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Infection of cultured striatal neurons with a defective HSV-1 vector: implications for gene therapy

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ABSTRACT

Several neurological diseases which affect the corpus striatum are candidates for gene therapy. We have developed a defective Herpes Simplex Virus (HSV-1) vector system to introduce genes into postmitotic cells, such as neurons. The prototype vector, pHSVlac, contains a transcription unit which places the *E. coli Lac Z* gene under the control of the HSV-1 immediate early (IE) 4/5 promoter, a constitutive promoter. We now demonstrate that a HSV-1 vector can deliver a gene into striatal neurons. Infection of cultured rat striatal neurons with pHSVlac virus resulted in stable expression of β-galactosidase for at least two weeks, without cell death. The potential to replace the *Lac Z* gene with other genes of interest, such as the gene responsible for Huntington's Disease, once it is isolated, may lead to insights about the pathogenesis of this genetic neurodegenerative disease, and may provide a method for performing gene therapy on this disease. Similarly, introduction of the tyrosine hydroxylase gene, which encodes the rate-limiting enzyme in the conversion of tyrosine to dopamine, into striatal neurons might provide a novel gene therapy approach towards treating Parkinson's Disease.

INTRODUCTION

A number of disease processes affect the function of the corpus striatum, including ischemic (usually caused by stroke), toxic, neurodegenerative, and metabolic disorders; also, several organic psychological disorders are hypothesized to affect the striatum. The most frequently occurring of these diseases are the two neurodegenerative diseases, Huntington's Disease (HD) and Parkinson's Disease (PD). HD, an autosomal dominant disorder, is characterized predominantly by selective nerve cell loss in the striatum (1,4,5). Although a genetic marker has been found for HD, and progress is being made towards isolating the gene defect, the actual biochemical deficit responsible for this disease remains unknown (10). In addition, effective therapy for HD is still lacking. PD is due to the death of dopaminergic neurons in the substantia nigra compacta which project to the striatum (16,17). In PD, the biochemical deficit in dopaminergic neurotransmission can be temporarily reversed with precursor (L-DOPA) or dopamine receptor agonist therapy, but the etiology remains a mystery and long-term response to L-DOPA therapy is suboptimal (15–17).

Animal models are available for both HD (4, 5) and PD (13); these models have yielded insights into the disease process and also provide attractive model systems for developing gene therapy approaches. It appeared desireable to develop a method by which one could introduce the HD gene, once isolated, into striatal neurons, *in vivo* or *in vitro*. Expression of the HD gene in such a model system might reveal the primary biochemical deficit responsible for the disease. Such a method also might be useful for achieving gene therapy for HD patients in the future. Much excitement has also been generated by the discovery that a neurotoxin, methylphenyltetrahydropyridine (MPTP), when systemically administered, results in a Parkinsonian syndrome in primates (13), but again, the pathogenesis of this common neurological condition remains obscure. Precursor therapy has proven effective in many cases of PD, but long-term efficacy is incomplete, and is limited by fluctuations in clinical response (3,7,15). Although PD is characterized neuroanatomically by destruction of the dopamine-containing neurons in the substantia nigra which project to the striatum, there exists therapeutic potential in introducing genes coding for growth factors or enzymes involved in catecholamine biosynthesis directly into neurons or glia in, or surrounding, the corpus striatum (2,12). Delivery of dopamine directly to the corpus striatum, using infusion pumps or other delivery systems, has been shown to result in behavioural recovery in animal models of PD (3,11). Also, fetal or adrenal-derived tissue transplants, which may direct tonic release of dopamine or growth factors, placed adjacent to or within the corpus striatum, have been demonstrated to result in behavioural recovery in animal and human clinical studies of PD (14).

We have recently developed a defective Herpes Simplex Virus (HSV-1) vector system which permits the introduction of genes into post-mitotic cells, such as neurons (6,8,9). Previously existing techniques for delivering DNA into cells have proven unable to deliver a gene directly into post-mitotic cells. Because of its ability to infect neurons, to be maintained in a latent state with expression of some genes, and to accommodate large genes,

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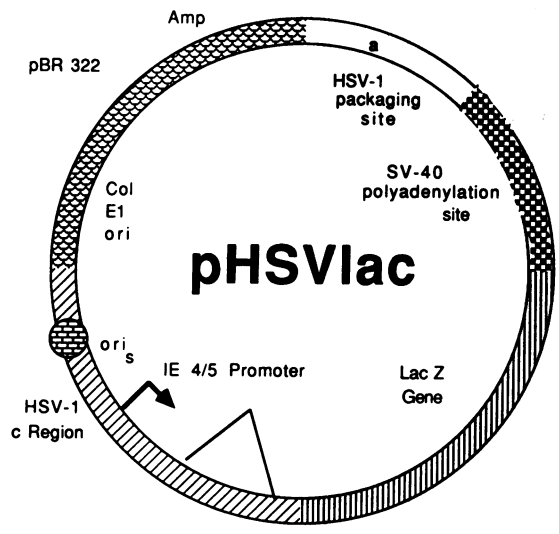

Fig. 1. The Structure of pHSVlac. The wavy line segment represents sequences from pBR322 including the ampicillin resistance gene and the Col E1 origin of DNA replication. The clear region contains the HSV-1 **a** sequence nucleotides 127–1132, the packaging site. The diagonal line region contains HSV-1 sequences from the **c** region (nucleotides 47–1066), which contains the following genetic elements: The HSV-1 origin of DNA replication ori_s, represented by a circle filled with rectangular bricks; the HSV-1 IE 4/5 promoter, represented by the arrow; and the intervening sequence following that promoter, represented by the triangle. The vertical line segment contains the *E. coli Lac Z* gene. The checkerboard portion contains the SV-40 early region polyadenylation site.

HSV-1 appeared to be an ideal vector to introduce genes into neurons. The prototype HSV-1 vector, pHSVlac, permits introduction of the *E. coli Lac Z* gene, encoding a β-galactosidase, into cells. We have shown that infection with this vector can result in the stable expression of high levels of β-galactosidase for at least two weeks, without causing cell death, in cultured peripheral and central nervous system (CNS) neurons (8,9). Our CNS studies were done primarily with cortical and hiippocampal neurons, which have different properties from striatal neurons. Due to the importance of the striatum in neurological diseases we undertook the present study to determine the behavior of HSV-1 vectors in striatal cells. We now demonstrate that i) pHSVlac can infect cultured striatal neurons and express β-galactosidase; ii) β-galactosidase is stably expressed in striatal neurons for at least two weeks; and iii) pHSVlac DNA persists in these cultures for at least two weeks and can be recovered following superinfection with HSV-1. Our results suggest that HSV-1 viral vectors can be used to deliver virtually any gene into striatal neurons offering the potential for gene therapy in either HD or PD, as well as other diseases affecting the striatum.

MATERIALS AND METHODS

(a) Enzymes and Chemicals

The restriction endonucleases *Eco RI* and *Hind III* were obtained from New England Biolabs (Beverly, MA). Mouse monoclonal anti-rat neurofilament antibody (SMI-33) was obtained from Sternberger-Meyer (Jarretsville, MD). Rabbit anti-*E.coli* β-

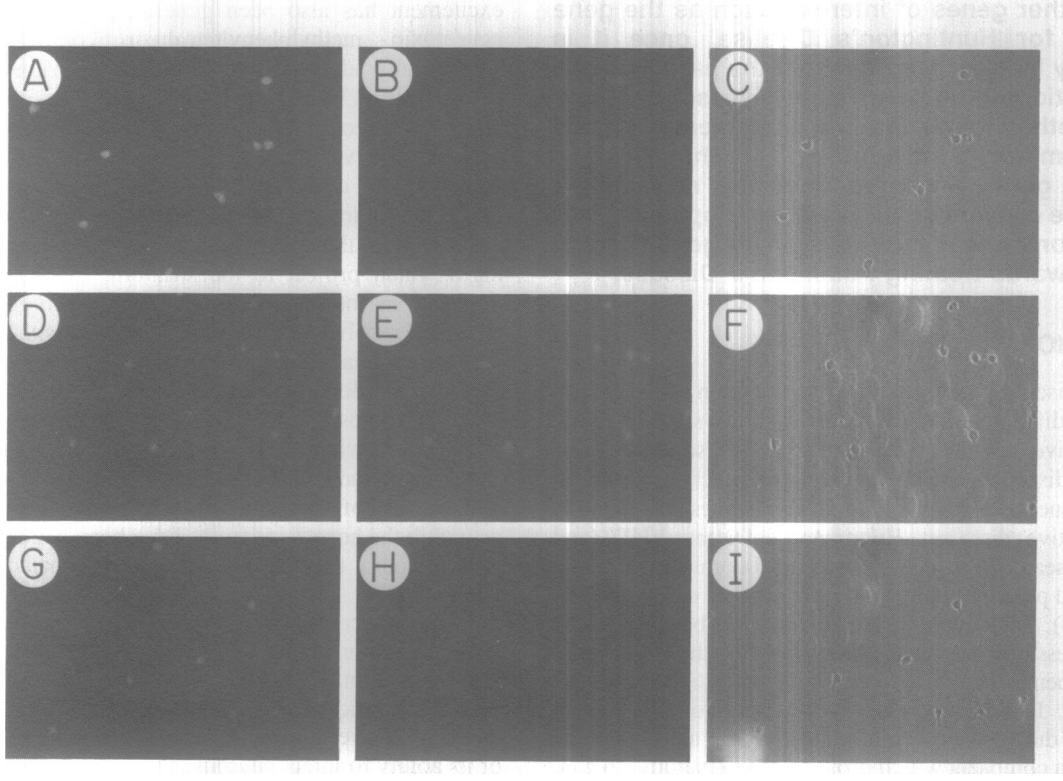

Fig. 2. Immunofluorescent colocalization of β-galactosidase-like immunoreactivity and neurofilament-like immunoreactivity in cultured striatal neurons 24 hours after infection with pHSVlac. Cultures of striatal neurons were infected with pHSVlac virus and incubated for 24 hours. Immunohistochemistry was performed as described using primary antibodies against *E. coli* β-galactosidase and neurofilament. β-Gal-IR was visualized with a rhodamine conjugated secondary antibody and Nf-IR with a fluorescein conjugated secondary antibody. The width of the photomicrograph represents 190 microns. A: Nf-IR, B: β-gal-IR, C: phase contrast of the same field; D: Nf-IR, E: β-gal-IR, F: phase contrast of the same field; G: Nf-IR, H: β-gal-IR, and I: phase contrast of the same field.

galactosidase antibody, flourescein isothiocyanate-conjugated goat $F(ab')_2$ antibody to mouse $F(ab')_2$, and rhodamine isothiocyanate-conjugated goat $F(ab')_2$ antibody to rabbit $F(ab')_2$ were obtained from Cooper Biomedical. Genetran was obtained from Plasco Co. (Woburn, MA). Pregnant Sprague Dawley rats were obtained from Zivic Miller (Zelienople, PA). Media components were obtained from Gibco (New York, NY); poly-L-lysine (75,000 – 150,000 MW) and cytosine arabinoside were obtained from Sigma (St. Louis, MO).

(b) Cell Culture and Viruses

CV1 monkey fibroblasts were grown in Dulbecco's modified minimum essential medium (DMEM) with 10% fetal bovine serum. HSV-1 strain 17 ts K was kindly provided by Dr. Subak-Sharpe and was grown on CV1 cells as previously described (8,9). Mixed striatal cultures, containing both glial and neuronal elements, were prepared from postnatal day 0 rat pups. Using sterile technique, each pup was decapitated, the brain was removed, and placed into a petri dish under a dissecting microscope. The anterior striatum was removed bilaterally and placed into a petri dish containing Tyrode's buffer (NaCl: 140 mM; glucose: 11 mM; KCl: 4 mM; NaH_2PO_4: 360 μM; KH_2PO_4: 180 μM; pH adjusted to 7.4 with bicarbonate) on ice. After all the rat pups were sacrificed and the brains dissected, the Tyrode's buffer was aspirated and approximately 1 ml of media (50% DMEM, 50% HAMS F12, 10% heat inactivated horse serum, supplemented with 100 units/ml Penicillin/Streptomycin and 4 g/L glucose) was added per 10 striata. The tissue was grossly dissociated by trituration five times with a 10 ml Pasteur pipette. 35 mm dishes were pretreated for three hours with 1 ml of 20 mg/ml poly-L-lysine and subsequently washed twice with distilled water and once with Tyrode's buffer. Approximately 1×10^6 cells were plated per dish and 1.5 ml of media was added. Cultures were then incubated at 37°C (5% CO_2) for 24 hours and the media was changed. After 5 days in culture, 3/4 of the media volume was aspirated and replaced with media containing 40 μM cytosine arabinoside, an inhibitor of mitosis, to prevent overgrowth by glial cells. Thereafter, two-thirds of the media volume in each dish was replaced every 3–4 days (5). Cultures were infected ten days after plating.

(c) Construction of pHSVlac and packaging into a HSV-1 Virus Stock

pHSVlac was constructed and packaged into HSV-1 particles as previously described (8,9). Briefly, 1.5×10^5 CV1 cells were seeded onto a 60 mm plate. The following day, the cells were transfected with an 0.5 ml calcium phosphate co-precipitate containing 1 μg pHSVlac DNA and 9 μg salmon sperm DNA. Four hours later, the cells were treated with 15% glycerol. Following a 24 hour incubation at 37°C, 1.5×10^6 plaque forming units (pfu) of HSV-1 ts K in 100 μl was added to each plate. After one hour at room temperature, 5 ml of medium was added to each plate. After incubation for 3 days at 31°C, virus was harvested. Virus stocks were subsequently passaged at a 1:2 dilution on CV1 cells at 31°C. The titer of the virus stock was 1×10^6 pfu of ts K per ml and 8×10^5 infectious particles of pHSVlac per ml.

(d) Immunofluorescent visualization of β-galactosidase and neurofilament

Immunofluorescent colocalization of β-galactosidase-like immunoreactivity (β-gal-IR) and neurofilament-like

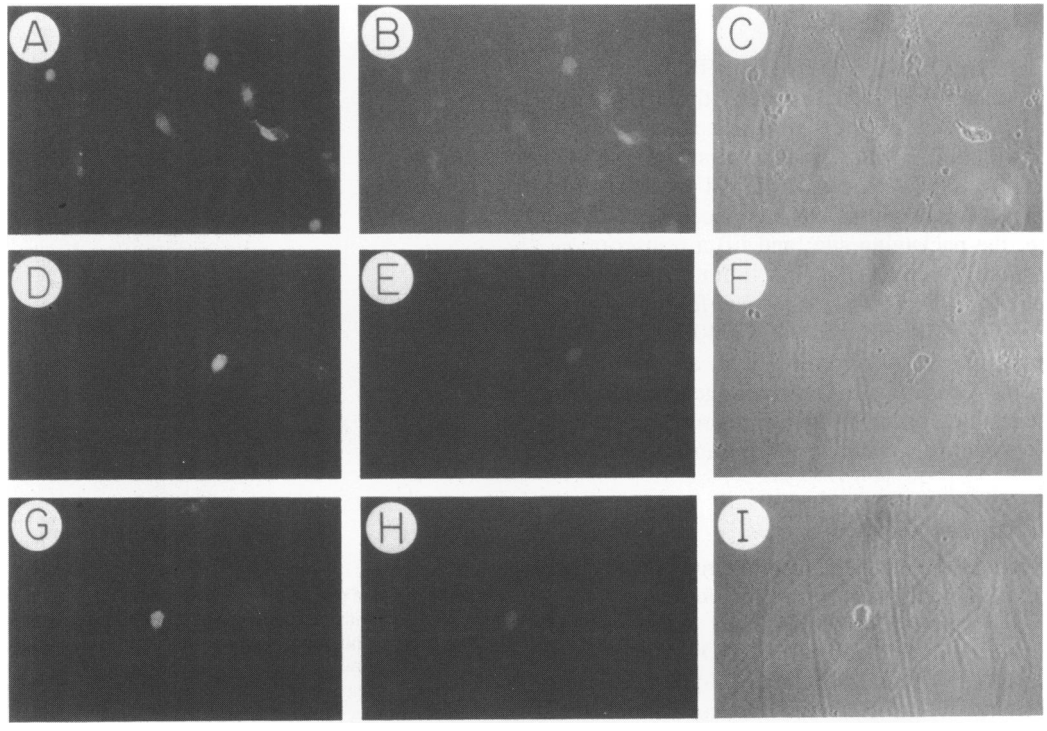

Fig. 3. Immunofluorescent colocalization of β-galactosidase-like immunoreactivity and neurofilament-like immunoreactivity in cultured striatal neurons two weeks after infection with pHSVlac virus. The experiment was performed as described in the legend to Fig. 2. A: Nf-IR, B: β-gal-IR, C: phase contrast of the same field; D: Nf-IR, E: β-gal-IR, F: phase contrast of the same field; G: Nf-IR, H: β-gal-IR, and I: phase contrast of the same field.

immunoreactivity (Nf-IR) in cultured striatal neurons was performed (8,9). Cultures were infected with 0.1 ml of pHSVlac virus stock, incubated for either 24 hours or two weeks at 37°C, and fixed with 4% paraformaldehyde in 0.1 M Phosphate Buffered Saline (PBS, pH 7.4) for 15 minutes. The primary antibodies were rabbit anti-*E. coli* β-galactosidase (1:800) and monoclonal mouse anti-rat neurofilament (1:800). The secondary antibodies were fluorescein isothiocyanate-conjugated goat $F(ab')_2$ antibody to mouse $F(ab')_2$ (1:200) and rhodamine isothiocyanate-conjugated goat $F(ab')_2$ antibody to rabbit $F(ab')_2$ (1:250). Controls included incubation in the absence of either of the primary antibodies. Verification of the neuronal elements of the primary striatal cultures have been described elsewhere (5).

(e) Recovery and analysis of pHSVlac DNA from cultures

Cultures previously infected with pHSVlac virus were infected with 5×10^5 pfu of ts K and incubated for two days at 31°C. The resulting virus stock was passaged three times on 2×10^6 CV1 monkey fibroblasts at 31°C to yield virus stocks Striatum 1 and Striatum 2. 1×10^7 CV1 cells were infected with 5×10^7 pfu of virus stock (Striatum 1, Striatum 2, ts K alone or mock infected) and incubated at 31°C for 24 hours. Total cellular DNA was prepared as described (8); 5 μg of DNA, or 2×10^{-4} μg of pHSVlac DNA isolated from *E. coli* HB101 as standard, was digested with 12.5 units of *Eco RI* overnight at 37°C, resolved on 0.7% agarose gels, and transferred to Genetran. Hybridization was performed using the 3.3 kb *Eco RI, Hind III* fragment from the plasmid pCH110 radiolabeled with ^{32}P. This fragment contains most of the *Lac Z* gene, except for 133 bp at the 3′ end. This procedure has been described in greater detail elsewhere (8,9).

RESULTS

(a) The Vector

The prototype defective HSV-1 vector, pHSVlac (8.9), schematically represented in Fig. 1, contains three types of genetic elements: i) sequences from pBR322 which permit propagation of pHSVlac in *E. coli*; ii) sequences from HSV-1 which permit packaging of the vector into a HSV-1 virus particle: the HSV-1 ori_s, an HSV-1 origin of DNA replication; and the HSV-1 a sequence, the packaging site; and iii) a transcription unit, the components of which are the HSV-1 Immediate Early (IE) 4/5 promoter, the intervening sequence following that promoter, the *E. coli Lac Z* gene, and the SV-40 early region polyadenylation site. The IE 4/5 promoter is a constitutive promoter active in most cell types. The *Lac Z* gene encodes a β-galactosidase normally absent from all mammalian cells, providing a simple assay for expression of the transcription unit in pHSVlac.

(b) Expression in Striatal Neurons

Primary cultures derived from neonatal rat striatum were infected with pHSVlac virus. Following incubation for one day or two weeks at 37°C, assays were performed to detect expression of *E. coli* β-galactosidase in neurons and pHSVlac DNA. Cultures were fixed and assayed for the colocalization of β-galactosidase-like immunoreactivity (β-gal-IR) and neurofilament-like immunoreactivity (Nf-IR) using immunofluorescent visualization. As shown in Fig. 2, phase-positive cultured striatal cells demonstrated staining for both β-gal-IR and Nf-IR. Approximately 70% of the Nf-IR positive cells were also β-gal-IR positive (sample size approximately 500 cells). Parallel cultures treated with pre-immune primary sera contained background levels of fluorescein and rhodamine fluorescence (results not shown), and parallel cultures treated with antibody against neurofilament and rabbit preimmune serum, followed by the fluorescent-conjugated antibodies contained Nf-IR but no β-gal-IR (results not shown). Cultures infected with ts K alone or mock infected, and treated with both primary and secondary antibodies exhibited Nf-IR, but again no β-gal-IR. As shown in Fig. 3, two weeks after infection with pHSVlac, striatal cells demonstrated both Nf-IR and β-gal-IR; approximately 70% of striatal neurons were β-gal-IR positive (sample size approximately 500 cells).

(c) Recovery of pHSVlac DNA from neurons

We devised a strategy to recover pHSVlac DNA from cultured neurons based on the observation that superinfection of a latently infected neuron results in a lytic infection, and both the latent genome and the superinfecting genome are represented in the progeny virus (6). After infection with pHSVlac virus and incubation for two weeks at 37°C, striatal cultures were then infected with ts K alone and incubated for two days at 31°C. Total cellular DNA was isolated from these virus stocks and digested with *Eco RI.* Southern analysis was used to detect pHSVlac DNA with a probe homologous to *Lac Z* DNA.

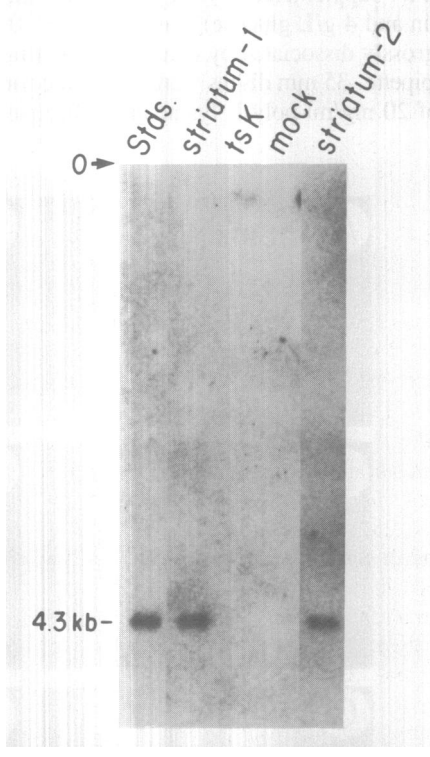

Fig. 4. Analysis of pHSVlac DNA recovered from cultured striatal cells two weeks after infection with pHSVlac virus. Cultures of striatum were infected with pHSVlac virus, incubated for two weeks at 37°C, and then infected with ts K and incubated for two days at 31°C. Total cellular DNA was isolated and subjected to Southern Analysis with a probe homologous to *Lac Z.* The origin of the gel is marked with an O, and the sizes of DNA are as shown. The lanes labeled Striatum 1 and Striatum 2 represent DNA from CV1 cells infected with virus stocks recovered from cultures of striatum, two weeks after infection with pHSVlac virus; lanes labeled ts K or mock represent DNA from CV1 cells infected with ts K or mock infected; and the lane labeled Stds. represents pHSVlac DNA isolated from *E. coli.*

pHSVlac contains three *Eco RI* sites, one at each end of the pBR segment and a third in the *Lac Z* gene 133 bp from the 3′ end of the fragment. As shown in Fig. 4, the 4.3 kb band which contains most of the transcription unit in pHSVlac is detected in virus stocks obtained from superinfection of neurons harboring pHSVlac for two weeks, but is absent from virus stocks of ts K alone and from mock infected cells. The 2.3 kb fragment contains the pBR sequences and the 1.5 kb fragment contains the 3′ end of *Lac Z*, the SV-40 polyadenylation site and the HSV-1 **a** sequence. The probe is not homologous to these two fragments. In summary, pHSVlac DNA is stably maintained in primary striatal cultured neurons for at least two weeks and directs the stable expression of β-galactosidase.

DISCUSSION

Several important neurological diseases affect the striatum. In order to perform gene therapy on these diseases it is necessary to develop a method for delivering genes into neurons and glia in the striatum. We have developed a defective HSV-1 vector system that can deliver genes into postmitotic cells (6,8,9). Previous studies have established that our prototype vector, pHSVlac, can efficiently transfect both peripheral and central neurons in culture; one particle is sufficient for expression in a neuron (8,9,13) at a moi of as low as 0.02 (13). Our CNS studies used primarily hippocampal and cortical neurons which are clearly different from striatal neurons. Therefore, we undertook the present study to determine if HSV-1 vectors could be used in a similar fashion on striatal cells. In this study, infection of neurons in primary culture derived from neonatal rat striatum with pHSVlac virus resulted in expression of β-galactosidase without causing cell death. Expression of β-galactosidase activity in those cells staining positive for neurofilament-like immunoreactivity, a marker for neurons, was maintained for at least two weeks. The rate of horizontal transmission of pHSVlac from an infected to a non-infected neuron was minimal by two criteria: First, the low virus titers in culture medium observed two weeks after infection (results not shown) and second, the presence of β-galactosidase negative cells two weeks after infection. Superinfection with HSV-1 of cells infected two weeks earlier with pHSVlac and analysis of the resulting virus stocks confirmed the presence of pHSVlac DNA. We conclude that pHSVlac can stably express β-galactosidase in striatal neurons.

The present study used cultured cells; however, we have shown that pHSVlac can stably express β-galactosidase in neurons and glia in the adult rat brain (manuscript submitted). Therefore, it should be possible to deliver genes into neurons and glia in the adult mammalian striatum. Furthermore, we have recently developed a deletion mutant packaging system for HSV-1 vectors (18) which may allow HSV-1 vectors to be introduced into humans.

These findings indicate the potential utility of HSV-1 vectors to introduce and express a gene selectively in striatal neurons to perform gene therapy on neurological diseases that affect the striatum. For application to Parkinson's Disease, localized delivery of a gene encoding either tyrosine hydroxylase or a growth factor to striatal neurons may be useful in restoring dopaminergic tone or synaptic connectivity within the corpus striatum (2,3,11,12,14,15). Furthermore, we have recently demonstrated that biologically active tyrosine hydroxylase can be expressed from a HSV-1 vector (manuscript in prepartion). For application to Huntington's Disease, delivery of the H.D. gene, once it has been isolated, to striatal neurons *in vitro* or *in vivo* may permit characterization of the biochemical and/or physiological alterations responsible for the pathogenesis of the disease process.

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