficacy of GDNF delivered via an rAAV vector as a possible treatment for Parkinson's disease.

Keywords: Glial cell-line derived neurotrophic factor, Parkinson's disease, marmoset monkey, neuroprotection, behaviour, gene therapy, adeno-associated viral vector.

Introduction

Evidence suggests that glial cell-line derived neurotrophic factor (GDNF) could be a valuable therapeutic agent for Parkinson's disease (PD). Not only is GDNF found in the substantia nigra (SN) (Del-Fiacco et al., 2002; Kawamoto et al., 2000) and the striatum (Kawamoto et al., 2000; Nosrat et al., 1996), but there is some evidence that PD patients may have reduced levels of GDNF in their SN (Chauhan et al., 2001). This suggests that loss of GDNF may contribute to the process of degeneration of dopamine (DA) cells within the SN.

GDNF ameliorates parkinsonian neuropathology and behavioural symptoms in animals with lesions of the DA system when given before (Kearns and Gash, 1995; Kirik et al., 2000) or after (Kordower et al., 2000; Costa et al., 2001) the delivery of DA neurotoxins.

The progressive nature of the pathology underlying PD suggests that the long-term efficacy of any treatment, including GDNF, will depend on its continuous long-term delivery into the nigrostriatal system (Zurn et al., 2001; Freeman, 1997). Gene therapy using viral vectors is a promising tool for the long-term delivery of GDNF into specific target sites in the brain. In a study by Kordower and colleagues (2000), a lenti-viral vector encoding GDNF injected into the striatum and substantia nigra of parkinsonian monkeys provided behavioural recovery and neuronal sparing after the lesion. There are, however, other suitable vector systems. Recombinant adeno-associated viral vectors (rAAV) in particular have several advantages. First, wild-type AAV is a non-pathogenic dependovirus that is unable to replicate without co-infection of the host by a helper virus, such as adenovirus or herpes simplex virus (Berns and Linden, 1995; Muzyczka, 1992). Second, it can infect non-dividing cells *in vivo* with very high efficiency making it an excellent tool for application in the adult brain (Samulski et al., 1999).

Before any viral vector method of gene delivery is to proceed to the clinic, it should ideally be assessed in non-human primates. Delivery of GDNF via rAAV has only been tested in rodent models of PD (e.g. Kirik et al., 2000; Wang et al., 2002; Mandel et al., 1999; Mandel et al., 1997). The goal of the present experiment was to assess the behavioural and anatomical efficacy of rAAV-GDNF when given four weeks prior to a unilateral 6-hydroxydopamine (6 OHDA) nigrostriatal bundle lesion in the common marmoset monkey (*Callithrix jacchus)*. rAAV-GDNF was given 4 weeks prior to the 6-OHDA lesion because it has been shown that GDNF is not expressed in appreciable quantities until at least two weeks after the viral injections (Mandel et al., 1999). It would therefore not have been appropriate to give rAAV-GDNF after the 6-OHDA lesion since this lesion has a very acute time course.

Methods

Experimental Design

Monkeys were assessed on a range of behavioural measures before surgery. rAAV-GDNF was injected unilaterally into the SN and striatum of one group of monkeys (group GDNF-L; n = 6). The other group (group CON-L; n = 5) did not undergo rAAV surgery. Both groups of monkeys were re-tested on all behavioural measures one and three weeks after the rAAV-GDNF surgery. On the fourth week, the monkeys in both groups received a unilateral 6-OHDA lesion of the nigrostriatal bundle. Monkeys were then re-tested one, three and five weeks after 6-OHDA surgery. The appropriate control for this, the first experiment to use marmoset monkeys to assess rAAV delivery of gene therapy, was a lesion-alone group because the injection of rAAV expressing a different protein could have had a deleterious effect in this species leading to an over-optimistic interpretation of the effects of rAAV-GDNF. See Fig. 1 for a summary of the experimental schedule. When behavioural testing was complete, monkeys were perfused and their brains removed and examined histologically.

Recombinant AAV-GDNF vector preparation

rAAV-GDNF was manufactured at the University of Florida Gene Therapy Center Vector Core. A chicken β-actin promoter with a cytomegalovirus enhancer with a down-stream chicken β-actin/rabbit β-globin hybrid intron drove the production of the transgene as described in detail elsewhere (Xu et al., 2001; Conway et al., 1997; Zolotukhin et al., 1999). The construct included the woodchuck post-translational regulatory element downstream of the GDNF gene. The vector had a final titre of 4×10^{11} IU/ml determined by the infectious centre assay.

Monkeys and surgery

Experimental procedures were carried out under a Project Licence in accordance with the U.K. Animals (Scientific Procedures) Act 1986. Eleven laboratory-bred, adult common marmosets (*Callithrix jacchus*), seven females and four males were used. The monkeys weighed 300-487g and were 37-63 months old at the start of the experiment. Each monkey was housed with a cage-mate of similar age. Six monkeys (group GDNF-L; three males, three females) received unilateral rAAV -GDNF injections in the striatum and substantia nigra, four weeks before receiving ipsilateral 6-OHDA lesions of the nigrostriatal bundle. Five monkeys (group CON-L; one male, four females) received only the unilateral 6-OHDA nigrostriatal bundle lesion. The coordinates for the rAAV-GDNF and 6-OHDA injections were derived from a stereotaxic atlas of the marmoset brain (Stephan et al., 1980) and are shown in Table 1.

In preparation for either the rAAV-GDNF or the 6-OHDA injections, monkeys were anaesthetized with 0.5ml alphaxalone-alphadolone (Saffan; Schering Plough Ltd, Welwyn Garden City, UK; 12mg/ml given intramuscularly). A supplementary dose of 0.2-0.3ml Saffan was given during surgery if necessary. The monkeys were given a post-operative analgesic (Finadyne; Schering-Plough Animal Health, Mildenhall, UK; 0.1mg/kg, subcutaneously) and

kept in a warm incubator until recovery and then returned to their home cages, usually on the day following surgery.

rAAV-GDNF surgery

The monkeys in group GDNF-L were injected unilaterally in the striatum and substantia nigra with rAAV-GDNF suspended in phosphate-buffered saline (PBS) solution. The injections were made with a 29g needle at a rate of 0.25µl per minute. Two injections of 3µl were made in the substantia nigra. Five injections of 3µl were made in the striatum. After each injection, the needle was kept in place for an additional four minutes before being withdrawn.

6-OHDA surgery

Four weeks after the rAAV-GDNF surgery, the monkeys in groups GDNF-L and CON-L underwent 6-OHDA surgery, as previously described (Annett et al., 1992). The injections were made unilaterally and, in group GDNF-L, were made ipsilateral to the rAAV-GDNF injections. 6-OHDA Hbr (4mg/ml free base weight dissolved in 0.01% ascorbate/saline) was freshly prepared and stored on ice immediately prior to use. Each injection was made using a 10µl Hamilton syringe at a rate of 0.5µl/min. The volume for each injection was 2µl except for the most posterior injection which was 3µl. After each injection, the needle was left in place for a further two minutes before being withdrawn.

Histology

All the monkeys were perfused for histological analysis of their brains after completing their last behavioural testing session. The monkeys were premedicated with 0.05ml ketamine (Vetalar; Shering-Plough, Welwyn Garden City, UK; 100mg/ml, intramuscularly) and deeply anaesthetised with 0.6ml sodium pentobarbitone (200mg/ml, intraperitoneally) prior to being perfused. Monkeys were perfused transcardially with 500ml 0.1M phosphate-buffered saline

pH7.4, followed by 1000ml of 4% paraformaldehyde in PBS. The brains were removed and placed in 4% paraformaldehyde solution for 24 hours and then transferred to 30% sucrose solution in PBS for 4 days at 4^{o}C.

Sections were cut on a freezing stage microtome at 40µm thickness and stained for tyrosine hydroxylase (TH), GDNF and vesicular monoamine transporter-2 (VMAT). Free-floating sections were treated for 10 min with 10% methanol/3% hydrogen peroxide in distilled water to remove endogenous peroxidase, then washed in potassium phosphate buffered saline, pH7.4 (KPBS) and incubated overnight at room temperature in the appropriate primary antibody with 2% serum in 0.25% Triton X-100 in KPBS. The primary antibodies used were rabbit anti-TH antibody (1:250, Pel-Freeze, Rogers, AR, USA), goat anti-GDNF antibody (1:2000, R&D systems, Minneapolis, MN), and rabbit anti-VMAT antibody (1:2000, Chemicon, CA). Sections were then washed in KPBS and incubated for one hour in the appropriate secondary antibody in 2% serum in KPBS. The secondary antibodies used were biotinylated goat anti-rabbit (1:400; Vector Labs, Burlingame, CA) for TH and VMAT immunochemistry and biotinylated horse anti-goat (1:400; Vector Labs, Burlingame, CA) for GDNF immunochemistry. Subsequently, sections were washed in KPBS and incubated for 2 hours in a streptavidin-biotin-peroxidase kit (ABC, Vector Laboratories, Burlingame, CA; in 2% serum in KPBS). After washes in KPBS, the sections were treated with 3,3'-diamino benzidine (1% in Tris buffer). The sections were washed in KPBS, mounted on 1% gelatin-coated microscope slides and left to dry over night. After rehydration and dehydration through graded alcohols, the slides were cleared in xylene and coverslipped using DPX mountant. A separate series of sections was stained for cresyl violet.

Quantification of dopamine cells in the SN pars compacta (SNpc)

Quantification was made separately for TH immunoreactive (TH-IR) and VMAT immunoreactive (VMAT-IR) cells in the SNpc. Prior to quantification, the borders of the SNpc were delineated in order to separate the DA cells in the SNpc from the DA cells in the ventral tegmental area (VTA; see Fig. 6a for example). In the anterior regions of the SN, all the visible immunoreactive cells were considered to be in the SNpc since the VTA is not present at this level. In the central and caudal sections the most lateral roots of the third nerve separated the SNpc and the ventral tegmental cell group (Stephan et al., 1980). At the most caudal level, the SNpc was the dense immunoreactive cell group found ventral to the medial lemniscus and the retrorubral area. The immunoreactive cells in the SN pars retriculata (SNpr) were included in the SNpc cell counts because in primates, including marmosets, these cells comprise fingers of DA cells belonging to the extensions of the SNpc that protrude deep into the SNpr (Lewis and Sesack, 1997). Typically seven or eight sections per monkey were used for each immunoreactive measure.

The number of DA cells was quantified using the optical fractionator method (West et al., 1991). This method is advantageous because the total number of cells estimated is not affected by the tissue shrinkage that occurs in immunochemical processing, nor is there a need to determine the volume of the area being sampled. Sampling was done as previously described (Kirik et al., 1998) using an Olympus CAST system (Olympus Danmark A/S, Denmark). After the region of interest was outlined for each slide under a 4X magnification, the first counting frame was randomly placed over this area. The computer-operated counting frame was then moved systematically through the area of interest until the whole area was sampled. The X-Y step length was chosen so that between 100 and 200 cells would be counted per SNpc for each immunoreactive measure. The total number of cells was estimated using the optical fractionator formula according to West *et al.* (1991). As far as we are aware, we are the only laboratory that has used the optical fractionator method of stereological counting to determine dopamine

populations in the nigrostriatal system in marmoset monkeys. However, this method has been widely used in rodent studies (e.g. Georgievska et al., 2002b; Kirik et al., 2001b; Winkler et al., 2002; Andsberg et al., 2002).

TH-IR cell size was determined using the Leica program WIN-Q under 40x magnification. One section for each side of each monkey was chosen for the analysis at the level of the third nerve. The size was determined for those cells located within a 150µm x 110µm area in the SNpc near the border of the third nerve. In this area TH-IR cells were located in the lesioned and unlesioned side of both groups of monkeys.

To determine whether GDNF treatment resulted in an increase in TH-IR cells in the striatum as previously observed in primates (Palfi et al., 2002), one section from each animal at the commissural level was observed under 20x magnification and the cells counted manually. Cells were counted separately for each side and for each caudate and putamen.

Body weights

GDNF administration been been shown to cause weight loss in experimental animals (Lapchak et al., 1997a; Lapchak et al., 1997b). Therefore, all monkeys were monitored for changes in weight prior to any experimental manipulation, one and three weeks after the rAAV-GDNF surgery and one, three and five weeks after the 6-OHDA surgery.

Behavioural Tests

Disability rating scale: This scale quantifies the degree to which each monkey shows 'parkinsonian' signs assessed both in spontaneous and induced behaviours (Eslamboli et al., 2003). All readings were made in the home room when other non-experimental monkeys in the room were active. For spontaneous behaviours, the experimenter stood approximately two

metres in front of the cage and observed each monkey's behaviour for three separate one minute intervals, assessing the following behaviours: 1) akinesia, 2) impaired climbing, 3) abnormal posture and 4) tremor. For induced behaviours, the experimenter tempted each monkey three times with a small piece of marshmallow (~5mm^3), and on each occasion gave a score for the following: 1) clumsiness, 2) bradykinesia, and 3) poor balance. Each monkey was given a score of zero to three for each measure for each minute interval, with zero representing normal behaviour and three representing the greatest impairment. The total score consisted of the sum of spontaneous and induced behavioural scores, with 63 representing the greatest possible level of impairment.

Head position bias: This measure quantifies the degree to which each monkey shows a positional head bias towards its ipsilesional side, a measure shown to be sensitive to unilateral DA loss in marmosets (Annett et al., 1992). For this measure, the experimenter recorded the position of each monkey's head (left, right or straight ahead) every second for three separate one-minute intervals to the beat of a metronome. The measure was made on four (usually consecutive) days. The score for each day consisted of the total number of seconds the monkey spent looking ipsilateral to its lesion, minus the total number of seconds spent looking contralaterally. The final score was an average across days.

Rotation: Each monkey was assessed for spontaneous rotation and after being challenged with the DA agonists amphetamine and apomorphine. For all rotations, each monkey was placed in a Plexiglass box (~ 20cm wide x 17cm deep x 17cm high) in a test room where its behaviour was videotaped after the experimenter had left the room. For spontaneous rotation, the monkey's behaviour was videotaped for 30 minutes. For amphetamine-induced rotations, each monkey was videotaped for 30 minutes, starting 30 minutes after they had been injected with the drug (Sigma, St Louis, MO, USA; 0.5mg/kg dissolved in saline, given intramuscularly). For

apomorphine-induced rotations, each monkey was videotaped for 60 minutes starting immediately after receiving the drug (Sigma, St Louis, MO, USA; 0.05mg/kg dissolved in 0.1% ascorbate in saline, given intramuscularly).

The behavioural tests were administered by one of two experimenters (AE/LC), who were blind as to the treatment group. In an inter-rater reliability test between the experimenters a high correlation coefficient was obtained on the rating scale (r = 1.0) and the head position bias task (r = 0.89).

Statistical analysis: Three comparisons were made between groups for the behavioural data. First, comparisons were made between groups prior to the rAAV-GDNF surgery, using *t*-tests. Second, the groups were compared for the two testing sessions after the rAAV-GDNF surgery, prior to the 6-OHDA lesion, by using repeated measures ANOVA. Third, the post 6-OHDA lesion data were analysed using a repeated measures ANOVA. Histological data were also analysed by ANOVA. *Post hoc* tests were performed after any significant interactions using Bonferroni t-tests.

Results

Behavioural Tests

Disability rating scale (see Fig. 2): Prior to the rAAV-GDNF surgery, group GDNF-L and group CON-L had low rating scores which did not differ from each other (t= 0.25, n.s.). One and three weeks after undergoing rAAV-GDNF surgery, both groups continued to have low scores which did not differ from each other (Group effect $F[1,9] = 0.01$, n.s.). Repeated measures ANOVA on disability scores after the 6-OHDA lesion showed that rAAV-GDNF treatment afforded functional protection (Group effect $F[1,9] = 8.56$, $P<0.05$). Although both groups showed some recovery with time (Week effect; $F[2,18] = 7.43$, $P<0.01$), only the GDNF-L group reached pre-

lesion levels by the end of the experiment (i.e. week 9). The disability observed after 6-OHDA surgery consisted mainly of hunched posture and a reduction in movement and climbing. No tremor was observed.

Head position bias (see Fig. 3): Behavioural testing before the rAAV-GDNF surgery showed that both monkeys in both groups spent the same amount of time with their heads towards the side to be lesioned as towards the control side (t = 1.53, n.s.). Following rAAV-GDNF surgery, group GDNF-L had a contralesional head bias, in comparison to group CON-L (Group effect $F[1,9] = 8.4$, $P<0.05$). After the 6-OHDA surgery, both groups had an ipsilesional bias. rAAV-GDNF treatment, however, afforded significant protection at all time points after the 6-OHDA surgery (Group effect; $F[1,9] = 16.102$, $P<0.01$).

Rotation (see Fig. 4A and 4B): Neither group rotated spontaneously prior to, or after, 6-OHDA surgery (data not shown). Prior to rAAV-GDNF surgery, both groups had low amphetamine-induced rotational scores which did not differ from each other (t = 0.146, n.s.). One and three weeks after undergoing rAAV-GDNF surgery, both groups continued to have low scores which did not differ from each other ($F[1,9] = 0.685$, n.s.). After both groups had received the 6-OHDA lesion, group CON-L at first rotated contralesionally (week 5) and then ipsilesionally (weeks 7 and 9) in response to amphetamine, in contrast to group GDNF-L where the rAAV-GDNF treatment protected against this functional impairment (Group effect; $F[1,9] = 5.906$, $P<0.05$, Fig. 4A). A week by lesion interaction indicated that the deficit in group CON-L developed as the weeks progressed ($F[2,18] = 9.296$, $P<0.01$).

Neither group rotated in response to apomorphine at any time prior to the 6-OHDA surgery (Fig. 4B). After 6-OHDA surgery, although both groups rotated contralesionally, rAAV-GDNF

treatment may have had some functionally beneficial effect (Group effect; $F[1,9] = 4.599$, $P = 0.06$).

Body weight

Neither experimental group experienced significant weight change during the experiment.

Histology

Similar needle tracks were identified, directed towards all the target areas, in both groups of animals. There was a very localised gliotic reaction surrounding each track.

Cell counts:

TH-IR: ANOVA revealed a significant difference between the lesioned and unlesioned sides ($F[1,9] = 151.62$, $P<0.001$) across all animals, as well as between groups ($F[1,9] = 17.97$, $P<0.01$). Although the group x side interaction was not significant, the number of TH-IR cells on the lesioned side in group CON-L was 21.1 ± 2.4 % of the number of cells on the unlesioned side, while in group GDNF-L the number of TH-IR cells on the lesioned side was 40.1 ± 5.9 % of the number of cells on the unlesioned side. This difference was statistically significant ($t = 2.755$, $P<0.05$) (Figs. 5A and 6).

The mean size of the TH-IR cells in the SN in the GDNF-L monkeys was greater than that of the TH-IR cells in the SN in the CON-L monkeys both on the intact (213.5 ± 20.7 μm^2 and 150.1 ± 9.7 μm^2, respectively) and the lesioned side (193.3 ± 15.8 μm^2 and 110.9 ± 8.2 μm^2, respectively; $F[1,9] = 14.53$, $P < 0.01$). In both groups, however, the cells found on the lesioned side were smaller then those on the intact side ($F[1,9] = 8.03$, $P < 0.05$). These results are consistent with previous studies showing an increase in cell size after GDNF treatment (Gash et al., 1996; Kordower et al., 2000).

Although Palfi *et al*. (2002) report that GDNF treatment in an MPTP model of PD increased the number of TH-IR cells in the lesioned striatum, GDNF treatment in the present study did not result in a similar increase.

VMAT-IR: ANOVA revealed a significant difference between the lesioned and unlesioned sides ($F[1,9] = 206.52$, $P<0.001$) across all animals, as well as between groups ($F[1,9] = 6.02$, $P<0.05$). The group x side interaction approached significance ($F[1,9] = 4.66$, $P = 0.059$). The number of VMAT-IR cells on the lesioned side in group CON-L was 16.1 ± 1.5 % of the number of cells on the unlesioned side, while in group GDNF-L the number of VMAT-IR cells on the lesioned side was 39.1 ± 6.5 % of the number of cells on the unlesioned side. This difference was statistically significant ($t = 3.181$, $P<0.05$; Figs. 5B and 7).

Microscopic fibres in the lesioned striatum:

The group GDNF-L monkeys displayed a fine network of TH-IR fibres particularly in the posterior parts of the caudate and putamen ipsilateral to the lesion. However, the fibre density was usually very low and we were unable to quantify it by optical density measures. In only one monkey in the GDNF-L group was there sustantial fibre density within the striatum on the lesion side (See Fig. 8). In contrast, no fibre networks could be detected ipsilateral to the lesion side in most group CON-L monkeys (Fig. 8). The same pattern was observed for VMAT-IR fibres (data not shown).

In the group GDNF-L monkeys an extensive network of disorganized TH-IR and VMAT-IR fibres were observed dorsal to the lesioned SN. At the level of the third nerve, this network extended laterally to the peripeduncular nucleus and dorsally to the ventral thalamus and was absent from the intact side (see Figs. 6 and 7).

GDNF expression:

Robust GDNF-immunoreactivity was observed through the greater rostro-caudal extent of the ipsilesional side of the GDNF-L group brains (Fig. 9). GDNF-immunoreactivity was found approximately 2mm anterior to the most rostral rAAV-GDNF injection site in the striatum (compare Figs. 9A and 9B). The GDNF expression extended caudally to the superior colliculus, leading to a distribution of 4mm from the most caudal rAAV-GDNF injection. In contrast group CON-L did not display any GDNF-immunoreactivity (data not shown).

Ipsilesionally, GDNF-immunoreactivity was found in the striatum at each rostro-caudal level and extended to surrounding structures. At the mid-striatal level, GDNF-immunoreactivity was found in the caudate and the putamen and extended to the surrounding white matter tracts (compare Figs. 9C and 9B). GDNF-immunoreactivity was also found in cortical areas along and adjacent to the rAAV-GDNF injection needle tracks. At the posterior striatal level, GDNF-immunoreactivity was observed in the caudate and the dorsolateral portion of the putamen, extending to the globus pallidus, the internal capsule and the thalamus (Fig. 9E). At the level of the SN, GDNF-immunoreactivity was found throughout the SN, extending laterally to the peripeduncular nucleus and dorsally to the medial portion of the thalamus (Fig. 9H).

GDNF expression was mostly extracellular (Fig 9I), although in some monkeys a few GDNF-immunoreactive cell bodies were found in the SN pars reticulata (Fig. 9J).

Discussion

In the present study, we investigated the behavioural and anatomical protection provided by rAAV vector mediated over-expression of GDNF against a unilateral 6-OHDA lesion of the nigrostriatal dopaminergic system in the common marmoset monkey. Anatomically, rAAV-

GDNF treatment increased survival of TH-positive cells in the SN and protected a fine network of TH-IR fibres, particularly in the posterior striatum and in the tegmentum dorsal to SN. Functionally, whereas the lesion-control group showed stable deficits in motor behaviour after the dopamine lesion, the lesioned but GDNF treated animals demonstrated a clear lack of, or reduction in, these lesion-induced impairments.

Anatomical protection:

Unilateral rAAV-GDNF delivery to the striatum and the SN resulted in robust extracellular GDNF-immunoreactivity in the brains of the GDNF-treated group. GDNF-immunoreactivity was found in the striatum and in structures beyond the injection targets, including the white matter tracts adjacent to the striatum, and in parts of the cortex. The latter immunoreactive areas corresponded to the injection tracks and were most likely due to the rAAV-GDNF infection of cells along these paths rather than GDNF diffusion from the deposit site. GDNF-immunoreactivity was found in the ventral thalamus and the peripeduncular nucleus as well as the contralateral VTA, suggesting that some SNpr cells were infected by the rAAV vector. A previous study in which GDNF was delivered via the rAAV vector in rodents also observed substantial distribution of the protein away from the vector deposit site (Kirik et al., 2000). In the present experiment, the GDNF protein was observed up to 4mm in the anterior-posterior axis to the deposit site, which was greater than that seen in the rodents (Kirik et al., 2000). The considerable GDNF distribution in the present study may have resulted from a large number of cells being infected, due to dispersion of the large volume of injected vector (15µl). However, levels of extracellular diffusion of GDNF may also have affected distribution. GDNF binds to heparin-sulphate side chains of the extracellular-matrix proteoglycans (Airaksinen and Saarma, 2002) which may determine the extent of diffusion (Hamilton et al., 2001).

In the present study we used the nigrostriatal 6-OHDA bundle model of PD in order to ensure a severe depletion of DA cells. This type of lesion produces a severe and stable behavioural deficit in the marmosets, allowing for the assessment of behavioural improvements due to therapeutic interventions. However, it is worth noting that with this model the degeneration following the administration of 6-OHDA is acute, in contrast to the progressive degeneration seen in the intra-striatal 6-OHDA rodent model or the primate 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model. The demonstration of beneficial effects of GDNF in this stringent model is particularly encouraging with respect to its clinical potential where disease progression is slow. A greater level of nigral protection has been obtained using viral vector mediated delivery of GDNF in more progessive models (e.g. Kordower et al., 2000; Kirik et al., 2000; Kirik et al., 2001a). An assessment of the efficacy of rAAV-GDNF in partial lesion models of PD in primates will be of interest.

Although this is the first study to report the effects of GDNF after nigrostriatal bundle lesions in monkeys, a series of experiments assessing the efficacy of GDNF (via protein injections) has been published with the equivalent model in rats. These studies reported a similar or greater degree of cellular protection (Kearns et al., 1997; Kearns and Gash, 1995; Sullivan et al., 1998).

We were unable to detect *gross* anatomical protection of the dopaminergic terminals resulting from GDNF over-expression within the striatum and SN as there were no quantitative differences between treated and control monkeys in TH-IR or VMAT-IR fibres on optical density measures. However, detailed microscopical analysis revealed a *fine* network of thin calibre TH-IR and VMAT-IR fibres in the GDNF treated group, particularly in the posterior striatum. The staining intensity of these fibres was faint and was therefore not detected by optical density measurement. The fibre sparing observed in this experiment is much less than that reported in the MPTP model by Kordower and colleagues (2000). In that study, lenti-viral

delivery of GDNF provided relatively complete protection to the dopaminergic fibers in the striatum as well as the DA cells in the SN. This difference from the present study may be due to the use of different lesion models. The nigrostriatal bundle lesion is acute and causes rapid damage to the dopaminergic cell bodies, followed by an anterograde degeneration of the projection fibres. In contrast, the MPTP treatment may initially induce striatal pathology followed by a retrograde degeneration of the nigral cells a few days to a week after the insult (Eberling et al., 1997). The MPTP model is therefore similar to the *striatal* 6-OHDA lesion model in rodents, where the degeneration also starts in the striatum and proceeds to the SN. This allows a time-window for intervention and indeed, data obtained in rats using both recombinant lentiviral vectors and rAAV vectors encoding GDNF indicate that robust fibre protection in the striatum can be obtained when using the intrastriatal 6-OHDA lesion model (Kirik et al., 2000). However, the mechanism of DA loss in PD is not fully understood and so the ability of GDNF treatment to protect DA cell bodies directly may be important (Georgievska et al., 2002a).

Aberrant sprouting of TH-IR fibres was observed, in GDNF treated monkeys, dorsal to the rAAV-GDNF injection site in the SN. This form of sprouting suggests a local response to the GDNF protein and has been observed in previous studies in rats, after injections of rAAV-GDNF or recombinant GDNF protein into the SN (Kirik et al., 2000).

Behaviour:

rAAV-GDNF treatment provided functional protection against the behavioural deficits occurring as a result of 6-OHDA lesion. After the 6-OHDA lesion, the lesion-control group showed parkinsonian deficits in a battery of spontaneous and drug-induced tests including the disability rating scale, head position bias, and the amphetamine rotation tests. Among these tests, head bias and amphetamine rotation were the most sensitive measures in detecting the partial protection of the DA system, since monkeys injected with the rAAV-GDNF vector were

significantly improved and showed near complete recovery on these measure, returning to prelesion levels five weeks after the 6-OHDA lesion.

In conclusion, we have shown that in marmoset monkeys rAAV delivery of GDNF was able to protect SN cells against the toxic effects of a 6-OHDA lesion of the nigrostriatal system and to protect against the functional deficits occurring as a result of the lesion. These results encourage the view that viral vector-mediated delivery of GDNF might be of use in the treatment of PD. However, delivery of GDNF resulted in the distribution of the protein beyond target structures. The functional behavioural consequence of unwanted distribution of gene products as well as the over-expression of the products in target areas may be a potential problem in future clinical application of this procedure. The determination of the quantity of a particular vector construct needed for appropriate distribution of a gene product and its dosing by regulatable vectors in the primate brain will be a necessary step towards safer clinical application of vector-mediated GDNF delivery. Although more work is needed with this method of delivery in monkeys, these data are encouraging and support further development of the rAAV vector system for treatment of PD, given the long-term progressive nature of the disease.

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Figure 1: Experimental schedule for surgery and behavioural assessments. Monkeys were assessed on head position, disability rating scale and spontaneous and drug-induced rotational biases at various time intervals. Group GDNF-L monkeys received rAAV-GDNF in the striatum and SN after the first assessment. Group CON-L monkeys did not undergo surgery at this time. Four weeks after the rAAV-GDNF surgery, both groups received a 6-OHDA lesion of the nigrostriatal bundle.

Figure 2: Disability ratings scale. After 6-OHDA lesion, rAAV-GDNF treatment afforded functional protection against the lesion-induced disability (Group difference: $F[1,9] = 8.56$, $P<0.05$) and produced recovery to prelesion levels.

Figure 3: Head position bias. After 6-OHDA lesion, rAAV-GDNF treatment afforded functional protection against the lesion-induced head bias (Group difference: $F[1,9] = 8.4$, $P<0.05$).

Figure 4: Drug induced rotation. (A) Amphetamine-induced rotation. After the 6-OHDA lesion, rAAV-GDNF treatment afforded functional protection against the amphetamine-induced rotation (Group difference: $F[1,9] = 5.91$, $P<0.05$) and produced recovery to prelesion levels. (B) Apomorphine induced rotation. After 6-OHDA lesion, rAAV-GDNF treatment afforded some functional protection against the apomorphine-induced rotation which aproached statistical significance (Group difference: $F[1,9] = 4.6$, $P = 0.06$). * group difference, $P < 0.05$.

Figure 5: (A) TH-IR and (B) VMAT-IR cell counts in the SN.

Figure 6: TH immunochemistry of the SN. A: intact side in a monkey from the CON-L group. B: lesioned side in the same monkey from the CON-L group. C: intact side in a monkey from the GDNF-L group. D: lesioned side in the same monkey from the GDNF-L group. E and F are magnifications of the area dorsal to the SN shown by arrowheads in A and B respectively. G and H are magnifications of the area dorsal to the SN shown by the arrowheads in C and D respectively. rAAV-GDNF treatment provided neuroprotection to TH-IR cells in the GDNF-L group monkeys (compare D with B). In the lesioned side of group GDNF-L monkeys, a dense network of TH-IR fibres was observed. This network was absent on the intact side (compare D with C). Bar = 1mm. Bar in magnifications = 100µm.

Figure 7: VMAT immunochemistry of the SN. A: intact side in a monkey from the CON-L group. B: lesioned side in the same monkey from the CON-L group. C: intact side in a monkey from the GDNF-L group. D: lesioned side in the same monkey from the GDNF-L group. E and F are magnifications of the area dorsal to the SN shown by arrowheads in A and B respectively. G and H are magnifications of the area dorsal to the SN shown by arrowheads in C and D respectively. rAAV-GDNF treatment provided neuroprotection to VMAT-IR cells in the GDNF-L group monkeys (compare D with B). In the lesioned side of GDNF-L group monkeys, a dense network of VMAT-IR fibres was observed. This network was absent on the intact side (compare D with C). Bar = 1mm. Bar in magnifications = 100µm.

Figure 8: TH immunochemistry of the striatum. A: intact side in a monkey from the CON-L group. B: lesioned side in the same monkey in the CON-L group. C: intact side in a monkey from the GDNF-L group. D: lesioned side in the same monkey from the GDNF-L group. E, F, G

and H are magnifications of the areas highlighted by the arrows in A, B, C and D, respectively. Microscopic fibres were observed in the injected side in the GDNF group monkeys (compare D and B, and H and F). Bar = 1mm. Bar in inset = 100µm.

Figure 9: GDNF immunochemistry in the intact and injected side in a monkey from the GDNF-L group. A and B: anterior striatum in the intact and injected side, respectively. Note GDNF in the caudate and surrounding fibre tracts of B. C and D: mid-striatum in the intact and injected side, respectively. Note GDNF in the striatum as well as internal capsule and tracts dorsal to the striatum in D. E and F: posterior striatum in the intact and injected side, respectively. GDNF is found in striatum, internal capsule (IC), thalamus and globus pallidus (GP) in F. G and H: GDNF immunochemistry of the SN in the intact and injected side, respectively, in a monkey from the GDNF-L group. GDNF expression was found throughout the injected SN and extended to the peripeduncular nucleus (pp) and dorsally to the thalamus. Note the general lack of GDNF expression in the intact side. I and J are magnifications of the area indicated by the asterisk and arrowhead in H respectively. Although staining was found mainly extracellularly, some intracellular staining was seen in the substantia nigra pars reticulata, SNpr, (compare extracellular staining in I with intracellular staining in J). The arrowheads in J point to cells found in the SNpr. Bar for A-H = 1mm. Bar for I and J = 50µm

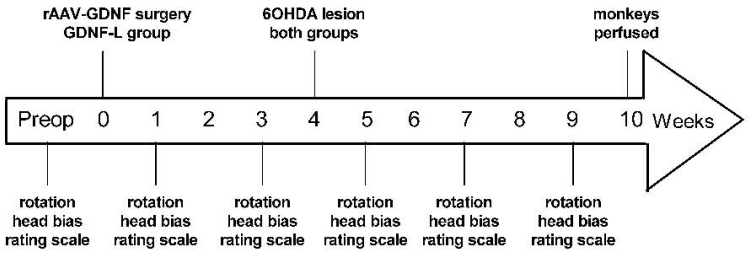
rAAV-GDNF surgery
GDNF-L group
6OHDA lesion
both groups
monkeys
perfused
Preop 0 1 2 3 4 5 6 7 8 9 10 Weeks
rotation head bias rating scale
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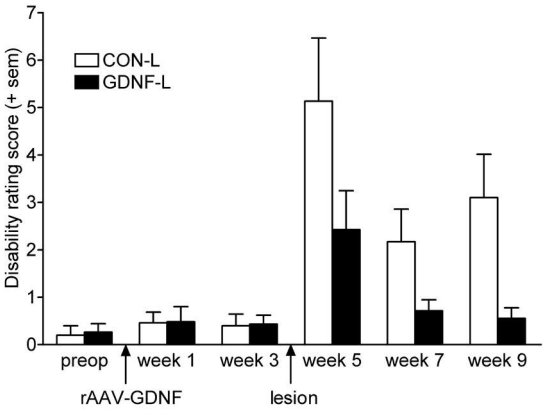
Fig 1


Disability rating score (+ sem)
CON-L
GDNF-L
0
1
2
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4
5
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7
preop
week 1
week 3
week 5
week 7
week 9
rAAV-GDNF
lesion

Fig 2

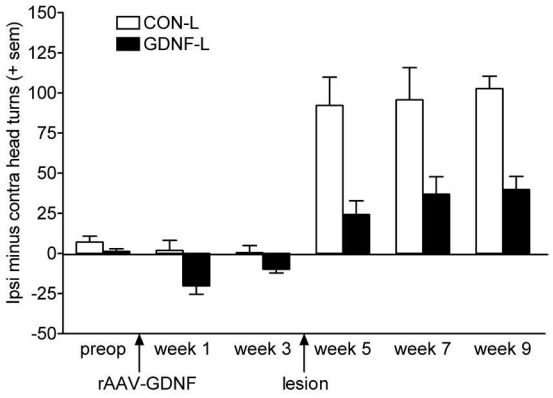

Fig 3

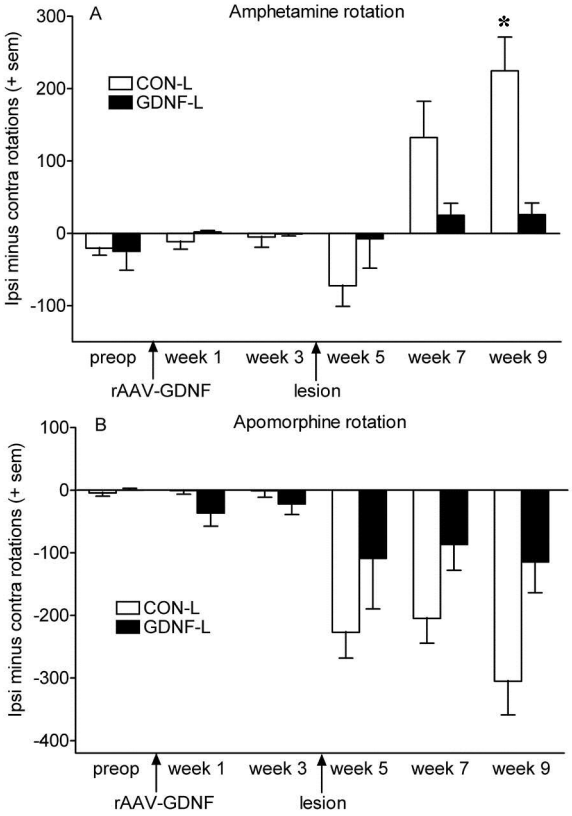

Fig 4

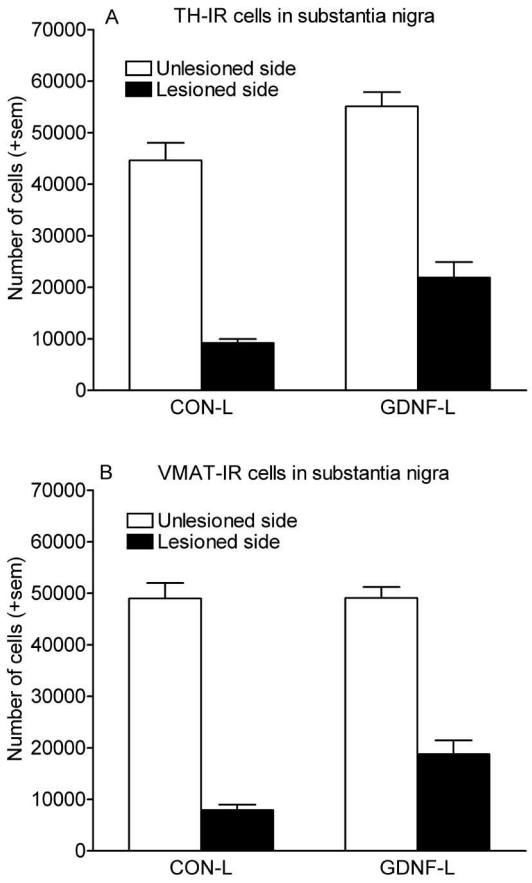

Fig 5

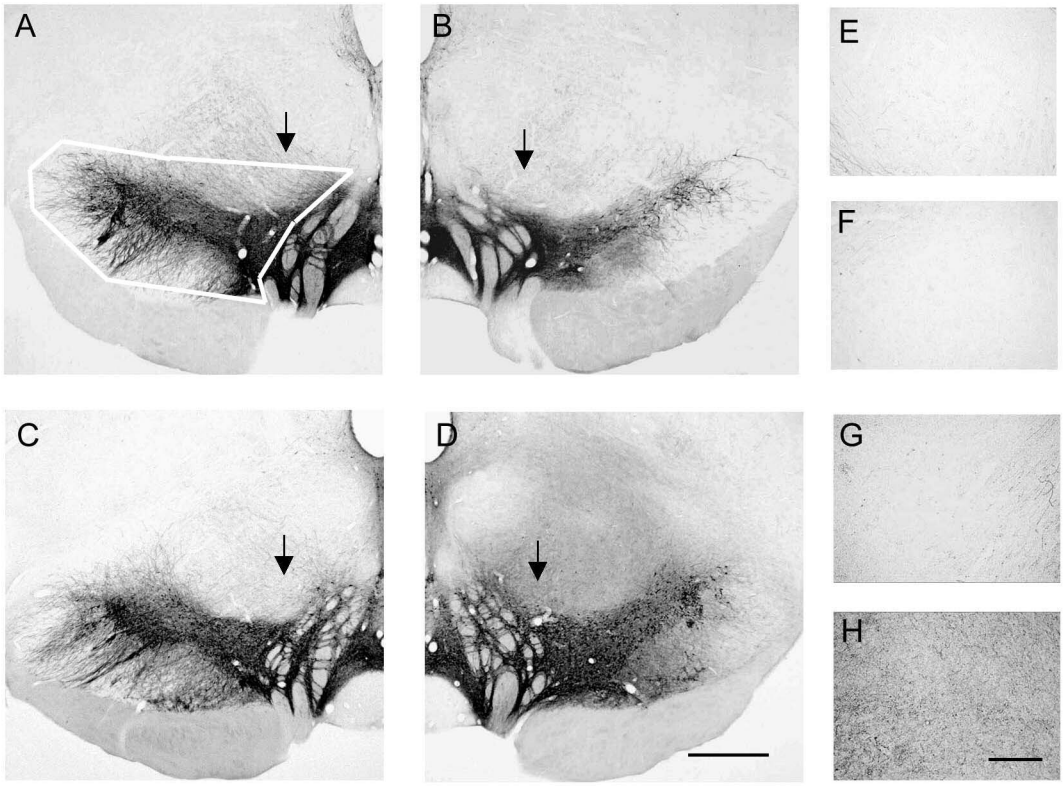

Fig 6

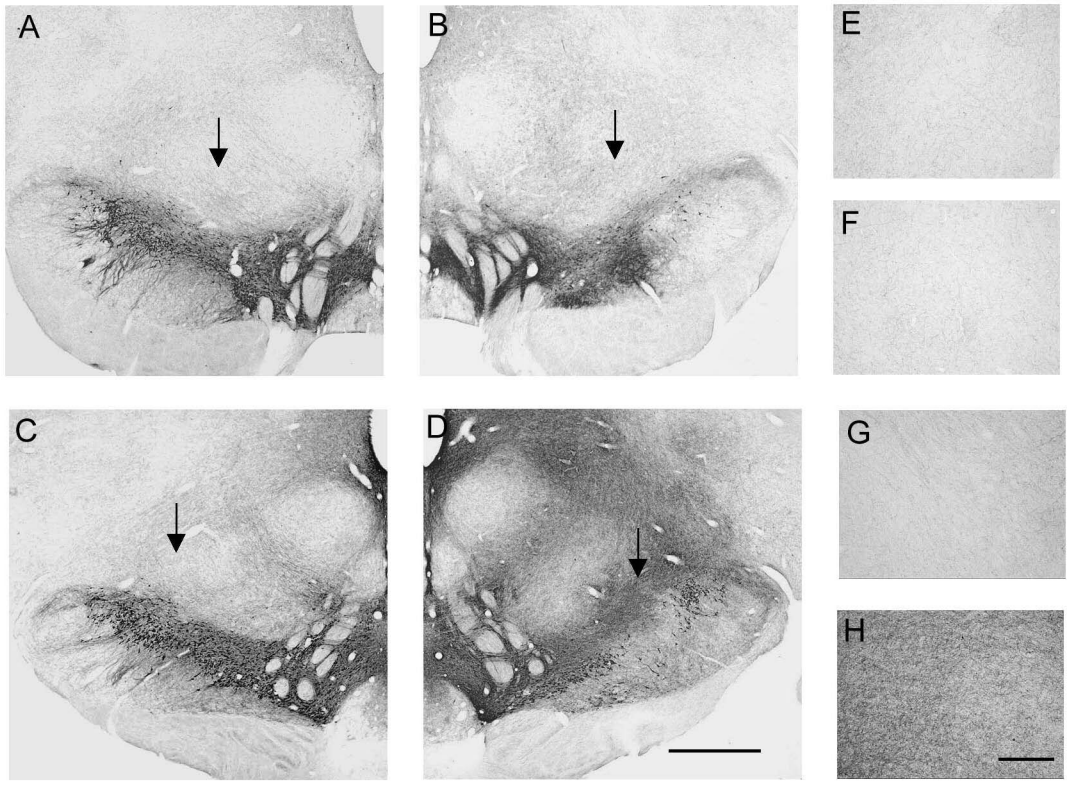

Fig 7

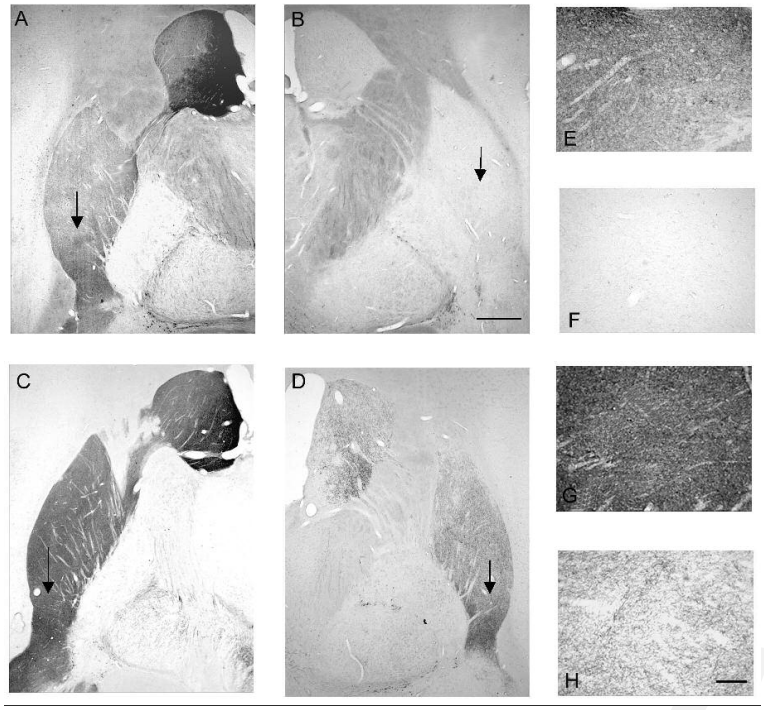

Fig 8

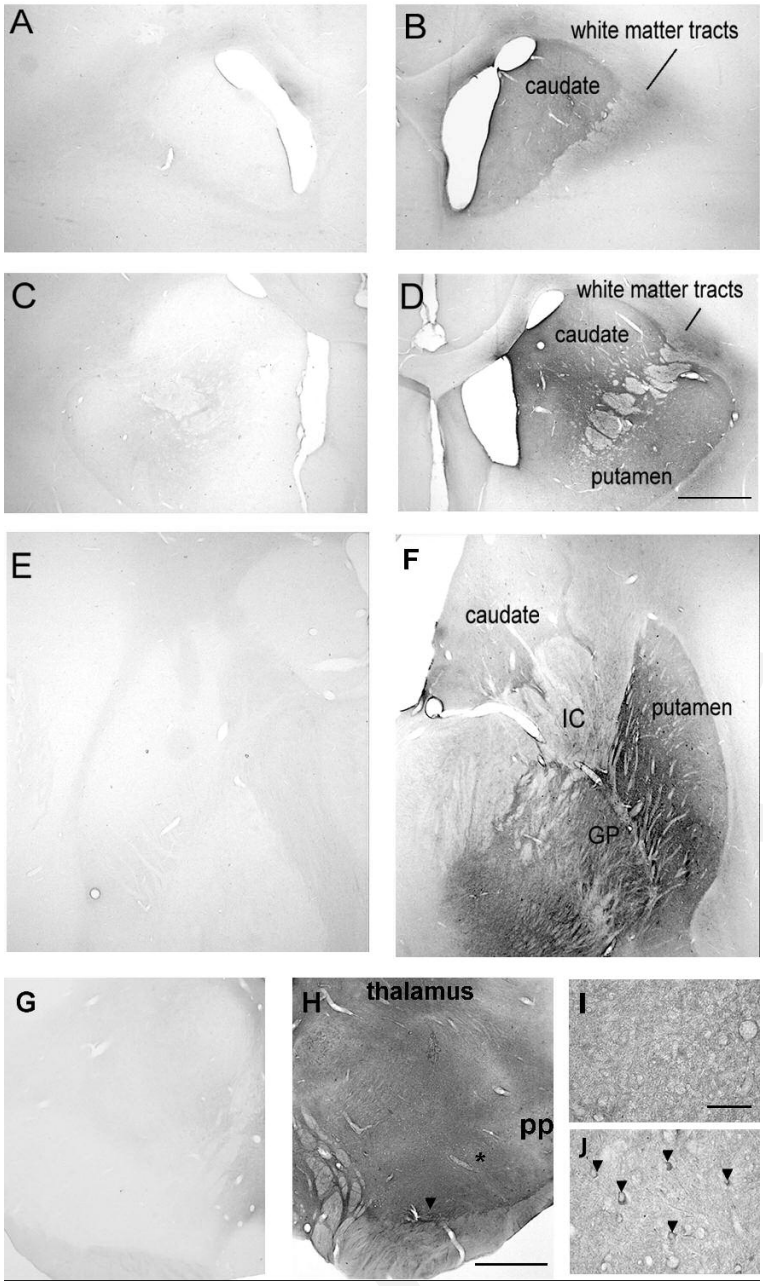

Fig 9

Table 1: Stereotaxic coordinates for the rAAV-GDNF surgery and the 6-OHDA nigro-striatal bundle lesion.

	AP	Lat	V
rAAV-GDNF (GDNF group)			
Substantia nigra			
1	5.5	2.5	6.6
2	4.0	2.5	6.6
Striatum			
1	11.5	3.0	12.0
2	10.0	3.0	12.0
3	10.5	5.2	10.2
4	9.0	6.0	10.5
5	7.5	6.3	10.3
6-OHDA nigro-striatal bundle (both groups)			
1	6.5	1.2	6.0
2	6.5	1.2	7.0
3	6.5	2.2	6.5
4	6.5	2.2	7.5
5	6.5	3.2	7.5