Reversal of dyskinesias in an animal model of Parkinson’s disease by continuous L-DOPA delivery using rAAV vectors

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Summary
Dyskinesias are a major complication of long-term L-3,4-dihydroxyphenylalanine (L-DOPA) treatment in Parkinson’s disease, and are believed to result from the intermittent and pulsatile supply of L-DOPA. Daily injections of L-DOPA can prime similar abnormal involuntary movements of the limb, orolingual and axial muscles in rats rendered parkinsonian by destruction of the nigrostriatal dopamine (DA) neurons. In this study we used 33 rats with severe nigrostriatal dopamine depletion and showed that in vivo gene transfer of the DA-synthetic enzymes tyrosine hydroxylase (TH) and GTP cyclohydrolase 1 (GCH1) using recombinant adeno-associated virus vectors can provide a constant source of DOPA production locally in the striatum, at a level that is effective in reducing L-DOPA-induced dyskinesias by >85%, and reverse lesion-induced motor impairments. Furthermore, the abnormal expression of ΔFosB, prodynorphin and preproenkephalin mRNA within the striatal projection neurons normally seen in dyskinetic animals was completely reversed by TH–GCH1 gene transfer. These findings form a strong basis for replacing, or supplementing, conventional systemic L-DOPA therapy by continuous intrastratial DOPA using in vivo gene transfer in the treatment of patients with advanced Parkinson’s disease.

Keywords: dyskinesia; gene therapy; GTP cyclohydrolase 1; Parkinson’s disease; tyrosine hydroxylase

Abbreviations: AIM = abnormal involuntary movement; AP = anterioposterior; DA = dopamine; GCH1 = GTP cyclohydrolase 1; KPBS = potassium-phosphate buffer; l-DOPA = l-3,4-dihydroxyphenylalanine; ML = mediolateral; 6-OHDA = 6-hydroxydopamine; PDyn = prodynorphin; PPE = preproenkephalin; rAAV = recombinant adeno-associated virus; TH = tyrosine hydroxylase


Introduction
Therapy with the dopamine (DA) precursor L-3,4-dihydroxyphenylalanine (l-DOPA) is the most effective treatment for Parkinson’s disease. However, while treatment response is excellent initially, over the course of several years most patients develop therapy-related adverse effects such as l-DOPA-induced dyskinesias (Obeso et al., 2000; Ahlskog and Mueuter, 2001). These complications are thought to arise from the intermittent and pulsatile stimulation of supersensitive DA receptors on striatal neurons (Chase, 1998; Nutt et al., 2000). In agreement with this, continuous DA receptor stimulation using either duodenal (Syed et al., 1998; Nyholm et al., 2003) or intravenous (Mouradian et al., 1990) infusion of l-DOPA, or subcutaneous infusion of the DA receptor agonist apomorphine (Poewe and Wenning, 2000), has been shown to markedly reduce the frequency and severity
of abnormal involuntary movements in Parkinson’s disease patients.

Similar to L-DOPA-induced dyskinesia seen in Parkinson’s disease patients and MPTP-treated monkeys, intermittent administration of L-DOPA in rodent models of Parkinson’s disease induces abnormal involuntary movements (AIMs) affecting orolingual, forelimb and trunk muscles (Lee et al., 2000; Cenci et al., 2002; Winkler et al., 2002; Steece-Collier et al., 2003). These behavioural abnormalities are accompanied by changes in the expression of opioid message RNAs [preproenkephalin (PPE) and prodynorphin (PDyn)] in striatal projection neurons (Cenci et al., 1998; Henry et al., 1999). Moreover, the rat dyskinesia model has provided evidence of a causal link between L-DOPA-induced movement abnormalities and changes in ΔFosB-like transcription factors in striatal projection neurons (Andersson et al., 1999, 2001).

Direct injection of viral vectors in the parkinsonian brain is particularly interesting since it provides a continuous and local production of DOPA centrally at a specific target site in the brain, i.e. in the DA-depleted striatum. Local DOPA delivery by in vivo gene therapy, using intrastriatal gene transfer of DA-synthetic enzyme tyrosine hydroxylase (TH), has been explored as a potential therapeutic intervention for Parkinson’s disease (Horellou et al., 1994; Kaplitt et al., 1994). It has been shown that the levels of DOPA production are very low unless expression of TH is combined with exogenous administration of tetrahydrobiopterin, the co-factor for TH, or with co-expression of its rate-limiting synthetic enzyme, GTP cyclohydrolase 1 (GCH1) (Bencsics et al., 1996; Mandel et al., 1998; Corti et al., 1999). The most promising long-term results so far have been obtained using recombinant adeno-associated viral (rAAV) vectors (Mandel et al., 1998; Szczypka et al., 1999; Kirik et al., 2002). We have shown that intrastriatal injection of high titre rAAV vectors encoding the genes for TH and GCH1 can provide pronounced behavioural recovery in rats rendered parkinsonian by injection of 6-hydroxydopamine (6-OHDA), provided that the level of striatal DOPA production exceeds a critical threshold (Kirik et al., 2002).

In the present study we investigated whether rAAV-mediated expression of the DOPA-synthesizing enzymes, TH and GCH1, in the striatum is capable of eliminating L-DOPA-induced dyskinesias in the rat Parkinson’s disease model. Our data demonstrate that in vivo gene therapy by rAAV-TH and rAAV-GCH1 vectors has dual action: (i) alleviation of dyskinesias induced by systemic intermittent L-DOPA treatment; and (ii) near complete reversal of the lesion-induced deficits in spontaneous motor behaviour. These changes are associated with a normalization of striatal opioid gene expression and reversal of the abnormal ΔFosB expression, both of which are considered as markers of maladaptive plasticity induced by the L-DOPA treatment.

Methods

Experimental design

Female Sprague–Dawley rats (B&K Universal, Stockholm, Sweden) were housed under a 12 h light/12 h dark cycle with free access to food and water. All surgical procedures were performed according to the rules set by the Ethical Committee for use of Laboratory Animals at Lund University. The experiment comprised four behaviourally balanced groups of animals with unilateral intrastriatal 6-OHDA lesions. While one group of lesioned rats (n = 7) was left untreated (drug-naïve lesion control), the others were treated with L-DOPA and later allocated into three balanced groups based on their dyskinesia scores to receive either a 1:1 mixture of the rAAV-TH and rAAV-GCH1 vectors (therapeutic vector group, n = 9), the rAAV-GCH1 vector alone (control vector group, n = 7) or sham operations (L-DOPA-treated control group, n = 10) (Fig. 1).

Fig. 1 Time-course of experiment. Following a unilateral striatal 6-OHDA lesion, the rats were screened for robust motor behavioural impairments using amphetamine-induced rotation and cylinder test. Selected animals received daily injections of L-DOPA or vehicle (drug-naïve controls) for 22 days (dark grey box), during which time AIMs were evaluated at four time-points. Among the L-DOPA-treated rats, three balanced groups were formed, where two groups received injections of either rAAV-GCH1 only or a 1:1 mix of rAAV-TH + rAAV-GCH1, while the third group remained as L-DOPA-treated controls. Further AIM tests were performed at 2, 6 and 12 weeks after viral transduction while the animals were kept on maintenance drug treatment (light grey box). At the end of week 12 an 8-day L-DOPA challenge (dark grey box) was performed and two additional AIM tests were conducted. A cylinder test was also performed at 10 weeks after transduction to evaluate the extent of recovery in spontaneous motor behaviours. Upon completion of the behavioural tests the animals were killed by decapitation for histological and biochemical analysis.
6-OHDA lesion

All 6-OHDA lesions were performed under Hypnorm (Apoteksbolaget, Sweden) and Dormicum (Apoteksbolaget, Sweden) anaesthesia. The surgery was carried out using a Stereotaxic frame (Stoelting, Wood Dale, IL, USA) and a 5 μl Hamilton syringe fitted with a glass capillary (outer diameter of 60–80 μm). All animals received injections of a total of 28 mg of 6-OHDA (3.5 mg/μl in 0.2 mg/ml ascorbic acid in saline; Sigma–Aldrich AB, Sweden) distributed over four sites in the striatum in order to obtain animals with severe nigrostriatal lesion. The coordinates were calculated with reference to bregma for the anterioposterior (AP) and the mediolateral (ML) coordinates using the rat brain atlas (Paxinos and Watson, 1998) were as follows: (1) AP + 1.3, ML −2.6; (2) AP +0.4, ML −3.2; (3) AP −0.4, ML −4.2; (4) AP −1.3, ML −4.5. The dorsoventral position of all injections was −5.0 mm below the dura and the tooth bar set to 0.0. 6-OHDA was injected at a rate of 1 μl/min and the injection syringe was left for an additional 3 min before it was slowly retracted.

Viral vector production and surgery

Both rAAV vectors (rAAV-TH and rAAV-GCH1) used in the present study were driven by a hybrid promoter consisting of an enhancer element from the cytomegalovirus promoter and the chicken β-actin promoter with a rabbit β-globin intron (Xu et al., 2001). Vectors were produced by a double-transfection method of rAAV plasmids and helper plasmid coding for the necessary genes, normally provided by the adenoavirus, where the plasmids were co-transfected into 293 cells using the calcium phosphate method. The virus was purified as described by Zolotukhin et al. (1999). Final titres, determined using an infectious centre assay (McLaughlin et al., 1988), were 1.5 × 1011 and 5.2 × 1011 infectious units/ml for the rAAV-TH and rAAV-GCH1 vectors, respectively.

All rAAV vector injections were performed under isoflurane anaesthesia using a 5 μl Hamilton syringe fitted with a glass capillary (outer diameter of 60–80 μm) mounted on a stereotaxic frame (Stoelting). Animals in the rAAV-GCH1 vector control group received a total of 15 μl of the rAAV-GCH1 vector stock, while the rAAV-TH + rAAV-GCH1-treated group was injected with 15 μl of a 1 : 1 mix of the rAAV-TH and rAAV-GCH1 vector stocks. The vector injections were distributed over 10 deposits along five needle tracts at the following coordinates with reference to bregma and the dorsoventral coordinates were as follows: (1) AP +1.3, ML −2.6; (2) AP +0.4, ML −3.2; (3) AP −0.4, ML −4.2; (4) AP −1.3, ML −4.5. The dorsoventral position of all injections was −5.0 mm below the dura and the tooth bar set to 0.0. The viral vectors were injected at a rate of 1 μl/min and the injection syringe was kept in place for an additional 3 min before it was slowly pulled up.

Behavourial analysis

Amphetamine-induced rotation

Two weeks after the 6-OHDA lesions an amphetamine-induced rotational behaviour was monitored in order to select animals with profound DA-denervating lesions in the striatum. Following an intraperitoneal injection of 2.5 mg/kg of di-amphetamine sulphate (Apoteksbolaget, Sweden), right and left full body turns were monitored over 90 min using automated rotometer bowls (Ungerstedt and Arbuthnott, 1970). The data are expressed as net full body turns per min ipsilateral to the lesion. Animals were included in the study when they exhibited at least 7 turns/min during the monitored observation time of 90 min.

Cylinder test

Spontaneous forelimb use was assessed using the cylinder test (Schallert and Tillerson, 1999) as described by Kirik et al. (2000). This test is designed to score weight-shifting movements initiated by the forelimb without handling by the experimenter. The animals were videotaped as they moved freely in a 20 cm diameter cylinder. Contacts made by each forepaw with the wall of the cylinder were scored from the videotape by an observer blinded to the identity of the animals. A total of 20 contacts were recorded for each animal.

The data are presented as left (impaired) paw use as percentage of the total number of touches, where an unbiased animal will receive a score of 50%. Performance in the cylinder test was monitored once at 4 weeks after the 6-OHDA lesion and prior to the daily l-DOPA treatment, and at a second time at 10 weeks after the viral vector injections. Animals were only included in the experiment when they exhibited ≥10% left paw use, which is indicative of severe behavioural deficit.

l-DOPA injections and AIMS test

l-DOPA methyl ester (Sigma–Aldrich) was given at a dose of 6 mg/ml combined with 15 mg/ml of the peripheral DOPA-decarboxylase inhibitor, benserazide-HCI (Sigma–Aldrich). The drugs were dissolved in physiological saline (1 ml per kg body weight) immediately prior to use. During the induction period, the animals received daily l-DOPA injections for 22 days, i.e. until AIM scores had reached a stable plateau. The rats were then kept on a maintenance regimen consisting of two l-DOPA injections/week for 12 weeks. At 12 weeks after vector injection, animals were again challenged with daily l-DOPA injections for 8 days. l-DOPA-induced AIMS were scored during the induction time, and at 2, 6 and 12 weeks after viral transduction according to a rat dyskinesia scale (Winkler et al., 2002). Animals were placed individually in transparent plastic cages and scored every 30 min for 4 h following a single dose of l-DOPA. Subtypes of AIMS were classified according to their topographic distribution as forelimb, orolingual, axial and locomotive. However, locomotive dyskinesia, i.e. circular locomotion, with side bias contralateral to the lesion, was not observed in the animals, which is in agreement with our previous observations in animals with partial intrastriatal lesions using the same dose of l-DOPA. Enhanced manifestations of otherwise normal behaviours such as grooming, gnawing, rearing and sniffing were not included in the rating. AIM severity was assessed using a score from 0 to 4 for each of the four AIM subtypes according to the time per monitoring period during which the AIM is present (0, absent; 1, occasional, i.e. present during <50% of the observation time; 2, frequent, i.e. present during >50% of the observation time; 3, continuous but interrupted by strong sensory stimuli, e.g. sudden noise, opening of the cage lid; 4, continuous, not interrupted by strong sensory stimuli). Borderline scores such as 1.5, 2.5 and 3.5 were allowed in order to increase the sensitivity of the rating. From the raw data, an integrated AIM score was computed for each animal and session as the area under the curve in a plot of AIM scores per monitoring period against the total observation time of 4 h.

Histological analysis

At the end of the experiment the animals were killed 2 days after the last l-DOPA injections. All animals were deeply anaesthetized with sodium pentobarbital (Apoteksbolaget) and killed by decapitation. The brains were rapidly removed and cut into two pieces at the level of the hypothalamus. The forebrain was immediately frozen on
powdered dry-ice and stored at −80°C until cutting on a cryostat
(HM500M, Microm); 16-μm thickness sections were mounted on
plus-charged glass slides (Superfrost+; Electron Microscopy
Sciences, PA, USA) and stored at −20°C until further use. The
midbrain was fixed in 4% paraformaldehyde (PFA), in 0.1 M
phosphate buffer, pH 7.4, for 24 h and dehydrated in 25% sucrose
and cut on a freezing microtome (SM2000R, Leica) at 40-μm
thickness.

**Immunohistochemistry**

Immunohistochemistry of midbrain sections was performed using
specific antibody for TH (mouse IgG, 1:4000; Chemicon, CA,
USA). The sections were quenched for 10 min in 3% H2O2, 10% 
methanol in potassium–phosphate buffer (KPBS), preincubated
for 1 h in 5% normal horse serum, 0.25% Triton-X in KPBS and
incubated with the primary antibody overnight at room temperature.
This was followed by incubation with biotinylated secondary
antibody [1:200 horse anti-mouse (BA2001); Vector Laboratories,
Burlingame, CA, USA]. After this incubation the sections were
further incubated for 1 h in avidin–biotin–peroxidase solution (ABC
Elite; Vector Laboratories) and visualized using the chromo-
further incubated for 1 h in avidin–biotin–peroxidase solution
(Burlingame, CA, USA]. After this incubation the sections were
preincubated for 1 h in 5% normal horse serum, 0.25% Triton-X in KPBS
and incubated with the primary antibody overnight at room temperature.
This was followed by incubation with biotinylated secondary
antibody [1:200 horse anti-mouse (BA2001); Vector Laboratories,
Burlingame, CA, USA]. After this incubation the sections were
further incubated for 1 h in avidin–biotin–peroxidase solution
(ABC Elite; Vector Laboratories) and visualized using the chromo-
gen 3’,3’-diaminobenzidine and 0.01% H2O2. The sections were
mounted on chrome-alum-coated glass slides, dehydrated in ascending
alcohol solution, cleared in xylene and coverslipped with Depex.

Two series of the slide-mounted sections through the striatum
was processed for FosB/ΔFosB (referred as FosB) and for TH
immunohistochemistry. Sections were air-dried, fixed for 30 min in 10% 
formalin (J. T. Baker, Deventer, Holland) and further rinsed with 3×
KPBS + 0.25% Triton-X (KPBS/T). Staining was performed accord-
ing to the protocol above but KPBS/T was used in all steps and the
quenching step was excluded [primary antibody for FosB (1:15 000;
polyclonal IgG; SC-48X; Santa Cruz), and secondary antibody
(1:250, horse anti-goat, BA9500; Chemicon)], while the procedures
for TH immunostaining were identical to the above.

**In situ hybridization**

The mRNA expression of PDyn and PPE was measured in the lateral
striatal areas from six equally spaced sections, representing the
majority of the striatal volume from the rostral tip to the level of
globus pallidus, using synthetic oligonucleotide probes (Scandinavian Gene
Synthesis AB, Köping, Sweden). The PDyn oligo was complementary to nucleotides 938–982 of the cloned rat
PDyn gene (Civelli et al., 1985) and the PPE oligo was com-
plementary to nucleotides 322–360 of the rat cDNA (Howells et al.,
1984). The oligos were labelled at the 3’ end with [35S]dATP (>37
Tbq/mmol; Amersham) using the terminal deoxynucleotidyltransfer-
ase (MBI Fermentas Inc., Amherst, NY, USA). Purification of the
oligonucleotides from non-incorporated [35S]dATPs was performed
using spin column chromatography (Chroma Spin Columns;
Clontech Laboratories, Palo Alto, CA, USA) and stored at 4°C
with 10 mM dithiothreitol (DTT). The slides were air-dried and
fixed in 4% ice-cold PFA in 0.1 M phosphate buffer (pH 7.4) for
30 min, rinsed 3× 5 min in PBS/DEPC and dehydrated in 70–95% 
ethanol before hybridization. Two hundred and fifty milliliters of
the hybridization buffer [50% deionized formamide, 1× Denhardt’s
solution, 1% N-lauroylsarcosine, 10% dextran sulphate, 500 μg/ml
sheared and denaturated salmon sperm DNA, 0.2 mg/ml heparin,
200 mM DTT, 4× standard sodium citrate buffer (SCC) and
10 c.p.m./ml labelled oligonucleotide] were added to each section.
The sections were then covered with parafilm and incubated for 18 h
in humid chambers at 42°C. After the hybridization the coverslips
were floated off in 1× SCC at 55°C, rinsed 4× 15 min in 1× SCC at
55°C followed by a further rinse in 1× SCC starting at 55°C and
cooling down to room temperature. The slides were rinsed in MilliQ-
water and dehydrated in 70–95% ethanol before exposing them, for
11 h for PPE and 24 h for PDyn, to 35S-sensitive Fuji imaging plates.
To obtain good illustrations, sections were also exposed to auto-
radiographic film (β-Max; Amersham) for 2–4 weeks and developed
using Kodak D-19 and Kodak Unifix. In order to confirm that the
signal on the films correspond to specific cellular labelling, the
sections were dipped in photographic LM-1 emulsion (dilution
1:1 in distilled water; Amersham), exposed for 6–12 weeks at
−20°C and developed in Kodak D-19 and fixed with Kodak Unifix.
The slides were counterstained in Cresyl Violet and coverslipped with DPX.

[3H]Mazindol binding assay

The [3H]Mazindol binding assay was performed according to Henry
et al. (1998). The slides containing six equally spaced sections, from
the rostral tip of the striatum to the level of globus pallidus, were
removed from the freezer, thawed at room temperature for 10 min
and preincubated in 50 mM Tris–HCl (pH 7.9) for 5 min at 4°C.
This was followed by 60 min incubation at 4°C in 50 mM Tris–HCl
(pH 7.9) containing 300 mM NaCl, 5 mM KCl, 10 mM [3H]mazindol
(Perkin-Elmer Life Sciences Inc., Boston, MA, USA) and 50 mM
desipramine (Sigma–Aldrich). The unspecific binding was defined
with sections incubated as above with addition of 100 mM of nomi-
fensine (Sigma–Aldrich). The sections were further rinsed 2× 1 min
in ice-cold Tris–HCl (pH 7.9) and dipped in ice-cold MQ-water and
dried thoroughly in a cold-room. The slides were exposed together
with radiolabelled standards to 1H-sensitive film (Hyperfilm; Amer-
sham) for 3 weeks at 4°C and developed and fixed using Kodak D-19
and Kodak Unifix.

**Image analysis and cell counts**

**Analysis of in situ hybridization, [3H]mazindol binding and TH transgene expression**

In order to analyse PDyn and PPE gene expression, the exposed
35S-sensitive Fuji imaging plates were scanned using a phosphorimagery (BAS-5000; Fujifilm AB, Sweden) to obtain
digitized autoradiographs. The hybridized sections were calibrated
against radioactive levels using simultaneously exposed 14C
standards (Amersham Pharmacia). The radioactive signal of each
animal was quantified using the Tina Adobe 2.10 software where
the striatum on both sides (lesioned versus intact side) was divided
into lateral and medial striatum. The results are presented as the
signal of the left (lesioned) striatum as percentage of the right (intact)
striatum. Quantitation of the [3H]mazindol binding assay was made
in the same way as the PDyn and PPE quantitation, using the Image
J v1.31 software for Macintosh OS X (National Institutes of Health,
Bethesda, MD, USA; http://rsb.info.nih.gov/ij/). TH transgene
expression was assessed by determining the volume fraction occu-
pied by the TH-positive cells and the processes as percentage of the
total striatal volume on the same side using the Image J program as
detailed above.

**Nigral cells counts**

Stereological estimation of the total TH-positive cell number in the
substantia nigra (SN) was performed using an optical fractionator as
described by West (1999). The brains were cut into five series leading to typically eight to 10 equally spaced sections covering the whole nigral region. All sections identified in a given series were used for quantitation. The SN was delineated from the rostral tip of the pars compacta to the caudal end of the pars reticulata. A vertical line passing through the medial tip of the cerebral peduncle and the medial terminal nucleus of the accumbens nucleus of the optic tract was used as the medial border of the SN in order to exclude the TH-positive cells in the ventral tegmental area. The sampling was performed using the Olympus CAST 2.0 system (Olympus Denmark A/S, Albertslund, Denmark). A counting frame was placed randomly on the first counting area and systematically moved through all counting areas until the delineated SN region was sampled. Guard volumes (5 μm from the top and 5 μm from the bottom of the section) were excluded from both surfaces, and only those profiles that come into focus within the counting volume were counted. The estimation of the total number of labelled cells was calculated according to the optical fractionators formula (West, 1999). The number of TH-positive neurons in the 6-OHDA-lesioned SN is expressed both as absolute numbers and as percentage of the values on the contralateral intact side in each animal.

Quantitation of FosB-positive cells
In order to count FosB-positive cells, the lateral striatal areas from six equally spaced sections, representing most of the striatal volume from the rostral tip to the level of globus pallidus, were delineated using the Olympus CAST 2.0 system (Olympus Denmark A/S). On each of the six sections FosB-positive cells were counted on the lesion side by placing a fixed counting frame, which is advanced through the whole cross sections by an XY stepper motor (400 × 400 μm) using a 40× objective with a numerical aperture of X. Thus 4.83% of the whole structure on six sections was sampled systematically. The final data points presented in the paper is calculated as: number of cell counted × 100 divided by 4.83.

Statistical analysis
Group comparisons were performed using repeated measures or factorial ANOVA where appropriate, followed by Tukey’s HSD post hoc test, using the JMP statistical software version 5.0.1.2 (SAS Institute Inc., Cary, NC, USA). Statistical significance was set at P < 0.05. For all comparisons in the cylinder test (see Fig. 3) and the in situ hybridization (see Fig. 5A and K), the percentage value was used without prior transformation.

Results
Reversal of L-DOPA-induced dyskinesia by in vivo gene transfer
The aim of the present study was to investigate whether continuous local production of DOPA in the striatum, mediated by co-transduction with rAAV vectors encoding the TH and GCH1 genes, can alleviate L-DOPA-induced dyskinesias in parkinsonian rats. In order to test this hypothesis, we generated rats with severe unilateral depletion of the nigrostriatal DA pathway by injection of 6-OHDA into four sites in the striatum (4 × 7 μg; see experimental design in Fig. 1). Lesioned rats were monitored for impairments of forelimb use in the cylinder test and amphetamine-induced rotation in order to select animals with severe behavioural deficits. Animals displaying <210% left paw touches and >7 full body turns/min were included in the study. This level of impairment is known to reflect >75% cell loss in the SN (Kirik et al., 2000). The selected animals were subjected to daily intraperitoneal injections of L-DOPA at a dose (6 mg/kg L-DOPA methyl ester + 15 mg/kg of the peripheral decarboxylase inhibitor benserazide) that is effective in inducing behavioural improvement in the lesioned animals, but subthreshold for induction of dyskinesias in non-primed animals (Winkler et al., 2002). Over the 3-week induction period, the 6-OHDA-lesioned animals gradually developed AIMS (equivalent to L-DOPA-induced dyskinesias seen in humans and primates) (Cenci et al., 2002; Lundblad et al., 2002) involving the limb, orolingual and axial muscles, primarily affecting the side of the body contralateral to the lesion. These movements were purposeless and could not be ascribed to enhanced manifestations of repetitive normal motor activities such as grooming, gnawing, rearing or sniffing, and disrupted or totally replaced normal behaviour. The abnormal behavioural profile seen in these animals was consistent with peak-dose dyskinesia seen in Parkinson’s disease patients, in that it was time-linked to the administration of the drug, peaked at 60–90 min, and disappeared by 180–210 min (Fig. 2A).

Daily L-DOPA-injections were continued for 22 days until AIM scores had reached a stable plateau, at which stage animals exhibiting moderate to severe AIMS (>250 on the integrated AIM scale) were allocated to three balanced groups, receiving either a 1:1 mixture of the rAAV-TH and rAAV-GCH1 vectors (therapeutic vector group), the rAAV-GCH1 vector alone (control vector group) or sham operation (L-DOPA-treated control group). A fourth group of lesioned rats was included as drug-naive control animals and received saline injections throughout the experiment. These saline-injected drug-naive lesioned control rats never displayed any of the behaviours seen in L-DOPA-treated animals, confirming the fact that the abnormal movements seen in the other groups were indeed a consequence of L-DOPA therapy (Fig. 2A–C).

After vector transduction, the animals received two L-DOPA injections/week for 12 weeks (Fig. 1). This injection regimen maintains dyskinetic animals in a stable response state, as was observed in the L-DOPA-treated lesion control group at the 2, 6 and 12 week time-points (Fig. 2C). The group injected with the rAAV-GCH1 vector did not differ from the L-DOPA-injected controls at any time, showing that transduction with the control vector (expressing the co-factor alone) did not have any effects on the AIM scores observed after peripheral bolus injection of L-DOPA. In the animals receiving injections of rAAV-TH + rAAV-GCH1 mixture, in contrast, the AIM scores gradually declined over time. Consistent with the delayed onset of transgene expression from the rAAV vectors, no difference was observed at the 2 week time-point. At 6 and 12 weeks after transduction, however,
the dyskinesias were significantly reduced by 63.9 ± 4.9% and 85.3 ± 8.9% as compared with the preoperative score (one-way ANOVAs [6 weeks: $F(3,32) = 7.41$, $P = 0.0008$; 12 weeks: $F(3,32) = 17.57$, $P < 0.0001$], followed by Tukey’s HSD post hoc analysis). At the 12 week time-point dyskinesias completely disappeared in four of the nine lesioned animals. The remaining animals had substantial reductions (between 58.6 and 84.9%) as compared with the pretransduction AIM scores (Fig. 2D). In these five animals, the abnormal movements of all types (limb, orolingual and axial) were consistently suppressed throughout the 4-h observation period (Fig. 2B).

**Fig. 3** Spontaneous forelimb use. Forelimb bias was evaluated using the cylinder test at two time-points, and the data are presented as left (impaired) paw use as percentage of the total paw contacts with the wall of the cylinder. The dashed line (at 50%) represents symmetric paw use that normal rats display. While all groups showed a strong bias in paw use prior to the vector injection (open bars), the group that received the combined $TH + GCH1$ gene transfer showed complete recovery in left paw use at 10 weeks after transduction (filled bars). *Significantly different from all other groups [one-way ANOVA, $F(3,32) = 15.65$, $P < 0.0001$; followed by Tukey’s HSD post hoc analysis].

**Normalization of lesion-induced functional impairments**

Prior to transduction with rAAV vectors, all animals included in this experiment had severe deficits in forelimb use on the left paw, i.e. contralateral to the side of the lesion in the brain, as assessed in the cylinder test (Fig. 3). At 10 weeks after transduction, all four groups were re-evaluated under off-medication conditions. The two lesion control groups and the rAAV-GCH1 control vector group displayed a similar degree of deficit, confirming that the behavioural impairment was stable in the lesioned rats and that the injection of the control vector provided no functional benefit. The animals injected with the rAAV-TH + rAAV-GCH1 mixture, on the other hand, showed complete behavioural recovery in this non-drug-induced paw-use test (Fig. 3).
Extent of the nigrostriatal DA lesion and in vivo transgene expression

The extent and consistency of the nigrostriatal DA lesion was confirmed in all animals by quantitation of nigral TH-positive cell numbers using stereological estimation tools and by quantitation of DA uptake sites using $[^3H]$mazindol binding (Table 1; Fig 4A and B). Injections of the dopaminergic neurotoxin 6-OHDA in the striatum led to a severe loss of the TH-positive cell bodies of the nigrostriatal projection neurons located in the SN pars compacta, preserving the mesolimbic projections neurons placed more medially in the ventral tegmental area (Fig. 4A). Consistent with previous data (Kirik et al., 1998; Winkler et al., 2002), TH-positive cell numbers in SN were reduced to between 8.9 ± 1.7% and 15.0 ± 2.4% of normal in the different experimental groups. Similarly, the striatal DA uptake sites, as assessed by $[^3H]$mazindol binding affinity, were consistently reduced to between 13.4 ± 1.2% and 17.7 ± 1.5% of normal in all groups. There were no differences between groups in these analyses ($P > 0.05$ for all comparisons). The expression of the $TH$ transgene was confirmed in all injected animals. Figure 4C–E shows the extent of vector-induced expression of the TH protein in the lesioned striatum at three rostrocaudal levels, appearing as columns of transduced striatal cells surrounding the injection sites. We estimated that ~38.6 ± 2.8% of the total striatum was occupied by the transduced cells and their processes, as assessed using the TH-stained sections.

Normalization of striatal gene expression by in vivo gene transfer

In both rat and non-human primate models of Parkinson’s disease, the development of L-DOPA-induced dyskinesia is associated with up-regulation of ΔFosB-like transcription

<table>
<thead>
<tr>
<th>Group</th>
<th>TH-positive cells in SN</th>
<th>$[^3H]$mazindol binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intact side</td>
<td>Lesion side</td>
</tr>
<tr>
<td>Les Ctrl (drug naive)</td>
<td>12 360 ± 1195</td>
<td>1852 ± 399</td>
</tr>
<tr>
<td>Les Ctrl</td>
<td>12 586 ± 941</td>
<td>1831 ± 327</td>
</tr>
<tr>
<td>GCH1</td>
<td>14 446 ± 1596</td>
<td>1335 ± 279</td>
</tr>
<tr>
<td>TH + GCH1</td>
<td>12 064 ± 1296</td>
<td>1262 ± 143</td>
</tr>
</tbody>
</table>

The extent of lesion-induced DA-denervation was quantified at the level of SN by stereological cell counting methods, and in the striatum as density of DA uptake sites obtained by $[^3H]$mazindol binding assay. There was no statistically significant group difference for either of the measures (one-way ANOVAs [SN cell no.: $F(3,32) = 2.32, P > 0.05$; $[^3H]$mazindol binding: $F(3,32) = 1.84, P > 0.05$]).

Fig. 4 The 6-OHDA lesion and transduction with rAAV vectors. The injection of the 6-OHDA in the striatum induced a pronounced loss of DA neurons in the SN pars compacta (SNpc) as visualized using midbrain sections stained with antibodies against TH, whereas the ventral tegmental area (VTA) remained well preserved (A). Similarly, loss of nigrostriatal DA fibres led to a pronounced reduction in the DA uptake sites as visualized by $[^3H]$mazindol binding (B). Injection of the rAAV vectors coding the human TH enzyme led to expression of the protein in the otherwise TH-depleted striatum (C–E). Scale bar = 2 mm.
factors, and PPE and PDyn mRNA in striatal projection neurons (Doucet et al., 1996; Tel et al., 2002; Henry et al., 2003). In the rat, expression of the PDyn and fosB genes in the lateral striatum is particularly well correlated with the severity of dyskinesia (Cenci et al., 1998; Andersson et al., 1999; Winkler et al., 2002). We thus hypothesized that reversal of l-DOPA-induced AIMs by combined TH + GCH1 gene transfer should be paralleled by normalization of PDyn mRNA levels and reversal of abnormal ΔFosB expression in the lateral striatum. As shown by quantitative in situ hybridization analysis, the 6-OHDA lesion induced a moderate decrease of the PDyn mRNA expression of 25.9 ± 8.3% compared with the side contralateral to the lesion (Fig. 5A and B), while pulsatile l-DOPA treatment over the 12-week test period induced a abnormal up-regulation in the PDyn signal to 178.6 ± 23.3% of normal values (Fig. 5A and C). This increase was maintained in animals receiving injections of the rAAV-GCH1 control vector (208.8 ± 34.5%; Fig. 5A and D), whereas in animals receiving the therapeutic vector mixture (rAAV-TH + rAAV-GCH1), PDyn mRNA up-regulation in the lateral striatum was completely normalized (94.5 ± 6.3% of normal values; Fig. 5A and E) [one-way ANOVA, $F(3,32) = 8.97, P = 0.001$; followed by Tukey’s HSD post hoc analysis]. The number of cells immunoreactive for FosB/ΔFosB-like proteins, systematically sampled within the lateral striatum from six serial sections per rat, was increased from 1245 ± 684 cells in the drug-naïve lesion control group to 3251 ± 433 and 3186 ± 684 in the non-transduced controls and the rAAV-GCH1-injected animals, respectively. Whereas, in animals that received TH + GCH1 gene transfer the number of FosB-positive cells was reduced to 1548 ± 579 and no longer differed from the values obtained in drug-naive lesion controls (Fig. 5F–J) [one-way ANOVA, $F(3,32) = 4.18, P = 0.01$; followed by Tukey’s HSD post hoc analysis].

We next analysed changes in the striatal expression levels of PPE, which has also been significantly correlated with l-DOPA-induced dyskinesia (Calon et al., 2000; Winkler et al., 2002). The 6-OHDA lesions induced a moderate increase of PPE mRNA expression in the lateral striatum to 124.1 ± 8.5% of normal, which was further exacerbated by pulsatile l-DOPA administration (141.9 ± 20% in l-DOPA-treated lesion controls, and 153.8 ± 6.4% in rAAV-GCH1-injected rats; Fig. 5K–N). As observed for PDyn expression, the PPE mRNA levels in the lateral striatum were significantly reduced and were not different from the contralateral side in animals transduced with the combination of TH and GCH1 genes (106.8 ± 4.3% of normal values; Fig. 5K and O) [one-way ANOVA, $F(3,32) = 20.98, P < 0.0001$; followed by Tukey’s HSD post hoc analysis].

**Discussion**

The present results show that intrastriatal rAAV-TH and rAAV-GCH1 gene transfer in the rat Parkinson’s disease model not only induces substantial functional recovery, but also reverses dyskinesias and normalizes gene expression changes in the striatum induced by intermittent systemic l-DOPA treatment. The magnitude of dyskinesias in these animals was reduced by 85% despite continued systemic l-DOPA treatment, and in four of the nine animals the dyskinesias were completely abolished. In our previous study using the same batches of rAAV vectors at precisely the same parameters used here (Kirik et al., 2002), we estimated that the vector-induced striatal DOPA production exceeded therapeutic levels after peripheral injection of l-DOPA by ~2-fold. When given repeatedly as daily bolus injections, the same dose of l-DOPA leads to induction of dyskinesias (Winkler et al., 2002). The fact that continuous production of DOPA within the striatum in the vector-treated animals did not worsen dyskinesias but rather reversed already existing dyskinesias suggests that the mode of DA receptor stimulation (continuous versus intermittent), and not the overall level of l-DOPA within the striatum, is the critical factor determining the emergence and maintenance of dyskinetic complications.

The development of dyskinesia induced by intermittent peripheral injections of l-DOPA is associated with profound changes in the mRNA expression levels of PDyn (178% of normal) and PPE (141% of normal) in projection neurons in the lateral striatum, accompanied by a marked increase in the FosB-expressing neurons in this area (Cenci et al., 1998; Andersson et al., 1999; Winkler et al., 2002). Intrastriatal DOPA production using combined expression of TH + GCH1 reversed all these changes. Strikingly, the expression levels of PDyn and PPE were not simply returned to the levels seen in l-DOPA-naïve lesioned animals, but they were normalized to the levels measured on the non-lesioned control side. Thus, whereas the 6-OHDA lesions induced a down-regulation of PDyn, to 74% of normal, the expression levels in the rAAV-TH + rAAV-GCH1-treated animals were normalized to 95% of the control side. Similarly, whereas PPE expression levels were increased to 124% of normal in the lesioned drug-naïve controls, combined rAAV-TH + rAAV-GCH1 vector treatment reversed PPE levels to 107% of normal.

It has been debated whether changes in the levels of opioid neurotransmitters are causal to the expression of parkinsonian symptoms or represent a homeostatic, compensatory response to the loss of DA afferents in the striatum (Maneuf et al., 1994; Schneider et al., 1999; Wade and Schneider, 2001). Increased opioid transmission in the striatum and its output structures, however, is regarded as an important event at the basis of l-DOPA-induced dyskinesias both in human Parkinson’s disease (Henry et al., 2003; Piccini et al., 1997) and in animal models of this disorder (Henry et al., 2001; Johansson et al., 2001). Our present data show that in vivo DOPA delivery by combined TH + GCH1 gene transfer normalizes the levels of opioid transmitters in the striatal projection neurons. This suggests that normalization of opioid-dependent information processing in striatal efferent pathways plays a role in reversal of the abnormal involuntary movements and recovery of normal motor function in the rat model of Parkinson’s disease.
Although L-DOPA treatment is highly effective during early stages of Parkinson’s disease, the efficacy of this therapy gradually declines in patients with advanced disease. This is mainly due to the fact that the appearance of dyskinesias limits the therapeutic window for L-DOPA treatment (Mouradian et al., 1988). Similarly, in rats with extensive lesions of the nigrostriatal DA projections, the efficacy of peripheral L-DOPA treatment is incomplete, which, at least

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**Fig. 5** PDyn mRNA expression (A–E), number of FosB-positive cells (F–J), and PPE mRNA expression (K–O) in the striatum. The dashed lines in A and K represent gene expression on the contralateral non-lesioned side. Digitized and pseudo-coloured photomicrographs are presented for PDyn mRNA expression (B–E), FosB-positive cells (G–J) and PPE mRNA expression (L–O). The lesioned side for PDyn and PPE expression is to the right of each panel. Note that transduction with rAAV-TH + rAAV-GCH1 vectors reverses all of the changes induced by peripheral L-DOPA treatment. *Significantly from the drug-naive control group and the rAAV-TH + rAAV-GCH1 vector group (one-way ANOVAs [PDyn: F(3,32) = 8.97, P = 0.001; PPE: F(3,32) = 20.98, P < 0.0001; FosB: F(3,32) = 4.18, P = 0.01]; followed by Tukey’s HSD post hoc analysis). Scale bar in B–E and L–O = 3 mm; G–J = 200 mm.

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in part, is due to the induction of dyskinesias at low doses of the drug (Chang et al., 1999; Metz et al., 2001; Lundblad et al., 2002; Winkler et al., 2002). L-DOPA-induced dyskinesias in patients are thought to result from the intermittent and pulsatile stimulation of supersensitive DA receptors by peripheral administration of the drug. This concept has provided the basis for development of alternative treatment strategies, which aim at continuously stimulating striatal DA receptors (Nutt et al., 2000). While duodenal infusion of DA agonists effectively reduces both the dyskinesias seen in the ‘on’ phase and the time period patients spend in ‘off’ phase (Pietz et al., 1998; Poewe and Wenning, 2000; Stocchi et al., 2002; Nyholm et al., 2003), motor performance as measured on the Unified Parkinson’s Disease Rating Scale is improved to a similar degree as seen after intermittent L-DOPA treatment. Interestingly, in the TH + GCH1 expressing animals, lesion-induced deficits in forelimb use, as seen in the cylinder test, were completely reversed following continuous production of DOPA locally in the striatum. The assessment of limb use in the cylinder test is particularly relevant since it measures explorative forelimb contacts with the walls of the test chamber in a setting where the animals are spontaneously active. Thus, recovery in symmetric paw use in the animals reflects normalization of spontaneous motor performance on the impaired paw, which is in direct cooperation with the intact paw for supporting the rat’s body against the wall of the cylinder.

Normalization of motor function following gene transfer in the rat Parkinson’s disease model is thus particularly striking, and suggests that intrastriatal transfer of the TH + GCH1 genes using viral vectors should be considered as a complement or alternative to systemic L-DOPA treatment, giving the combined benefit of reducing dyskinesias and improving motor function, i.e. providing an efficient means for widening of the therapeutic window in severely affected Parkinson’s disease patients. Furthermore, local intrastriatal DOPA production has the advantage that unwanted side-effects, such as psychosis and hypotension, induced by stimulation of DA receptors outside the striatum are less likely to occur after targeted expression of the transgene (Pietz et al., 1998; Stocchi et al., 2002). With ensured safety, and optimizing and up-scaling of the delivery parameters to match the large size of the primate/human striatum, this gene therapy approach should hold great promise for clinical use in patients with advanced Parkinson’s disease.

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N.M. is an inventor on patents related to recombinant AAV technology and owns equity in a gene therapy company that is commercializing AAV for gene therapy applications.

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