

Differentiation and Transcription Factor Gene Therapy in Experimental Parkinson's Disease: Sonic Hedgehog and Gli-1, but Not Nurr-1, Protect Nigrostriatal Cell Bodies from 6-OHDA-Induced Neurodegeneration

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We tested the activity of the dopaminergic neuron differentiation factor sonic hedgehog, its downstream transcription factor target Gli-1, and an orphan nuclear receptor, Nurr-1, necessary for the induction of the dopaminergic phenotype of nigrostriatal neurons, in an *in vivo* model of nigrostriatal neurodegeneration. Our preliminary experiments demonstrated that all three constructs expressed the proper molecules and that these had the predicted biological activities *in vitro*. We expressed the N-terminal of sonic hedgehog (ShhN) and the Gli-1 and Nurr-1 entire coding regions from highly purified, and quality controlled, replication-defective adenoviral vectors injected into the brains of rats and used the dopaminergic growth factor GDNF as a positive control. The neurotoxin 6-hydroxydopamine was used to lesion the nigrostriatal dopaminergic innervation; RAd-ShhN and RAd-Gli-1 protected dopaminergic neuronal cell bodies in the substantia nigra, but not axonal terminals in the striatum, from 6-OHDA-induced cell death, while RAd-Nurr-1 was ineffective in protecting either cell bodies or axons. RAd-GDNF was able to protect both the dopaminergic cell bodies and the striatal axon terminals. Our results establish for the first time, to the best of our knowledge, that gene transfer of ShhN and one of its target transcription factors can selectively protect dopaminergic nigrostriatal neuronal cell bodies from a specific neurotoxic insult. Selective protection of nigrostriatal dopaminergic cell bodies by the differentiation factor ShhN and the transcription factor Gli-1 was achieved in a neurotoxic model that eliminates more than 70% of the nigral neurons under consideration. Differentiation and transcription factors can thus be used for the treatment of neurodegeneration by gene therapy.

Key Words: Parkinson's disease, neurodegeneration, gene therapy, differentiation factors, transcription factors, stem cells

INTRODUCTION

Treatments for Parkinson's disease, although effective, do not halt the progressive loss of substantia nigra dopaminergic neurons. Eventually, clinical symptoms become resistant to treatments relying on the integrity of nigrostriatal neurons, such as L-DOPA [1]. Preserving viable nigrostriatal neurons would delay disease progression and thus prolong treatments' efficacy [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].

GDNF has been delivered into the brain using adenovirus (RAd)-, adeno-associated virus-, herpes simplex virus type 1 (HSV-1)-, or lentiviral-derived vectors or by direct peptide injection [5,7–9]. Despite its neuroprotective actions, GDNF can have untoward effects, i.e., reduction of tyrosine hydroxylase mRNA in nigrostriatal neurons, aberrant morphologies of striatal tyrosine hydroxylase-immunoreactive axons, and increased cell death following experimental stroke [10–12].

Thus, we explored potential neuroprotective effects of other factors important for dopamine neuron development, i.e., sonic hedgehog (Shh) N-terminal peptide (ShhN), Gli-1, and Nurr-1 [13–16]. Shh, secreted by the floor plate, ventralizes the developing neural tube and induces differentiation of midbrain nigrostriatal dopamine neurons [17]. Shh interacts with its receptor patched (*ptc*) and smoothened (*smo*) [18], leading to the phosphorylation and nuclear translocation of the transcription factor Gli-1 [19,20] and activation of downstream genes [21–24].

ShhN protects cultures of fetal dopamine neurons from MPP+ toxicity [25] and regulates the differentiation and proliferation of neuronal stem cells [13,14,26]. Further, Shh peptide injected directly into the brains of rodents and marmosets has beneficial effects in experimental models of Parkinson's disease [27–29]. Nurr-1 is an orphan nuclear receptor necessary for the expression of the dopaminergic phenotype of developing nigrostriatal neurons, e.g., tyrosine hydroxylase, dopamine transporter [16]. Shh, *ptc*, *smo*, Gli-1, and Nurr-1 are present in the adult rodent brain [30–32].

To test the hypothesis that Shh, Gli-1, or Nurr-1 protects dopamine nigrostriatal neurons from neurotoxin-induced neurodegeneration we constructed RAd vectors expressing ShhN (RAd-ShhN), Gli-1 (RAd-Gli-1), or Nurr-1 (RAd-Nurr-1) under the control of the major immediate early human cytomegalovirus promoter (hCMV) and compared these to GDNF (RAd-GDNF) and a control vector expressing β -galactosidase (RAd-35).

RAd-ShhN and RAd-Gli-1 protected nigrostriatal dopaminergic cell bodies, but not their striatal terminals, from 6-OHDA-induced neurodegeneration, while RAd-Nurr-1

was ineffective. Our results indicate that nigrostriatal dopaminergic cell bodies can be protected from neurotoxin-induced cell death independent of the maintenance of their axonal terminals. ShhN and Gli-1 may be neuroprotective through the activation of mechanisms different from those of GDNF, which protects both cell bodies and striatal terminals.

RESULTS

Molecular Characterization of Recombinant Adenoviral Vectors

We cotransfected the shuttle vectors encoding GDNF, ShhN, Gli-1, or Nurr-1 with the adenovirus 5 (Ad5) genomic plasmid pJM17 into 293 cells; the structure of the expected recombinant vectors is shown in Fig. 1a. After the onset of cytopathic effect (CPE), we collected infected cells and extracted their DNA to characterize the recombinant adenoviruses and confirm the presence of the transgenes within the adenoviral genome (RAd-GDNF, Figs. 1b and 1c; RAd-ShhN, Figs. 1d and 1e; RAd-Gli-1, Figs. 1f and 1g; the construction of RAd-Nurr-1 is not illustrated in detail).

Fig. 1b shows the restriction pattern and Fig. 1c the Southern blot hybridization of the shuttle vector pALGDNF (lane 1), RAd-GDNF (lane 2), the Ad5 genomic plasmid pJM17 (lane 3), and the molecular weight (MW) markers (lane 4). We extracted DNA and digested it with *Hind*III to release the insert (0.7 kb) together with the major immediate early hCMV promoter (0.7 kb), resulting in the generation of a 1.4-kb fragment (Figs. 1b and 1c) from the shuttle vector (lanes 1), and the RAd-GDNF (lanes 2), but not from pJM17 (lanes 3). We used Southern blot hybridization, utilizing a GDNF-specific 0.7-kb DIG-labeled probe corresponding to the full-length GDNF cDNA, to identify the 1.4-kb band. As shown in Fig. 1c the shuttle vector (lane 1) and RAd-GDNF (lane 2) exhibit the expected 1.4-kb positive hybridization signal, whereas this signal is absent from the plasmid pJM17 (lane 3).

We also constructed a recombinant adenovirus encoding ShhN under the control of the hCMV promoter (RAd-ShhN). We extracted the viral DNA and digested it with *Hind*III. Fig. 1d shows the restriction patterns of RAd-ShhN (lane 1) and the shuttle vector pALShhN (Fig. 1d, lane 2) used as positive control. The *Hind*III digestion resulted in the release of a 0.9-kb fragment that corresponds to the ShhN coding region together with the hCMV promoter (Fig. 1d, lanes 1 and 2). We confirmed the identity of the 0.9-kb band by Southern blot hybridization using a homologous DIG-labeled probe corresponding to the ShhN cDNA. As shown in Fig. 1e the probe specifically hybridized with the 0.9-kb band of RAd-ShhN (Fig. 1e, lane 1) and the shuttle vector pALShhN (Fig. 1e, lane 2).

Following the cotransfection of the shuttle vector pAL-Gli-1 together with the Ad5 genomic plasmid pJM17 into 293 cells, and the onset of CPE, we extracted the viral DNA

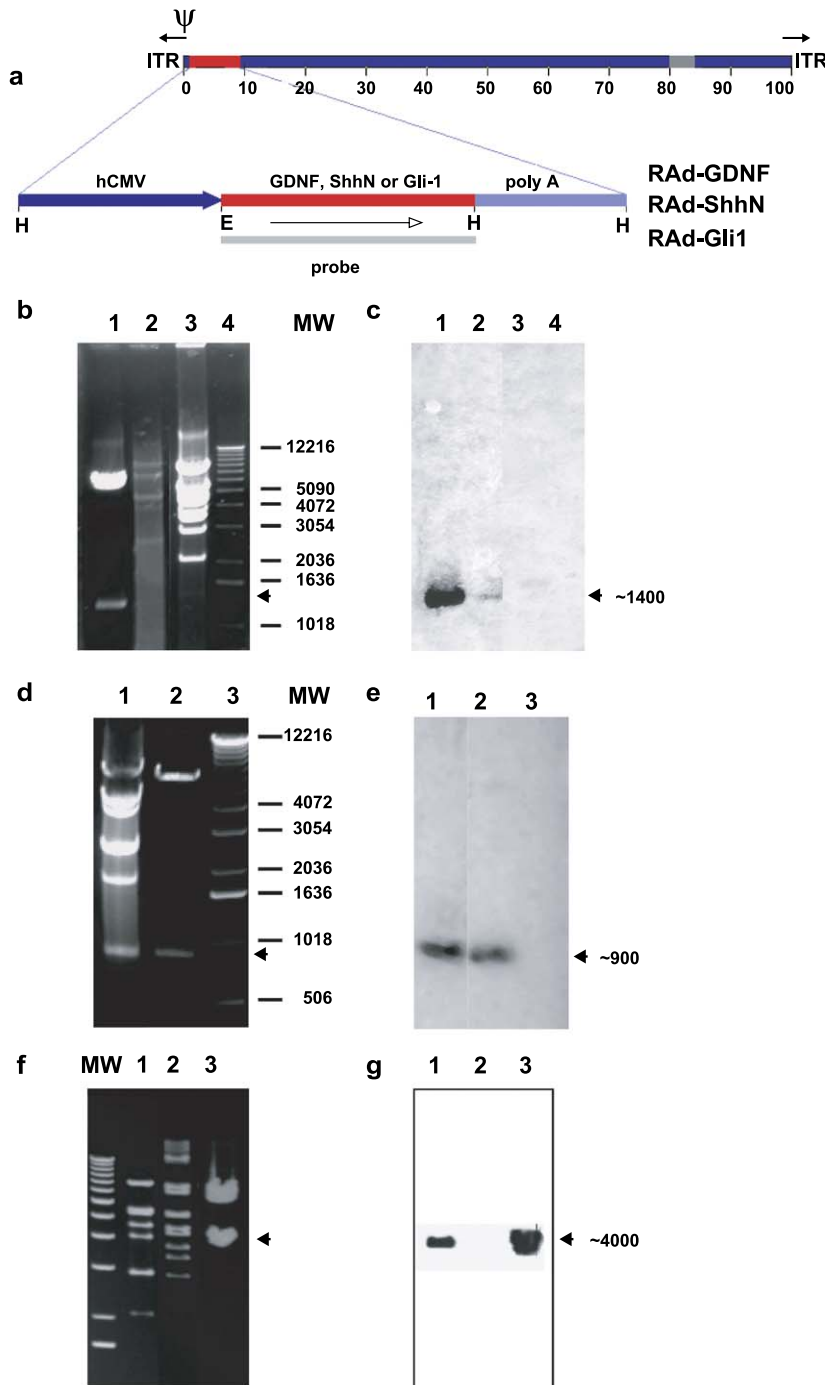


FIG. 1. Genomic structures of RAD-GDNF, RAD-ShhN, and RAD-Gli-1. Recombinant adenoviruses (RAD) were generated by homologous recombination after cotransfection into 293 cells of a shuttle expression plasmid encoding ShhN, Gli-1, or Nurr-1 together with the Ad5 genomic plasmid pJM17. The shuttle plasmid contained adenoviral DNA sequences encoding the left-end replication origin/packaging elements and the overlap–recombination region. Restriction patterns of adenoviral vectors digested with *Hind*III confirmed the presence of the transgenes. Identity of the transgenes was confirmed by Southern blot hybridization, using specific DIG-labeled probes. (a) The schematic structure of the new vectors described herein. (b, c) The characterization of RAD-GDNF, with (b) the restriction analysis and (c) the Southern blot hybridization indicating the presence of the expected transgene band (1.4 kb), in lanes 1 and 2. In b and c the lane numbers indicate 1, the shuttle vector used to construct the recombinant virus, pALGDNF (as positive control); 2, RAD-GDNF; 3, the Ad5 genomic plasmid pJM17 (as negative control); 4, molecular weight markers. (d, e) The characterization of RAD-ShhN, with (d) the restriction analysis and (e) the Southern blot hybridization indicating the presence of the band containing the transgene ShhN (0.9 kb), in lanes 1 and 2. In d and e the lane numbers indicate 1, RAD-ShhN; 2, the shuttle plasmid pALShhN (as positive control); 3, molecular weight markers. (f, g) The characterization of RAD-Gli-1, with (f) the restriction analysis and (g) the Southern blot hybridization indicating the presence of the band containing the transgene Gli-1, of approx 4.0 kb, in lanes 1 and 3. In f and g the lane numbers indicate MW, molecular weight markers; 1, RAD-Gli-1; 2, pJM17 (as negative control); 3, pALGli-1 (as positive control). The construction of Nurr-1 followed identical principles but has not been illustrated.

and digested it with *Hind*III to confirm the presence of the Gli-1 transgene within the Ad5 genome. Fig. 1f shows the restriction pattern of RAD-Gli-1 in lane 1. The digestion released the insert (3.6 kb) together with the poly(A) signal (0.4 kb) that results in the generation of a 4.0-kb fragment. We used the shuttle plasmid pALGli-1 digested with *Hin*

dIII as positive control for the presence of the expression cassette (Fig. 1f, lane 3) and the *Hind*III-digested Ad5 genomic plasmid pJM17 (Fig. 1f, lane 2) as negative control. To confirm the identity of the 4.0-kb band we performed a Southern hybridization, using as a probe the Gli-1 cDNA fragment labeled with DIG. A positive hybrid-

ization signal was detected in the lane corresponding to RAD-Gli-1 (Fig. 1g, lane 1) as well as in the lane corresponding to the shuttle vector pALGli-1 digested with *Hind*III (Fig. 1g, lane 3); in contrast, no hybridization signal was detected in the digested viral plasmid pJM17 (Fig. 1g, lane 2) used as negative control.

Release of ShhN by BHK or Primary Cultures of Glial Cells Infected with RAD-ShhN

Previously, it was demonstrated that by expressing the ShhN-terminal sequence, it is possible to obtain a functional and soluble peptide [33–37]. To evaluate whether RAD-ShhN expressed a soluble form of the ShhN peptide, we infected BHK cells with RAD-ShhN at m.o.i. 300 for 6 h, after which we replaced the supernatant and incubated the cells for a further 48 h. After 48 h we collected the supernatant and concentrated the proteins. We used BHK cells infected with a RAD expressing β -galactosidase (RAD-35), uninfected cells, or uninfected cells grown in the absence of serum as negative controls. The presence of ShhN in the supernatant was detected by Western blot (Figs. 2a and 2b). A 20-kDa band corresponds to the predicted molecular weight of ShhN. This band was immunoreactive for the specific anti-Shh monoclonal antibody 5E1 in the sample obtained from RAD-ShhN-infected cells (Fig. 2b, lane 4). Such an immunoreactive band was not detected in the samples obtained from BHK cells infected with RAD-35 (Fig. 2b, lane 3), uninfected cells (Fig. 2b, lane 2), or uninfected cells cultured in the absence of serum (Fig. 2b, lane 1).

Dot-blot analysis also further confirmed that ShhN was secreted into the medium, whereas it was not detected in the media from RAD-35-infected cells, uninfected cells, or cell uninfected and cultured in the absence of serum (Fig. 2c). Finally semiquantitation of the levels of ShhN peptide released to the medium using an ELISA technique demonstrated a m.o.i.-dependent increase in secreted ShhN, reaching its peak at 300 m.o.i. (Fig. 2d).

In vivo, mostly glial cells will be infected by RAD. Thus, to determine if glial cells could produce ShhN and release it into the medium, we infected primary cultures of glial cells with increasing concentrations of RAD-ShhN and either immunostained for ShhN (Figs. 2e and 2f) or evaluated the culture medium for its content of released ShhN (Fig. 2g). These data showed that glial cells do express ShhN and can release it into the medium, supporting the use of RAD-ShhN in *in vivo* experiments.

In Vitro Bioactivity of ShhN: Conditioned Media from Cultures Infected with RAD-ShhN Promote the Survival of Dopamine Neurons in Primary Cells

In vivo ShhN would be expected to be released and then act on dopamine neurons to exert its effects. Thus, we tested this model first in culture. To determine whether the ShhN peptide encoded by RAD-Shh and released from infected cells would protect dopaminergic neurons

in vitro, we maintained ventral-mesencephalic (VM) cultures in 50% conditioned medium (CM) from BHK cells infected with RAD-ShhN (CM-ShhN) or RAD-35 (CM-35) or uninfected. We maintained VM cultures under stringently serum-free conditions (i.e., at no time were the cells exposed to serum). Further, to assess the specificity of the effects of CM-ShhN on DA neuronal survival, we incubated conditioned medium from either mock- or RAD-infected BHK cells with or without a monoclonal anti-Shh blocking antibody before adding it to the VM cultures. We kept the cultures in different conditioned media with or without Shh-blocking antibody for 3 days and then processed them for tyrosine hydroxylase (TH) immunocytochemistry to determine the effects of the treatments on dopaminergic neuron survival.

Fig. 3 illustrates the response of VM-TH⁺ neurons to the different treatments. Figs. 3a–3f illustrate that the survival of TH⁺-immunoreactive neurons in culture is increased only in cultures treated with CM from RAD-ShhN-infected cells (Fig. 3f) and that this increase is blocked by pretreatment of the CM with anti-ShhN antibodies (Fig. 3e). Further, Fig. 3 shows that control CM maintained a lower number of TH⁺-immunoreactive neurons in culture and that this effect could not be inhibited by anti-ShhN antibodies.

The quantification of these experiments is shown in Fig. 3g. CM from cells infected with RAD-ShhN enhanced dopaminergic neuron survival by approx 160%, and preincubation with anti-ShhN antibodies resulted in the complete inhibition of such trophic effects. This demonstrates that the increase in DA neuronal survival in CM removed from BHK cells infected with RAD-ShhN is due to the presence of ShhN in the CM. In the absence of ShhN-blocking antibodies, only the CM from BHK infected with RAD-ShhN significantly improved survival of TH⁺ neurons in VM cultures. In contrast, in the presence of ShhN-blocking antibodies, all CM provided comparable levels of TH⁺ neuronal survival, indicating that the levels of uncharacterized BHK-derived trophic factors were similar in all CM and that these uncharacterized activities did not include ShhN.

In Vitro Bioactivity of ShhN and Gli-1: Infection of the Pluripotent Cell Line C3H10T1/2 with RAD-ShhN or RAD-Gli-1 Induces Osteoblastic Differentiation

Although the mechanism of action of Shh is not fully understood, biochemical and genetic data suggest that the receptor for Shh is the product of the tumor suppressor gene *patched* (*ptc*) [38,39]. The Shh signal is received and transduced at the membrane via a receptor complex consisting of *ptc* and *smo*. *Ptc* is a 1500-amino-acid glycoprotein with 12 membrane-spanning domains [40] and two extracellular loops that are required for Shh binding [39,41]. *Smo* is a 115-kDa protein [42]. In absence of Shh, *Smo* and *Ptc* form an inactive complex. When Shh binds to *Ptc* the complex is altered and *Smo* is

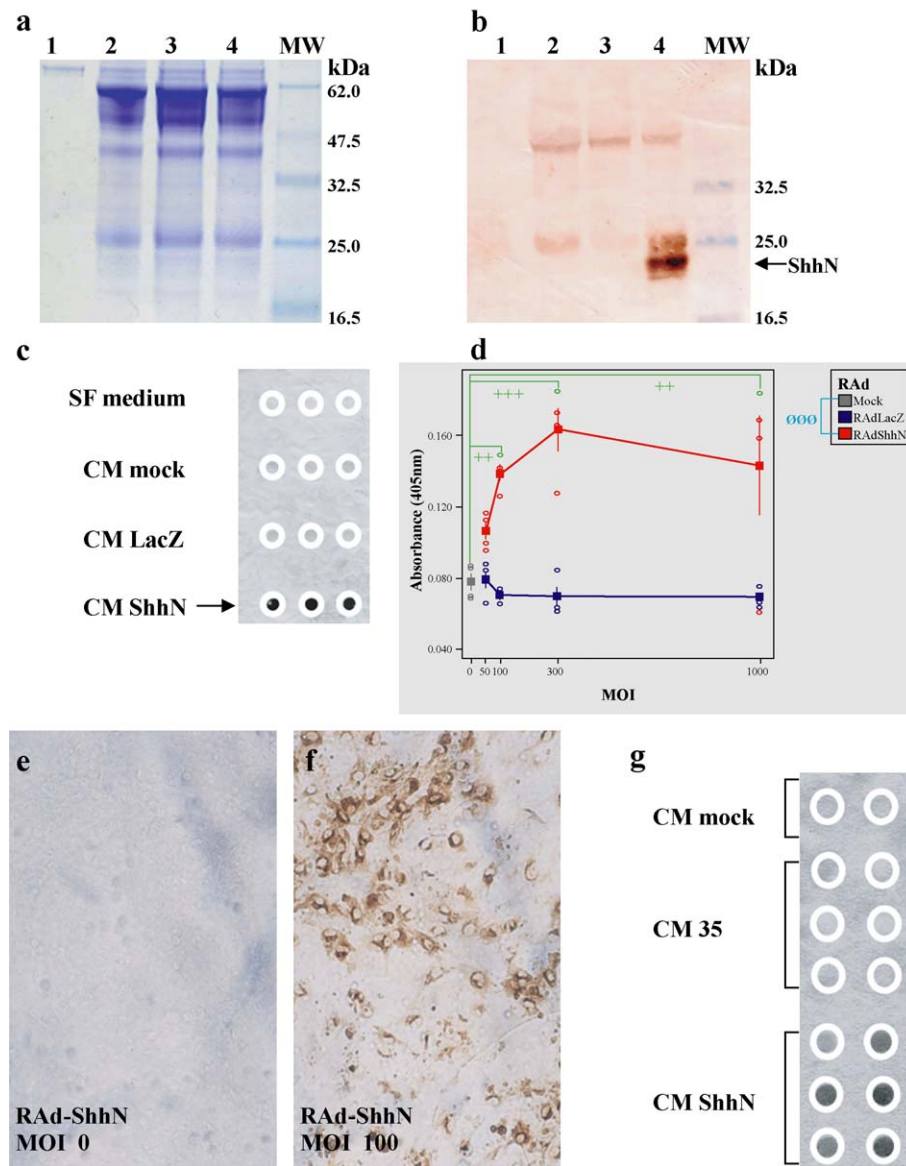


FIG. 2. ShhN is produced and released by BHK or glial cells infected with RAD-ShhN. The presence of ShhN in the supernatant of BHK cells infected with RAD-ShhN is shown in (a, b) a Western blot analysis, (c) a dot blot, and (d) an ELISA. Electrophoretic separation of conditioned medium (CM) from RAD-infected BHK cells on 10% Nu-PAGE gel is shown in (a). Lanes 1–4 contain 25-fold-concentrated samples from the different CM: (1) serum-free (SF) medium, (2) CM mock, (3) CM LacZ, (4) CM ShhN. MW corresponds to molecular weight standards. Western blot analysis (b) confirmed that ShhN (approx 20 kDa) was released into culture medium after RAD-ShhN infection. Dot-blot analysis is illustrated in (c): 200 μ l of 50% conditioned medium from RAD-infected BHK cells was immunoreacted with anti-ShhN antibodies. This assay demonstrated that ShhN was detected only in the conditioned medium from BHK cells infected with RAD-ShhN. Note that this assay was carried out using conditioned medium at the same concentration used for bioactivity assays. Conditioned medium from BHK cells infected with increasing m.o.i. of 0–1000, and assayed for ShhN using an ELISA, is shown in (d). Two way ANOVA: m.o.i. F327 = 1.586, $P \geq 0.05$. Rad F127 = 72.423, $P \leq 0.001$. Rad*m.o.i. F327 = 3.231, $P \leq 0.05$ (+). Dunnett t (two-tailed) *post hoc* test for RAD effects: RAD-CMV-ShhN vs mock, $P \leq 0.001$, but RAD-35 vs mock, $P \geq 0.05$. Dunnett t (two-tailed) *post hoc* test for RAD*m.o.i. interaction: RAD-ShhN 100 vs mock, $P \leq 0.05$ (++) ; RAD-ShhN 300 vs mock, $P \leq 0.01$ (+++); and RAD-ShhN 1000 vs mock, $P \leq 0.01$ (++). The other possible RAD*m.o.i. combinations were not significant compared to mock-infected cultures. This illustrates that the release of ShhN into the medium, following RAD-ShhN-infection of BHK cells, increased proportional to RAD-ShhN m.o.i. and reached its peak at 300 m.o.i.; this m.o.i. was selected for production of the conditioned media for further bioactivity studies. In addition, to test whether ShhN would also be produced and released from rodent glial cells, primary cultures of glial cells were infected with RAD-ShhN. The control cells are illustrated in (e) and infected cells expressing ShhN are shown in (f). Release of ShhN into the supernatant, analysis by dot blot, is shown in (g). Two hundred microliters of 50% conditioned medium from mock or RAD-infected glial cells was immunoreacted with a specific anti-ShhN antibody. This assay demonstrated that ShhN was released only into the conditioned medium originating from glial cells infected with RAD-ShhN.

released from inhibitory control to transduce an activating signal to the nucleus [18] (Fig. 4a); this activates Gli-1, a transcription factor proposed to be a major mediator of

the Shh signal [21–24] (Fig. 4a). Previous studies [43] demonstrated that conditioned medium containing ShhN was able to induce differentiation of the pluripo-

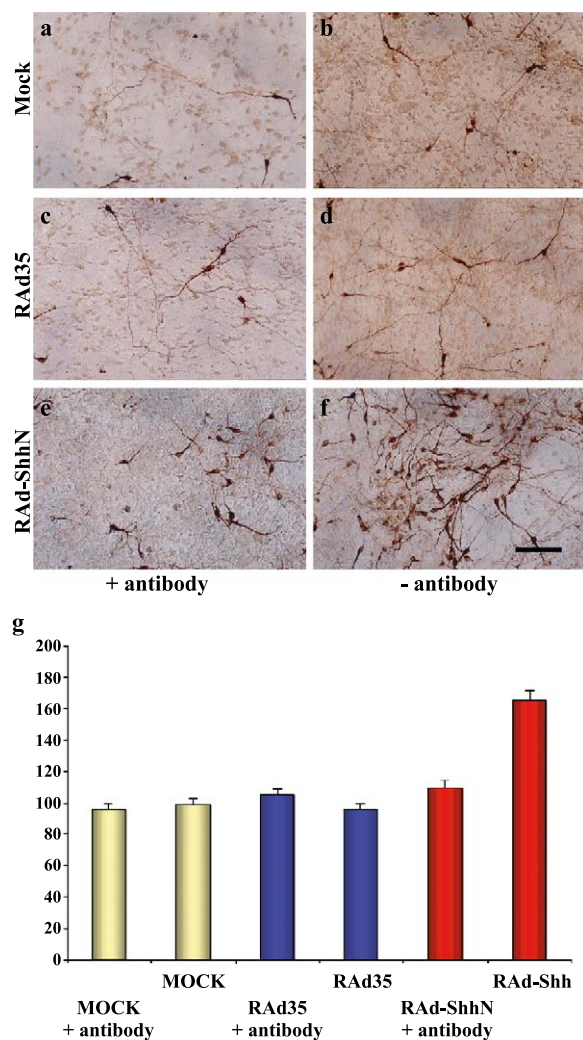


FIG. 3. Rad-ShhN increases the survival of dopaminergic neurons (TH⁺ neurons) in ventral-mesencephalic (VM) cultures. E14 VM cultures were incubated for 4 days with (a, b) CM from mock-infected BHK cells or CM from BHK cells infected with (c, d) Rad-35 or (e, f) RAD-ShhN. In parallel cultures cells were pretreated with the anti-ShhN blocking antibody 5E1 (the effects of anti-Shh Ab are not illustrated; a, c, e). Anti-ShhN antibody was coadministered to the different conditioned media to a final dilution of 1:500. Neuron numbers were assessed and are expressed as the percentage of TH⁺ neurons per well, relative to mock-infected treatment in the absence of anti-ShhN antibody (means ± SEM); this is illustrated in (g). Compared with the other treatments, CM ShhN significantly increased the number of TH⁺ neurons in the absence but not in the presence of anti-Shh antibody (g). CM-mock + antibody ($n = 12$), 86.68 ± 6.47 ; CM-mock ($n = 12$), 100.00 ± 6.73 ; CM-RAD-35 + antibody ($n = 12$), 103.62 ± 9.84 ; CM-RAD-35 ($n = 12$), 97.46 ± 4.12 ; CM-Shh + antibody ($n = 12$), 112 ± 8.22 ; CM-Shh ($n = 12$), 159.82 ± 9.51 . Two way ANOVA followed by Tukey post hoc analysis indicated that the group treated with RAD-ShhN was statistically significantly different ($P \leq 0.001$) from all other groups, and there was no statistically significant difference between any of the other treatments. This experiment was repeated at least three times. a–f and g originate from different experiments. Images were chosen to illustrate neuronal morphology; quantitation was chosen to determine the statistical significance of the results.

tential fibroblast-like cell line C3H10T1/2 into osteoblasts, as determined by the induction of alkaline phosphatase (AP) activity, an early marker of bone differentiation. Based on these observations we designed an experiment to test whether RAD-Gli-1 could replicate the morphogenetic properties of Shh. In this experiment we mock infected 50% confluent C3H10T1/2 cells with PBS or infected them with RAD-Gli-1 at an m.o.i. 200. AP activity was detected 8 days after infection using the Fast Red Kit (Sigma). C3H10T1/2 cells infected with RAD-ShhN at m.o.i. 200 were used as a positive control. As shown in Figs. 4b and 4c infection with RAD-Gli-1 and RAD-ShhN was able to induce differentiation of the pluripotent cell line C3H10T1/2 into osteoblasts as inferred from the detection of AP activity. As expected from a protein targeted to the secretory pathway, ShhN immunoreactivity outlined the ER/Golgi compartments (Fig. 4b). Gli-1 has been shown to shuttle between the cytoplasm and the nucleus. Thus, the immunoreactivity detected in both the cytoplasm and the nucleus is expected from previous knowledge of the subcellular distribution and function of Gli-1 (Fig. 4b; note that due to the different levels of expression in either compartment the figure shows higher cytoplasmic localization). In contrast, cells incubated with PBS did not induce osteoblast differentiation of this cell line. These results demonstrated that RAD-Gli-1 encodes a transcription factor that is biologically active and, more importantly, that it is able to mimic the effects of ShhN *in vitro*.

In Vitro Bioactivity of Rad-Nurr-1

We tested the bioactivity of RAD-Nurr-1 using COS-7 cells transiently transfected with a reporter plasmid containing the Nurr-1-responsive element NBRE upstream of the prolactin promoter (Pro36) as illustrated in Fig. 4d. Expressed Nurr-1 binds to the NBRE and stimulates Pro36-driven luciferase expression. Infection of transfected COS-7 cells with RAD-Nurr-1 increased luciferase expression two to three times over control values. Uninfected cells, or cells infected with a vector expressing the antisense noncoding strand of Nurr-1, did not induce luciferase expression over the basal activity of the reporter construct even in the absence of infection with RAD-Nurr-1 (Fig. 4e). Immunohistochemistry of control COS-7 cells infected with RAD-Nurr-1 indicates strong immunoreaction for Nurr-1 in the nucleus of infected cells (Fig. 4f).

Distribution of Transgenes throughout the Rostrocaudal Extent of the Substantia Nigra Following the Injection of RAds into the Striatum

To determine the distribution throughout the rostrocaudal extent of the substantia nigra of an intracellular transgene expressed from an RAd, we injected RAd-TK into the

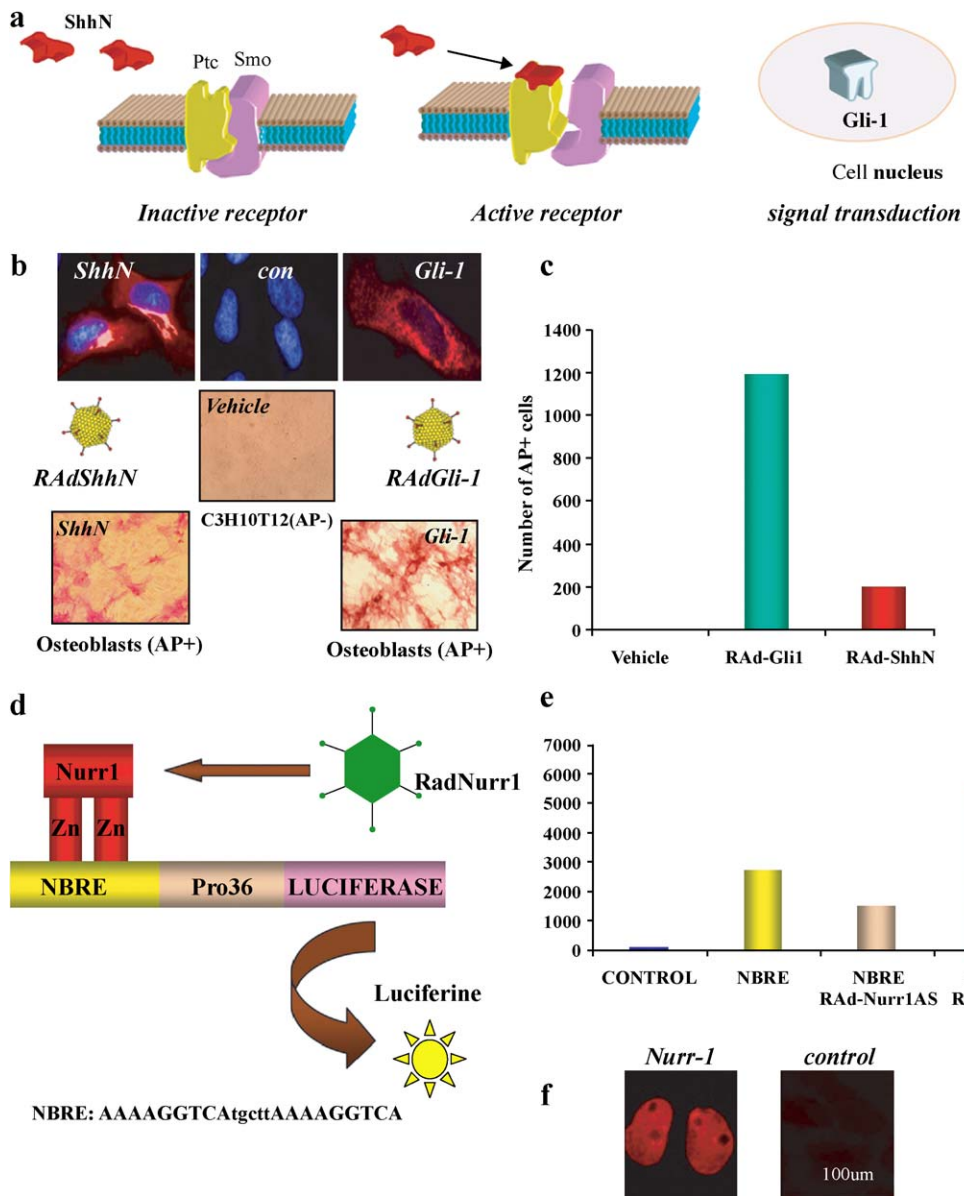
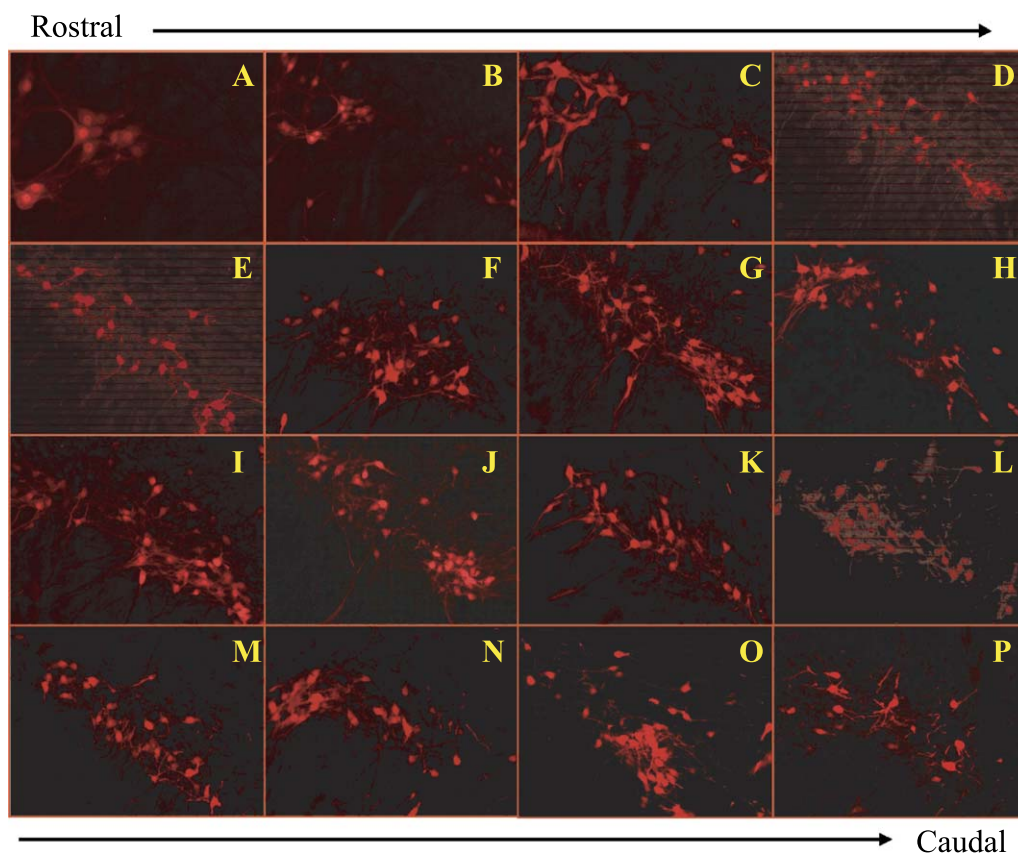


FIG. 4. (a) *In vitro* bioactivity of RAD-ShhN, RAD-Gli-1, and RAD-Nurr-1. A schematic view of the mechanism of action of ShhN, and Gli-1, is shown. This illustrates the interaction of ShhN with Ptc and Smo, the release of the inhibition on Smo, and the eventual stimulation of activated Gli-1 translocation into the nucleus to activate further downstream target genes. (b) The bioactivity of RAD-ShhN and RAD-Gli-1. HeLa cells were infected with RAD-ShhN, or RAD-Gli-1, or a negative control vector, at m.o.i. 200 IU/cell, and 48 h later cells were fixed and the proteins detected with specific primary antibodies and immunofluorescently labeled secondary antibodies. Most of the ShhN immunoreactivity highlighted the Golgi apparatus, compatible with the intracellular distribution of a secretory protein, while Gli-1 showed both a cytoplasmic and a nuclear localization, as expected from a transcription factor that has been shown to shuttle between the cytoplasm and the nucleus. Differentiation of the pluripotential cell line C3H10T1/2 into osteoblasts was induced upon infection with RAD-ShhN, or RAD-Gli-1, following infection at m.o.i. 200. Alkaline phosphatase (AP) activity was used as a marker for osteoblast differentiation. Both vectors induced AP activity, and the quantitative analysis of AP⁺ cells is illustrated in (c). (d, e) The transcriptional activation mediated by RAD-Nurr-1 is shown. COS-7 cells were transfected with the reporter plasmid NBRE-Luciferase (d), containing the binding site for Nurr-1, and infected with RAD-Nurr-1 in sense orientation or RAD-Nurr-1 in antisense orientation (RAD-Nurr-1AS), used as negative control. (e) Luciferase activity was measured 48 h after. The transcription factor Nurr1 binds to the canonical NBRE domain and induces expression of luciferase. (f) COS-7 cells were infected with RAD-Nurr-1, or a negative control vector, at m.o.i. 200 IU/cell, and 48 h later cells were fixed and immunostained with antibodies recognizing Nurr1.

FIG. 5. Retrograde targeting of nigrostriatal dopaminergic neurons throughout the rostrocaudal extent of the substantia nigra. A recombinant adenovirus encoding the reporter gene thymidine kinase (RAd-TK, 3.2×10^7 IU) was stereotactically injected into the rat dorsal striatum (AP + 1.0 mm, ML + 3.2, DV -5.0 mm) as described. Retrograde transport of this vector to the substantia nigra pars compacta was verified 1 week after the injection, by immunostaining of TK protein using specific anti-HSV TK antibodies. (A–P) The expression of TK throughout the rostrocaudal axis of the substantia nigra pars compacta from (A) AP - 4.8 to (P) AP - 6.30.



striatum and assessed the immunocytochemical distribution of the transgene in retrogradely labeled neurons in the substantia nigra. RAd-TK encodes the full-length herpes simplex virus type 1 thymidine kinase, HSV-1 TK [44,45]. The wide distribution throughout the rostrocaudal extent of the transgene TK detected immunocytochemically throughout the substantia nigra is illustrated in Fig. 5.

Combined Retrograde Targeting of Substantia Nigra Dopaminergic Neurons with both Fluoro-Gold and Adenoviral Vectors

Fig. 6 illustrates the injection site and distribution of fluoro-gold (green) in the striatum, as well as the site of injection of RAd, detected by immunocytochemistry for the transgene (red) (Fig. 6a). The overlap between both labels indicates that fluoro-gold and RAd have distributed over an equivalent area of striatal tissue. Fig. 6b shows the detection of retrogradely transported fluoro-gold in neurons also expressing a RAd-encoded transgene. This demonstrates that RAd-encoded therapeutic transgene expression occurs in nigral neurons that project to the striatum. Finally, Figs. 6c–6g indicate that all neurons containing the retrogradely transported dye fluoro-gold are TH⁺ nigral neurons and thus identifies these as *bona fide* dopaminergic nigrostriatal neurons.

Rad-ShhN and Rad-Gli-1, but Not Rad-Nurr-1 Protect Dopaminergic (DA) Nigrostriatal Neurons against 6-OHDA-Induced Neurodegeneration *in Vivo*

We evaluated the ability of RAd-Shh and RAd-Gli-1 to protect DA neurons from 6-OHDA neurotoxicity using a modification of the rat model of Parkinson's disease previously reported by Choi-Lundberg [46] (illustrated in Fig. 6). We injected fluoro-gold intrastrially on both sides of the brain; during the same surgical intervention we injected 1×10^8 IU of RAd-ShhN, RAd-Gli-1, RAd-Nurr-1, the negative control vector RAd-35, or the positive control RAd-GDNF into the right striatum. One week later, we induced retrograde degeneration of the nigrostriatal pathway by unilateral (right dorsal striatum) administration of 16 μ g of 6-OHDA-HCl using the same coordinates used for the delivery of fluoro-gold and RAd (referred to as the "ipsilateral site"), and 4 weeks later, we injected the rats with an overdose of anesthetic, perfused-fixed them through the left ventricle of the heart, post-fixed them overnight, and cut 25- μ m-thick sections on a vibratome and analyzed them using an Olympus AHBS fluorescence photomicroscope. We counted the surviving neurons on the ipsilateral side (exposed to the neurotoxin) and expressed them as a percentage of neurons of the contralateral (control) hemisphere.

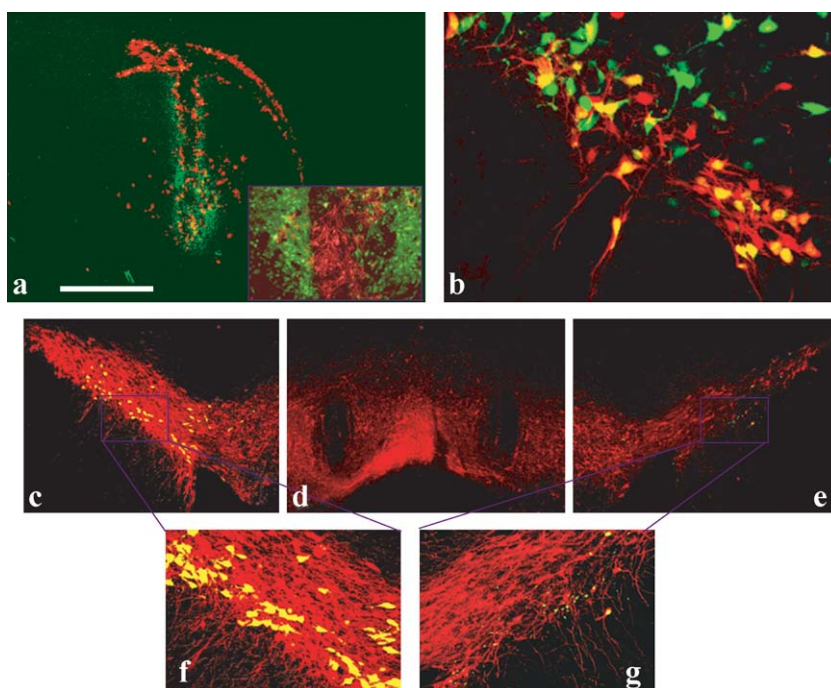


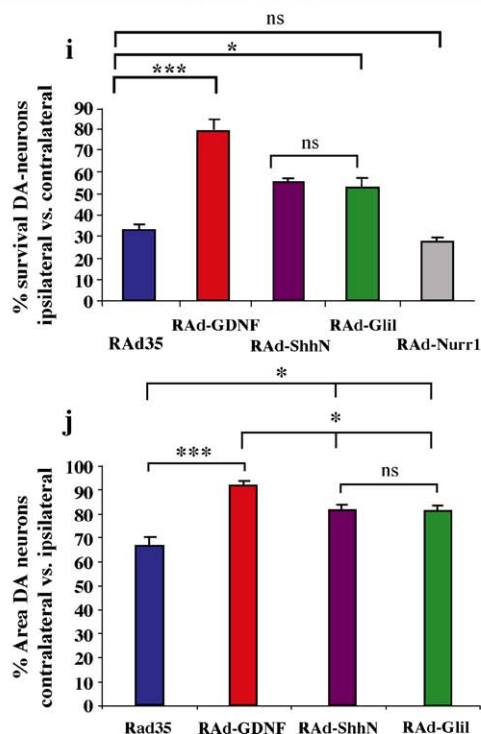
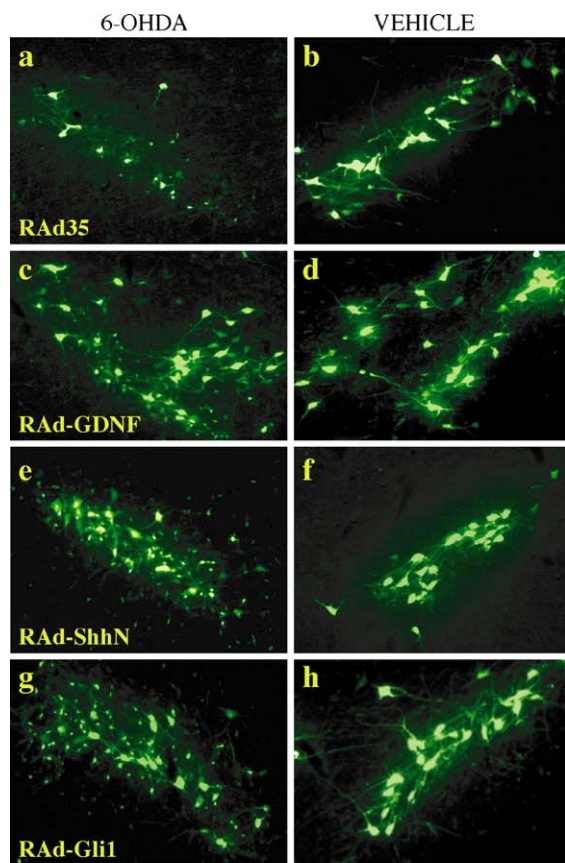
FIG. 6. Method for combined retrograde targeting of substantia nigra dopaminergic neurons with both fluoro-gold and adenoviral vectors. The method used to retrograde target RAD and fluoro-gold to anatomically overlapping areas in the substantia nigra is illustrated. The method was exhaustively optimized to label a comparable amount of nigrostriatal neurons (range 30–50) by each method. Also illustrated is the degeneration of fluoro-gold⁺ cells after administration of 6-OHDA and the fact that all neurons retrogradely labeled with fluoro-gold indeed are TH-positive. All neurons retrogradely labeled by RAD were also TH-positive (not illustrated). (a) A coronal section through the striatum showing colocalization of the fluorescent tracer fluoro-gold (green) and RAD-expressed transgene (red), following their stereotaxic injection into the rat brain at the level of the dorsal striatum, using coordinates identical to those used for the neurotoxicity experiments (scale bar, 1 mm). For these experiments RAD-TK (encoding an intracellular gene product) was injected into the striatum, as described for Fig. 5. One week later, the progressive degeneration of the nigrostriatal pathway was induced by injection of 6-OHDA at the same coordinates used for fluoro-gold and RAD. (b) A coronal section of the substantia nigra. Fluoro-gold has been retrogradely transported to the substantia nigra (green), and RAD-expressed transgene is detected by immunocytochemistry (red). Double labeling demonstrates colocalization (yellow) of fluoro-gold (green) and RAD-encoded TK (red) in the neurons of the SNpc. Expression of the encoded marker transgene was detected by indirect immunofluorescence. Sections illustrated in (c–g) show coronal sections of the substantia nigra immunoreacted for TH and evaluated for the presence of fluoro-gold. Dopaminergic neurons were detected by immunofluorescence using a specific anti-TH antibody. Notice the degeneration of fluoro-gold⁺ neurons after intrastriatal injection of 16 μ g of 6-OHDA (e.g., e, low-power view of the substantia nigra; g, high-magnification view). In the contralateral side vehicle (saline) injection in the striatum does not induce degeneration (c and f, c, low-power view of the substantia nigra; f, high-magnification view). Colocalization (yellow) indicates that every fluoro-gold⁺ neuron (green) is TH⁺ (red).

Animals injected with the negative control vector RAD-35 preserved only $33.4 \pm 1.83\%$ of nigrostriatal neurons (Figs. 7a, 7b, and 7i). Neuronal counts indicated that throughout the substantia nigra pars compacta (SNpc) of rats injected with RAD-ShhN, $53.3 \pm 1.29\%$ of dopaminergic neurons survived on the lesioned site in comparison with the intact contralateral site (Figs. 7e, 7f, and 7i). In animals injected with RAD-Gli-1 $52.9 \pm 3.31\%$ of fluoro-gold-labeled neurons survived (Figs. 7g, 7h, and 7i), while $79 \pm 3.7\%$ of retrogradely labeled dopaminergic neurons were protected from neurodegeneration by RAD-GDNF (Figs. 7c, 7d, and 7i). Animals injected with RAD-Nurr-1 retained only $27 \pm 1.22\%$ of labeled striatonigral neurons, a number not statistically different from that of animals injected with the negative control vector RAD-35 (Fig. 7i). These results demonstrate that intrastriatal de-

livery of 1×10^8 IU of RAD-ShhN and RAD-Gli-1 can protect a statistically significant proportion of nigrostriatal neurons susceptible to being killed by 6-OHDA [analysis of variance (ANOVA), $F = 38.33$, $P \leq 0.01$, $n = 7$]; RAD-GDNF protected a higher proportion of nigrostriatal neurons (ANOVA, $F = 38.33$, $P \leq 0.001$, $n = 7$), while RAD-Nurr-1, however, was ineffective.

RAD-ShhN or RAD-Gli-1 does not Protect Dopamine Neurons' Tyrosine-Hydroxylase-Immunoreactive Terminals in the Striatum: a Comparison with RAD-GDNF

Having demonstrated that a single injection of 1×10^8 IU of RAD-Shh and RAD-Gli-1 is neuroprotective for retrogradely labeled dopaminergic neurons in the SNpc, we examined whether these vectors could prevent 6-OHDA-



induced dopaminergic denervation of the striatum. We processed forebrain coronal sections from animals injected with 1×10^8 IU of RAD-ShhN or RAD-Gli-1 by immunohistochemistry to detect TH-immunoreactive (TH-IR) fibers in the striatum. We evaluated the extent of striatal denervation produced by 6-OHDA by measuring the density of TH-IR fibers in the entire ipsilateral striatum and expressed it as a percentage of the contralateral site. The density of TH-IR fibers in the striatum of rats treated with RAD-ShhN and RAD-Gli-1 decreased to 42.4 ± 1.26 and 50.62 ± 4.25 of the contralateral site, respectively (Fig. 8). In rats treated with RAD-35 the density of TH-IR fibers decreased to $40 \pm 0.25\%$ of controls, while in rats treated with RAD-GDNF they decreased only to $64\% \pm 6.72$ of control values (Fig. 8).

Statistical analysis of these data indicated that neither RAD-Shh nor RAD-Gli-1 was able to protect striatal axonal DA terminals from degeneration 4 weeks after injection of 6-OHDA (ANOVA, $F = 25.22$, $P \geq 0.05$, $n = 4$). Only the injection of RAD-GDNF resulted in a statistically significant increase (ANOVA, $F = 25.22$, $P \leq 0.01$, $n = 4$) in the density of TH-IR in comparison with RAD-35-treated rats. These results indicate that while RAD-mediated gene transfer of ShhN and Gli-1 results in the protection of DA neurons from 6-OHDA neurotoxicity, denervation of striatal DA terminals is not prevented.

RAD-ShhN or RAD-Gli-1 Partially Protects Dopamine Striatonigral Neuronal Cell Bodies from Atrophy Induced by Intrastriatal Injection of the Neurotoxin 6-OHDA

If GDNF could protect the striatal dopaminergic innervation, it could also possibly protect the decrease in cell body size of dopaminergic neurons in the substantia nigra that is caused by dopaminergic denervation, with cells

FIG. 7. Effects of gene transfer on substantia nigra dopaminergic neurons. Adenovirus-mediated gene transfer of 1×10^8 IU of (a, b) RAD-35, (c, d) RAD-GDNF, (e, f) RAD-ShhN, (g, h) RAD-Gli-1, or RAD-Nurr-1 (not illustrated) was tested against 6-OHDA-induced neurodegeneration of nigrostriatal cells retrogradely labeled with fluoro-gold. The side injected with 6-OHDA is shown on the left, and the control side is shown on the right. Injection of RAD-GDNF, RAD-ShhN, and RAD-Gli-1 protected a significant amount of nigrostriatal neurons compared to animals injected with the negative control vector RAD-35. Note the survival of large fluoro-gold⁺ neurons in the ipsilateral site of animals injected with RAD-ShhN (e), RAD-Gli-1 (g), and RAD-GDNF (c) compared with RAD-35 (a). The quantitative analysis is shown in (i) and also indicates the analysis of the animals injected with RAD-Nurr-1. Survival of nigrostriatal neurons was expressed as a percentage of unlesioned contralateral neurons. (j) The area occupied by dopamine neurons' cell bodies protected from degeneration after treatment with 1×10^8 IU of RAD-ShhN, RAD-Gli-1, or RAD-GDNF was quantified and expressed as a percentage of the neuron soma area in the contralateral site. RAD-GDNF, RAD-ShhN, and RAD-Gli-1 all protected cell body size compared with RAD-35. RAD-GDNF showed the strongest effect. Cell body protection by ShhN and Gli-1 was statistically significantly different from that of animals injected with RAD-35. The treatment groups were compared by repeated-measures ANOVA with *post hoc* Tukey or Dunnett multiple comparison test; * $P \leq 0.05$; *** $P \leq 0.005$.

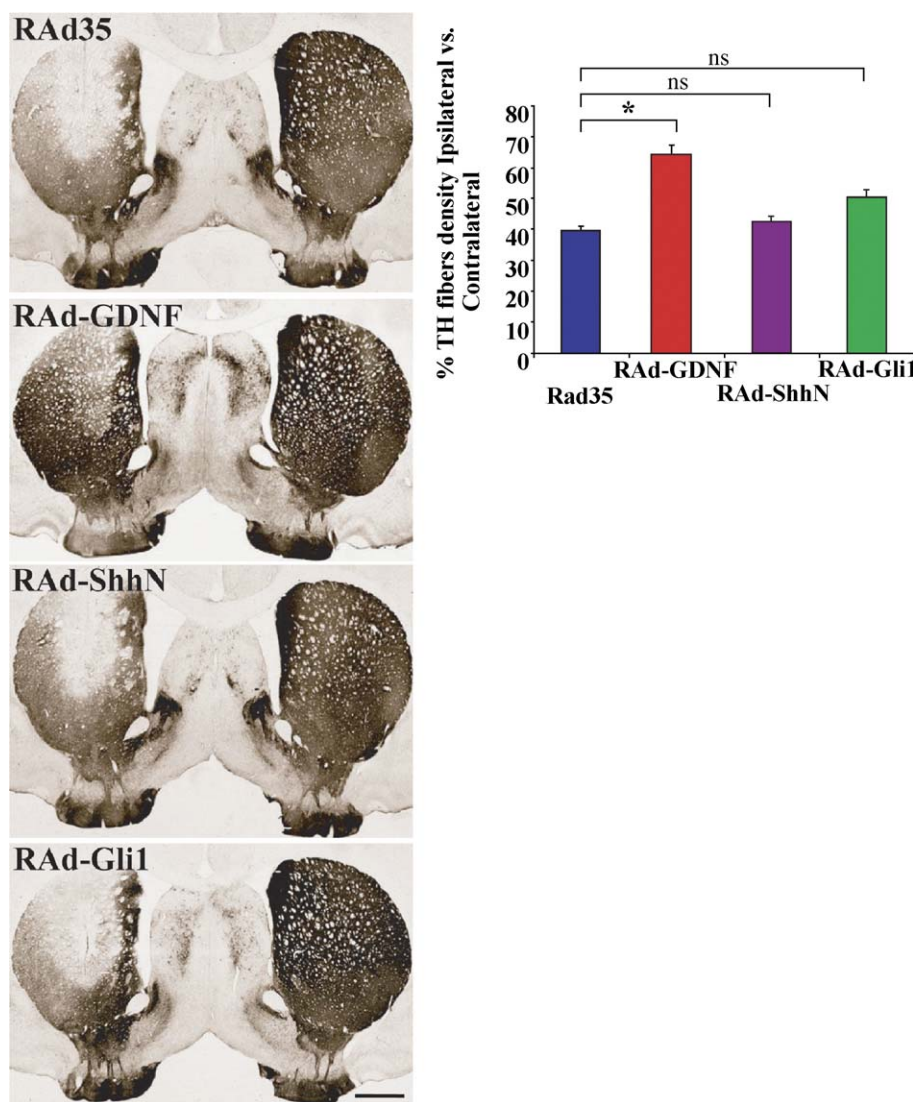


FIG. 8. Effects of gene transfer on striatal dopaminergic innervation. The column on the left illustrates sections throughout the striatum of treated animals immunoreacted with an antibody raised against TH, to reveal the density of TH-immunopositive fibers in the striatum. The lesioned side is on the left, the control side, on the right. Notice that only animals injected with RAD-GDNF showed a protection of the striatal dopaminergic fibers. The graph shows the densitometric analysis of TH⁺ fiber density in the striatum of rats treated with 1×10^8 IU of RAD-Shh, RAD-Gli-1, RAD-GDNF, or RAD-35. Degeneration of axonal terminals in the striatum after administration of 6-OHDA is not prevented following the injection of RAD-ShhN, RAD-Gli-1, or RAD-Nurr-1 (not shown). Only RAD-GDNF is able to protect TH⁺ fibers significantly. The treatment groups were compared by repeated-measures ANOVA with *post hoc* Tukey or Dunnett multiple comparison test; * $P \leq 0.05$. Scale bar, 1 mm.

with larger terminal fields displaying larger sizes and vice versa. To test this hypothesis we measured the area occupied by nigrostriatal cell bodies ($n = 100$ TH⁺ neurons on both the control and the neuroprotected substantia nigra) and expressed values of ipsilateral DA neuronal cell body area (neuroprotected) as a percentage of the cell body area of neurons in the contralateral substantia nigra (Fig. 7j). In rats injected with RAD-GDNF, cell body size decreased to $92 \pm 1.26\%$ of the contralateral site. In contrast, in RAD-ShhN- and RAD-Gli-1-treated rats the cell body area of nigrostriatal neurons was reduced to 82% of the contralateral side neurons. Soma size in rats injected with the control vector RAD-35 decreased to $67 \pm 5.24\%$ of the contralateral side. The reduction in size of dopaminergic neurons in rats treated with RAD-ShhN and RAD-Gli-1 was statistically significant ($F = 51.49$, $P \leq 0.01$, $n = 100$ dopaminergic neurons analyzed in total or $F = 14.054$,

$P \leq 0.05$, $n = 7$ when the data are analyzed per number of animals studied) compared with the size of those in animals injected with RAD-GDNF. The reduction in cell body size in animals injected with the control vector RAD-35 was statistically significant compared with RAD-ShhN-, RAD-Gli-1- (ANOVA, $F = 51.49$, $P \leq 0.01$, $n = 100$ or $F = 14.054$, $P \leq 0.05$, $n = 7$), and RAD-GDNF-treated rats (ANOVA, $F = 51.49$, $P \leq 0.001$, $n = 100$ or $F = 14.054$, $P \leq 0.001$, $n = 7$).

These results indicate that, despite comparable striatal denervation seen in animals injected with 6-OHDA and treated with either RAD-ShhN and RAD-Gli-1 or the control vector RAD-35, only RAD-ShhN and RAD-Gli-1 treatment partially prevented the progressive decrease of nigrostriatal cell body size induced by the neurotoxin. This indicates that ShhN and Gli-1 can protect the size of dopamine neurons in the substantia nigra, independent

from trophic effects at the level of the striatum and/or the striatal axonal terminals.

Expression of ShhN and Gli-1 in the Substantia Nigra at 1 and 4 Weeks Following Their Injection into the Dorsal Striatum

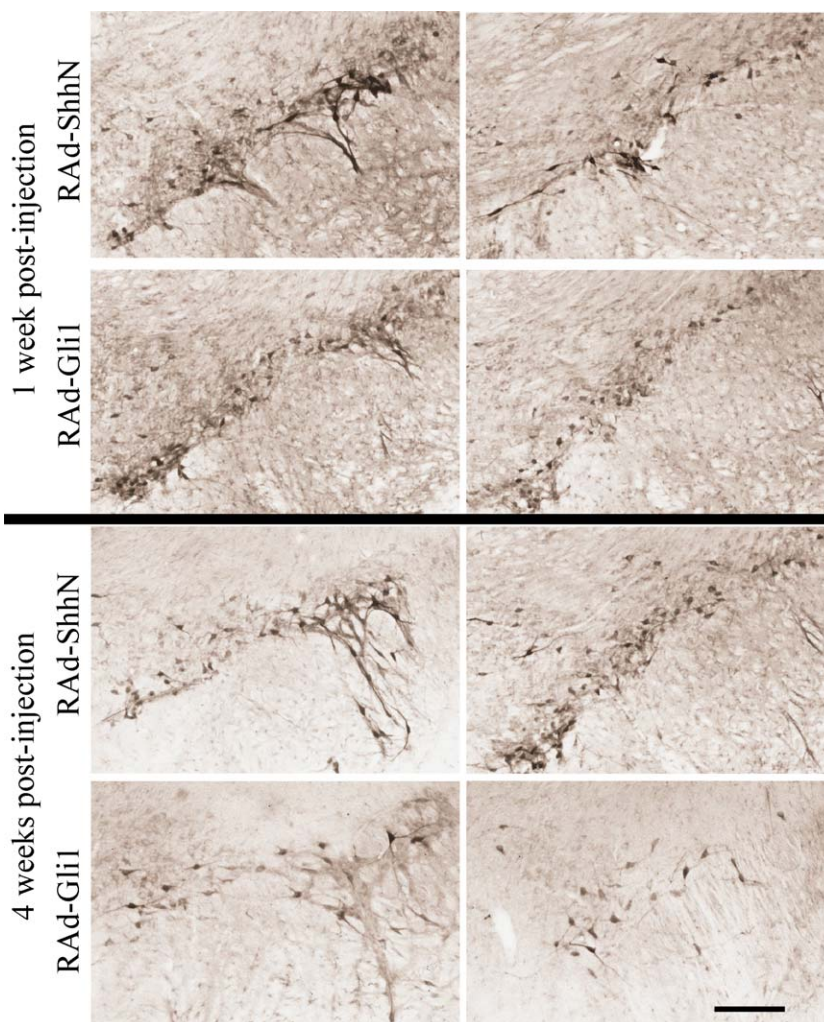
To confirm that the transgenes were present throughout the substantia nigra during the experimental procedure, we determined the expression of ShhN and Gli-1 in the substantia nigra at 1 and 4 weeks after their intrastriatal injection. Using specific immunohistochemical techniques, both transgenes could be detected in the substantia nigra at 1 or 4 weeks after the injection of viruses into the striatum (Fig. 9). This indicates that the potential neurotrophic factors were available in the substantia nigra at the proper time to exert their pharmacological effects. There was no positive immunoreaction in the contralateral substantia nigra (not shown). Immunoreactivity for Nurr-1 was

present, but it was also present in many other areas of the rat brain, including the contralateral substantia nigra (not shown). Thus, due to the high levels of basal expression of Nurr-1 we could not detect an increase due to RAd-Nurr-1 expression.

DISCUSSION

Our experiments demonstrate that the differentiation factor ShhN can act as a trophic factor for embryonic dopaminergic neurons in primary cultures, and, similar to its downstream transcriptional activator, Gli-1, they both protect a significant percentage of adult dopaminergic nigrostriatal neuronal cell bodies from 6-OHDA-induced neurotoxicity. Interestingly, ShhN and Gli-1 protected only the nigral dopaminergic cell bodies from neurodegeneration and not the striatal axonal terminals of these neurons. Our positive control vector expressing GDNF, on the other hand, protected both the nigral cell

FIG. 9. Transgene expression in the substantia nigra. RAd-ShhN or RAd-Gli-1 was injected into the striatum and 1 or 4 weeks later animals were perfusion-fixed and brains were sectioned and probed with specific antibodies for either ShhN or Gli-1. Two rostrocaudal levels are shown for each condition. There was no specific immunostaining in either the contralateral substantia nigra or the uninjected animals. Scale bar, 200 μ m.



bodies and the dopaminergic terminals. Whether the neuroprotective effect of GDNF is at the level of individual neurons (e.g., nigral neuronal cell bodies protected by GDNF provide axons to the striatum), or whether GDNF preserves neuronal numbers in the substantia nigra while causing sprouting of remaining axons in the striatum (e.g., protected nigral neuronal cell bodies in the nigra do not provide axons to the striatum), awaits studies that examine the effects of GDNF at the single-cell level. Those studies have not yet been attempted. However, it is important to note that GDNF protected a higher percentage of nigral cell bodies ($\approx 80\%$) compared to the percentage of striatal dopaminergic innervation ($\approx 60\%$). Our negative control vector expressing the reporter β -galactosidase and the RAD-Nurr-1 vector, however, were unable to protect either the cell bodies or the dopaminergic axon terminals in the striatum. The use of internal positive and negative control vectors provided us with stringent mechanisms to identify both active and inactive factors able to protect nigral cell bodies from neurotoxic degeneration.

As expected from a neurotoxin that produces a lesion of the axon terminals, which causes retrograde atrophy and/or degeneration, 6-OHDA reduced the size of dopaminergic neuronal cell bodies in the substantia nigra. RAD-GDNF, which showed the greatest capacity to maintain the axonal terminals of nigrostriatal neurons, also achieved the greatest protection of cell body size in the substantia nigra. While ShhN and Gli-1 were less potent, they significantly protected against the neurotoxin-induced soma atrophy. Thus, while GDNF was more effective in preserving nigral dopamine neuron numbers, striatal tyrosine hydroxylase axon terminal density, and nigral dopamine soma size, ShhN and Gli-1 had a protective effect on neuronal numbers and nigral soma area, but were completely ineffective in protecting the striatal dopaminergic innervation from neurotoxin-induced degeneration. Thus, ShhN and Gli-1 protected partially against the denervation-induced soma atrophy, without preserving the density of dopaminergic striatal innervation.

The effect of ShhN and Gli-1 in primarily protecting the nigral dopaminergic cell bodies is expected. Administration of GDNF into the substantia nigra has been previously shown to protect mainly the nigral dopaminergic cell bodies [8] but not the striatal axonal terminals. Delivery of GDNF to the striatum has been shown to provide neuroprotection to both nigral cell bodies and axonal terminals, as seen by us here and as described by others [4,8,46–48]. Further, the antiapoptotic protein XIAP has also been shown to protect predominantly nigral cell bodies upon delivery using recombinant adenovirus into the striatum of mice [49]. Thus, there is precedence for the selective neuroprotection of nigral dopaminergic cell bodies, even when using experimental paradigms very comparable to ours.

The increased effectiveness of GDNF may be related to its capacity to preserve the striatal axon terminals [47,50]. Although recently untoward effects of GDNF have been described at long times following GDNF delivery [10,12], our experimental design was not designed to examine these specifically, and thus, they were not uncovered by our designs. Intra-striatal 6-OHDA causes a slow degeneration of nigral neurons. Hence, nigral neuron survival could depend on the continued retrograde transport of the adenoviruses, or the growth factors encoded by them, to the substantia nigra. It has been recently demonstrated that Shh and its receptor Ptc can also be endocytosed in neural plate explants, suggesting that this event may be linked to the mechanism of Shh signal transduction [51]. Again, these mechanisms will operate only in the presence of intact axon terminals, and the loss of striatal axons will thus compromise the effectiveness of RAD-ShhN and RAD-Gli-1. Retrograde transport of RADs has been demonstrated by us in this paradigm and has also been described by others [46,52].

Nurr-1, despite being indispensable during early brain development for the expression of the dopaminergic phenotype [16], had no effect on the survival of nigral dopamine neurons in our experimental paradigm. Interestingly, Nurr-1 is a powerful factor that contributes to the determination of the dopaminergic phenotype during neuronal differentiation, most possibly by heterodimerization with the retinoid X receptor [53]. Thus, Nurr-1 activates dopaminergic-specific genes in neuronal stem cells, and allows the differentiation of neuronal stem cells along a dopaminergic pathway, and has also been shown to have a neuroprotective effect in mouse neural stem cells [15,26,54–57]. In summary, the role of Nurr-1 in promoting the development of the dopaminergic phenotype of midbrain neurons has been studied during ontogenesis, but its role in adults has not been thoroughly evaluated. That it does play a role can be surmised by its being essential for the expression of Ret, a central component of GDNF signaling, in midbrain dopamine neurons [58]. Thus, the lack of effect of Nurr-1 could be due to the high constitutive levels of Nurr-1 already present in the adult brain, its exclusive cell-autonomous function, or its lack of neuroprotective action against neurotoxins [59,60].

Previously, GDNF has been successfully used in several animal models of Parkinson's disease [61]. However, in a clinical trial, intraventricular administration of GDNF protein failed to prevent nigrostriatal degeneration [62]; it did not improve parkinsonism, and side effects including weight loss, paresthesias, and hyponatremia were reported [63]. In a more recent clinical trial in which GDNF was injected directly into the affected striatum of Parkinson's disease patients, potentially therapeutic results were forthcoming [7]. This indicates that direct delivery of neuronal growth factors into target brain areas may be effective in human patients. Methods such as

gene therapy, by providing sustained and regulated growth factor, may well become the delivery method of choice for the treatment of Parkinson's disease.

There has been one previous report suggesting that ShhN peptide protected DA neurons in primary cultures from MPP⁺-induced neurodegeneration [25]. More recently ShhN peptide has been proposed to act as a neuro-modulator in the adult subthalamic nucleus [27] and to protect partially from 6-OHDA-induced neurotoxicity in rodents [29] and marmosets [28]. Although some of our data have been presented by us earlier in abstract form [64], the detailed results described by us in this article demonstrate for the first time, to the best of our knowledge, that *in vivo*, ShhN or the transcription factor downstream of Shh signaling, Gli-1, expressed by a recombinant viral gene transfer vector, protects dopaminergic nigrostriatal neurons from neurodegeneration. In our experiments, RAD-GDNF was more effective than either RAD-ShhN or RAD-Gli-1, similar to results obtained when ShhN peptide was injected directly into the striatum and compared to infused GDNF [29].

In our experiments RAD-ShhN or RAD-Gli-1 protected a significant percentage of dopaminergic nigrostriatal neurons from neurodegeneration, although we could not detect behavioral protection with RAD-GDNF, RAD-ShhN, or RAD-Gli-1, because we could not detect any rotational asymmetries in response to the lesion; thus, the model did not allow us to test for any behavioral protection and is thus a model of neuroanatomical neuroprotection (data not shown). Importantly, this correlated with the lack of neuroprotection of striatal dopaminergic terminals, since behavioral neuroprotection of striatal function is contingent on the preservation of the dopaminergic striatal innervation. However, it was recently reported that ShhN peptide injected directly into the striatum prevented behavioral modifications induced by 6-OHDA and protected some of the dopaminergic innervation density, but was less powerful compared to GDNF [29]. Different results obtained by both groups could be explained by the repeated and presumably higher doses of ShhN peptide injected directly into the striatum [29] and/or other experimental differences between both sets of experiments. The detailed mechanisms of action and dose dependence of Shh in different neuroanatomical sites remain to be analyzed in detail. Alternatively, the dose of ShhN expressed from RAD-ShhN may not have been optimal, or the cell type expressing ShhN could affect its ultimate biological effect.

Differences in the effectiveness between GDNF and ShhN/Gli-1 could be attributed to differences in the density of receptors and signaling pathways activated by either factor. The receptors for ShhN, Ptc and Smo, have been identified in the adult rat brain in several regions, including striatum and midbrain [32]. However, the density of these receptors in the adult brain has not been determined. For GDNF, however, it is clear that its recep-

tors are expressed at physiologically relevant levels in the adult brain of various species, including human [65–69].

Differences in signaling pathways activated by GDNF or ShhN/Gli-1 most likely explain their different activities. GDNF signals through binding to GRF1 α and its interactions with the Ret-receptor tyrosine kinase. The expression of GRF1 α is regulated by TGF β , and *ret* is under the control of Nurr-1 [58,70,71]. More recently, it has also been demonstrated that GDNF can bind to heparan sulfate proteoglycans to activate the Met-receptor tyrosine kinase or bind to NCAM, leading to the activation of *Fyn* and *FAK*. The rescuing of nigrostriatal neurons from neurotoxin-induced toxicity has not yet been linked to individual signaling pathways.

Shh signals through its interactions with Ptc and Smo, leading to the activation of a macromolecular complex consisting of Suppressor of fused, Fused, and protein kinase A, which phosphorylates and activates Gli proteins [19]. Although the overall signaling pathways are not completely elucidated in the CNS, our data suggest that Gli-1 is downstream of Shh signaling.

In view of the recent description of the existence of progenitor cells within the substantia nigra [72] that in certain paradigms give rise to neurons [73], and the activity of Shh in inducing neural progenitor proliferation in the hippocampus *in vivo* [13], would it be possible that the effects we detected are due to an effect of Shh on neural progenitors? We believe this to be unlikely, since the effect we described was seen on neurons that were projecting to the striatum (fluoro-gold⁺). In our experimental paradigm there would not be enough time for cells to be born in the nigra, project to the striatum, and pick up the retrograde tracer. However, this potential effect of ShhN could be explored in the future.

In conclusion, we demonstrated that a single intrastriatal injection of a RAD encoding ShhN or its downstream transcriptional activator Gli-1 protects dopamine neurons against 6-OHDA neurotoxicity *in vivo* and also protected these cells from neurotoxin-induced cell body atrophy. Nevertheless, this treatment did not prevent the dopaminergic denervation of the striatum. This is the first report that demonstrates the feasibility of using transcriptional gene therapy to mimic neuroprotective signals to bypass any limited availability of receptors or signaling cascades of neurotrophic factors. This strategy can be used both in the context of therapeutic applications and to determine which signaling pathway mediates particular effects of a given neurotrophic factor. Our results suggest that adenovirus-mediated gene transfer using Shh or downstream elements of its signaling pathways represents a new strategy to prevent progressive degeneration of dopamine-containing neurons in the substantia nigra in disorders like Parkinson's disease.

MATERIALS AND METHODS

Cell lines. Human embryonic kidney cells (293 transformed with E1 from Ad5) were obtained from Microbix, Biosystems Inc. (Toronto, Ontario, Canada); HeLa and BHK cells were purchased from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). These cells were grown using complete minimal essential medium Eagle (MEM; fetal bovine serum 10%, penicillin/streptomycin 1%, L-glutamine 1%, MEM nonessential amino acids) and incubated at 37°C with 5% CO₂ [74]. C3H/10T1/2 cells, a mouse embryo mesenchymal cell line that can be differentiated into cartilage and bone, were used to test the bioactivity of ShhN and Gli-1. Differentiation induces alkaline phosphatase expression detected histochemically. C3H/10T1/2 cells were grown in basal Eagle medium with Earle's BSS 90%, supplemented with 10% heat-inactivated fetal bovine serum in 25-cm² flasks and incubated at 37°C with 5% CO₂ and passaged at 60% confluency.

VM primary cultures. Pregnant Sprague–Dawley rats were killed by neck dislocation on day E14.5 [75]. The uterus was removed and transferred to ice-cold buffer, where fetuses were removed until dissection under a stereomicroscope as described in detail elsewhere [75,76]. Neocortical cultures were described by us in detail before [76], and midbrain cultures were prepared as in Shimoda *et al.* [75]. Glial cells from the neocortical cultures were immunostained with antibodies against glial fibrillary acidic protein, and VM midbrain cultures were shown to be enriched in dopaminergic neurons by immunostaining with antibodies to tyrosine hydroxylase. Serum-containing or serum-free media were prepared as follows. Serum-free medium consisted of Dulbecco's modified Eagles medium (DMEM)–F12 (1:1; Life Technologies) containing 2 mM L-glutamine (Life Technologies), 100 units of penicillin/ml, 100 units of streptomycin/ml (Life Technologies), 33 mM glucose (Sigma), and the N1 supplements [5 µg/ml insulin (Sigma), 5 µg/ml transferrin (Sigma), 2 × 10⁻⁸ M progesterone (Sigma), 100 µM putrescine (Sigma), and 3 × 10⁻⁸ M selenium (as Na₂SeO₃) (Sigma)]. Serum containing medium was DMEM–F12 (1:1; Life Technologies) containing 10% fetal bovine serum (Life Technologies), 4.0 mM glutamine (Life Technologies), 100 units of penicillin/ml, and 100 units of streptomycin/ml (Life Technologies). To prepare conditioned media (CM) from either BHK cells or glial cultures, cells were infected for 6 h with an m.o.i. of 300 for each virus and then incubated for a further 48 h. At this time medium was removed, centrifuged, and filtered with a 0.2-µm Sartorius filter. Conditioned medium was diluted 1:1 in fresh medium to maintain cell viability.

Construction of recombinant adenovirus. The cDNA encoding sonic hedgehog amino-terminal gene product was excised by enzymatic digestion with *EcoRI/HindIII* from a recombinant pBluescript II plasmid provided by Dr. P. Beachy, Johns Hopkins University (Baltimore, MD, USA). The full-length (3.6 kb) *HindIII/XbaI* insert of human (*Homo sapiens*) Gli-1 cDNA clone pGLK12 was kindly provided by Dr. Bert Volgestein, Johns Hopkins University, and the full-length (0.7 kb) *BamHI/XhoI* insert of rat GDNF cDNA clone pCDNA-GDNF was made available by Dr. Ira Black (UMDNJ, NJ). These inserts were then cloned into the shuttle vector pAL119, yielding pALShhN, pALGli-1, and pALGDNF containing the ShhN, the Gli-1, or the GDNF coding region, respectively, in the sense orientation with respect to the major immediate early hCMV promoter of pAL119 [76]. The orientation of the cloned transgenes was determined by restriction analysis with *EcoRI* or *HindIII* for ShhN and *HindIII* or *SacI* for Gli-1 [77]. Recombinant adenoviruses RAD-ShhN, RAD-Gli-1, and RAD-GDNF expressing ShhN, Gli-1, and GDNF, respectively, under the control of the major immediate early hCMV promoter were generated by cotransfection of the shuttle plasmid pALShhN, pALGli-1, or pALGDNF with the Ad5 genomic plasmid pJM17 into 293 human embryonic kidney cells as we previously described [74,76,78]. After molecular characterization of the RADs, they were purified by three rounds of dilution limiting assay, scaled up, and purified by CsCl₂ gradient, and the presence and identity of the transgenes were confirmed by restriction and Southern blot analysis.

The construction and use of RAD-35 (expressing β-galactosidase) and RAD-TK (expressing HSV-1 TK), both under the control of the hCMV promoter, were described by us earlier [44,45,76]. Quality control of viral stocks was assayed by detection of contaminating replication-competent adenovirus (RCA) [79] or endotoxin (LPS), using the Multitest *Limulus* Amebocyte Lysate Pyrogen Kit (Biowhittaker, Inc.) All viral stocks used in this study were RCA and LPS free [80].

Analysis of Shh expression and secretion. The expression and secretion of ShhN into the culture medium after infection of BHK cells with RAD-ShhN was evaluated by dot blot, Western blot, and ELISA. Media from RAD-infected cells, i.e., CM, were prepared as described above. For dot-blot analysis 200 µl of CM from uninfected or RAD-ShhN- or RAD-35-infected BHK cells was bound to nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech) using a Bio-Dot apparatus (Bio-Rad), and the nitrocellulose membrane was probed using the 5E1 anti-ShhN monoclonal antibody (1:1000 [81]) as primary antibody and biotinylated anti-mouse antibody (1:1000; DAKO) as secondary antibody. Colorimetric detection of Shh immunoreactivity was carried out using the biotin–avidin–horseradish peroxidase detection kit Vectastain ABC from Vector Laboratories. For Western blot analysis CM from uninfected or RAD-35- or RAD-ShhN-infected BHK cells was concentrated 25-fold using the Ultra-free-0.5 centrifugal kit from Millipore (Biomax 5-kDa NMWL-membrane). Concentrated CM samples were fractionated by Nu-PAGE and transferred to a nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech) for 30 min at 15 V using a semidry blot transfer system (Hoeffer Scientific Instruments). The membrane was probed using a specific goat anti-ShhN polyclonal antibody (1:100; Santa Cruz Biotechnology) as primary antibody and a biotinylated anti-goat antibody (1:1000; DAKO) as secondary antibody. Colorimetric detection of ShhN was carried out using the ABC detection kit from Vector Laboratories. ELISA was carried out as follows: 96-well ELISA plates (Greiner) were coated with 50 µl of coating solution (coating antibody Shh 5E1 [81] diluted 1:500 in PBS, pH 7.2) overnight at room temperature. Coating solution was discarded the next day. Nonspecific binding sites were blocked by incubating with 100 µl of 3% BSA–PBS solution for 2 h at 37°C. The plate was then washed three times with 0.2% Tween 20–PBS solution. Conditioned medium from RAD-infected BHK cells (100 µl/well) was added to the plate and incubated at 37°C for 2 h. After the incubation period, the plate was washed three times using a 0.1% Tween 20–PBS solution. The plate was then incubated with 50 µl/well of secondary antibody (goat anti-Shh polyclonal antibody diluted 1:200 in 3% BSA–PBS solution) for 1 h at room temperature and washed, and 35 µl of detection antibody [anti-goat biotinylated antibody (DAKO) diluted 1:5000 in 3% BSA–PBS solution] was added to each well. The plate was then incubated for another hour at room temperature and washed thoroughly to remove any remaining unbound antibody. The plate was incubated with 100 µl of ABC (Vector Laboratories) for 30 min and washed. Finally, 100 µl of 1 mg/ml ABTS substrate from Sigma was added. The reaction was developed in the dark for 30 min at room temperature. The presence of ShhN in conditioned medium was determined by reading the absorbance at 405 nm.

In vitro assessment of ShhN-mediated survival of dopaminergic midbrain neurons. To assay ShhN-mediated survival of dopaminergic neurons, VM cell primary cultures were plated and maintained in 50% conditioned medium from infected and control BHK cells for 7 or 4 days, respectively. VM primary culture cells were then fixed with 4% paraformaldehyde and 0.2 M sucrose in PBS, pH 7.4, and processed for TH immunocytochemistry. The number of TH-positive cells present in each condition was counted. To confirm the specific effects of ShhN on dopaminergic neuron survival, an immunoblocking experiment was performed by adding to the conditioned medium from mock-, RAD-35-, or RAD-ShhN-treated BHK cells the 5E1 anti-ShhN antibody (to a final dilution of 1:500 [81]) for 30 min at 4°C before addition of the mixture to VM cultures. VM cultures were incubated for 4 further days after which they were processed for TH immunocytochemistry using the ABC kit. The number of TH-immunoreactive neurons was counted using a total mag-

nification of 200 ×. Data regarding the survival of dopaminergic neurons in culture, as well as ShhN-blocking experiments, were confirmed in three independent experiments in triplicate, values were expressed as means ± SEM, and differences in the survival of TH-immunoreactive neurons among the treatments were analyzed statistically [82]. Differences in TH⁺ neuronal survival were evaluated by the Student *t* test. The differences between the effects of each treatment were assessed by one-way ANOVA. When the ANOVA showed significant differences, pair-wise comparisons between means were tested using either Tukey or Dunnett multiple comparisons test [82]. Statistical tests were performed using the SPSS statistical package for Windows version 9 from SPSS, Inc., USA.

In vitro bioactivity of Rad-ShhN, Rad-Gli-1, and Rad-Nurr-1, determined as ShhN- or Gli-1-induced differentiation of C3H10T1/2 cells or Nurr-1-induced luciferase expression from a Nurr-1-responsive promoter.

Differentiation of the pluripotential cell line C3H10T1/2 into the osteoblastic lineage in response to Rad-ShhN or Rad-Gli-1 infection was carried out as follows: 1×10^5 cells per well were seeded in two six-well plates and later infected with either Rad-ShhN or Rad-Gli-1 at m.o.i. 200. Uninfected cells or a control adenovirus (Rad-35; m.o.i. 200) was used as negative control. The induction of osteoblast phenotype in response to the viral treatment was determined by detecting AP activity, a marker of bone differentiation, using the histochemical detection afforded by the kit Fast Red TR/Naphtol AS-MX (Sigma). AP-positive cells (red reaction product) in each well were visualized under light microscopy and three independent experiments were carried out to confirm the results. To test the bioactivity of Rad-Nurr-1, a COS-7 cell line transiently transfected with a plasmid containing a specific Nurr-1-responsive element, NBRE-LUC (kindly provided by Dr. J. Milbrandt), and thus responding to the presence of Nurr-1 with an increase in luciferase activity was used.

Detection of TH immunoreactivity by immunohistochemistry. Midbrain cultures or brain sections were permeabilized in 2 ml of TBS/Triton X-100 (0.5% v/v) at room temperature and washed, and endogenous peroxidase activity was inactivated by adding 3 ml of 0.3% H₂O₂ for 15 min. Nonspecific antibody binding sites and Fc receptors were blocked by incubating the sections with 10% horse serum (HS) in 1 ml of TBS/Triton for 1 h and washed, and sections were incubated with a specific rabbit anti-TH polyclonal antibody (Pharmingen) diluted 1:1000 in TBS/Triton/1% HS. This incubation was carried out overnight at room temperature. Sections were then washed and incubated for 4 h with rabbit biotinylated secondary antibody (1:200) in TBS/Triton/1% horse serum. TH immunoreactivity was revealed using ABC (Vector Laboratories), and diaminobenzidine tetrahydrochloride (Sigma) was the substrate for horseradish peroxidase. Sections were washed, mounted onto gelatin-coated slides, air-dried, dehydrated, and coverslipped with DPX as previously described [83].

Immunocytochemical detection of β-galactosidase, HSV-1 TK, ShhN, Gli-1, and Nurr-1. The striatum or substantia nigra of animals injected with Rad-35, Rad-TK, Rad-ShhN, Rad-Gli-1, or Rad-Nurr-1 was immunostained as described in detail above. ShhN was detected using a mouse monoclonal antibody raised against the amino-terminal of Shh (University of Iowa Hybridoma Bank), Gli-1 and Nurr-1 were detected using rabbit polyclonal antibodies raised against the amino-terminal of Gli-1 or against Nurr-1 (Santa Cruz Biotechnology). HSV-1 TK was detected with a specific polyclonal rabbit antibody raised against a TK-specific peptide and produced by us and published elsewhere [45,84].

Intrastriatal delivery of RAdS in the 6-OHDA experimental model of Parkinson's disease and quantification of RAd-mediated protection of dopaminergic neurons in the SNpc. Adult male Fisher 344 rats of 200–250 g body weight (Charles River Breeding Laboratories, UK) were used. All animals had free access to food and water, a 12-h light/dark cycle, and constant housing temperature and humidity, and experiments followed approved local regulations guiding experimental research. The ability of Rad-ShhN, Rad-Gli-1, or Rad-Nurr-1 to protect DA neurons from 6-OHDA

neurotoxicity was evaluated using a modification of a robust experimental model of Parkinson's disease in rats [46]. Stereotaxic neurosurgery was performed on the animals under gaseous anesthetic as previously described [85], using the following stereotaxic coordinates from bregma: AP + 1.0 mm, ML + 3.2 mm, DV –5.0 mm for the right hemisphere injection and AP + 1.0 mm, ML –3.2 mm, DV –5.0 mm for the left hemisphere injection. Using a 0.5-μl Hamilton syringe, a total volume of 0.02 μl of the retrograde tracer fluoro-gold (FG) (2% diluted in saline 0.9% w/v) was injected bilaterally, over a total of 6–7 min. The volume of FG was optimized to label a number of cells comparable to those labeled in the substantia nigra following the injection of RAdS into the striatum. The following volumes of FG were tested: 0.2, 0.1, 0.05, and 0.02 μl of 2% FG. The tracer was injected into the striatum and the total number of retrogradely labeled striatal neurons was counted. The injection of 0.02 μl of 2% FG consistently labeled a range of 30–50 neurons per 25-μm-thick midbrain section throughout the nigra (a total mean of 1647 neurons for all sections throughout the substantia nigra); this number was comparable to the number of neurons retrogradely labeled following the injection of RAd. Therefore, this volume of FG was chosen for the experiments.

Following the FG injection, 3 μl of RAd (1×10^8 IU) was injected into the right hemisphere, using a 10-μl Hamilton syringe, at the identical coordinates used for the first injection of FG. One week later, the animals were prepared for a second surgery, during which 2 μl of 6-OHDA–HCl, resuspended in ascorbic acid 0.2 mg/ml and diluted in saline 0.9% w/v to a final concentration of 8 μg/μl, was injected over 6 min into the right striatum in exactly the same anatomical site previously injected with FG and RAd. Animals were sacrificed 4 weeks after injection of 6-OHDA and the brains fixed by cardiac perfusion with oxygenated tyrode followed by 4% paraformaldehyde, pH 7.4, as previously described in detail [83,85]. Brains were postfixed for 6 h at 4°C, washed with PBS, and stored in PBS containing 0.1% sodium azide at 4°C until required. Coronal sections (25 μm thickness) of the midbrain or the forebrain (40 μm) were cut using an electronic Vibratome (Leica). The midbrain was sectioned from AP –4.52 mm to AP –6.30 mm. The forebrain was sectioned from AP 3.20 mm to AP –1.30 mm according to the stereotaxic rat brain atlas [86].

The extent of neuroprotection was measured by counting, using the 20× objective, the number of FG-positive retrogradely marked nigrostriatal neurons throughout the rostrocaudal axis (AP –4.8 mm to AP –6.04) of the ipsilateral (lesioned) SNpc and expressed as a percentage of the number of fluoro-gold-marked nigrostriatal neurons in the contralateral hemisphere (unlesioned); *n* = 7 per group. The medial terminal nucleus of the accessory optic tract was used to define the border between the SNpc and the VTA. Rats injected with Rad-GDNF or Rad-35 (β-galactosidase) were used as positive and negative controls, respectively. All neurons present in all 25-μm sections cut throughout the extent of the substantia nigra were counted. Any counting method in which the dependent variable enters into the statistical calculations, e.g. in which the percentage of neuronal survival and the counts are performed by someone who does not know the experimental manipulation or potential outcomes can be used instead of stereology; stereology is useful mainly for unbiased estimations of very large numbers of cells. The operator performing the neuronal counts was blind to the identity of the sections (e.g., from either any of the control or any of the experimental groups). The estimation of the percentage of protected susceptible neurons (PSN) was calculated using the following mathematical correction:

$$PSN = \frac{\bar{x} \% \text{ protected cells} - \bar{x} \% \text{ survivor cells (negative control)}}{100 - \bar{x} \% \text{ survivor cells (negative control)}} \times 100.$$

Quantification of the cell body area of dopamine nigral neurons within the SNpc. The size of ipsilateral (lesioned side) dopaminergic neurons in RAd-treated animals and controls was measured as square micrometers of cell body area and expressed as a percentage of the cell body area of dopaminergic neurons in the contralateral site (unlesioned side). One hundred dopaminergic neurons were randomly chosen at the level of the rostral SNpc and used to estimate the area of neuronal somata; seven animals per group were used. The experimenter selected the neurons and was blind to the treatment groups. Measurements were made with a

Leica Quantimet Q600 Image Analysis System controlled by QWIN software (Leica Microsystems, Cambridge, UK) connected to a Leica RMD8 microscope.

Quantification of the density of striatal dopaminergic, TH-immunoreactive, fibers. The extent of striatal dopaminergic denervation produced by the injection of the neurotoxin 6-OHDA, and the effect of the potentially therapeutic RAdS, was evaluated by measuring the density of TH-IR fibers in the striatum. Six representative forebrain sections corresponding to the coordinates (from bregma) AP 1.60, AP 1.20, AP 1.00, AP 0.70, AP 0.48, and AP 0.20 were used to measure the density of TH-IR in the entire ipsilateral striatum and expressed as a percentage of an equivalent area in the contralateral site ($n = 4$). All measurements were made with a Leica Quantimet Q600 Image Analysis System controlled by QWIN software (Leica Microsystems) connected to a Leica RMD8 microscope.

Statistical analysis. The treatment groups (RAd-ShhN, RAd-Gli-1, RAd-Nurr-1, RAd-35, RAd-GDNF) were compared by ANOVA or repeated-measures ANOVA with Tukey–Kramer (multiple comparisons test) or Dunnett post hoc pair-wise comparisons. Statistical calculations were made using the Graphpad Instat v2.00 statistical package.

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