

RESEARCH ARTICLE

Neuronal expression of the transcription factor *Gli1* using the *T α 1* α -tubulin promoter is neuroprotective in an experimental model of Parkinson's disease

D Suwelack^{1,2,4}, A Hurtado-Lorenzo¹, E Millan¹, V Gonzalez-Nicolini¹, K Wawrowsky¹, PR Lowenstein^{1,2,3} and MG Castro^{1,2,3}

¹Gene Therapeutics Research Institute, Cedars-Sinai Medical Center, Los Angeles, CA, USA; ²Molecular Medicine and Gene Therapy Unit, Department of Medicine, University of Manchester, Manchester, UK; and ³Department of Medicine and Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA, USA

Nigrostriatal neurons degenerate during Parkinson's disease. Experimentally, neurotoxins such as 6-hydroxydopamine (6-OHDA) in rodents, and MPTP in mice and non-human primates, are used to model the disease-induced degeneration of midbrain dopaminergic neurons. Glial-cell-derived neurotrophic factor (GDNF) is a very powerful neuroprotector of dopaminergic neurons in all species examined. However, recent reports have indicated the possibility that GDNF may, in the long term and if expressed in an unregulated manner, exert untoward effects on midbrain dopaminergic neuronal structure and function. Although GDNF remains a powerful neurotrophin, the search for alternative therapies based on alternative and complementary mechanisms of action to GDNF is warranted. Recently, recombinant adenovirus-derived vectors encoding the differentiation factor Sonic Hedgehog (*Shh*) and its downstream transcriptional activator (*Gli1*) were shown to protect dopaminergic neurons in the substantia nigra pars compacta from 6-OHDA-induced neurotoxicity in rats *in vivo*. A pancellular human CMV (hCMV) promoter was used to drive the expression of both *Shh* and *Gli1*. Since *Gli1* is a

transcription factor and therefore exerts its actions intracellularly, we decided to test whether expression of *Gli1* within neurons would be effective for neuroprotection. We demonstrate that neuronal-specific expression of *Gli1* using the neuron-specific *T α 1* α -tubulin (*T α 1*) promoter was neuroprotective, and its efficiency was comparable to the pancellular strong viral hCMV promoter. These results suggest that expression of the transcription factor *Gli1* solely within neurons is neuroprotective for dopaminergic neurons *in vivo* and, furthermore, that neuronal-specific promoters are effective within the context of adenovirus-mediated gene therapy-induced neuroprotection of dopaminergic midbrain neurons. Since cell-type specific promoters are known to be weaker than the viral hCMV promoter, our data demonstrate that neuronal-specific expression of transcription factors is an effective, specific, and sufficient targeted approach for neurological gene therapy applications, potentially minimizing side effects due to unrestricted promiscuous gene expression within target tissues.

Gene Therapy (2004) 11, 1742–1752. doi:10.1038/sj.gt.3302377

Keywords: adenoviral vectors; GDNF; neurodegeneration; neuron-specific promoter; dopamine; substantia nigra

Introduction

In spite of pharmacological advances, current treatments for Parkinson's disease fail to halt the continued loss of substantia nigra dopaminergic neurons. Eventually, even if initially responsive, with time, clinical symptoms become resistant to pharmacological treatments, such as L-DOPA or dopaminergic agonists, all of which depend on the integrity of nigrostriatal neurons.¹ Halting the progressive degeneration of nigrostriatal neurons would delay disease progression, and thus prolong the efficacy of available pharmacological treatments.^{2–5} Glial-

cell-derived neurotrophic factor (GDNF) protects nigral dopaminergic cell bodies and their striatal axon terminals from *in vitro* and *in vivo* neurotoxicity induced by 6-hydroxydopamine (6-OHDA),⁴ MPTP,² or metamphetamine,⁶ and possibly also in Parkinson's patients.⁷ Gene therapy using GDNF could thus be employed as a complementary treatment to pharmacological approaches. GDNF has been delivered into the brain using recombinant adenovirus (RAd), adenovirus-associated virus, herpes simplex virus type 1, lentiviral-derived vectors, or by direct peptide injection.^{5,7–9}

In spite of its powerful neuroprotective actions, recent reports of potentially deleterious effects, that is, reduction of tyrosine hydroxylase (TH) mRNA in nigrostriatal neurons, aberrant morphologies of striatal TH-immunoreactive axons, and increased cell death following experimental stroke,^{10–12} have stimulated the search for novel alternative gene therapeutic approaches, and/or regulated GDNF expression.

Correspondence: Dr MG Castro and Dr PR Lowenstein, Gene Therapeutics Research Institute, Cedars-Sinai Medical Center, 8700 Beverly Blvd., Davis Building, Research Pavilion, Room 5090, Los Angeles, CA 90048, USA

⁴Current address: Neurology Clinic, Alfried Krupp Hospital, Alfried Krupp St. 21, D-45131 Essen, Germany

Received 11 June 2004; accepted 16 July 2004

Recently, the morphogen Sonic Hedgehog amino-terminal (ShhN) moiety¹³ has been suggested to have neurotrophic and neuroprotective properties for dopaminergic neurons *in vitro*, since it was able to increase survival of these neurons in ventral mesencephalic cultures and protect them against MPP⁺ neurotoxicity.^{14–17} Shh is a secreted neurodifferentiation factor that binds to a receptor complex, patched (*ptc*)–smoothed (*smo*). Binding of Shh to *ptc* inhibits *smo*, an inhibitory receptor,¹⁸ stimulating the transduction of the intracellular signaling pathway that leads to the activation of its main downstream transcriptional mediators,^{19–22} that is, the transcription factor Gli1^{23,24}, these proteins are all present within the adult rodent brain.^{25–27} Ectopic expression of Shh or its downstream effector Gli1 in the dorsal neural tube induces the expression of ventral neuronal markers such as TH, the rate-limiting enzyme in the synthesis of the neurotransmitter dopamine, and a marker for ventral midbrain nigrostriatal dopaminergic neurons. ShhN protects cultures of fetal dopamine neurons from MPP⁺ toxicity,²⁸ and regulates the differentiation and proliferation of neuronal stem cells.^{14,15,29} Further, Shh peptide injected directly into the brain of rodents and marmosets has beneficial effects in experimental models of Parkinson's disease.^{30–32}

We have recently demonstrated that adenoviral vectors encoding ShhN, or Gli1 under the control of pancellular viral promoters, have a neuroprotective effect on nigrostriatal dopaminergic neurons in an experimental model of dopamine neurodegeneration induced by 6-OHDA.^{33,34} Gli1 is a transcription factor and is thus needed intracellularly in order to be able to exert its neuroprotective effects. Having previously demonstrated the specificity³⁵ and therapeutic effectiveness³⁶ of cell-type specific promoters within adenoviral vectors, we hypothesized that expression of Gli1 from a neuronal-specific promoter could increase its effectiveness and safety. We chose the neuronal-specific tubulin $\alpha 1$ (*T α 1*) promoter to drive expression of both the marker and potentially therapeutic transgenes. We demonstrate that the *T α 1* promoter restricts the expression of transgenes encoded within RAd vectors to neurons. Therefore, expression of Gli1 within neurons alone is effective and sufficient to promote neuroprotection in a rat model of Parkinson's disease. Our data also demonstrate that the use of a cell-type specific neuronal promoter can uncover expression in other brain areas that receive the vector by retrograde transport from the striatal injection site, which may not be apparent when a viral pancellular promoter is used. Our experiments also demonstrated the presence of altered axonal morphologies in the striata of animals injected with RAd-GDNF, where GDNF is under the control of the pancellular human CMV (hCMV) promoter, urging the implementation of regulated promoter elements when employing this powerful neurotrophin for the treatment of neurodegenerative diseases.

Results

Construction of RAd-*T α 1*-Gli1 and RAd-*T α 1*-lacZ: neuron-specific transgene expression

T α 1 is a neuronal-specific promoter, the specificity of which has been demonstrated during normal development and in transgenic animal experiments.^{37,38} We

constructed first-generation RAd vectors expressing Gli1 or the control marker gene *lacZ* with a nuclear localization signal. A diagram illustrating the construction of the RAd vectors and the molecular analysis of the recombinant genomes is shown in Figure 1. To construct RAd-*T α 1*-lacZ and RAd-*T α 1*-Gli1, the *T α 1* promoter sequence was inserted into p Δ E1 upstream of either the *lacZ* or Gli1 sequence. Restriction fragment analysis and Southern blotting were used to confirm the correct orientation of the cDNAs for *lacZ* and Gli1 in the resulting plasmids, p Δ E1-*T α 1*-lacZ and p Δ E1-*T α 1*-Gli1. These were then cotransfected together with pBHG10 into 293 cells to construct the recombinant viruses, that is, RAd-*T α 1*-lacZ and RAd-*T α 1*-Gli1. The RAd vectors were isolated, purified, and tested for quality control as described by us in detail.^{39,40}

Expression from the *T α 1* promoter is neuronal specific in the brain *in vivo*

Injection of 6.1×10^7 IU of RAd-*T α 1*-lacZ into the striatum transduced mostly neurons. Also, retrogradely labeled nuclei expressing β -galactosidase were found in areas distant from the striatum. These cells were labeled by retrograde transport of RAd-*T α 1*-lacZ from the striatal injection site to these distant, anatomically connected areas. Cellular nuclei expressing β -galactosidase immunoreactivity were found within the cingulate cortex, somatosensory cortex, and paraolfactory cortex. Further, immunoreactive nuclei were also found within the substantia nigra pars compacta (SNpc), a nucleus that projects directly to the striatum (Figure 2).

Owing to the nuclear localization of β -galactosidase protein, morphological characterization of the cells expressing this transgene was insufficient to characterize the nature of the transduced cells. To determine the cell type transduced by the RAd-*T α 1*-lacZ vectors, histological sections were double immunoreacted with antibodies to β -galactosidase and cell-type specific antibodies (Figure 3). We used the neuronal nuclear marker Neu-N to double label cells in the striatum and neocortex, given the predicted cellular specificity of the *T α 1* promoter. This analysis demonstrated that the vast majority (approx. >95%) of cells immunoreactive for β -galactosidase were also positive for Neu-N, demonstrating that these cells are neurons. To identify cells expressing the transgene in the substantia nigra, sections were double immunoreacted with antibodies to the neurotransmitter synthesizing enzyme TH, which identifies dopaminergic neurons within the substantia nigra. All cells expressing β -galactosidase were also immunoreactive for TH, indicating that only dopaminergic neurons had been transduced by the RAd vectors.

Neuronal-specific expression in the rat brain using the *T α 1* promoter in RAd vectors: characterization of cell-type specific expression and effectiveness in protecting dopamine neurons in an experimental model of Parkinson's disease

The vectors RAd-GDNF and RAd-hCMV-Gli1 have been described by us recently,^{33,34} as has the vector expressing the control marker transgene β -galactosidase under the control of the hCMV promoter.^{41–44} The neuroprotective paradigm used was adapted to our particular needs from the detailed 6-OHDA rat model of Parkinson's disease

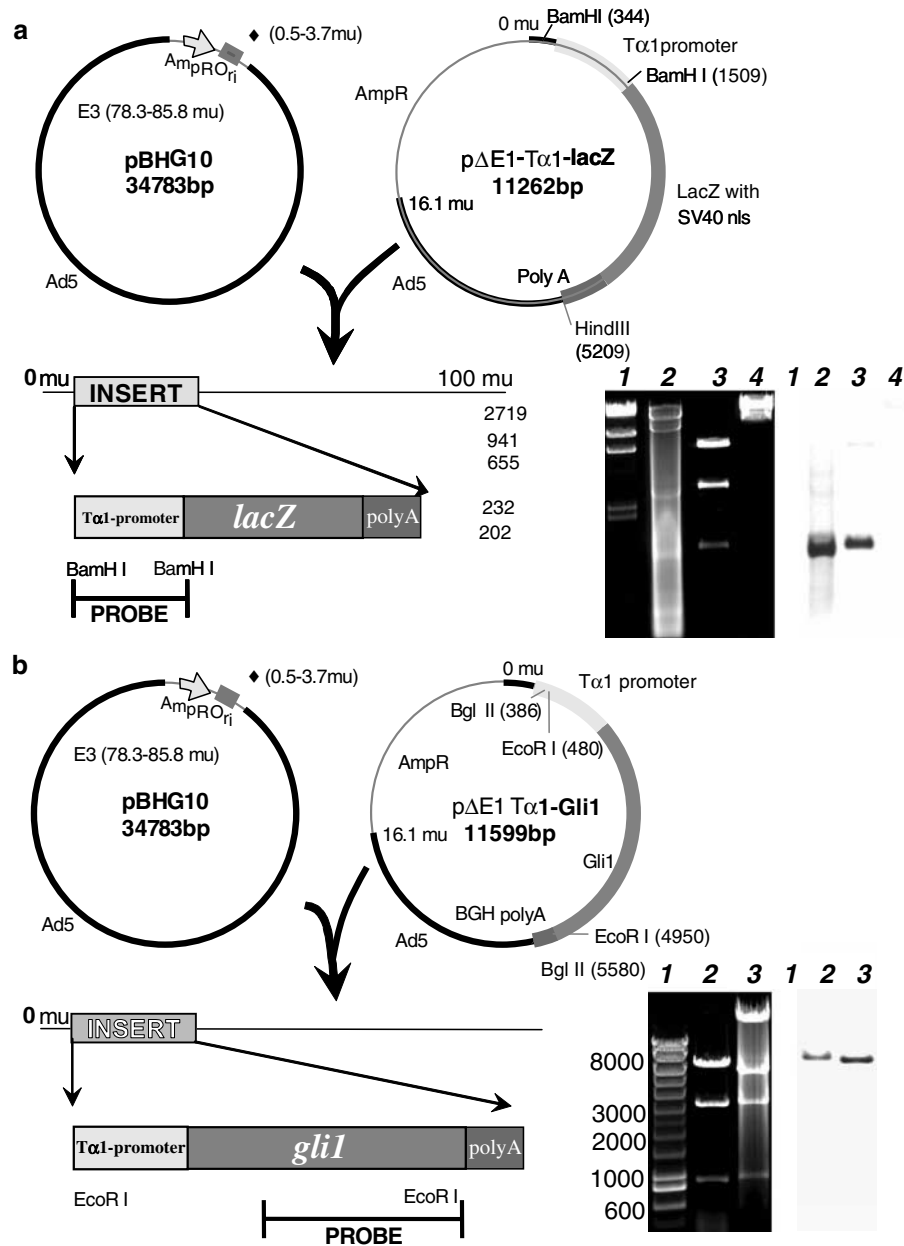


Figure 1 Construction and molecular characterization of the novel RAD vectors, RAD-Tx1-lacZ, and RAD-Tx1-Gli1. RADs were generated by homologous recombination after cotransfection into 293 cells of the shuttle plasmid encoding the promoter transgene cassette, and pBHG10, containing all necessary sequences of the adenoviral genome, apart from the E1 and E3 deletions, needed to construct a recombinant vector. The diagram to show the genetic structure of corresponding plasmids, the results of the restriction analysis, and Southern blot, used for the construction of RAD-Tx1-LacZ are all shown in (a). For the restriction analysis and Southern blot, lanes are as follows: lane 1, molecular size markers from a HindIII digest of λ DNA; lane 2, RAD-Tx1-LacZ; lane 3, shuttle plasmid pΔE1-Tx1-LacZ; and lane 4, pBHG10, all digested with BamHI. Next to the restriction analysis is the corresponding Southern blot using a specific probe for the Tx1 promoter sequence, as illustrated in the schematic drawing. The diagram to show the genetic structure of corresponding plasmids, the results of the restriction analysis, and Southern blot, used for the construction of RAD-Tx1-Gli1 are all shown in (b). For the restriction analysis and Southern blot, lanes are as follows: lane 1, molecular size DNA markers; lane 2, shuttle plasmid pΔE1-Tx1-Gli1; lane 3, RAD-Tx1-Gli1, all digested with EcoRI. Next to it is the corresponding Southern blot showing the results utilizing a probe recognizing sequences within transgene Gli1, as illustrated.

previously reported by the research team of Martha Bohn and collaborators.^{2,3} In our experiments, the RADs were injected stereotactically into the rat striatum using the same coordinates as used for the retrograde tracer fluorogold (FG) (Figure 4, and described in detail in Hurtado-Lorenzo *et al*^{33,34}). After 1 week, we induced degeneration of the nigrostriatal pathway by injecting 16 μ g of 6-OHDA at the same coordinates of FG and

virus injection. We used a dose of 0.02 μ l of FG in order to reduce the target neuronal population to be examined to a specific population of dopaminergic neurons within the SNpc, which would be exposed both to the RADs and FG, as described by us in detail recently^{33,34} (Figure 4).

In control experiments, we demonstrated that all FG+ neurons are TH+, and that they correspond to the same

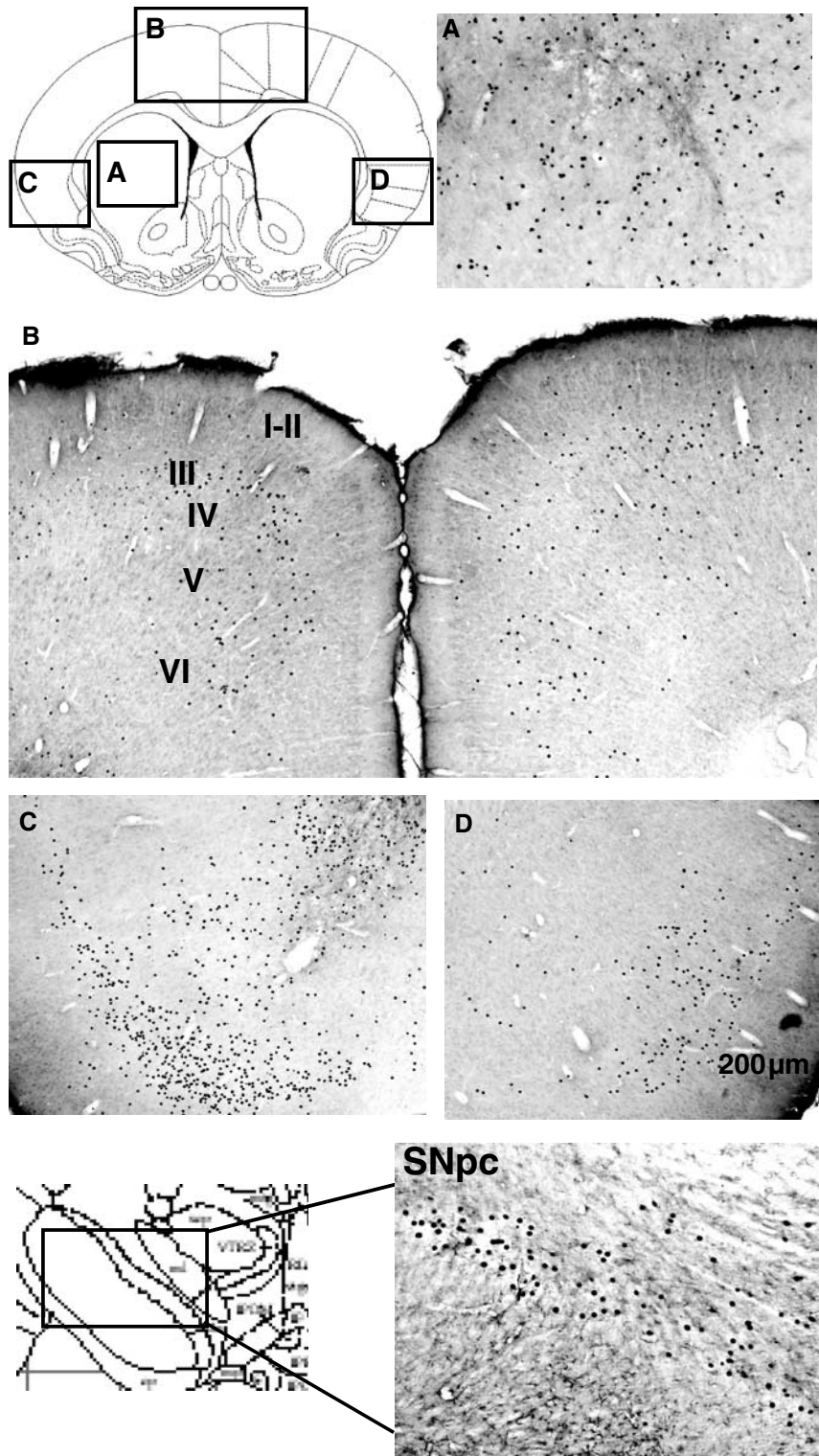


Figure 2 Expression of nuclear β -galactosidase in the neocortex, striatum, and SNpc following the injection of RAd-T α 1-lacZ into the striatum. RAd-T α 1-lacZ(ns) was injected into the striatum, and 7 days later, animals were perfused fixed and immunoreacted with antibodies recognizing β -galactosidase protein. Labeled nuclei were detected both in the striatum (A), cingular/somatosensory cortex (B), perolfactory cortex (C, D), and the SNpc. Thus, nuclear β -galactosidase immunoreactivity was detected within the striatum, and within cells projecting to the striatum. To determine the identity of transduced cells, tissues were double immunoreacted for β -galactosidase and cell-type specific markers, as shown in Figure 3.

population of dopaminergic neurons that are retrogradely infected with RAd and/or express the encoded protein; they also constitute the same population of neurons that degenerate after the injection of 6-

OHDA.^{33,34} At 4 weeks after injection of 6-OHDA, the animals were killed, the brains sectioned, and the FG+ and TH+ neurons were counted along the rostrocaudal axis of the substantia nigra, in the ipsilateral side and the

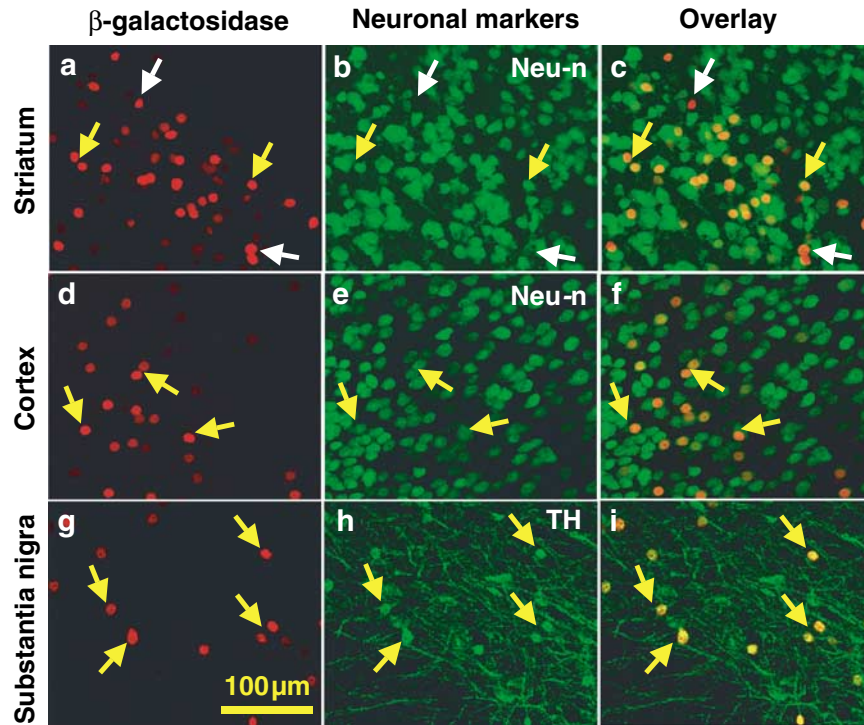


Figure 3 Identification of cells expressing nuclear β -galactosidase as neurons in the neocortex, striatum, and SNpc following the injection of RAD-T α 1-lacZ(ns) into the striatum. TH labels dopaminergic neurons and NeuN reacts with most neuronal cell types throughout the brain. (a) and (d) show nuclear β -galactosidase, visualized with Texas red, and (b, e and h) show neurons marked with NeuN and TH, respectively, each detected with secondary antibodies labeled with Fluorescein. Note the different labeling pattern of TH – it labels the cytoplasm and neurites – and NeuN, staining mainly the nuclei. (c) is the overlay of (a and b); (f) is the overlay of (d and e); (i) is the overlay of (g and h). Scale bar in (g) is 100 μ m. The number of double labeled cells in each case was >95% in each of the tissues. The <5% of cells not expressing the neuronal marker were not identified further.

contralateral side. The survival of dopaminergic neurons was expressed as the percentage of the contralateral side and analyzed statistically using ANOVA analysis. Cell numbers were assessed in three ways (Figure 4): either by counting all double-labeled (FG+/TH+) cells in all sections, counting them every sixth section and estimating the number of cells present in the tissue sections that were not counted, or by unbiased quantitative stereology using StereoInvestigator™ on an upright Zeiss microscope. All these methods provided identical results. The comparison of cell counts in every single section or every sixth section is shown in Figure 4b.

To test the effectiveness of Gli1 expressed exclusively within neurons in protecting dopaminergic neurons from 6-OHDA-induced injury, RAD-T α 1-Gli1, RAD-T α 1-lacZ, RAD-GDNF, and RAD-hCMV-Gli1 were compared in our experimental model of nigral neuronal degeneration induced by 6-OHDA. As predicted, RAD-T α 1-lacZ (Figure 5a, b, and i) had no neuroprotective effect, while GDNF did (Figure 5c, d, and i). Also, RAD-hCMV-Gli1 was effective in neuroprotecting nigral neurons from neurodegeneration (Figure 5e, f, and i). Importantly, RAD-T α 1-Gli1 (Figure 5g, h, and i) was as effective as RAD-hCMV-Gli1.

Also, the immunostaining for TH-immunoreactive fibers in the striatum indicated that, as had been described by us recently,^{33,34} only GDNF (Figure 6b) was able to partially preserve striatal dopaminergic fibers (Figure 6). Only in animals injected intrastrially with RAD-GDNF did we detect a halo of intense TH-immunoreactive fibers surrounding the intrastriatal

injection site (Figures 6b and 7). In animals injected with either the RADhCMV-Gli1 or RADT α 1-Gli1, the denervated striatum can be clearly delineated as an area of very sparse density of TH-immunoreactive fibers (Figure 6c and d). Thus, RAD-T α 1-Gli1 was as effective as RAD-hCMV-Gli1, demonstrating that a neuronal-specific promoter is efficient to drive expression of a transcription factor as a neuroprotective agent in experimental Parkinson's disease. Further, these experiments demonstrate that neuronal expression is effective to elicit the neuroprotective effect of Gli1, and that expression in other brain cells is unlikely to be necessary to enable the neuroprotective effect of Gli1 upon the survival of dopaminergic TH-immunoreactive neurons within the substantia nigra.

Fiber morphology of TH-immunoreactive fibers in the striatum of animals treated with GDNF

In view of previous reports indicating that GDNF may have some untoward effects on parameters of dopamine neuron function, such as TH-immunoreactive fiber morphology,^{10–12} we examined the morphology of TH-immunoreactive fibers in animals injected with RAD-GDNF in which we were able to detect strong TH immunoreactivity surrounding the injection site. We detected various regions of differential TH-immunoreactive fiber density in the area surrounding the injection site in animals injected with RAD-GDNF. Importantly, only in animals injected with RAD-GDNF did we detect an area of increased TH immunoreactivity surrounding

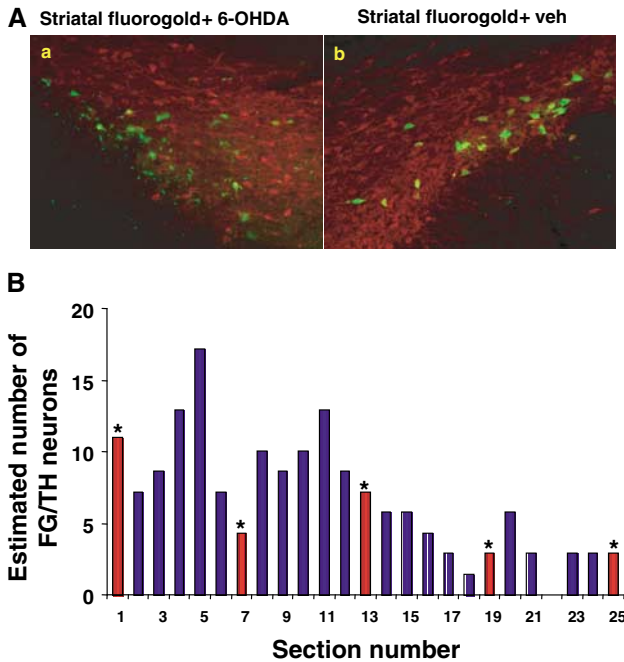
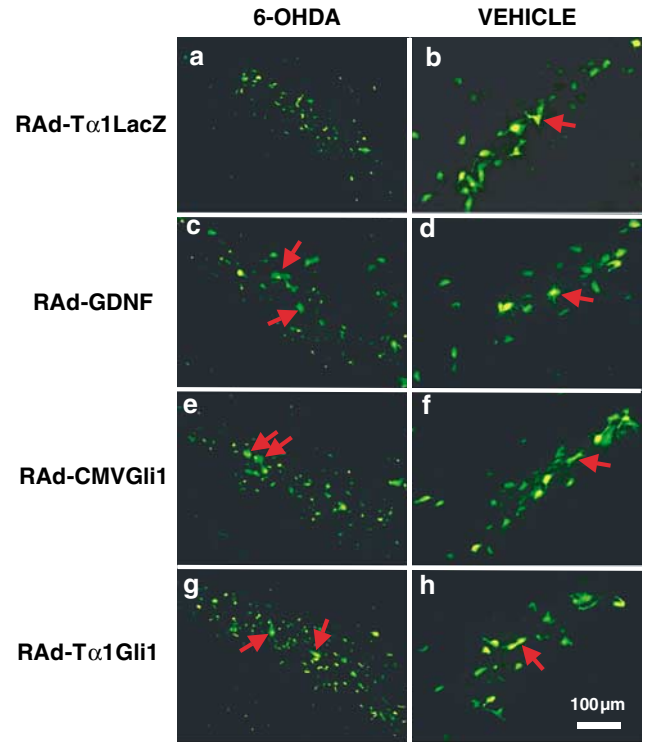


Figure 4 Labeling of nigrostriatal neurons with FG and the stereological quantitative unbiased stereological estimation of their numbers. Retrograde labeling of nigrostriatal dopaminergic neurons with FG, their lesioning with intrastriatal 6-OHDA, and their double immunoreaction with antibodies to TH is shown in (A). (B) shows the results of the quantitative unbiased stereological analysis, demonstrating that the number of double-labeled neurons for FG and TH is equally revealed if all sections are counted, or if only every sixth one (*) is analyzed.

the RAD injection site in the striatum (Figure 7), strongly suggesting that GDNF expression either preserved the density of pre-existing TH-immunoreactive fibers or caused sprouting of TH fibers following the action of 6-OHDA.

In animals injected with RAD-GDNF, we could detect the following areas of differing morphology and TH immunoreactivity within the striatum: the needle tract was evidenced by hemosiderin staining (area 1 in Figure 7), and was surrounded by an area devoid of TH immunoreactivity (area 2 in Figure 7), indicating an area of cell loss. The needle tract and the area of absence of immunoreactivity can also be seen in the animals injected with RAD-hCMV-Gli1 or RAD-T α 1-Gli1 (illustrated in Figure 6c and d). However, only in the animals injected with RAD-GDNF could we detect a significant halo of increased TH immunoreactivity (Figures 6b and 7 (area 3)). This was surrounded by an area of reduced TH immunoreactivity (area 4 in Figure 7) indicating the extent of the area of 6-OHDA caused denervation. This area of denervation extends until reaching the most peripheral areas of the striatum where the dopaminergic innervation had not been compromised by 6-OHDA and thus looks preserved. In animals injected with RAD-hCMV-Gli1 or RAD-T α 1-Gli1, as described previously, only the area of denervation can be identified (Figure 6c). In animals injected with RAD-GDNF, many axons appeared thicker and more bulbous than those found in nondenervated areas of the striatum. These atypical axonal morphologies were detected in all animals injected with RAD-GDNF (illustrated in Figure 7b–e).



→ neurons
green punctate staining: fluorogold within microglia

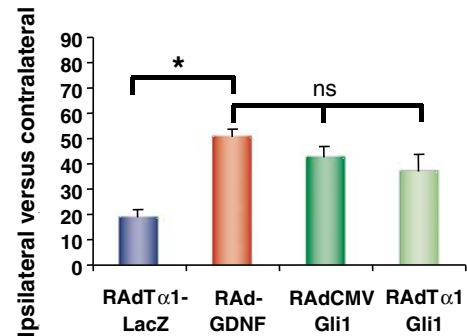


Figure 5 Neuroprotection of SNpc cell bodies following the injection of RAD vectors expressing GDNF or Gli1 into the striatum. All four treatment groups are represented: the left panels, (a, c, e, and g) show the ipsilateral side exposed to RAD at a dose of 6×10^7 IU and subsequent 6-OHDA-induced neurodegeneration. In this paradigm, as FG is administered preceding 6-OHDA-induced neurodegeneration, microglial cells on the ipsilateral side are labeled with FG, possibly by phagocytosing degenerated neurons. Microglial cells made visible by the engulfed FG can be distinguished morphologically from the polygonally shaped neurons and their long neurites by a smaller cell body and fine reticular processes. The small microglial cells never expressed TH immunoreactivity. The right panels (b, d, f, and h) show the contralateral untreated control side, only exposed to FG and vehicle. On this side exclusively neurons are labeled with FG. Scale bar in (h) is 100 μ m and applies to images (a–h). (i) shows the quantitation of the survival of nigral dopaminergic neurons containing FG.

Notice that within the area of GDNF-induced strong TH immunoreactivity (area 3 in Figure 7a), we could detect axons that were generally thicker, appeared more tortuous (Figure 7b–e), and contained enlarged bulbous terminations (Figure 7c and e) compared to those detected in areas of the striatum where the TH

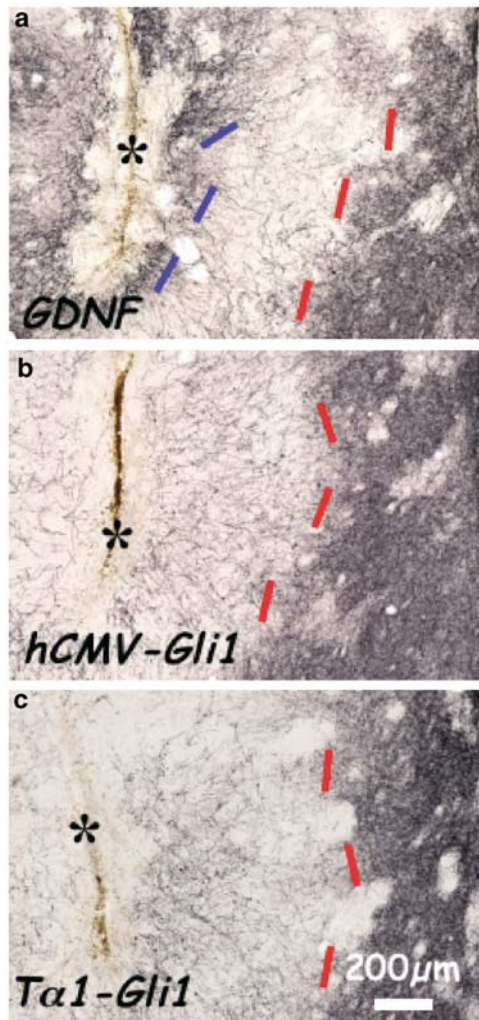


Figure 6 Effects of RAAd-GDNF (a), RAAd-Gli1 (hCMV-Gli1) (b), or RAAd-T α 1-Gli1 (c) on the density of striatal TH-immunoreactive fibers in the striatum. Injection site, area of denervation, and the boundary with normal striatum are illustrated. The asterisks overlies the injection site, the red broken line indicates the boundary between the denervation caused by 6-OHDA (to the left of the red line), and the remaining unaffected striatum (to the right). Only in animals injected with RAAd-GDNF did we detect a halo of immunoreactive fibers (to the left of the broken blue line; shown in more detail in Figure 7). No such halo was detected in any of the animals injected with the vectors expressing Gli1. The scale bar in (c, 200 μ m) applies to all images.

immunoreactivity had not been compromised by 6-OHDA (Figure 7f).

Discussion

We have previously demonstrated that the differentiation factor ShhN is able to protect dopaminergic neurons from degeneration induced by intrastriatal 6-OHDA.^{33,34} We also determined that Gli1, a transcription factor thought to mediate many of the effects of Shh, is also able to protect nigral neurons from the neurotoxic insult. We had previously used pancellular promoter elements (eg hCMV) to express Shh and Gli1. ShhN is secreted, and can thus act independently of the cell that synthesizes it. Gli1, however, is a transcription factor that most likely

acts intracellularly within dopaminergic TH-immunoreactive neurons in the substantia nigra. We wished to test the hypothesis that neuronal expression of Gli1 (eg by using a neuronal-specific promoter) will be effective in eliciting the neuroprotective actions of Gli1 in an animal model of Parkinson's disease.

We utilized the T α 1 promoter element that has been shown to mediate neuronal-specific expression in transgenic animals and in neurons *in vitro*.^{37,38,45,46} To determine whether the T α 1 promoter would retain its neuronal-specific pattern of expression within the context of an adenoviral vector, we constructed a recombinant adenoviral vector where T α 1 was cloned upstream of the gene encoding β -galactosidase containing a nuclear localization signal. Double labeling of neurons following the injection of the T α 1-lacZ vector into the striatum indicated that more than 95% of cells transduced and expressing the reporter gene also expressed the neuronal marker Neu-N indicating the neuronal phenotype of transduced cells. Importantly, cells expressing β -galactosidase within distant areas, for example, neocortex, or the substantia nigra were also immunoreactive for the appropriate neuronal markers. This confirms that cells that had been infected by the vector injected directly into the striatum, or retrogradely labeled from the injection site, were effectively neurons. This demonstrates the strict neuronal selective expression provided by the T α 1 promoter. This promoter has not been used previously in the context of gene transfer and adenoviral vectors to drive neuronal-specific expression of transgenes in the brain *in vivo*.

Expression in brain areas labeled by retrograde transport from the striatal injection site is not usually observed when a viral promoter, for example, hCMV promoter, is used to drive expression of the lacZ gene.⁴⁷ Injection of RAAd-T α 1-lacZ leads to expression of transgene in cells within the striatum, and also expression in the neocortex and substantia nigra. These data indicate that the T α 1 promoter permits high-level expression in the striatum, and within the cell bodies of neurons that had been infected retrogradely from the striatal injection site.

These results demonstrate that use of a neuronal-specific promoter, while effectively restricting expression to a single-cell type, does not restrict expression to a predetermined anatomical area, since RAdS are taken up by axonal terminals and transported back to the neuronal cell bodies. These results highlight that the use of such cell-type specific promoters will allow expression of transgenes in a predetermined target area and its selective afferent neuronal projections. We also demonstrate that the use of neuronal-specific promoters uncovered expression of β -galactosidase in neurons that provide afferents to the striatum; expression in neuronal afferents to the striatum is not seen when the pancellular hCMV promoter is used to drive expression of lacZ.⁴⁷ Thus, although cell-type specific promoters are effective, their anatomical pattern of distribution of transgene expression needs to be assessed for each vector and experimental paradigm, as highlighted by the expression in afferents projecting axons to the injection site.

The data demonstrate the effectiveness of Gli1 as a neuroprotective transgene in the 6-OHDA model of nigrostriatal dopamine neuron degeneration, when expressed selectively in neurons. Both the promiscuous

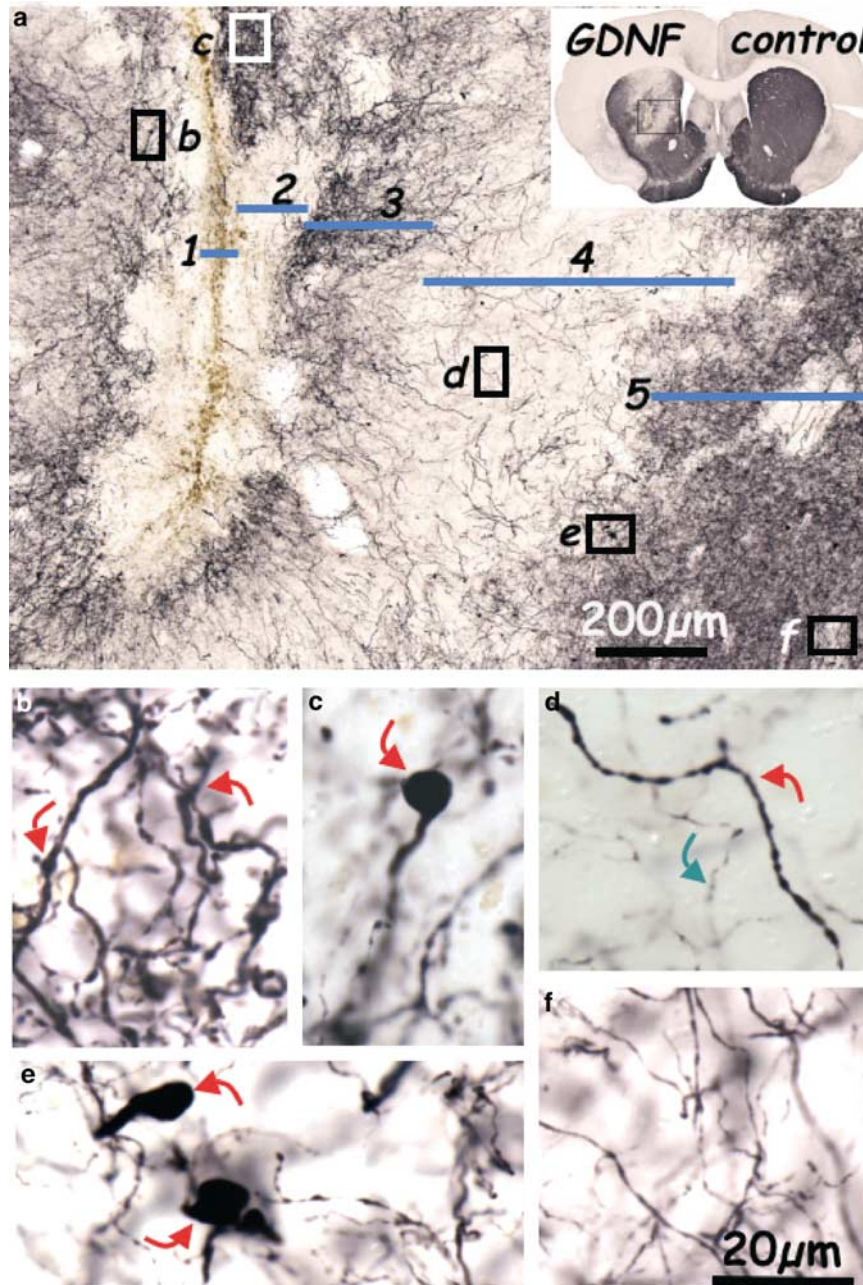


Figure 7 Neuropathology of TH-immunoreactive fibers following the injection of RAD vectors expressing GDNF into the striatum of animals subsequently injected with the neurotoxin 6-OHDA. (a) shows a larger representation of the area surrounding the needle tract in the ipsilateral striatum of an animal treated with 6×10^7 IU RAD-GDNF and subsequent 6-OHDA. TH was detected immunohistochemically. Five distinctive zones surrounding the needle tract can be distinguished microscopically. 1 is the site of injection filled with macrophages, 2 corresponds with an area of necrosis, 3 shows neurites protected from 6-OHDA neurotoxicity, 4 demonstrates the effect of 6-OHDA toxicity on DA neurons 28 days after its injection and 5 represents an area unaffected by administration of all previously mentioned substances. (a–f) show examples of the axon morphology in the different zones. Of particular note are swollen dystrophic axon terminals only found in zone 3, see image (c), and in the transition between zone 4 and 5, see image (e). Scale bar in (f) applies to images (b)–(f) and measures 20 μ m.

hCMV and the neuron-specific T α 1 promoter elicited significant neuroprotective effects on dopaminergic neurons, when challenged by 6-OHDA-induced neurodegeneration. These results can be explained by the fact that Gli1 needs to be expressed intracellularly in order to protect nigral dopaminergic neurons from neurodegeneration. The number of substantia nigra neurons that can be protected must depend on the number of neurons that become retrogradely infected from the striatal injection,

and this number is unlikely to be altered by the promoter used to drive transgene expression.

Since we used identical viral doses of all vectors, the same number of neurons should be transduced by either vector. Taking into account that cell-type specific promoters are several fold less potent at driving expression of transgenes from Rads,^{35,36,48–51} the fact that RAD-hCMV-Gli1 and RAD-T α 1-Gli1 were equally effective suggests that neuronal expression of Gli1 is a very

effective targeted therapeutic approach in this model. We did not utilize $T\alpha 1$ to drive GDNF because GDNF is supposed to be active extracellularly, and therefore, in principle the cell-type expressing GDNF is not thought to play an important role. However, it is possible that expression of GDNF directly in neurons could lead to a more physiologically regulated release that may be absent if GDNF is being released constitutively from non-neuronal cells. Whether expression of GDNF selectively from neurons leads to different neuroprotective or neurotoxic outcomes remains to be determined in future experiments.

Our results also confirm previously suggested untoward effects of GDNF on the morphology of dopaminergic TH-immunoreactive fibers. In all animals injected with GDNF, while we could observe a distinct corona of increased innervation near the site where RAD-GDNF had been injected, we could also detect that the normal axonal morphology was affected. Whether GDNF is protecting pre-existing TH-immunoreactive fibers, or causing their sprouting following injection of 6-OHDA, and whether the detected anatomical characteristics have any bearing on the function of these axons remains to be determined.

In summary, our results demonstrate that transcriptional targeting to neurons of Gli1 expressed from a RAD in the 6-OHDA model of nigral degeneration is neuroprotective. Our results also demonstrate that the use of a neuronal-specific promoter can be sufficient to achieve a therapeutic effect. Further, the fact that the $T\alpha 1$ promoter, upon injection into the striatum, transduces not only striatal neurons but also other striatal afferents may provide the possibility of transducing interconnected neuronal circuits that may provide further therapeutic advantages.

Materials and methods

Construction of RAD- $T\alpha 1$ -Gli1 and RAD- $T\alpha 1$ -lacZ

The construction, characterization, and *in vitro* and *in vivo* effectiveness of RAD-hCMV-GDNF and RAD-hCMV-Gli1 has been described in detail previously.^{33,34} To construct RAD- $T\alpha 1$ -lacZ and RAD- $T\alpha 1$ -Gli1, the cDNA for lacZ or human Gli1 was cloned into the shuttle plasmid p Δ E1 under the control of the $T\alpha 1$ promoter (kindly provided by Freda Miller, Toronto, Canada). RAD- $T\alpha 1$ -lacZ and RAD- $T\alpha 1$ -Gli1 were generated by homologous recombination after cotransfection in 293 cells of recombinant shuttle vectors with the Ad5 viral genome plasmid pBHG10 (Microbix Inc., Toronto, Canada). RAD- $T\alpha 1$ -lacZ and RAD- $T\alpha 1$ -Gli1 were purified by three rounds of plaque purification by end point dilution assay to ensure that the virus stock derived from a single infectious viral particle. Then, large-scale preps of RAD were performed and purified using CsCl gradients. All vector preparations were tested for endotoxin (LPS, lipopolysaccharide) contamination using the multitest limulus amoebocyte lysate pyrogen^R kit (BioWhittaker) according to the manufacturer's instructions. A two-step assay was used to detect contamination of vector preparations with replication-competent adenovirus, as described by Dion *et al*⁵² and utilized by us elsewhere.^{39,40}

Detection of transgene expression

Expression of β -galactosidase was detected using primary rabbit antibodies against β -galactosidase, biotinylated secondary antibodies, and the ABC method to detect antigen presence using light microscopic immunocytochemistry, as described by us in detail elsewhere.^{33,34,39,40}

Bioactivity of Gli1 and GDNF

The bioactivity of Gli1 and GDNF were tested and reported previously.^{33,34}

Surgical methods

A measure of 200 g male Fisher 344 rats were kept in the Comparative Medicine Department at Cedars Sinai Medical Center, Los Angeles, USA. For stereotaxic surgery, animals were maintained under gaseous anesthesia (Fluothane). Two holes were drilled in the skull and 0.02 μ l of 2% FG (Fluorochrome Inc.) diluted in saline 0.9% (w/v) was bilaterally injected at the level of the dorsal striatum in the following coordinates: AP: +1 mm; ML: ± 30 ; and DV: -5 mm at a rate of 0.01 μ l/min. The needle was left in place for 5 min and retracted 1 mm/min. After injection of FG, RAD was injected unilaterally at: AP: +1 mm; ML: +3; and DV: -5 mm. A total of 6×10^7 IU in 3 μ l of PBS pH 7.4 was administered at a rate of 1 μ l/min. The needle was left in place for 5 min and retracted at 1 mm/min. After 1 week, degeneration of the nigrostriatal pathway was induced by injection of 16 μ g in 6-OHDA hydrochloride (Sigma) at the same coordinates of FG and RAD injection. The 6-OHDA was resuspended in ascorbic acid (2 mg/ml in saline 0.9% (w/v)) and the working aliquot of 6-OHDA was replaced every 2 h during the surgical procedure. Animals were killed 4 weeks after injection of 6-OHDA and their brains were fixed by transcardiac perfusion with 4% paraformaldehyde. Brains were postfixed for an additional 6 h and 25 and 50 μ m coronal sections were cut using a vibratome. FG+ cells were counted throughout the rostrocaudal axis of the substantia nigra from AP: -4.80 mm to AP: -6.04 mm.

Immunocytochemistry

Detection of TH+ neurons in the substantia nigra or TH fibers in the striatum was carried out by immunohistochemistry. Briefly, brain sections were washed with TBS (with 0.5% Triton X-100), incubated with 0.3% hydrogen peroxide, and blocked with 10% horse serum. Brains were incubated with primary antibody using anti-TH polyclonal antibody (1:1000-1:2500) (Calbiochem, USA) diluted in 1% horse serum (diluted in TBS 0.5% triton). Total immunoglobulins conjugated with Texas red (Jackson Research Labs, USA) or Biotin (Dakopatts, USA) were used as secondary antibodies. Biotinylated antibodies were detected with the avidin-biotin-horse radish-complex detection kit (ABC kit, Vector Labs, US). DAB (Sigma) was used as the colorimetric substrate. Sections were mounted on gelatine-coated slides, dehydrated with alcohol gradients, cleared with xylene, and coverslipped using DPX.^{33,34,39,40,44}

Quantitative unbiased stereological analysis

An optical fractionator protocol was used for unbiased stereological cell estimation in the SNpc. With this

counting technique, systematic random sampling is performed in a three-dimensional space and cell estimates are unaffected by tissue shrinkage.⁵³ In the anterior–posterior plain sections from bregma -4.7 to -6.2 mm⁵⁴ were included. We defined the medial border of the SNpc in the rostrocaudal axis by the medial terminal nucleus of the accessory optic tract or fibers of the medial lemniscus, excluding TH+ neurons in the ventral tegmental area, according to the rat atlas of Paxinos. Only TH+ cells within the SNpc were counted. Every sixth coronal section was sampled, yielding five sections per animal. Analysis was performed using Stereo Investigator software version 5.00 (MicroBrightfield Inc., Colchester, VT, USA) and a Zeiss Axioplan 2 microscope controlled by a Ludl electronic MAC 5000 XY stage control (Ludl Electronics Products Ltd, Hawthorne, NY, USA), and Axioplan Z-axis control (Carl Zeiss Inc., Thornwood, NY, USA).

The region of interest to be sampled was traced with a $\times 5$ objective. To arrive at sufficiently precise cell estimates, the number of FG+ and TH+ cells in the ipsilateral SNpc, which tended to be somewhat below 300 were counted exhaustively on the ipsi- and contralateral side. Therefore, we designed both the counting-frame and grid-size at $125 \times 125 \mu\text{m}^2$ leaving no space between counting frames. To estimate the number of TH+ cells in the SNpc, we designed counting frames sized $100 \times 100 \mu\text{m}^2$ with a grid size of $120 \times 120 \mu\text{m}^2$. The thickness of all counting frames was $14 \mu\text{m}$, and every sixth section was counted. To avoid the problem of lost caps and to exclude tissue that may be distorted at the surfaces of a section, top and bottom guard zones of $3 \mu\text{m}$ each were chosen.

Cells were assessed with a $\times 63$ oil-immersion objective with a numerical aperture of 1.4 and were included in the count if they were coming into focus while focusing down from the top of the counting frame and their top was within all boundaries of the counting frame, that is, in the x -, y -, and z -axis. The estimate of the total number of neurons was calculated according to the optical fractionator formula (see West *et al*⁵³ for details).

Acknowledgements

This work in the GTRI is funded by NIH Grants 1 RO1 NS44556 (MGC), 1 RO1 NS42893 (PRL), U54 4 NS04-5309 (PRL), R21 NS47298 (PRL), and the Kane Fellowship in Gene Therapy for Cancer Research. Dr D Suwelack was supported during part of the work described herein by a doctoral fellowship from The Wellcome Trust, UK. Drs Andres Hurtado-Lorenzo and Enrique Millan, during their PhD work, were generously supported by pre-doctoral fellowships from the UK Academic Council for Foreign Graduates, the University of Manchester, England, and the National Research Council of Venezuela. We thank Freda Miller for providing the plasmid containing the T α 1 promoter. PRL is a holder of the Bram and Elaine Goldsmith Chair in Gene Therapeutics. We are very grateful to the Board of Governors at Cedars-Sinai Medical Center for their vision and very generous creation and support of the GTRI. We also thank Dr Shlomo Melmed for his support and academic leadership, Mr Richard Katzman for his excellent administrative support, and Mr Nelson Jovel for the

skillful editing and preparation of the figures and manuscript for publication.

References

- 1 Mandel S *et al*. Neuroprotective strategies in Parkinson's disease: an update on progress. *CNS Drugs* 2003; **17**: 729–762.
- 2 Kordower JH *et al*. Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. *Science* 2000; **290**: 767–773.
- 3 Kirik D, Rosenblad C, Bjorklund A, Mandel RJ. Long-term rAAV-mediated gene transfer of GDNF in the rat Parkinson's model: intrastratial but not intranigral transduction promotes functional regeneration in the lesioned nigrostriatal system. *J Neurosci* 2000; **20**: 4686–4700.
- 4 Choi-Lundberg DL *et al*. Dopaminergic neurons protected from degeneration by GDNF gene therapy. *Science* 1997; **275**: 838–841.
- 5 Castro MG *et al*. Gene therapy for Parkinson's disease: recent achievements and remaining challenges. *Histol Histopathol* 2001; **16**: 1225–1238.
- 6 Cass WA. GDNF selectively protects dopamine neurons over serotonin neurons against the neurotoxic effects of methamphetamine. *J Neurosci* 1996; **16**: 8132–8139.
- 7 Gill SS *et al*. Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. *Nat Med* 2003; **9**: 589–595.
- 8 Bjorklund A *et al*. Towards a neuroprotective gene therapy for Parkinson's disease: use of adenovirus, AAV and lentivirus vectors for gene transfer of GDNF to the nigrostriatal system in the rat Parkinson model. *Brain Res* 2000; **886**: 82–98.
- 9 Burton EA, Glorioso JC, Fink DJ. Gene therapy progress and prospects: Parkinson's disease. *Gene Therapy* 2003; **10**: 1721–1727.
- 10 Georgievska B, Kirik D, Bjorklund A. Aberrant sprouting and downregulation of tyrosine hydroxylase in lesioned nigrostriatal dopamine neurons induced by long-lasting overexpression of glial cell line derived neurotrophic factor in the striatum by lentiviral gene transfer. *Exp Neurol* 2002; **177**: 461–474.
- 11 Arvidsson A *et al*. Elevated GDNF levels following viral vector-mediated gene transfer can increase neuronal death after stroke in rats. *Neurobiol Dis* 2003; **14**: 542–556.
- 12 Rosenblad C, Georgievska B, Kirik D. Long-term striatal overexpression of GDNF selectively downregulates tyrosine hydroxylase in the intact nigrostriatal dopamine system. *Eur J Neurosci* 2003; **17**: 260–270.
- 13 Hynes M *et al*. Induction of midbrain dopaminergic neurons by Sonic Hedgehog. *Neuron* 1995; **15**: 35–44.
- 14 Lai K, Kaspar BK, Gage FH, Schaffer DV. Sonic Hedgehog regulates adult neural progenitor proliferation *in vitro* and *in vivo*. *Nat Neurosci* 2003; **6**: 21–27.
- 15 Matsuura N *et al*. Sonic Hedgehog facilitates dopamine differentiation in the presence of a mesencephalic glial cell line. *J Neurosci* 2001; **21**: 4326–4335.
- 16 Sakurada K, Ohshima-Sakurada M, Palmer TD, Gage FH. Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells derived from the adult brain. *Development* 1999; **126**: 4017–4026.
- 17 Zetterstrom RH *et al*. Dopamine neuron agenesis in Nurr1-deficient mice. *Science* 1997; **276**: 248–250.
- 18 Kalderon D. Transducing the hedgehog signal. *Cell* 2000; **103**: 371–374.
- 19 Hui CC *et al*. Expression of three mouse homologs of the *Drosophila* segment polarity gene cubitus interruptus, Gli, Gli-2, and Gli-3, in ectoderm- and mesoderm-derived tissues suggests multiple roles during postimplantation development. *Dev Biol* 1994; **162**: 402–413.

- 20 Lee J, Platt KA, Censullo P, Ruiz i Altaba A. Gli1 is a target of Sonic Hedgehog that induces ventral neural tube development. *Development* 1997; **124**: 2537–2552.
- 21 Platt KA, Michaud J, Joyner AL. Expression of the mouse Gli and Ptc genes is adjacent to embryonic sources of hedgehog signals suggesting a conservation of pathways between flies and mice. *Mech Dev* 1997; **62**: 121–135.
- 22 Sasaki H, Hui C, Nakafuku M, Kondoh H. A binding site for Gli proteins is essential for HNF-3beta floor plate enhancer activity in transgenics and can respond to Shh *in vitro*. *Development* 1997; **124**: 1313–1322.
- 23 Ruiz IAA, Palma V, Dahmane N. Hedgehog-Gli signalling and the growth of the brain. *Nat Rev Neurosci* 2002; **3**: 24–33.
- 24 Hardy RJ. Dorsoventral patterning and oligodendroglial specification in the developing central nervous system. *J Neurosci Res* 1997; **50**: 139–145.
- 25 Charytoniuk D et al. Sonic Hedgehog signalling in the developing and adult brain. *J Physiol Paris* 2002; **96**: 9–16.
- 26 Traiffort E et al. Discrete localizations of hedgehog signalling components in the developing and adult rat nervous system. *Eur J Neurosci* 1999; **11**: 3199–3214.
- 27 Traiffort E, Charytoniuk DA, Faure H, Ruat M. Regional distribution of Sonic Hedgehog, patched, and smoothened mRNA in the adult rat brain. *J Neurochem* 1998; **70**: 1327–1330.
- 28 Miao N et al. Sonic Hedgehog promotes the survival of specific CNS neuron populations and protects these cells from toxic insult *In vitro*. *J Neurosci* 1997; **17**: 5891–5899.
- 29 Kim TE et al. Sonic Hedgehog and FGF8 collaborate to induce dopaminergic phenotypes in the Nurr1-overexpressing neural stem cell. *Biochem Biophys Res Commun* 2003; **305**: 1040–1048.
- 30 Bezard E et al. Sonic Hedgehog is a neuromodulator in the adult subthalamic nucleus. *FASEB J* 2003; **17**: 2337–2338.
- 31 Dass B et al. Behavioural and immunohistochemical changes following supranigral administration of Sonic Hedgehog in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated common marmosets. *Neuroscience* 2002; **114**: 99–109.
- 32 Tsuboi K, Shults CW. Intrastratial injection of Sonic Hedgehog reduces behavioral impairment in a rat model of Parkinson's disease. *Exp Neurol* 2002; **173**: 95–104.
- 33 Hurtado-Lorenzo A, Millan E, Castro M, Lowenstein PR. Adenovirus-mediated gene transfer of Shh, Gli-1 and Nurr-1 in rat model of Parkinson's disease. *30th Annual Meeting of the Society for Neuroscience*. New Orleans, LA, 2000.
- 34 Hurtado-Lorenzo A et al. Differentiation- and transcription factor gene therapy in experimental Parkinson's disease: Sonic Hedgehog and Gli-1, but not Nurr-1, protect nigro-striatal cell bodies from 6-OHDA induced neurodegeneration. *Mol Ther* 2004; **10**: 507–524.
- 35 Smith-Arica JR et al. Cell-type-specific and regulatable transgenesis in the adult brain: adenovirus-encoded combined transcriptional targeting and inducible transgene expression. *Mol Ther* 2000; **2**: 579–587.
- 36 Southgate TD et al. Transcriptional targeting to anterior pituitary lactotrophic cells using recombinant adenovirus vectors *in vitro* and *in vivo* in normal and estrogen/sulpiride-induced hyperplastic anterior pituitaries. *Endocrinology* 2000; **141**: 3493–3505.
- 37 Bamji SX, Miller FD. Comparison of the expression of a T alpha 1:nlacZ transgene and T alpha 1 alpha-tubulin mRNA in the mature central nervous system. *J Comp Neurol* 1996; **374**: 52–69.
- 38 Gloster A et al. The T alpha 1 alpha-tubulin promoter specifies gene expression as a function of neuronal growth and regeneration in transgenic mice. *J Neurosci* 1994; **14**: 7319–7330.
- 39 Southgate T, Kingston P, Castro MG. Gene transfer into neural cells *in vivo* using adenoviral vectors. In: Gerfen CR, McKay R, Rogawski MA, Sibley DR, Skolnick P (eds). *Current Protocols in Neuroscience*. John Wiley and Sons: New York, NY, 2000, pp 4.23.21–24.23.40.
- 40 Thomas CE et al. Gene transfer into rat brain using adenoviral vectors. In: Gerfen JN, McKay R, Rogawski MA, Sibley DR, Skolnick P (eds). *Current Protocols in Neuroscience*. John Wiley and Sons: New York, NY, 2000, pp 4.23.21–24.23.40.
- 41 Shering AF et al. Cell type-specific expression in brain cell cultures from a short human cytomegalovirus major immediate early promoter depends on whether it is inserted into herpesvirus or adenovirus vectors. *J Gen Virol* 1997; **78**: 445–459.
- 42 Thomas CE et al. Acute direct adenoviral vector cytotoxicity and chronic, but not acute, inflammatory responses correlate with decreased vector-mediated transgene expression in the brain. *Mol Ther* 2001; **3**: PG-36–PG-46.
- 43 Thomas CE et al. Preexisting antiadenoviral immunity is not a barrier to efficient and stable transduction of the brain, mediated by novel high-capacity adenovirus vectors. *Hum Gene Ther* 2001; **12**: 839–846.
- 44 Thomas CE et al. Peripheral infection with adenovirus causes unexpected long-term brain inflammation in animals injected intracranially with first-generation, but not with high-capacity, adenovirus vectors: toward realistic long-term neurological gene therapy for chronic diseases. *Proc Natl Acad Sci USA* 2000; **97**: 7482–7487.
- 45 Kügler S et al. Neuron-specific expression of therapeutic proteins: evaluation of different cellular promoters in recombinant adenoviral vectors. *Mol Cell Neurosci* 2001; **17**: 78–96.
- 46 Mathew TC, Miller FD. Induction of T alpha 1 alpha-tubulin mRNA during neuronal regeneration is a function of the amount of axon lost. *Dev Biol* 1993; **158**: 467–474.
- 47 Zermansky AJ et al. Towards global and long-term neurological gene therapy: unexpected transgene dependent, high-level, and widespread distribution of HSV-1 thymidine kinase throughout the CNS. *Mol Ther* 2001; **4**: PG-490–PG-498.
- 48 Windeatt S et al. Adenovirus-mediated herpes simplex virus type-1 thymidine kinase gene therapy suppresses oestrogen-induced pituitary prolactinomas. *J Clin Endocrinol Metab* 2000; **85**: 1296–1305.
- 49 Davis JR et al. Cell type-specific adenoviral transgene expression in the intact ovine pituitary gland after stereotaxic delivery: an *in vivo* system for long-term multiple parameter evaluation of human pituitary gene therapy. *Endocrinology* 2001; **142**: 795–801.
- 50 Gerdes CA, Castro MG, Lowenstein PR. Strong promoters are the key to highly efficient, noninflammatory and noncytotoxic adenoviral-mediated transgene delivery into the brain *in vivo*. *Mol Ther* 2000; **2**: 330–338.
- 51 Castro MG et al. Regulatable and cell-type specific transgene expression in glial cells: prospects for gene therapy for neurological disorders. In: *Progress in Brain Research*. Elsevier Science Publishers: Amsterdam, 2001, pp 665–691.
- 52 Dion LD, Fang J, Garver Jr RI. Supernatant rescue assay versus polymerase chain reaction for detection of wild type adenovirus-contaminating recombinant adenovirus stocks. *J Virol Methods* 1996; **56**: 99–107.
- 53 West MJ, Slomianka L, Gundersen HJ. Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. *Anat Rec* 1991; **231**: 482–497.
- 54 Paxinos G, Wilson C. *The Rat Brain in Stereotaxic Coordinates*. Academic Press: San Diego, 1988.