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Targeted gene transfer to nigrostriatal neurons in the rat brain by helper virus-free HSV-1 vector particles that contain either a chimeric HSV-1 glycoprotein C-GDNF or a gC-BDNF protein

Xiaodan Wang, Lingxin Kong, Guo-rong Zhang, Mei Sun, and Alfred I. Geller*

Department of Neurology, Research Building 3, West Roxbury VA Hospital/Harvard Medical School, 1400 VFW Parkway, West Roxbury, MA 02132, USA

Abstract

Direct gene transfer into neurons has potential for both studying neuronal physiology and for developing gene therapy treatments for specific neurological conditions. Due to the heterogeneous cellular composition of the brain, cell-type-specific recombinant gene expression is required for many potential applications of neuronal gene transfer. The two prevalent approaches for achieving cell-type-specific expression are to use a cell-type-specific promoter to control recombinant gene expression or to modify a virus vector particle to target gene transfer to a specific cell type. Targeted gene transfer to multiple peripheral cell types has been described, but targeted gene transfer to a specific type of neuron in the brain has yet to be reported. Targeted gene transfer approaches with Herpes Simplex Virus (HSV-1) vectors have focused on modifying glycoprotein C (gC) to remove the heparin binding domain and add a binding activity for a specific protein on the cell surface. This study was designed to develop HSV-1 vectors that target gene transfer to cells that contain receptors for either glial-cell-line-derived neurotrophic factor (GDNF) or brain-derived neurotrophic factor (BDNF), such as nigrostriatal neurons. We isolated chimeric gC-GDNF or chimeric gC-BDNF constructs, and the resulting proteins were incorporated into HSV-1 virus particles. We performed helper virus-free HSV-1 vector packaging in the presence of each chimeric protein. The resulting vector stocks supported 2.2- to 5.0-fold targeted gene transfer to nigrostriatal neurons in the rat brain, compared to vector particles that contained wild-type (wt) gC. Gene transfer to nigrostriatal neurons by vector particles that contained chimeric gC-BDNF was reduced by preincubation with an anti-BDNF antibody. Targeted gene transfer to neurons that contain specific neurotrophic factor receptors may benefit specific physiological and gene therapy studies.

Keywords

Targeted gene transfer; Glycoprotein C; Herpes Simplex Virus vector; Glial-cell-line-derived neurotrophic factor; Brain-derived neurotrophic factor; Nigrostriatal neuron; Heparin sulfate

1. Introduction

Direct gene transfer into neurons using virus vectors has potential for both studying neuronal physiology and for developing gene therapy treatments for specific neurological conditions. However, because of the heterogeneous cellular composition of the brain, cell-type-specific recombinant gene expression is required for many potential applications of neuronal gene transfer. The two prevalent approaches for achieving cell-type-specific expression are to use

* Corresponding author. Fax: +1 617 363 5563. E-mail address: alfred-geller@hms.harvard.edu (A.I. Geller).

a cell-type-specific promoter to control recombinant gene expression or to modify a virus vector particle to target gene transfer to a specific cell type [17,28,37,46,47,51]. These two approaches are complementary, and a higher level of cell-type-specific expression may be achieved by using these two approaches in combination. In the brain, neuronal-specific or neuronal subtype-specific promoters have been used to restrict recombinant gene expression to neurons or a specific type of neuron, respectively [37,51]. However, in virus vectors, cell-type-specific promoters usually support a low level of ectopic expression in inappropriate cell types. Thus, modifying a vector particle to target gene transfer to a specific type of neuron may be a useful approach for restricting recombinant gene expression to a chosen type of neuron. Modified vector particles have been used to target gene transfer to multiple peripheral cell types and organs, but targeted gene transfer to a specific type of neuron in the brain has yet to be reported.

Targeted gene transfer with modified vector particles has been developed using classical retrovirus, lentivirus, adeno-associated virus (AAV), adenovirus, and Herpes Simplex Virus (HSV-1) vectors [2,6,13,17,22,32,46–48]. The targeting strategies modify the surface of a virus vector particle to add a new cell tropism to the vector particle, reduce the normal cell tropism, and preserve efficient vector particle assembly. The most direct targeting strategy is to genetically modify a vector particle protein to add a specific binding capability; one of the first reports used retrovirus vector particles that contained a chimeric erythropoietin (EPO)-virus envelope (*env*) protein to target gene transfer to cells that contained EPO receptors [17]. Because addition of a large polypeptide to a vector particle protein may disrupt vector particle assembly, another approach has been to add a bridging molecule that binds to both the vector particle and a cell surface ligand [46,48]. As an indication of the potential utility and specificity of vector targeting, AAV particles with a desired targeting specificity have been genetically selected by expressing a library of binding sites at a specific site in the AAV capsid protein and subjecting the resulting virus particles to repeated rounds of infection of the chosen cell type [28].

HSV-1 vectors are attractive because they have a large capacity and can efficiently transduce neurons [9,11]. The HSV-1 particle contains four components: (i) The ~152 kb genome is contained within (ii) an icosahedral protein capsid, which is surrounded by (iii) a layer of proteins termed the tegument, which is enclosed within (iv) the envelope, a lipid bilayer that contains 10 glycoproteins encoded by the virus [33]. HSV-1 infection proceeds in two stages [39]. Initial binding to cells is mediated by specific domains on glycoprotein C (gC) and gB that bind to glycosaminoglycans, principally heparin sulfate, on cell surface proteoglycans [23,26,35,42]. Subsequent entry requires the binding of gD to a receptor. Receptors for gD include the herpesvirus entry mediator (HVEM), a member of the tumor necrosis receptor family; nectin-1 or nectin-2, members of the immunoglobulin superfamily; and specific sites in heparin sulfate produced by particular isoforms of 3-*O*-sulfotransferases [39]. Entry occurs by fusion of the envelope to the cell membrane and requires gB, gD, gH, and gL.

Targeted gene transfer approaches with HSV-1 vectors have focused on modifying gC to remove the heparin binding domain and add a ligand for a specific protein on the cell surface. The first study [22] isolated a recombinant HSV-1 virus that contained a chimeric gC-EPO protein and deleted the heparin sulfate binding domains on both gC and gB. This virus showed enhanced binding to cells that contained EPO receptors, but targeted infection was not reported. In the other study [13], an HSV-1 plasmid (amplicon) vector that expressed a chimeric gC-His tag was packaged using a helper virus that contained a deletion in gC, and the vector stocks targeted infection to a cultured cell line that contained an artificial pseudo-His-tag receptor. Both of these reports [13,22] used specific HSV-1 viruses that grow productively and kill infected cells, confounding potential gene transfer studies.

In this study, we performed helper virus-free HSV-1 vector packaging in the presence of either a chimeric gC-glia-cell-line-derived neurotrophic factor (GDNF) protein or a chimeric gC-brain-derived neurotrophic factor (BDNF) protein. Microinjection of either vector stock into the midbrain resulted in 2.2- to 5.0-fold targeted gene transfer to nigrostriatal neurons, which contain GDNF receptor α -1 (GFR α -1 [34,43]) and the high-affinity BDNF receptor, *TrkB* [29,49]. Preincubation of a vector stock that contained the chimeric gC-BDNF protein with an anti-bdnf antibody blocked the targeted gene transfer.

2. Materials and methods

2.1. Materials

Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs and Roche. Dulbecco's modified minimal essential medium, fetal bovine serum, G418, lipofectamine, and OPTI-MEM I were obtained from Invitrogen. 5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside (X-Gal) was obtained from Sigma. Rabbit anti-GDNF and rabbit anti-BDNF antibodies were obtained from Santa Cruz Biotechnology, mouse anti-HSV-1 gC was obtained from QED Bioscience Inc., rabbit and mouse anti-*E. coli* β -galactosidase (β -gal) and mouse monoclonal anti-flag were obtained from Sigma, rabbit and mouse anti-tyrosine hydroxylase (TH) were obtained from Chemicon, rabbit anti-glutamic acid decarboxylase (GAD) was obtained from Chemicon, and rabbit anti-glia fibrillary acidic protein (GFAP) was obtained from Santa Cruz Biotechnology. Fluorescein or rhodamine isothiocyanate-conjugated goat anti-rabbit immunoglobulin (Ig) G and fluorescein or rhodamine isothiocyanate-conjugated goat anti-mouse IgG were obtained from Jackson ImmunoResearch Laboratories. Horseradish-peroxidase (HRP)-conjugated-goat anti-mouse IgG was obtained from Dakocytomation. SDS-polyacrylamide gels and Immuno-Blot PVDF membranes were from BIO-RAD, and ECL Western Blotting Detection Reagents were from Amersham.

2.2. Cells

The growth of baby hamster kidney fibroblast (BHK21) cells [40] and 2-2 cells [36] has been described.

2.3. Plasmids

Plasmids that contain the gC promoter, either a GDNF cDNA or a BDNF cDNA, and an in-frame fusion to gC deleted in the N-terminus were generated in two steps using PCR.

In the first step, a plasmid was isolated that contains the gC promoter and a deletion of the N-terminus of gC. The template for the PCR was HSV-1 cosmid cos56 [5]. The primers for the gC promoter fragment were 5'GGCATC-GATCAGGGACTGCTTTTCCAGGGCTTGC 3' (contains a *Cla*I site; HSV-1 nucleotides 95,392 to 95,416 [27]) and 5'CGCGGATCCAAGCTTAAATGCCCGACGCCT-CCCCCTCGCGAGG 3' (contains *Hind*III and *Bam*HI sites; antisense to nucleotides 96,287–96,312; this primer destroys the gC ATG). A fragment of gC was isolated that started at amino acid (aa) 153 and extended to the C-terminus; the upstream primer was 5' GCGGGATCC-GAGTTCCGCCTCCAGATATGGCGTTACTCC 3' (contains a *Bam*HI site; nucleotides 96,767–96,797), and the downstream primer was 5'CCGGTTCGACTCGCATGGG-GAAAGTGGCCACGAGG 3' (contains a *Sal*I site; anti-sense to nucleotides 98,185–98,210). The gC promoter PCR products were digested with *Cla*I and *Bam*HI, the gC gene PCR products were digested with *Bam*HI and *Sal*I, and these two PCR fragments were inserted into pBR322 that had been digested with *Cla*I and *Sal*I to yield pBRgC-153.

In the second step, either a GDNF cDNA or a BDNF cDNA with a five aa spacer was inserted into pBRgC-153 to yield either pBRgC-GDNF or pBRgC-BDNF, respectively. A human

GDNF cDNA [25] was isolated by PCR: The template was plasmid GDNF3a (kindly provided by Dr. M. Bohn [4]). The upstream primer was 5' CGCAAGCT-TACGGGACTCTAAGATGAAGTTATGG 3' (contains a *Hind*III site and the GDNF ATG), and the downstream primer (contains a *Bam*HI site and the C-terminus of GDNF, lacks the termination codon, and adds five aa) was 5' GCCGGATCCTGCTCCTCCGATACATCCACACCGTT-TAGCGGAATGC 3' (adds gly, gly, ala, gly, ser). A human BDNF cDNA was isolated by PCR: The template was pINS-TH-NFHbndf/ires/lac [41]. The upstream primer was 5' GGGGAAGCTTACCATGACCATCCTTTTCCTTAC-TATGG 3' (contains a *Hind*III site and the BDNF ATG), and the downstream primer (contains a *Bam*HI site and the C-terminus of BDNF, lacks the termination codon, and adds five aa) was 5' GCCGGATCCTGCTCCTCCTCTCCCT-TTTAATGGTCAATGTAC 3' (adds gly, gly, ala, gly, ser). These two PCR products were digested with *Hind*III and *Bam*HI, and each PCR product was inserted into pBRgC-153 that had been digested with the same enzymes to yield pBRgC-gdnf or pBRgC-bdnf.

DNA sequencing, performed by the core facility at Brigham and Women's Hospital, confirmed the sequences of the gC promoter, the GDNF or BDNF cDNAs, and the gC coding region.

2.4. Cosmids

Cosmid set C (cos6, cos14, cos28, cos48, cos56, [5]) represents the HSV-1 genome; the a sequence was deleted from the two cosmids that contained it (cos6Δa, cos48Δa [8]).

The chimeric gC-GDNF or gC-BDNF genes were introduced into cos56 by homologous recombination cloning in *E. coli*. Recombination cloning in *E. coli* BJ5183 has been used to manipulate 35 kb adenovirus vectors [3], and we modified this method [20] to allow the use of up to 6 DNA fragments to reconstruct an HSV-1 cosmid. Each transformation contained 30 μl *E. coli* BJ5183, 3 ng vector, and the two other fragments (1:1 molar ratio relative to vector). The vector fragment was the 26.8 kb *Spe*I and *Xba*I fragment from cos56 (nucleotides 79,442 to 80,722 and 97,669 to 115,152, also contains SuperCos 1), and the insert fragments were the 17.3 kb *Eco*RI fragment from cos56 (nucleotides 79,442 to 96,751), and either the chimeric gC-GDNF or the gC-BDNF gene (6.7 kb *Eco*RI and *Sal*I fragments from either pBRgC-gdnf or pBRgC-bdnf, respectively; the *Eco*RI site is 23 bp from the *Cla*I site used to insert the gC promoter fragment). Candidates were analyzed by digestion with specific restriction enzymes (*Spe*I, *Xba*I, *Hind*III, *Eco*RI). Correct cosmids were transferred into SURE cells. DNA sequencing, performed by the core facility at Brigham and Women's Hospital, confirmed the sequences of the gC promoter, the GDNF or BDNF cDNAs, and the gC coding region in the cosmids.

2.5. Immunofluorescent costaining for either gC and GDNF or gC and BDNF

HSV-1 viruses that contained the chimeric gC-GDNF gene, or the chimeric gC-BDNF gene, or wild-type (wt) gC were derived as described [50] by cotransfecting 2-2 cells with cos6, cos14, cos28, cos48, and each of the three cos56 (containing the gC-GDNF chimeric gene, or the gC-BDNF chimeric gene, or wt gC). Virus stocks were titrated by standard plaque assay (Table 1). BHK cells (5×10^5 cells in 0.5 ml, plated 4 h before infection) were infected with an ~0.15 multiplicity of infection (moi) of each virus stock, 1 day later, the cells were fixed with 4% paraformaldehyde, and immunofluorescent costaining was performed as described [51] using either rabbit anti-GDNF (1:500 dilution) or rabbit anti-BDNF (1:500 dilution) and mouse anti-HSV-1 gC (1:1000 dilution). Primary antibodies were visualized using a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG and a rhodamine isothiocyanate-conjugated goat anti-mouse IgG.

Slides were examined using a Nikon Eclipse E600 microscope, and photomicrographs were taken using an Optronics videocamera. There was minimal overlap between the rhodamine and fluorescein filters, and specific figures contain cells that are positive under one filter and negative under the other filter.

2.6. Western blot analysis of HSV-1 particles for gC

HSV-1 virus particles were purified as described [24]. Proteins were extracted from HSV-1 virus particles (titers in Table 1) following the manufacturer's instruction (Bio-Rad): each sample was mixed with Laemmli sample buffer (1:1), treated at 95 °C for 5 min, and 20 µl was loaded onto each lane of a gel. SDS-polyacrylamide gel electrophoresis (PAGE) was performed using a 4–15% gradient gel under nonreducing conditions, and proteins were semi-dry transferred to an Immuno-Blot PVDF membrane. The membrane was incubated with mouse monoclonal anti-HSV-1 gC (1:200 dilution) followed by an HRP-conjugated-goat anti-mouse IgG (1:1000 dilution) and visualized using ECL Western Blotting Detection Reagents.

2.7. Vectors

pHSVlac [10] contains the *LacZ* gene under the control of the HSV-1 immediate early (IE) 4/5 promoter. pHSVpkcΔGG [38] contains the pkcΔGG gene under the control of the IE 4/5 promoter. PkcΔ is a flag-tagged catalytic domain of rat protein kinase C (PKC) βII, and PkcΔGG contains a point mutation in an evolutionarily conserved lys residue that abolishes PKC enzyme activity [38].

2.8. Vector packaging and titering

Helper virus-free packaging [8] was performed using a modified protocol [40] that improves the efficiency. 2-2 cells were cotransfected with a vector and an HSV-1 cosmid set that lacked a packaging site (cos6Δa, cos14, cos28, cos48Δa, and each of the three cos56 (containing the gC-GDNF chimeric gene, or the gC-BDNF chimeric gene, or wt gC)). Vector stocks were purified [24]. Vector stocks were titered by counting the numbers of positive cells at 1 day after transduction of BHK cells. pHSVlac stocks were titered by staining with X-gal, and pHSVpkcΔGG stocks were titered using an anti-flag antibody [38]. The titers of vector genomes ((VG)/ml [50]) were determined by extracting DNA from the vector stocks and measuring the amounts of vector DNAs using PCR and primers for the *LacZ* gene [50] or primers for the pkcΔGG gene (5' CTACAAAGACGATGACGATAAATCG 3' (from flag) and 5' TTCGAGTTTCTCCCAGTCGATATACC 3' (complementary to nucleotides 1942–1967 of the rat PKCβII cDNA [19,38])). No wt HSV-1 was detected in these vector stocks (<10 plaque forming units (pfu)/ml).

2.9. Gene transfer experiments in the brain

These studies were approved by the West Roxbury VA Hospital IACUC. Male Sprague–Dawley rats (175–200 gm) were used for these experiments. The titers of the vector stocks are in Table 1. Appropriate volumes of either pHSVlac/gC-gdnf and pHSVpkcΔGG/gC-wt or pHSVlac/gC-bdnf and pHSVpkcΔGG/gC-wt were mixed together to yield the same IVP/ml for the two vectors. This mixture of vector stocks was delivered by stereotactic injection (2 sites, 3 µl/site) into the midbrain proximal to the substantia nigra of the right hemisphere (anterior–posterior (AP) –5.5, medial–lateral (ML) –1.9, dorsal–ventral (DV) –7.1; AP –5.5, ML –2.3, DV –6.8). AP is relative to bregma, ML is relative to the sagittal suture, and DV is relative to the bregma-lambda plane [31]. Rats were perfused, brains were sectioned, and immunofluorescent costaining was performed, as described [51]. For experiment (expt.) 1 using pHSVlac/gC-gdnf and pHSVpkcΔGG/gC-wt, alternating sections were costained with either rabbit anti-β-gal (1:200 dilution) and mouse anti-TH (1:1000 dilution) or mouse anti-flag (detects PkcΔGG, 1:500 dilution) and rabbit anti-TH (1:1,000 dilution). For all the other

experiments, alternating sections were costained with either mouse anti- β -gal (1:200 dilution) or mouse anti-flag and an antibody that recognizes a specific cell marker; rabbit anti-TH, or rabbit anti-GAD (1:500 dilution), or rabbit anti-GFAP (1:500 dilution). Primary antibodies were visualized using fluorescein or rhodamine isothiocyanate-conjugated goat anti-rabbit IgG and fluorescein or rhodamine isothiocyanate-conjugated goat anti-mouse IgG.

2.10. Competition experiment with an anti-BDNF antibody

18 μ l of a mixture of equal IVP/ml of pHSVlac/gC-bdnf and pHSVpkc Δ GG/gC-wt was incubated with 2 μ l of rabbit anti-BDNF antibody (1:20 dilution) for 30 min on ice. This mixture of vector stocks was then used immediately for gene transfer.

2.11. Cell counts

Twenty-five μ m coronal sections that contained the midbrain were prepared, and recombinant gene products were detected in ~20 of these sections. Alternating sections were analyzed for costaining of either β -gal-immunoreactivity (IR) or Pkc Δ GG (flag-IR) and TH-IR, or GAD-IR, or GFAP-IR. Photomicrographs were taken under 40 $\times$ magnification using a video camera. The positive cells (β -gal-IR or flag-IR) in each section were scored for costaining with a specific cell marker, and all the positive cells in each section were scored. Each section was counted at least two times, on different days, and the two values differed by <10% for each section.

2.12. Statistical analyses

Statistical comparisons were performed using Student's unpaired *t* test (Excel).

3. Results

3.1. Construction of HSV-1 cosmids that encode chimeric gC-GDNF or gC-BDNF proteins

Our strategy to target gene transfer to nigrostriatal neurons was to modify the HSV-1 vector particle to preferentially bind to either GDNF receptors (Fig. 1) or BDNF receptors. GDNF and BDNF are neurotrophic factors for nigrostriatal neurons [16,25], and these neurons contain GDNF receptor α -1 (GFR α -1 [34,43]) and the high-affinity receptor for BDNF, *TrkB* [29,49]. The targeting proteins were designed to fuse either GDNF to gC (Figs. 1a and b) or BDNF to gC. A deletion in HSV-1 gC was isolated that removed the heparin sulfate binding domain from gC (aa 33–160; [26,35,42]), which plays a major role in the initial attachment of HSV-1 virus particles to the surface of cells; the deletion endpoint was located at aa 153. This deletion preserves the gC transmembrane domain and the internal C-terminal domain. Either a GDNF cDNA or a BDNF cDNA was inserted inframe at the N-terminus of the gC deletion, with a spacer of 5 aa separating the neurotrophic factor and gC domains. GDNF and BDNF are secreted proteins, and each contains a signal sequence [16,25] to support proper posttranslational processing of chimeric gC-GDNF or gC-BDNF protein. The gC deletion retained the transmembrane domain to support insertion of the chimeric proteins into the envelope of HSV-1 particles. These constructs retained the gC promoter and polyadenylation site to enable transcription of these constructs to be regulated in the same manner as the wt gC gene. In vivo homologous recombination cloning [20] was used to replace the wt gC gene in HSV-1 cosmid cos56 with each of these two constructs. The integrity of the gC promoter, neurotrophic factor cDNAs, and gC coding region were confirmed by DNA sequence analysis, in both the initial plasmids and the modified cos56 cosmids. All the other HSV-1 glycoproteins were not modified. Thus, following binding of an HSV-1 vector particle that contains either chimeric gC-GDNF (Fig. 1c) or gC-BDNF protein to the cognate receptor on the surface of cells, specific HSV-1 glycoproteins (gB, gD, gH, and gL) might support entry by the same mechanisms used for entry by wt HSV-1 virus [39].

3.2. Expression of chimeric gC-GDNF or gC-BDNF proteins and incorporation into HSV-1 particles

An experiment was designed to show that the gC-GDNF construct expressed both GDNF-IR and gC-IR and that the gC-BDNF construct expressed both BDNF-IR and gC-IR. We isolated HSV-1 viruses that contained each construct by transfecting 2-2 cells with an HSV-1 cosmid set that contains the **a** sequence (contains the packaging site) and each construct (cos6, cos14, cos28, cos48, and cos56 containing gC-GDNF, or gC-BDNF, or wt gC). BHK cells were infected with the resulting virus stocks (Table 1), 1 day later, the cells were fixed, immunocytochemistry was performed [51] using either anti-GDNF and anti-HSV-1 gC antibodies or anti-BDNF and anti-gC antibodies, and these antibodies were visualized using rhodamine- or fluorescein-conjugated secondary antibodies. The results showed that HSV-1/gC-GDNF supported costaining for both GDNF-IR and gC-IR (Figs. 2a–c), and HSV-1/gC-wt supported staining for only gC-IR (Figs. 2d–f). Furthermore, the results showed that HSV-1/gC-BDNF supported costaining for both BDNF-IR and gC-IR (Figs. 2g–i), and HSV-1/gC-wt supported staining for only gC-IR (Figs. 2j–l). As a control for the specificity of the staining, following infection with HSV-1/gC-GDNF, omission of the primary antibodies resulted in no staining (Figs. 2m–o). Cell counts (Table 2) showed that HSV-1/gC-GDNF and HSV-1/gC-BDNF supported >90% costaining for gC-IR and either GDNF-IR or BDNF-IR, respectively. The cell counts also showed that, using HSV-1/gC-wt, 5% of the gC-IR cells also contained either BDNF-IR or BDNF-IR; these viruses kill cells, and dead cells support a low level of nonspecific antibody binding.

To show that each chimeric gC was incorporated into HSV-1 particles, proteins were extracted from purified HSV-1 virus particles, displayed on a non-denaturing gel (to enable detection of noncovalent interactions, such as homodimers of gC) and subjected to Western blot analysis using an anti-gC antibody. The results (Fig. 3) showed that the lanes that contained HSV-1/gC-GDNF, or HSV-1/gC-BDNF, or HSV-1/gC-wt each contained a band at ~50 kDa. wt gC has a calculated molecular weight of ~51 kDa. The lane that contained HSV-1/gC-wt (lane 3) contained a second minor band at ~75 kDa; gC is known to form homodimers and to bind to other HSV-1 glycoproteins [14]. The gC-GDNF construct or the gC-BDNF construct add a neurotrophic factor domain of 134 aa or 119 aa, respectively (preproGDNF, 211 aa, mature GDNF 134 aa [25]; pre-proBDNF, 247 aa, mature BDNF 119 aa [16]), but delete the first 153 aa of gC (511 aa total), and the lanes that contained HSV-1/gC-GDNF or HSV-1/gC-BDNF (lanes 1 or 2) each contained a band with a size consistent with the respective construct. HSV-1 virus particle assembly is a complex process, and the final envelope of the virus particle is added by budding through the plasma membrane [33]. The gC-GDNF or gC-BDNF proteins in the plasma membrane are likely the mature forms of the proteins, after cleavage of both the pre and pro domains. Thus, the likely structure of the gC-GDNF or gC-BDNF protein in the virus particle is the mature neurotrophic factor domain (mature GDNF 134 aa; mature BDNF 119 aa) followed by the C-terminal 358 aa of gC (aa 153 to 511).

3.3. Helper virus-free packaging using gC-GDNF, or gC-BDNF, or wt gC

Helper virus-free packaging was performed (Fig. 1c [8,41]) by cotransfecting 2-2 cells with a vector (pHSVlac or pHSVpkcΔGG) and an HSV-1 cosmid set that lacked a packaging site and contained a specific gC (cos6Δa, cos14, cos28, cos48Δa, and one of the three cos56 containing the gC-GDNF chimeric gene, or the gC-BDNF chimeric gene, or wt gC). Vector stocks were purified [24] and titered by counting the numbers of positive cells at 1 day after transduction of BHK cells (infectious vector particles (IVP)/ml). pHSVlac expresses β-gal, which was detected by staining with X-gal. pHSVpkcΔGG expresses PkcΔGG, which was detected using an anti-flag antibody (PkcΔGG is a flag-tagged catalytic domain of rat PKC βII that contains a point mutation that abolishes PKC activity [38]). The titers of vector genomes ((VG)/ml [50]) were determined by extracting DNA from the vector stocks and measuring the amounts

of vector DNAs using PCR and primers for either the *LacZ* or *pkcΔGG* gene. The titers (Table 1) showed that vector stocks prepared using either gC-GDNF or gC-BDNF contained similar titers (IVP/ml or VG/ml) as vector stocks prepared using wt gC.

3.4. pHSVlac/gC-gdnf or pHSVlac/gC-bdnf targeted gene transfer to nigrostriatal neurons in the rat brain

We chose nigrostriatal neurons as a model system to determine if HSV-1 vector particles that contain gC-gdnf or gC-bdnf protein can target gene transfer, in the rat brain, to neurons that contain GDNF receptors or BDNF receptors, respectively. Nigrostriatal neurons, the principal type of neuron in the substantia nigra pars compacta (SNc), contain GDNF receptor α -1 (GFR α -1 [34,43]) and the high-affinity receptor for BDNF, *TrkB* [29,49]. These neurons also contain TH [7], enabling identification using anti-TH antibodies. Due to the complex and heterogeneous cellular composition of the midbrain, stereotactic injections of HSV-1 vectors into the midbrain proximal to the substantia nigra can support gene transfer to differing populations of cells in different rats, complicating comparisons between rats. To control for any variability in injection sites between the rats, we co-injected mixtures of equal titers (IVP/ml) of vector particles that contained either a chimeric gC or wt gC. The experimental vector particles were either pHSVlac/gC-gdnf or pHSVlac/gC-bdnf. The control vector particles were pHSVpkcΔGG/gC-wt.

In the first experiment (expt. 1), we co-injected equal titers of pHSVlac/gC-gdnf and pHSVpkcΔGG/gC-wt into the midbrain proximal to the substantia nigra, the rats were perfused at 4 days after gene transfer, the brains were sectioned, and alternating sections were costained for either β -gal-IR and TH-IR or flag-IR (PkcΔGG contains the flag tag) and TH-IR. Low power photomicrographs showed that most of the β -gal-IR cells were located in a narrow band that contained TH-IR neurons in the SNc (Figs. 4a–c; pHSVlac/gC-gdnf expresses β -gal). High power views showed that most of the β -gal-IR cells also contained TH-IR (Figs. 4d–f). In contrast, most of the flag-IR cells lacked TH-IR (Figs. 4g–i; pHSVpkcΔGG/gC-wt expresses PkcΔGG). No staining was observed if the primary antibodies were omitted (Figs. 4j–l; section from a rat that received pHSVlac/gC-gdnf). Cell counts from 3 rats (Table 3) showed that an average of 80% of the β -gal-IR cells also contained TH-IR, but only 16% of the flag-IR cells also contained TH-IR ($P < 0.001$, t test). Any differences in the sensitivities of the β -gal-IR and flag-IR assays are unlikely to explain the difference in the % TH-IR costaining; a previous study showed that, following delivery of pHSVlac/gC-wt (helper virus-containing packaging system) into the midbrain, only 4% of the β -gal-IR cells also contained TH-IR [37]. Thus, pHSVlac/gC-wt and pHSVpkcΔ/gC-wt each supported a low level of costaining for TH-IR, and the use of pHSVlac/gC-gdnf supported a large increase in the costaining for TH-IR.

A second experiment used the same gene transfer design as the first experiment, but different vector stocks (Table 1); this experiment quantified the level of targeted gene transfer to nigrostriatal neurons and the levels of untargeted gene transfer to either GABAergic neurons or astrocytes. The costaining for either β -gal-IR and TH-IR or flag-IR and TH-IR was similar to that observed in expt. 1 (not shown). Cell counts from 3 rats (Table 3) showed that an average of 78% of the β -gal-IR cells also contained TH-IR, but only 29% of the flag-IR cells also contained TH-IR ($P < 0.002$, t test). To detect untargeted gene transfer to GABAergic neurons, sections were costained for β -gal-IR and GAD-IR. Some costained cells were observed, but most of the β -gal-IR was located in cells that lacked GAD-IR (Figs. 5a–c). Additionally, to detect untargeted gene transfer to astrocytes, sections were costained for β -gal-IR and GFAP-IR. Some costained cells were observed, but most of the β -gal-IR was located in cells that lacked GFAP-IR (Figs. 5d–f). Cell counts (Table 3) showed that 3.9% of the β -gal-IR cells also contained GAD-IR, and 4.6% of the β -gal-IR cells also contained GFAP-IR.

Thus, results from two different experiments showed that pHSVlac/gC-gdnf supported a 2.5- to 5.0-fold targeting of gene transfer to nigrostriatal neurons in the rat brain compared to pHSVpkcΔGG/gC-wt.

An experiment with pHSVlac/gC-bdnf used the same gene transfer design as expt. 2 with pHSVlac/gC-gdnf. Low power photomicrographs showed that most of the β-gal-IR was located in the SNc, a narrow band that contained TH-IR neurons (Figs. 6a–c). High power views showed that most of the β-gal-IR cells also contained TH-IR (Figs. 6d–f). In contrast, low power views showed that most of the flag-IR (detects PkcΔGG) was located dorsal or ventral to the SNc (Figs. 6g–i), and high power views showed that most of the flag-IR was located in cells that lacked TH-IR (Figs. 6j–l). Cell counts from 4 rats (Table 4) showed that an average of 75% of the β-gal-IR cells also contained TH-IR, but only 34% of the flag-IR cells also contained TH-IR (2.2-fold targeted gene transfer; $P < 0.001$, t test). To detect untargeted gene transfer to GABAergic neurons, sections were costained for β-gal-IR and GAD-IR, and the results showed some costained cells, but most of the β-gal-IR was located in cells that lacked GAD-IR (Figs. 7a–c). Similarly, to detect untargeted gene transfer to astrocytes, sections were costained for β-gal-IR and GFAP-IR, and some costained cells were observed, but most of the β-gal-IR was located in cells that lacked GFAP-IR (Figs. 7d–f). Cell counts (Table 4) showed that 6.9% of the β-gal-IR cells also contained GAD-IR, and 12% of the β-gal-IR cells also contained GFAP-IR.

Expt. 2 with pHSVlac/gC-gdnf was performed in parallel with the experiment with pHSVlac/gC-bdnf. Both experiments contained the same internal control, pHSVpkcΔGG/gC-wt, and the percentage of the flag-IR cells that costained for TH-IR was similar in these two experiments (pHSVlac/gC-gdnf expt. 2, 29% (Table 3); pHSVlac/gC-bdnf experiment, 34% (Table 4); $P > 0.05$). Because the internal control in these two experiments supported a similar level of expression in TH-IR neurons, the results from these two experiments can be compared. pHSVlac/gC-gdnf expt. 2 and pHSVlac/gC-bdnf supported a similar level of targeted gene transfer to TH-IR neurons (pHSVlac/gC-gdnf expt. 2, 78% (Table 3); pHSVlac/gC-bdnf experiment, 75% (Table 4); $P > 0.05$) and a similar level of untargeted gene transfer to GAD-IR neurons (pHSVlac/gC-gdnf expt. 2, 3.9% (Table 3); pHSVlac/gC-bdnf experiment, 6.9% (Table 4); $P > 0.05$). However, pHSVlac/gC-gdnf supported a lower level of untargeted gene transfer to GFAP-IR cells than pHSVlac/gC-bdnf (pHSVlac/gC-gdnf expt. 2, 4.6% (Table 3); pHSVlac/gC-bdnf experiment, 12% (Table 4); $P < 0.01$).

3.5. Pretreatment of a pHSVlac/gC-bdnf vector stock with an anti-BDNF antibody reduced targeted gene transfer to nigrostriatal neurons

To determine if the BDNF domain in the chimeric gC-bdnf protein played a role in the targeted gene transfer to nigrostriatal neurons, we performed an experiment to interfere with the potential binding of these vector particles to the high-affinity BDNF receptor (*TrkB* [29,49]) by preincubation with an anti-BDNF antibody. A mixture of equal titers (IVP/ml) of pHSVlac/gC-bdnf and pHSVpkcΔGG/gC-wt was incubated with an anti-BDNF antibody for 30 min, gene transfer was performed, and the fraction of targeted gene transfer to nigrostriatal neurons was analyzed, as above. Low power photomicrographs showed that most of the β-gal-IR was located either dorsal or ventral to the TH-IR neurons in the SNc (Figs. 8a–c). High power views showed that most of the β-gal-IR cells lacked TH-IR (Figs. 8d–f). Similar results were observed with the flag-IR (not shown). Cell counts (Table 5) showed that only 16% of the β-gal-IR cells contained TH-IR, and 14% of the flag-IR cells contained TH-IR ($P > 0.05$).

4. Discussion

Chimeric gC-GDNF or gC-BDNF proteins contained both gC sequences and a specific neurotrophic factor, and each chimeric protein was incorporated into HSV-1 particles. Helper

virus-free HSV-1 vector packaging could occur in the presence of each chimeric protein. The resulting vector stocks supported 2.2- to 5.0-fold targeted gene transfer to neurons that contain GFR α -1 receptors or *TrkB* receptors, nigrostriatal neurons, in the rat brain. Targeted gene transfer by vector particles that contained gC-BDNF was blocked by an anti-BDNF antibody.

4.1. Titers and efficiency of gene transfer

pHSVlac packaged in the presence of either gC-GDNF or gC-BDNF protein displayed similar biological (IVP/ml) and physical (VG/ml) titers compared to pHSVpkc Δ GG/gC-wt. Thus, any modest decreases in the packaging efficiency caused by either gC-GDNF or gC-BDNF protein are unlikely to complicate the use of these vector stocks for gene transfer studies. In the rat brain, equal titers of vector particles that contained either a specific chimeric gC-neurotrophic factor protein or wt gC supported similar numbers of β -gal-IR or flag-IR cells, respectively, in adjacent sections. However, because the immunofluorescent assays used for costaining are relatively unstable compared to the horseradish peroxidase reaction product, we did not perform stereological counts to quantify the total numbers of β -gal-IR or flag-IR cells. Nonetheless, these results suggest that, in the presence of cells that contain either GDNF receptors or BDNF receptors, vector particles that contained either gC-GDNF or gC-BDNF protein supported similar efficiencies of gene transfer compared to vector particles that contained wt gC.

4.2. Targeted gene transfer

pHSVlac/gC-gdnf supported 2.5- to 5-fold targeted gene transfer to nigrostriatal neurons in the rat brain compared to pHSVpkc Δ GG/gC-wt. Similarly, pHSVlac/gC-bdnf supported 2.2-fold targeted gene transfer to nigrostriatal neurons compared to pHSVpkc Δ GG/gC-wt. Together, these results show that vector particles that contained either gC-GDNF or gC-BDNF protein targeted gene transfer to cells that contained either GDNF or BDNF receptors, respectively.

However, vector particles that contained either gC-GDNF or gC-BDNF protein still supported some gene transfer to cells that lacked GDNF or BDNF receptors, respectively, including BHK cells (used for titering) or astrocytes in the midbrain. The remaining portion of gC in the gC-GDNF or gC-BDNF protein lacks the heparin sulfate binding domain [26,44]; but these vector particles still contain wt gB, and gB contains a heparin sulfate binding domain [15,23]. Using HSV-1 virus particles, deletion of the gC heparin sulfate binding domain reduced binding to fibroblast cells ~65%, deletion of the gB heparin sulfate binding domain reduced binding ~20%, and deletion of both domains is roughly additive and reduced binding ~80% [23]. An L-cell line that does not synthesize any glycosaminoglycans showed an ~1000-fold decrease in HSV-1 wt virus infection [1]. Thus, a mutated gB that lacks a heparin sulfate binding domain (gBpK⁻ [23]) could be introduced into the HSV-1 cosmid set used for helper virus-free packaging. Vector particles that contained both gBpK⁻ and either chimeric gC-GDNF or gC-BDNF protein might show reduced gene transfer to cells that lack GDNF or BDNF receptors, respectively, which might increase the fold targeting to cells that contain the specific cognate receptor.

Comparing vector particles that contained chimeric gC-GDNF or gC-BDNF protein, we did not observe any significant differences in the targeted gene transfer to nigrostriatal neurons or the untargeted gene transfer to GABAergic neurons. A relatively low percentage of GABAergic neurons in the substantia nigra contain GDNF receptors [34,43], and these low levels of GDNF receptors did not appear to support significant levels of targeting of vector particles containing gC-GDNF to GABAergic neurons. Interestingly, vector particles that contained gC-GDNF supported a lower level of untargeted gene transfer to astrocytes than vector particles that contained gC-BDNF. More sensitive cell binding assays might reveal

additional differences between the binding capabilities of vector particles that contain gC-GDNF or gC-BDNF. Higher affinity binding to GDNF or BDNF receptors might be obtained by incorporating multimers of either GDNF or BDNF, respectively, into a chimeric gC-neurotrophic factor protein and/or by adding a large spacer between the neurotrophic factor and the gC domains to potentially enable the neurotrophic factor domain to bind more readily to the cognate receptor.

The 75 to 80% targeted gene transfer to nigrostriatal neurons obtained using vector particles that contained either the chimeric gC-GDNF or gC-BDNF protein compares favorably to the nigrostriatal neuron-specific expression supported by HSV-1 vectors that contain a TH promoter. A vector that contained a TH promoter, packaged using a helper virus-containing system, supported 40% nigrostriatal neuron-specific expression [37], and the same vector, packaged using the helper virus-free system, supported 61% nigrostriatal neuron-specific expression [45]. Further improvements in nigrostriatal neuron-specific expression might be obtained by using this vector targeting strategy and a cell-type-specific promoter in combination; a vector that contains a TH promoter could be packaged into vector particles that contain either gC-GDNF or gC-BDNF protein.

4.3. Implications for physiological studies

Nigrostriatal neuron-specific expression might be useful both for studies on the basic physiology of these neurons and for specific gene therapy approaches to Parkinson's disease. In particular, clinical trials that infused recombinant GDNF protein into the brain were recently halted due to side effects [12,21,30], suggesting that more restricted expression of GDNF is desirable. Furthermore, a study showed that expression of GDNF in the striatum supports a higher level of protection of nigrostriatal neurons than expression of GDNF in multiple types of cells in the midbrain [18]. Thus, targeted gene transfer to nigrostriatal neurons of a vector that expresses a specific neurotrophic factor might be beneficial.

More generally, the capability to target gene transfer to a specific type of neuron that contains a receptor for a particular neurotrophic factor may have advantages for specific studies in basic neuronal physiology or specific gene therapy treatments.

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Fig. 1.

Schematic diagrams of gC, the chimeric gC-GDNF construct, and helper virus-free packaging to produce vector particles that contain the chimeric gC-GDNF protein. (a) gC contains a signal peptide (sp) at the N-terminus, a heparin sulfate binding domain (aa 33–160; [26,35,42]) which plays a major role in the initial attachment of HSV-1 virus particles to the surface of cells, a transmembrane domain (tm), and an internal C-terminal domain. Glycosylation sites located within the major heparin binding domain are shown as ovals, and the other glycosylation sites are shown as circles. (b) The chimeric gC-GDNF construct contains the gC promoter, a GDNF cDNA (encoding preproGDNF, aa 1–211), a spacer encoding 5 aa, and a gC deletion from aa 153 to the C-terminus (aa 511). This construct retained the gC promoter and the gC polyadenylation site to enable transcription of the construct to be regulated as the wt gC gene. GDNF is a secreted protein that contains a signal sequence [25] to support proper posttranslational processing, and the gC deletion retains the transmembrane domain to support insertion into the envelope of HSV-1 particles. (c) Helper virus-free packaging [8,40] was performed using this chimeric gC-GDNF construct. An HSV-1 vector and an HSV-1 cosmid set that contained the chimeric gC-GDNF construct and lacked an *a* sequence (contains the packaging site) were cotransfected into 2-2 cells [36]. The resulting HSV-1 vector particles should contain the vector, chimeric gC-GDNF, and all the other HSV-1 envelope

glycoproteins. Thus, these vector particles are designed to enable initial binding to GDNF receptors on the surface of cells followed by cell entry using the same mechanisms used by wt HSV-1 virus particles [39].

Fig. 2.

Immunofluorescent costaining for either GDNF-IR and gC-IR or BDNF-IR and gC-IR at 1 day after infection of BHK cells with HSV-1 viruses that express gC-GDNF, gC-BDNF, or wt gC. Immunocytochemistry was performed [51] using either rabbit anti-GDNF or rabbit anti-BDNF, and mouse anti-HSV-1 gC, and these antibodies were visualized using rhodamine- or fluorescein-conjugated secondary antibodies. (a–c) HSV-1/gC-gdnf; GDNF-IR (a), gC-IR (b), and merge (c). (d–f) HSV-1/gC-wt; GDNF-IR (d), gC-IR (e), and merge (f). (g–i) HSV-1/gC-bdnf; BDNF-IR (g), gC-IR (h), and merge (i). (j–l) HSV-1/gC-wt; BDNF-IR (j), gC-IR (k), and merge (l). (m–o) HSV-1/gC-gdnf, no primary antibodies. Scale bar: 50 μ m.

Fig. 3.

Western blot analysis for gC epitopes in HSV-1 virus particles that contain gC-GDNF, or gC-BDNF, or wt gC. Proteins were extracted from purified HSV-1 virus particles, displayed on a non-denaturing gel, the proteins were transferred to a membrane, Western blot analysis was performed using a mouse anti-HSV-1 gC antibody, and antibody binding was visualized, as detailed in Materials and methods. Lane 1, HSV-1/gC-GDNF; lane 2, HSV-1/gC-BDNF; and lane 3, HSV-1/gC-wt.

Fig. 4. Costaining for nigrostriatal neuron-specific recombinant gene expression in rats sacrificed at 4 days after co-injection of pHSVlac/gC-gdnf and pHSVpkcΔGG/gC-wt into the midbrain. Alternating sections were costained using either rabbit anti-β-gal and mouse anti-TH or mouse anti-flag (detects PkcΔGG) and rabbit anti-TH, and these antibodies were visualized using rhodamine- or fluorescein-conjugated secondary antibodies. (a–f) pHSVlac/gC-gdnf supported expression of β-gal in nigrostriatal neurons: Low power (a–c); β-gal-IR (a), TH-IR (b), and merge (c). High power (d–f); β-gal-IR (d), TH-IR (e), and merge (f). Arrows indicate examples of costained cells. (g–i) Most expression of PkcΔGG, from pHSVpkcΔGG/gC-wt, was in cells that lacked TH-IR; flag-IR (g), TH-IR (h), and merge (i). Arrowheads indicate examples of flag-IR only. (j–l) pHSVlac/gC-gdnf, primary antibodies omitted from the assay. Scale bars: (a–c and g–l) 50 μm, (d–f) 20 μm.

Fig. 5.

Costaining for GABAergic neuron-specific or astrocyte-specific expression of β -gal in rats sacrificed at 4 days after co-injection of pHSVlac/gC-gdnf and pHSVpkc Δ GG/gC-wt into the midbrain. To detect GABAergic neuron-specific expression, sections were costained using mouse anti- β -gal and rabbit anti-GAD; to detect astrocyte-specific expression, sections were costained using mouse anti- β -gal and rabbit anti-GFAP; and these antibodies were visualized using rhodamine- or fluorescein-conjugated secondary antibodies. (a–c) pHSVlac/gC-gdnf supported only limited expression of β -gal in GABAergic neurons; β -gal-IR (a), GAD-IR (b), and merge (c). (d–f) pHSVlac/gC-gdnf supported only limited expression of β -gal in astrocytes; β -gal-IR (d), GFAP-IR (e), and merge (f). Arrows indicate examples of costained cells; arrowheads indicate examples of β -gal-IR only. Scale bar: 20 μ m.

Fig. 6.

Costaining for nigrostriatal neuron-specific recombinant gene expression in rats sacrificed at 4 days after co-injection of pHSVlac/gC-bdnf and pHSVpkcΔGG/gC-wt into the midbrain. Alternating sections were costained for either rabbit anti-β-gal and mouse anti-TH or mouse anti-flag (detects PkcΔGG) and rabbit anti-TH, and these antibodies were visualized using rhodamine- or fluorescein-conjugated secondary antibodies. (a–f) pHSVlac/gC-bdnf supported expression of β-gal in nigrostriatal neurons: Low power (a–c); β-gal-IR (a), TH-IR (b), and merge (c). High power (d–f); β-gal-IR (d), TH-IR (e), and merge (f). Arrows indicate examples of costained cells; arrowheads indicate examples of β-gal-IR or flag-IR only. (g–l) Most expression of PkcΔGG, from pHSVpkcΔGG/gC-wt, was in cells that lacked TH-IR: Low power (g–i); flag-IR (g), TH-IR (h), and merge (i). High power (j–l); flag-IR (j), TH-IR (k), and merge (l). Scale bars: (a–c and g–i) 50 μm, (d–f and j–l) 20 μm.

Fig. 7.

Costaining for GABAergic neuron-specific or astrocyte-specific expression of β -gal in rats sacrificed at 4 days after co-injection of pHSVlac/gC-bdnf and pHSVpkc Δ GG/gC-wt into the midbrain. GABAergic neuron-specific expression and astrocyte-specific expression were detected as in Fig. 5. (a–c) pHSVlac/gC-bdnf supported only limited expression of β -gal in GABAergic neurons; β -gal-IR (a), GAD-IR (b), and merge (c). (d–f) pHSVlac/gC-bdnf supported only limited expression of β -gal in astrocytes; β -gal-IR (d), GFAP-IR (e), and merge (f). Arrows indicate examples of costained cells; arrowheads indicate examples of β -gal-IR only. Scale bar: 20 μ m.

Fig. 8.

Costaining for nigrostriatal neuron-specific expression of β -gal in rats sacrificed at 4 days after co-injection, into the midbrain, of pHSVlac/gC-bdnf and pHSVpkc Δ GG/gC-wt that had been preincubated with anti-BDNF antibody. Sections were costained using rabbit anti- β -gal and mouse anti-TH, and these antibodies were visualized using rhodamine- or fluorescein-conjugated secondary antibodies. Preincubation with an anti-BDNF antibody reduced gene transfer to nigrostriatal neurons. Low power (a–c); β -gal-IR (a), TH-IR (b), and merge (c). High power (d–f); β -gal-IR (d), TH-IR (e), and merge (f). Arrows indicate examples of costained cell; arrowheads indicate examples of β -gal-IR only. Scale bars: (a–c) 50 μ m, (d–f) 20 μ m.

Table 1

Titers of the virus and vector stocks

Virus or vector stock	Purified titers ^a			
	pfu/ml	VG/ml	IVP/ml	VG/IVP
HSV-1/gC-GDNF	6×10^7			
HSV-1/gC-BDNF	2×10^7			
HSV-1/gC-wt	3×10^8			
pHSVlac/gC-gdnf (expt. 1) ^b		9×10^7	7×10^6	13
pHSVlac/gC-gdnf (expt. 2)		9×10^7	2×10^6	45
pHSVlac/gC-bdnf		9×10^7	8×10^6	11
pHSVpkcΔGG/gC-wt (expt. 1)		2×10^7	2×10^7	1
pHSVpkcΔGG/gC-wt (expt. 2)		9×10^8	2×10^7	45

^aThe titers of each virus or vector stock following purification and concentration. pfu/ml is plaque forming units/ml. VG/ml is vector genomes/ml. IVP/ml is infectious vector particles/ml.

^bpHSVlac/gC-gdnf (expt. 1) and pHSVpkcΔGG/gC-wt (expt. 1) were used in the first experiment that co-injected these vector stocks into the brain, and expt. 2 vector stocks were used in expt. 2 (Table 3).

Table 2

Counts of the costaining for either gC-IR and GDNF-IR or gC-IR and BDNF-IR at 1 day after infection of cultured fibroblast cells with HSV-1/gC-gdnf, or HSV-1/gC-bdnf, or HSV-1/gC-wt

Virus	Numbers of positive cells				% Costained
	gC-IR	GDNF-IR	BDNF-IR	Costained	
HSV-1/gC-gdnf	106	116		102	96
HSV-1/gC-wt	178	10		8	5
HSV-1/gC-bdnf	143		139	130	91
HSV-1/gC-wt	153		9	8	5

BHK cells were fixed at 1 day after infection (moi 0.15), and immunofluorescent costaining was performed to detect the indicated antibodies. Randomly chosen fields were examined until >100 gC-IR cells were counted, and all the positive cells in each field were counted.

Table 3
The numbers of β -gal or Pkc Δ GG positive cells that contained TH-IR, or GAD-IR, or GFAP-IR in rats sacrificed at 4 days after co-injection of pHSVlac/gC-gdnf and pHSVpkc Δ GG/gC-wt into the midbrain

Rat	Numbers of positive cells			% Costained	Numbers of positive cells		
	β -gal-IR	TH-IR	GAD-IR		GFAP-IR	Flag-IR	TH-IR
Expt. 1							
1	396	320		81	321	58	18
2	410	331		81	268	47	18
3	382	302		79	382	50	13
Average				80			16
Expt. 2							
1	600	468		78	732	232	32
2	716	592		83	292	112	38
3	684	500		73	160	28	18
Average				78			29
1	604		32	5.3			
2	380		24	6.3			
3	96		4	4.2			
Average				3.9			
1	436			7			
2	456			3.5			
3	276			2.9			
Average				4.6			

A mixture of equal IVP/ml of pHSVlac/gC-gdnf and pHSVpkc Δ GG/gC-wt (for titers, see Table 1; for injection conditions, see Materials and methods) was injected into the midbrain proximal to the substantia nigra. The rats were perfused at 4 days after gene transfer, the brains were sectioned, and alternating sections were costained for either β -gal-IR or flag-IR and a specific cell marker. Sufficient sections were examined to count at least ~100 cells that contained either β -gal-IR or flag-IR in each rat for each cell marker. In the sections that were examined, all of the β -gal-IR or flag-IR cells were scored for containing or lacking a specific cell marker.

Table 4
The numbers of β -gal or Pkc Δ GG positive cells that contained TH-IR, or GFAP-IR, or GAD-IR, or GFAP-IR in rats sacrificed at 4 days after co-injection of pHSVlac/gC-bdnf and pHSVpkc Δ GG/gC-wt into the midbrain

Rat	Numbers of positive cells			GAD-IR	GFAP-IR	% Costained	Numbers of positive cells		% Costained
	β -gal-IR	TH-IR					Flag-IR	TH-IR	
1	440	348				79	272	104	38
2	388	304				78	268	96	36
3	448	372				83	204	64	31
4	560	324				58	148	44	30
Average						75			34
1	156		20			13			
2	92		4			4.3			
3	152		16			11			
Average						6.9			
1	396				48	12			
2	60				8	13			
3	152				16	11			
Average						12			

This experiment was performed as in Table 3, except pHSVlac/gC-bdnf replaced pHSVlac/gC-gdnf. There were technical difficulties with the costaining for GAD-IR or GFAP-IR in one rat, and no positive cells were observed (not shown).

Table 5

The numbers of β -gal or Pkc Δ GG positive cells that contained TH-IR in rats sacrificed at 4 days after co-injection of pHSVlac/gC-bdnf and pHSVpkc Δ GG/gC-wt, preincubated with anti-BDNF, into the midbrain

Rat	Numbers of positive cells		% Costained	Numbers of positive cells		% Costained
	β -gal-IR	TH-IR		Flag-IR	TH-IR	
1	172	40	23	80	16	20
2	168	16	9.5	112	8	7.1
Average			16			14

A mixture of equal IVP/ml of pHSVlac/gC-bdnf and pHSVpkc Δ GG/gC-wt was incubated with an anti-BDNF antibody prior to injection into the midbrain proximal to the substantia nigra (for titers, see Table 1; for antibody incubation and injection conditions, see Materials and methods). The rats were perfused at 4 days after gene transfer, the brains were sectioned, and alternating sections were costained for either β -gal-IR and TH-IR or flag-IR and TH-IR. In the sections that were examined, all of the β -gal-IR or flag-IR cells were scored for containing or lacking TH-IR.