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Recombinant AAV Viral Vectors Serotype 1, 2, and 5 Mediate Differential Gene Transfer Efficiency in Rat Striatal Fetal Grafts

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Intrastriatal grafts of fetal ganglionic eminences (GE) can reverse symptoms of striatal lesions in animal models of Huntington's disease. On the other hand, neurotrophic factors have been shown to protect host striatal neurons from ongoing degeneration. Neurotrophic gene transfer into GE prior to grafting could combine the benefits of striatal neuron replacement and in situ delivery of neurotrophic factors. Here we evaluate the potency of recombinant adeno-associated viruses (rAAV) as vectors for gene delivery into rat embryonic (E15) GE using the eGFP reporter gene under the control of the strong cytomegalovirus (CMV) promoter. We observed a very efficient expression of the eGFP reporter gene in organotypic cultures of GE infected with rAAV serotype 1 from 4 days until at least 4 weeks postinfection. In contrast, transduction was low and absent when using serotype 2 and serotype 5 rAAV, respectively. Two months after transplantation of rAAV2/1-infected embryonic GE in adult rat striatum, more than 20% of grafted cells expressed eGFP. The majority of transduced cells in the graft were neurons as indicated by colabeling of GFP-immunoreactive cells with the NeuN marker. Our study suggests that GE transduced by rAAV-serotype 1 vectors could be an interesting tool to mediate efficient expression of a gene coding a neurotrophic factor in Huntington's disease.

Key words: Ganglionic eminence; Fetal graft; Huntington's disease; Gene transfer; Adeno-associated virus

INTRODUCTION

Huntington's disease (HD) is an autosomal dominant inherited neurodegenerative disorder involving primary GABAergic medium spiny projection neurons in the striatum (26) and cerebral cortex (27). The disease is characterized by devastating motor, cognitive, and psychiatric disturbances, which usually appear in adult life and progress over one to two decades. *Huntingtin*, the gene causing HD, has been cloned in 1993 and the mutation identified as an unstable (CAG)n repeat in the coding region of the protein (30). Although the mechanisms accounting for the selective striatal degeneration are intensively studied, to date pharmacological therapies show low efficiency and do not provide reconstruction of brain neural circuit or prevent progression of the disease (12,13,28,31). In various experimental animal models of HD, intrastriatal implantation of striatal fetal neurons can reverse a number of symptoms and reestablish the functional connections between afferent axons and the grafted cells (9). Moreover, clinical benefits have been demonstrated in patients after striatal grafts (3,17). However, after 6 years, motor and metabolic benefits decreased (4), presumably because the patient's neurons continue to degenerate.

Experimental studies, in vitro (24) as well as in animal models (1), demonstrated significant benefits of neurotrophic factors (NF). While neurotrophic factors have shown promising results, the method of their delivery may be critical in establishing potent neuroprotection. Indeed, systemic administration of NF is associated with important side effects and a low detectable concentration in the central parenchyma (20). These data have led to the investigation of delivery systems that allow continuous administration of NF directly into the brain (mechanical minipumps, gene delivery) (2,5,15,21). In-

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trastriatal administration of glial cell line-derived neurotrophic factor (GDNF) (15) and ciliary neurotrophic factor (CNTF) (21) has been shown to rescue striatal neurons from quinolinic acid-induced and 3-nitropropionic acid damage, respectively, suggesting that increased neurotrophic factor expression may prevent or reduce neuronal loss in the HD brain. A combined substitutive and neuroprotective therapy could enhance graft survival as well as halt or slow down the host's neuron degeneration.

In the present study, we tested the efficiency of recombinant adeno-associated viruses (rAAV) as vectors for gene transfer into striatal grafts. Recombinant AAV lacks all viral genes and mediates long-term gene expression in vivo in adult neurons with a low and transient immune response and no toxicity (23). Moreover, rAAVs have been used to deliver therapeutical genes in patients without toxicity (19). Recently, several different serotypes of AAV have been used to derive vectors with varying efficiencies in different tissues (25). AAV-1 and -5 have been shown to be particularly efficient in adult rat striatum (6). However, no data are available about the gene transfer efficiency of rAAVs in fetal striatum. Therefore, we evaluated the gene delivery potential of vectors based on AAV serotype 1, 2, and 5 into rat embryonic ganglionic eminences (GE), with the aim to improve the survival of grafted striatal neurons and to protect the host striatum neurons from ongoing degeneration by delivering genes coding for NF.

MATERIALS AND METHODS

Recombinant AAV Vectors

The rAAV2/1-CMV-eGFP, rAAV2/2-CMV-eGFP, and rAAV2/5-CMV-eGFP were produced at the "GVPN" (Laboratoire de Thérapie Génique, Nantes, France) and had titers of 2.0tiimx10^{11}, 7.0×10^{11}, and 4.2×10^{11} viral particles per milliliter, respectively.

Animals

Adult female Wistar rats of ~200 g (Charles River, France) were housed three to four in a cage with free access to rat chow and water during a 12-h light/dark cycle.

The housing and treatment of the animals were performed according to rules set by the Ethical Committee for Use of Laboratory Animals at ULB.

Rat embryos were obtained from pregnant Wistar rats (Charles River, France) at 15 days of gestation. The pregnant dam was terminally anesthetized with a mixture of ketamine (Ketalar® 50 mg/ml) and xylazin (Rompun® 2% 23 mg/ml, Bayer), and the uterine horns were removed by caesarian section and collected in Hank's balanced salt solution (HBSS; Life Technology, USA).

Preparation of Ganglionic Eminences Cell Suspensions

Dissection of the lateral (LGE) and medial (MGE) ganglionic eminences from E15 embryos was performed in HBSS. LGE and MGE fragments from both hemispheres of all embryos were pooled. Fragments (~1 mm^3) were infected with rAAV2/1-CMV-GFP vectors for 2 h at 37°C. Cell suspensions were prepared according to a protocol described by Watts and collaborators (33) with minor modifications. Fragments were washed in isotonic saline containing 0.6% glucose and trypsinized for 20 min at 37°C in saline containing 0.1% trypsin (Worthington) and 0.05% DNAase. Fragments were then mechanically dissociated in a 1:1 mixture of trituration medium [1% bovine serum albumin (BSA; Sigma), 0.001% deoxyribonuclease (Sigma), 0.5% soybean trypsin inhibitor in saline (Sigma), and Dulbecco's minimal essential medium (DMEM; Life Technologies) supplemented with 10% fetal calf serum (Biowhitakker)]. The cells were centrifuged at 800 rpm for 10 min and the pellet was resuspended in a volume of 8 µl per eminence of trituration medium. The cell survival rate was determined by trypan blue dye exclusion using a Bürker chamber and was always higher than 90%.

Organotypic Cultures

LGE and MGE fragments from four to five fetuses were infected with 10^9 viral particles of rAAV2/1-CMV-eGFP, rAAV2/2-CMV-eGFP, or rAAV2/5-CMV-eGFP for 2 h at 4°C. Fragments were then transferred on the membrane of Transwells (Costar) and maintained with 1.1 ml serum-free minimum essential medium (MEM; Life Technologies, Belgium) containing 1% glucose, 5 mM Tris-HCl, pH 7.4, 100 µl/ml BSA, 100 µl/ml tranferrin, 16 ng/ml putrescin, 40 ng/ml Na-selenium, 30 ng/ml tri-iodothyronin, 5 µg/ml insulin, and 60 ng/ml progesterone at 37°C and 5% CO_2 in a humidified atmosphere with three changes of medium a week. All supplements were from Sigma.

At the indicated time points, tissue was washed in PBS at 37°C for 15 min, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer at 37°C for 15 min. The fragments were then rinsed in PBS, mounted on slides using Vectashield mounting fluid (Vector laboratories, Burlingame, CA, USA), and observed under fluorescence microscope to detect native GFP fluorescence.

Stereotaxy

The animals were anesthetized with a mixture of ketamin (50 mg/ml, Ketalar®) and xylazine hydrochlorid (23 mg/ml Rompun® 2%). After anesthesia, animals were placed into stereotaxic frames (Kopf). All injections were made using a 10-µl Hamilton microsyringe that was attached to a motorized injection system (Nanoinjector, Stoelting) according to coordinates defined

by Paxinos and Watson (22). Experiments were approved by the local committee for animal care (Commission d'éthique du bien-être animal, ULB).

Cell Suspensions. The anterio-posterior (AP) and medial-lateral (ML) stereotaxic coordinates (AP: +0.5, ML: −2.5) were calculated from bregma. A burr hole was drilled in the skull. The dorso-ventral (DV: −5.5/−4.5) coordinates were calculated from dural surface. All animals received two injections of 2 µl of embryonic day 15-infected striatal cells. The injection rate was 0.2 µl/min in the striatum. One minute after the cessation of the infusion, the needle was retracted 1 mm and left in place for an additional 2 min before it was slowly retracted from the brain. Animals were sacrificed 2 months posttransplantation.

Recombinant Viruses. Viral particles diluted in D-PBS (BioWittaker) were infused using a motor-driven Hamilton syringe (0.2 µl/min) with a 30-gauge needle. The injection coordinates were, respectively, AP +;0.5 , L −2.8 , DV −5.0 for AAV2/1 (one deposit of 2 µl); AP +0.7, L −3.5, DV −4.0, −5.0, and −6.0 (three deposits of 1 µl) for AAV2/2; and AP +0.5, L −2.8, DV −4.5 and −5.5 for AAV2/5 (two deposits of 1 µl). After injection, the needle was left in place for 5 min in order to allow diffusion of the viral suspension in the parenchyma. The needle was then slowly lifted 1 mm up and left in place 5 min, then slowly removed. Animals were sacrificed 1 month after surgery.

Immunofluorescence

Rats were anesthetized with a mixture of ketamin (50 mg/ml, Ketalar®) and xylazine hydrochlorid (23 mg/ml Rompun® 2%) and transcardially perfused with physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PF4). Brains were removed and postfixed overnight in PF4 at 4°C. Coronal sections were cut using a vibratome (Leica) at 50 µm and labeled by immunofluorescence as follows.

Sections were sequentially incubated in: (i) THST [50 mM Tris, 0.5M NaCl, 0.5% Triton X-100 (Merck) pH 7.6] containing 10% horse serum (Gibco BRL, Life Technologies, Belgium) for 30 min; (ii) mouse monoclonal antibodies [anti-NeuN (1:200, Chemicon) or anti-glial fibrillary acidic protein (GFAP, 1:200, Chemicon)] together with polyclonal rabbit anti-GFP (1:200, Molecular Probes, Merck Eurolab, Belgium) diluted in THST containing 5% horse serum for 48 h at 4°C; (iii) donkey anti-rabbit coupled to biotin (Jackson ImmunoResearch, West Grove, PA) diluted 1:100 in TBS containing 0.1% Triton X-100 and 5% horse serum for 2 h at room temperature; (iv) streptavidin-cyanine 2 and donkey anti-mouse coupled to cyanine3 (both 1:100; Jackson Immuno Research, West Grove PA) in TBS containing 5% horse serum; five washings in TBS containing 0.1% Triton X-100 were performed between each step; (v) cell nuclei were stained with a nuclear dye, Hoechst 33258, to reveal all the grafted cells. Sections were then mounted on gelatin-coated slides and coverslipped with FluorSave reagent (Calbiochem, La Jolla, CA, USA).

Immunohistochemistry

For GFP stainings, vibratome sections (50 µm) were sequentially incubated in: (i) 3% H_2O_2 in TBS (Tris 10 mM, 0.9% NaCl, pH 7.6) for 30 min; (ii) THST (50 mM Tris, 0.5 M NaCl, 0.5% Triton X-100, pH 7.6) containing 10% horse serum for 1 h; (iii) polyclonal rabbit anti-GFP (Clonetech, Palo Alto, CA, USA) diluted 1:3000 in THST containing 5% horse serum overnight at 4°C; (iv) donkey anti rabbit-biotin (Amersham, Belgium) 1:600 in THST containing 5% horse serum, 2 h at room temperature. The peroxidase staining was revealed using the ABC Elite vectastain kit and diaminobenzidine (Vector, NTL Laboratories, Brussels, Belgium), according to the manufacturer's protocol. Sections were mounted on gelatin-coated slides, dehydrated, and mounted using DPX mounting fluid (Sigma). Sections were photographed using a Zeiss Axiophot 2 microscope.

Optical Microscopy

Cells harboring native GFP green fluorescence were evidenced using a Zeiss Axioplan II microscope equipped with an UV lamp and a FITC filter. Images were acquired using the KS300 software (Zeiss).

Confocal Microscopy

Cell counts of immunopositive cells stained for GFP, NeuN, and GFAP were performed on six sections covering the graft site using an automatic image analysis system [Lasersharp version 3.2 (Biorad) coupled to Axiovert 100 microscope, Zeiss, Germany].

To determine the proportion of GFP-expressing cells in the graft, the number of GFP-immunoreactive cells was divided by the total number of cells revealed by Hoechst staining in each section. The double-labeled tissue was used to determine the percentage of GFP-immunoreactive cells that were labeled for NeuN or GFAP in the grafts.

RESULTS

AAV-Mediated Gene Transfer in Organotypic Cultures of E15 GE

To investigate the ability of gene transfer by three serotypes of AAV vectors (AAV2/2, -2/1, and -2/5) into embryonic GE tissue, we incubated solid fragments from 8 to 10 rat embryonic GE with 10^9 viral particles of (i) rAAV2/1-eGFP, (ii) rAAV2/2-eGFP, and (iii) rAAV2/5-eGFP, and maintained them in organotypic

culture. After rAAV2/1-eGFP infection, cells started to express GFP as early as 4 days postinfection (data not shown) and their number increased up to at least 4 weeks postinfection (Fig. 1A, D). In contrast, after rAAV2/2-eGFP infection, only a few cells expressed GFP after 7 days and 4 weeks postinfection (Fig. 1B, E). In contrast, surprisingly, no green fluorescent cells were observed after rAAV2/5-eGFP infection (Fig. 1C, F). The rAAV-eGFP viral preparations were also evaluated in the adult rat striatum (Fig. 2). Confirming previous studies (6), rAAV2/1- and rAAV2/5-mediated gene transfer was efficient and widespread (Fig. 2A, C) whereas in the case of rAAV2/2, the transduced area was limited to the vicinity of the needle tract (Fig. 2B).

AAV2/1-Mediated Transgene Expression in Transplanted Rat GE

Embryonic rat GE fragments were incubated in rAAV2/1-EGFP viral suspension (10^9 viral particles per 6 GE) for 2 h at 4°C, pooled, and trypsinized for 20 min at 37°C after infection. Animals received 4 µl of cells/rat unilaterally into the right striatum. The cell viability before transplantation was more than 95% (data not shown). Two months after transplantation, the graft was well integrated. GFP-positive cells with a neuronal morphology were observed (Fig. 3). No GFP-immunoreactive cells were observed in control animals (with noninfected grafts; data not shown). The proportion of GFP-immunoreactive cells in the graft, as evaluated by co-staining with Hoechst, was $22 \pm 0.2\%$ ($n = 4$).

Characterization of Transducted Cells

In all animals, the vast majority ($93.6 \pm 1.6\%$) ($n = 4$) of GFP-immunoreactive cells in the graft colabeled with NeuN (Fig. 4A–C). No colabeling (<2%) with the astrocytic GFAP marker was observed (Fig. 4D–F).

DISCUSSION

HD is an autosomal dominant disorder caused by an expanded polyglutamine (CAG) tract at the IT15 locus on chromosome 4 causing degeneration of striatal and cortical neurons with severe motor and cognitive consequences. There is currently no treatment that can halt the progression of this disease. Fetal striatal transplants are currently explored as a novel therapeutic approach. Grafts of embryonic GE survive long term in the diseased brain, induce significant and lasting motor and cognitive therapeutic effects (4), and improve cortical metabolism (10). An autopsy study has suggested that the disease does not affect the transplanted cells that

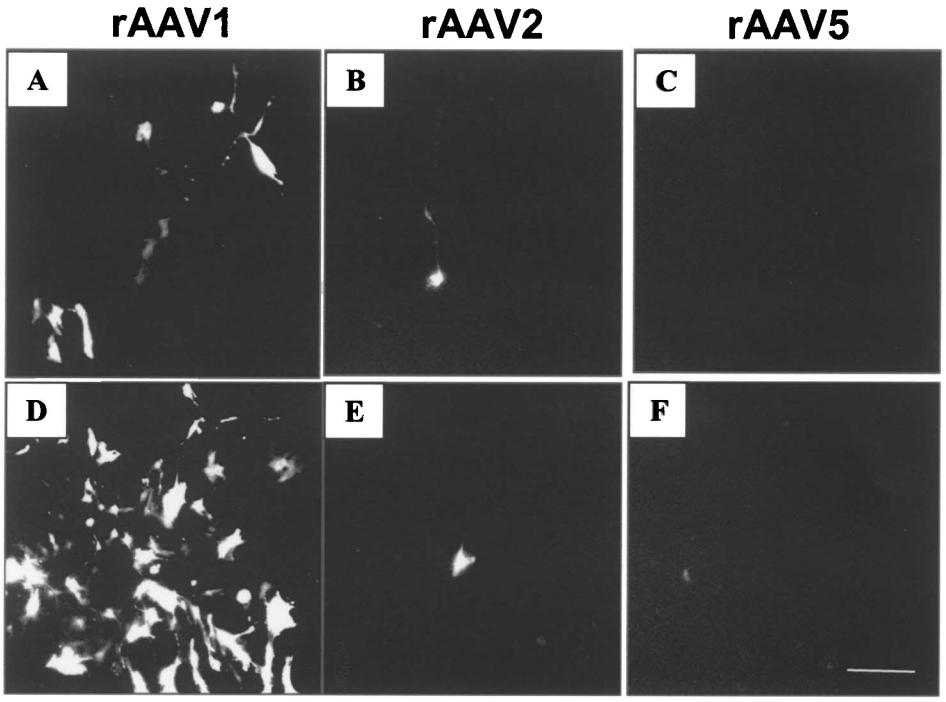

Figure 1. rAAV2/1, 2/2, and 2/5-mediated gene expression in organotypic cultures of rat E15 ganglionic eminences. Freshly explanted solid fragments of rat E15 ganglionic eminences (GE) were incubated with 10^9 viral particles of rAAV2/1-eGFP (A, D), rAAV2/2-eGFP (B, E), and rAAV2/5-eGFP (C, F). Native GFP fluorescence 1 week (A, B, C) and 1 month (D, E, F) after infection. Scale bar: 30 µm.

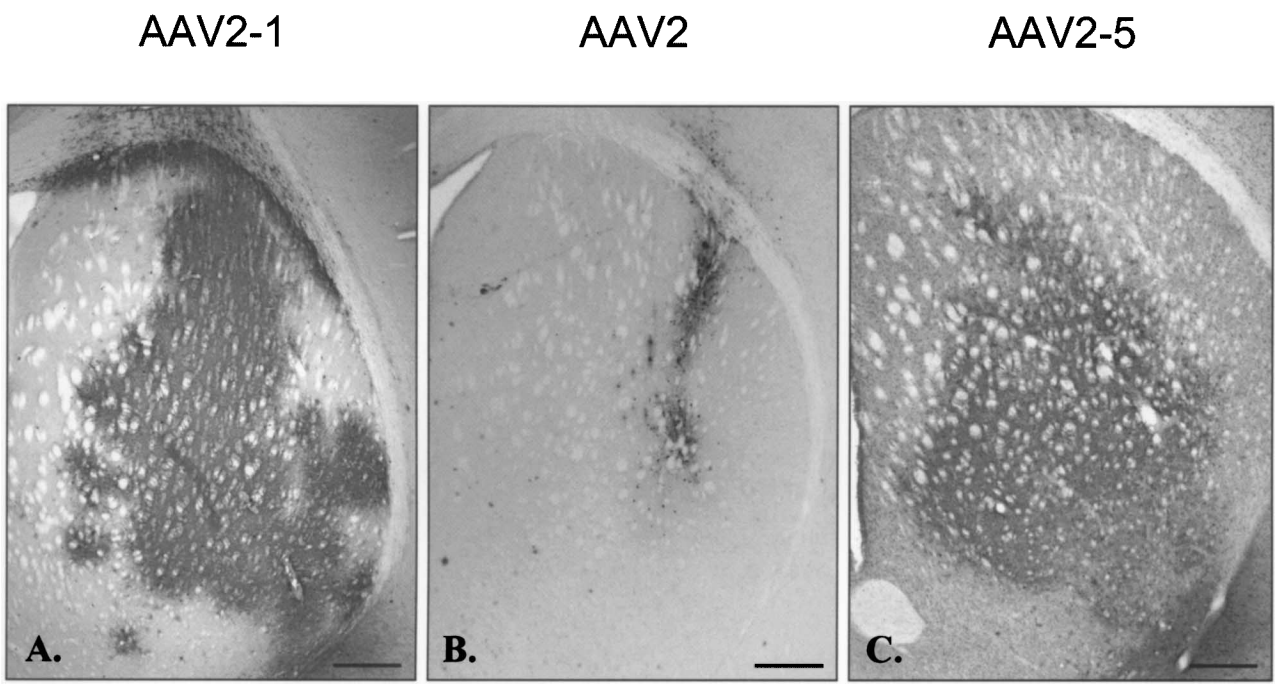

Figure 2. rAAV2/1, 2/2, and 2/5-mediated gene expression in the adult rat striatum. Four weeks after injection of rAAV2/1 (A), rAAV2 (B) or rAAV2/5 (C) vectors carrying the CMV-eGFP expression cassette into the striatum, 50 µm vibratome brain sections were labeled with GFP antibodies. Scale bar: 800 µm.

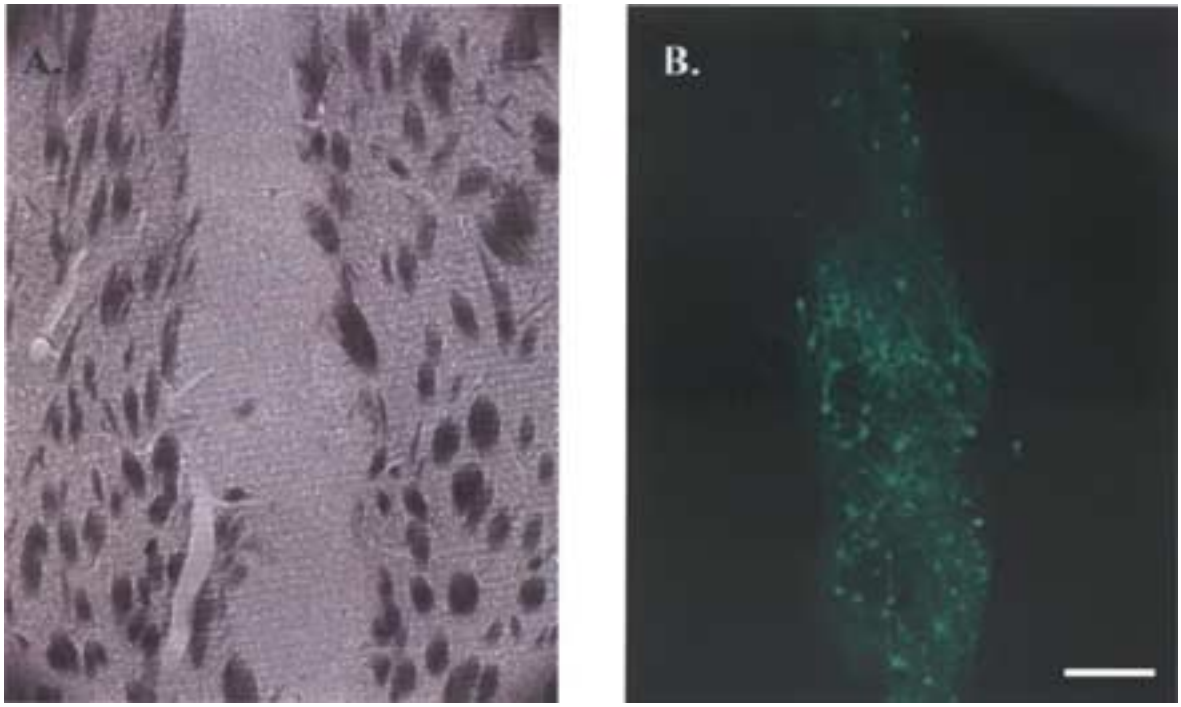

Figure 3. rAAV2/1-mediated transduction of transplanted embryonic (E15) striatal neurons. Two months after transplantation, the graft shows numerous GFP-immunoreactive cells with a neuronal morphology. Scale bar: 200 µm.

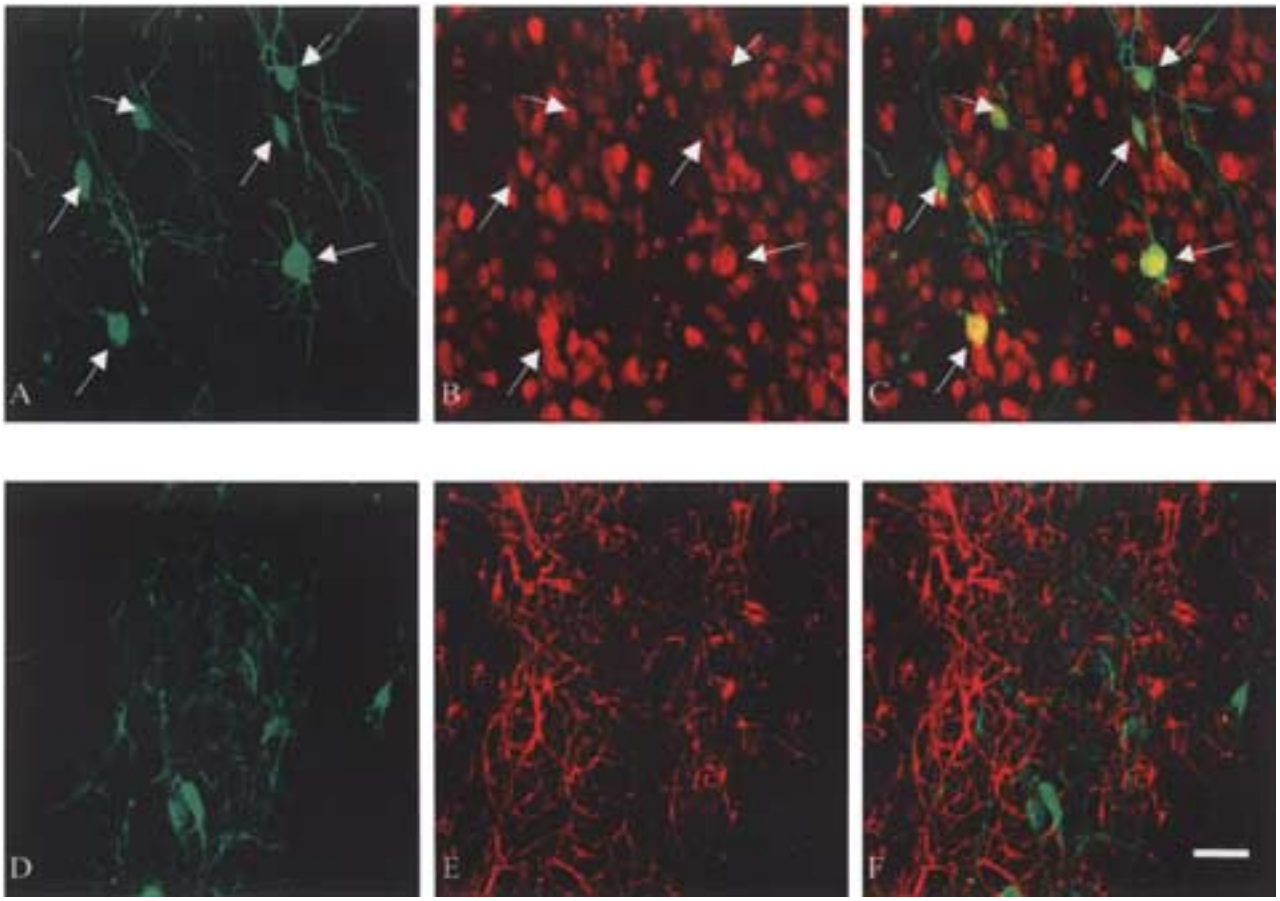

Figure 4. Cellular tropism of rAAV2/1 in transplanted embryonic (E15) striatal neurons. (A–C) Immunofluorescent colabeling with GFP and NeuN. The vast majority (93.6 ± 1.6%) of GFP-immunoreactive cells (green labeling) in the graft were colabeled with NeuN (red labeling). Arrows indicate double-labeled cells. Some nonneuronal cells also expressed GFP fluorescence. (D–F) Absence of colabeling of GFP-immunoreactive cells (green labeling) and GFAP-positive cells (red labeling).

lack the mutant *huntingtin* gene (11). In contrast, the graft might not provide protection of host striatal neurons. Accordingly, patients who initially benefited from fetal striatal grafts declined at 6 years posttransplantation (4). Therefore, neuroprotection might reinforce the therapeutic effect of cell transplantation in HD. Furthermore, HD is a good candidate for neuroprotection because HD patients can be identified prior to the onset of neuronal degeneration and functional decline. Knowing a patient's genetic profile could allow therapy to be applied before the initial anatomical changes occur and behavioral sequelae appear.

Recent studies in animal models have shown that virally mediated gene transfer could be a powerful means by which NF can be delivered to the central nervous system over long periods. Several vectors, including rAAV (7), lentiviral (14), and adenoviral vectors (18), have allowed the efficient transfer of genes to cells of the CNS. In contrast to other gene delivery systems, rAAVs lack all viral genes and show long-term gene expression in vivo in various anatomical regions of the adult CNS without toxicity and with limited immune response to viral capsids.

In this report, we demonstrated that rAAV2/1 vectors efficiently transduce rat striatal cells for at least 4 weeks postinfection in vitro in organotypic cultures. We furthermore showed that gene transfer efficiency of rAAV2/1 is higher than rAAV2/2 and that rAAV2/5 does not transduce fetal GE. These results are in striking contrast with the relative transduction efficiencies of adult rat striatum by these three serotypes (rAAV2/1 ≅ rAAV2/5 > rAAV2/2) [(6); our data]. This discrepancy could possibly be due to a differential expression of AAV type 5 receptor or co-receptor (8,32) in adult and fetal striatum.

After grafting of rAAV2/1-infected rat striatal fetal cells in the rat striatum, transgene expression was efficient (more than 20% of the cells expressed the eGFP transgene) and stable (up to 2 months posttransplantation). Therefore, rAAV2/1-transduced grafts could be used as a stable cellular vector to deliver beneficial fac-

tors such as NF to the host striatum. In contrast to direct viral gene delivery, this ex vivo strategy avoids intracerebral injection of viral capsids that elicit immune responses, in particular in AAV-seropositive subjects.

The cellular tropism of rAAV vectors has already been extensively characterized in adult rat brain. However, only one study is currently available about their cellular tropism for fetal nervous tissue (16). This study demonstrated the efficiency of AAV2 vectors for the transduction of fetal ventral mesencephalic grafts. Similar efficiency of rAAV2 for gene transfer into human fetal mesencephalon (29) suggests that using viral vectors to enhance the survival and/of functionality of fetal grafts might be clinically relevant. In the present study, we observed for the first time in vivo that more than 20% of GE cells were transduced by rAAV2/1 vector. The vast majority of transduced cells were neurons. In contrast to fetal ventral mesencephalic cells, GE cells were poorly tranduced by AAV2.

Our study suggests that rAAV2/1 vector could constitute a safe gene delivery system allowing efficient and sustained transduction of embryonic GE cells. This method of gene delivery could prove useful to achieve continuous and local secretion of NF at relevant doses to treat HD.

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