Altering central nervous system physiology with a defective herpes simplex virus vector expressing the glucose transporter gene

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ABSTRACT Because of their postmitotic nature, neurons are difficult subjects for gene transfer. To circumvent this, we have used a defective herpes simplex virus vector to overexpress the rat brain glucose transporter (GT) gene under the control of the human cytomegalovirus iel1 promoter. This vector, designated vIElGT, was propagated using a herpes simplex virus type 1 temperature-sensitive mutant, ts756. GT expressed from vIElGT was readily immunoprecipitated from membrane fractions of vIElGT-infected Vero cells. By using indirect double immunofluorescence techniques, vIElGT was shown to be capable of enhancing GT expression in cultured hippocampal neurons and glia. Glucose transport in such vIElGT-infected cultures was increased ~2-fold relative to controls. The efficacy of this system in vivo was then tested by microinjection of vIElGT into adult rat hippocampus. When examined 2 days later, GT expression from vIElGT was demonstrated in hippocampal neurons by in situ hybridization; a small but significant increase in glucose transport was detected in tissue immediately surrounding the injection site by 2-deoxy[14C]glucose uptake and autoradiography. Such injections did not cause marked cytopathology. Thus, this approach can be used to alter central nervous system physiology in vitro and in vivo.

Because of the postmitotic nature of neurons, gene transfer to these somatic nondividing cells requires innovative approaches; use of herpes simplex virus (HSV) vectors may represent such an approach. HSV-1 and HSV-2 are well-known neurotropic agents capable of replication and latency in the peripheral nervous system. In addition, work in experimental animals and in the natural human host has shown that these viruses also interact with neurons in the central nervous system (CNS). Recombinant HSVs have been employed as vectors of foreign genes in cultured cells (1–5) and have been used to express an indicator gene (Escherichia coli lacZ gene) in sensory neurons or in CNS during acute growth and latency (6–11).

Recent development of HSV vectors has capitalized on the natural occurrence of defective interfering particles that arise during high-multiplicity propagation of HSV (12). These defective viruses contain DNA molecules that are the same size as standard virus (152 kbp) but consist of head-to-tail concatamers of small regions of the viral genome including an origin of DNA replication (oriS or oriL) and a cleavage/packageing signal (a sequence). Defective viruses replicate and package only in the presence of a replication-competent helper virus. This phenomenon was extended to the construction of cloning-amplifying vectors (termed amplicons) consisting of a prokaryotic plasmid for amplification in bacterial host along with the important cis-acting HSV regulatory sequences for propagation in eukaryotic cells (13). Any eukaryotic transcription unit may be included on amplicons and propagated with helper virus to transfer genes into eukaryotic cells. Such defective HSV vectors have been used to express foreign genes in cultured cells, including neurons (14, 15). Although Kaplitt et al. (16) have used a defective HSV vector to express an indicator gene in adult rat brain, to our knowledge, no one has reported the use of HSV vectors, albeit intact or defective, to alter the physiology of the CNS in adult animals. In this study, we have employed a defective HSV vector to deliver the brain-type glucose transporter (GT) into primary hippocampal neurons in vitro and into the hippocampus. This particular gene was chosen as the first step in a strategy to deliver more glucose to hippocampal neurons, to buffer them from the neurotoxic effects of a number of metabolically disruptive neurological insults (see Discussion). We find that in vitro and in vivo, expression of the newly introduced GT gene is associated with increased glucose uptake.

MATERIALS AND METHODS

Cells and Virus. Mixed neuronal/glial cultures and enriched glial cultures were prepared from the hippocampi of fetal Sprague–Dawley rats on the 18th day of gestation as described (17). Mixed neuronal/glial cultures were maintained in MEM-PAK (a modified minimal essential medium purchased from the cell culture facility of University of California, San Francisco) supplemented with 10% (vol/vol) horse serum (HyClone) and were used at ~10 days of age when the neuron/astrocyte ratio was ~50:50, as determined by immunocytochemical staining (data not shown). The glial cultures were maintained in Dulbecco’s modified Eagle’s medium (MEM; GIBCO/BRL) supplemented with 5% (vol/vol) fetal calf serum (HyClone). Vero cells (ATCC CCL81) were maintained in MEM/10% (vol/vol) NuSerum (Collaborative Research). All cells are maintained in a 5% CO2/95% air atmosphere.

ts756 (HSV-1 KOS) has a temperature-sensitive mutation in an essential regulatory gene encoding ICP4 and was provided by R. G. Hughes, Jr. (Rosewell-Park Cancer Institute, Buffalo, NY).

Construction of Amplicons. The amplicon pIElGTori was constructed as follows: The GT coding sequence was isolated from pG310 (kindly provided by Morris J. Birnbaum, Harvard University) as an EcoRI–Bgl II fragment and cloned into pG310, an expression vector provided by Lidia Sambucetti (Stanford University). pG310 contains the promoter, exon 1 (untranslated), and intron 1 (from nt −1021 to +947), and the poly(A) signal (from nt +3270 to +3430) of the human cytomegalovirus (HCMV) iel1 gene in a pGEM-2 background. This plasmid was derived from pON308G (18) by first deleting the pGEM-2 sequences between EcoRI and BamHI, deleting the iel1 coding sequences (exons 2–4) between the

Abbreviations: HSV, herpes simplex virus; GT, glucose transporter; β-Gal, β-galactosidase; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; pfu, plaque-forming unit; CNS, central nervous system; HCMV, human cytomegalovirus; MAP2, microtubule-associated protein 2; GFAP, glial fibrillary acidic protein.
Pst I site (nt +947) and the BamHI site (nt +3270), and inserting a linker with a unique EcoRI site downstream of the splice receptor of intron 1. The GT gene was cloned into the EcoRI site and transcription of GT is terminated by the iel poly(A) signal. The HVS-1 ori site (295 bp, from strain F) and the a sequence (330 bp, from strain KOS) were then isolated from pON812 as an Xba I fragment and inserted downstream of the poly(A) signal. pON812 was constructed by Jeffrey Viera (Stanford University) by cloning the a sequence (19, 20) isolated from pUC18-19 (kindly provided by James Smiley, McMaster University) as a BamHI fragment and the ori site was cloned from pON103 (21) as a HindIII–EcoRI fragment into a cloning vector pIC-20H (22). The construction of pIE1βgalori is similar to that of pIE1GTori, except that the GT gene and the HCMV iel poly(A) signal were replaced by an EcoRI–Xba I fragment from pON3 (23) containing the E. coli lacZ gene and the poly(A) signal of simian virus 40.

Propagation of Defective HSV Vectors. To generate defective HSV vectors, 3 μg of ampiclon DNA and 10 μg of helper virus genomic DNA were transfected into Vero cells (1 × 10^6) using the calcium phosphate/glycerol–shock method (6). When 100% cytopathic effect was observed, the cells were then frozen, thawed, and sonicated to release the infectious virus. The virus stocks were then serially passaged onto fresh Vero cells by using a 1:4 diluter. Titers of ts756 in viral stocks were determined by plaque assays performed at 33°C on Vero cells. Titers of defective virus carrying ampiclon were estimated by quantitating the number of cells expressing GT immunocytochemically or expressing β-galactosidase (β-gal) by staining with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside [X-Gal, a chromogenic substrate of β-Gal (6)]. Preliminary Southern blot analyses did not detect any integration of vector sequences into the helper genome. The ratio of defective vs. helper virus varied during passage but always peaked at passage 4 or 5 with the maximal helper/defective ratio reaching =1:1. Viral stocks from passages 4 or 5 were used and unless otherwise stated, experiments in cell culture were performed at 37°C, a temperature that retarded but did not eliminate ts756 growth and cytopathic effects.

Immunoprecipitation. Vero cells grown in 25-cm² flasks were mock-infected or infected with VIE1GT or with VIE1βgal at =1 plaque-forming unit (pfu) per cell. The next day, the inoculum was replaced with fresh DMEM, 10% Nusera. When indicated, tunicamycin was added to 10 μg/ml and was maintained during the subsequent labeling of cells. At 13 h after infection, the cells in each flask were washed twice with methionine-free DMEM (Irvin Scientific) and labeled with 120 μCi of [35S]methionine (1102 Ci/mmol, 15 mCi/ml; 1 Ci = 37 GBq; ICN Biomedicals) in 1.5 ml of methionine-free DMEM. The cells were harvested 16 h after infection. Membrane fractions were isolated by differential centrifugation and immunoprecipitated (24) with a rabbit polyclonal serum to a synthetic peptide corresponding to the 13 aa of the C terminus of GT (1:400 dilution; East Acres Biologicals, Southbridge, MA). Membrane fractions were then separated by SDS/PAGE on a 12% gel without heating and the proteins were visualized by autoradiography.

Immunofluorescence. GT with microtubule-associated protein 2 (MAP2) or glial fibrillary acidic protein (GFAP) were colocalized on 10-day-old mixed neuronal/glial cultures grown on chamber slides. The cultures were mock-infected, infected with VIE1GT, or infected with VIE1βgal at =0.1 pfu per cell. Sixteen hours after infection, the cells were fixed with acetone/methanol, 3:1 (vol/vol), and GT expression was detected with anti-GT rabbit serum (1:400 dilution), followed by rhodamine-conjugated goat anti-rabbit IgG antibody (1:80 dilution; Tago). Neurons were identified using a mouse MAP2 monoclonal antibody (1:100 dilution; Sigma) and astrocytes were identified using a mouse GFAP monoclonal antibody (1:200 dilution; Sigma). MAP2 and GFAP immunoreactivities were visualized by fluorescein-conjugated goat anti-mouse IgG antibody (1:40 dilution; Tago).

In Vivo 2-Deoxy[14C]Glucose Uptake. Cells were mock-infected, infected with VIE1GT, or infected with VIE1βgal at =1 pfu per cell at 37°C or 39°C as indicated. Glucose uptake was then measured 16–20 h after infection (26). Cells in each well were washed twice with phosphate-buffered saline (PBS) = 137 mM NaCl/4 mM KCl/8 mM Na₂HPO₄/1.5 mM KH₂PO₄, warmed to 37°C, and then pulsed with a tracer dose (1 μCi/ml) of 2-deoxy[14C]glucose (55 mCi/ml; American Radiolabeled Chemicals, Inc.) in warm PBS. After 5 min, the uptake of glucose was terminated by rinsing the cells twice with 0.4 mM phosphate (Sigma) in ice-cold PBS (4°C) and twice with ice-cold PBS. The cells were then lysed in 1% SDS in PBS. Radioactivity of the lysate was standardized to protein concentration as determined by the o-phenthaldehyde method (Pierce) and expressed as the percent of mock-infected values.

In Vivo 2-Deoxy[14C]Glucose Uptake. Male Sprague–Dawley rats (375–450 g; n = 4) were catherized in the femoral vein and artery. Two days later they were microinjected unilaterally in the hippocampus with VIE1GT (2 μl; 1 × 10^5 pfu) and contralaterally with a similar amount of VIE1βgal (coordinates with bregma = lambda: AP, 4.1; ML, 2.1; DV, 3.0). Rats were decapitated 2 days later and brains were frozen in 2-methylbutane (−20°C). Coronal sections (15 μm thick) were prepared for hybridization as described (25). The sections were hybridized in 50% (vol/vol) formamide/4× standard saline citrate (SSC)/1× Denhardt’s solution/10% (wt/vol) dextran sulfate/tRNA (250 μg/ml)/takelwarm DNA (500 μg/ml) containing the oligonucleotide probe end-labeled with 35S-labeled dATP (5 × 10⁵ cpm per section) for 16 h at 37°C. After hybridization, the sections were washed (25), coated with emulsion, exposed for 15 days at 4°C, developed, and stained with 0.1% thionin.

RESULTS

Construction of Amplicons and Generation of Defective Vectors. We constructed an amplicon carrying a cDNA encoding the Glut-1 isoform of GT, initially isolated from rat brain endothelium (27, 28). The GT gene was put under the control of the HCMV iel promoter-enhancer, which allows constitutively strong expression in many different cell types. A test amplicon (pIE1GTori) and a control amplicon (pIE1βgalori, containing the E. coli lacZ gene in place of GT) were constructed (Fig. 1). We used ts756 (29), an HSV-1 temperature-sensitive mutant, as the helper virus. ts756 has
Fig. 1. pIE1GTori. The transcriptional unit is driven by the HCMV iel promoter-enhancer. Exon 1 of iel is untranscribed and intron 1 is spliced. The poly(A) signal inserted downstream of the GT gene is derived from the HCMV iel gene. The oris and the a sequences provide the necessary HSV replication signals. The ampicillin-resistance gene of the parent plasmid pGEM-2 is also indicated. The structure of pIE1galori is similar to that of pIE1GTori, except that the GT gene and the iel polA signal are replaced by lacZ terminated with the simian virus 40 poly(A) signal.

a mutation in ICP4, an essential regulatory α gene of the virus and is completely replication-defective at 39°C. Defective virus vectors were generated by cotransfecting pIE1GTori or pIE1βgalori along with genomic DNA of ts756 into Vero cells and then serially passaging the viral stocks at 33°C by using a 1:4 dilution to encourage growth of defective virus. The virus stocks thus generated from pIE1GTori and pIE1βgalori are referred to as vIE1GT and vIE1βgal, respectively.

Immunoprecipitation of GT from vIE1GT-Infected Vero Cells. We first confirmed that vIE1GT enhanced GT expression in Vero cells. A new species of ~41 kDa was immunoprecipitated from membrane fractions from vIE1GT-infected cells (Fig. 2, lane 3) but not in mock-infected or vIE1βgal-infected controls (Fig. 2, lanes 1 and 4, respectively). In the presence of tunicamycin, this product was reduced to ~38 kDa (Fig. 2, lane 2), the reported size of the primary translation product of Glut-1 (28). The endogenous GT in rat brain endothelium is known to be heterogeneous glycosylated with a mean molecular mass of ~52 kDa (27), and alternative GT species of 45–47 kDa also occur in rat brain (27, 28). Thus, the 41-kDa GT that we observed probably results from differential glycosylation in Vero cells.

Efficacy of Infection with vIE1GT in Cultured Neurons and Astrocytes. We next tested the ability of vIE1GT to express GT in primary cultures of hippocampal neurons and astrocytes by using immunofluorescence. GT immunoreactivity was observed in vIE1GT-infected cultures (Fig. 3) but not in vIE1βgal-infected or mock-infected cultures (data not shown). By using double immunofluorescence methods to colocalize neuron-specific antigen MAP2 and glia-specific antigen GFAP with GT immunoreactivity, both neurons and astrocytes were found to be capable of expressing GT (Fig. 3). Similarly, β-Gal-expressing neurons or astrocytes were observed in vIE1βgal-infected but not in vIE1GT-infected or mock-infected cultures (data not shown).

Given that our goal was to manipulate cellular function with these interventions, we tested whether increased GT expression was associated with an increased rate of glucose transport. Infection with vIE1GT doubled the rate of 2-deoxy[14C]glucose transport in Vero cells, mixed hippocampal cultures, and primary astrocyte cultures (Fig. 4). Mock infection or infection with vIE1βgal did not alter transport.

Efficacy of Infection with vIE1GT and vIE1βgal in Vivo. We next tested the ability of vIE1GT to express GT when injected into the hippocampus of adult rats by using in situ hybridization techniques. To distinguish the viral vector-
derived GT mRNA from the endogenous version, a 75-nt oligonucleotide probe antisense to the iel promoter from nt -35 to +40 was used. This probe hybridized to the 5’ untranslated end of both the GT transcripts from vIE1GT and the lacZ transcripts from vIE1βgal. Hybridization signals were readily detected in hippocampus microinfused with vIE1GT (Fig. 5A) or vIE1βgal (Fig. 5B); in contrast, no such signal was detected after injection with the helper virus or with DMEM alone (data not shown). The signals were clustered around the injection site and highly concentrated along the dentate gyrus. The pattern of vIE1βgal expression as visualized by in situ hybridization was similar to that visualized by histochemical staining with X-Gal (Fig. 5C).

![Image](image.png)

Fig. 5. Expression of vIE1GT and vIE1βgal in hippocampus as detected by in situ hybridization and X-Gal staining. (A and B) Dark-field microscopy of hybridization on the dorsal blade of the dentate gyrus from vIE1GT-infected (A) and vIE1βgal-infected (B) animals. The ventral blade of the dentate gyrus is outlined with arrows. (C) Bright-field microscopy showing β-Gal-expressing cells as darkly stained cells on the dorsal blade of the dentate gyrus.

We further characterized the parameters of expression with the HSV system by injecting vIE1βgal and staining with X-Gal. β-Gal expression was analyzed 2, 4, and 7 days after injections; expression peaked at day 2. At that time, expression was localized immediately surrounding the injection site, with most positive cells within a 150-μm radius. Within that area, ≈5% of cells expressed β-Gal. Of those cells, ≈82% were found to be neuronal by morphological criteria. The number of cells that expressed β-Gal within the 150-μm radius of the injection site was ≈50% of the peak value by 4 days after injection and declined to zero by 7 days.

We then tested whether injection of vIE1GT altered glucose uptake in the CNS of adult rats. vIE1GT was microinfused unilaterally into the hippocampus and vIE1βgal was microinfused contralaterally; 2-deoxy[14C]glucose uptake was assessed 2 days later. Because of the data for the spatial distribution of β-Gal-positive cells, we analyzed glucose uptake within a small sphere of tissue, with a radius of ≈150 μm, surrounding the injection site (corresponding to the dorsal blade of the dentate gyrus).

Glucose transport was enhanced a significant 10% at the injection site on the vIE1GT side, relative to the contralateral side. This enhancement declined with increasing distance from the infusion site, and uptake was equivalent to that on the contralateral side within ≈180 μm of the injection site (Fig. 6). There were no differences in uptake in hippocampal regions more distant from the injection site (data not shown).

**DISCUSSION**

The use of defective HSV vectors to deliver genes to neurons and glia shows great potential, and a number of investigators have exploited this system in recent years. In some reports, HSV vectors were used with cultured cells (e.g., rat neurona cell lines, mouse ovary cells, Vero cells, or HEP-2 cells). In all of these cases, efficacy of the vector was documented by enhanced levels of mRNA or protein for a reporter gene or enhanced activity of the reporter enzyme (2, 3, 14, 30). In some instances, HSV vector systems have been used for in vivo studies. Pallela et al. (3) overexpressed mRNAs for the HPRT gene in mouse brain by such an approach. Chiocca et al. (11), Kaplitt et al. (16), Huang et al. (8), and Fink et al. (9) delivered lacZ as a reporter gene to the rodent brain, and Ho and Mocarski (6, 7) and Dobson et al. (10) used a similar

![Graph](graph.png)

Fig. 6. Effect of vIE1GT infection on 2-deoxy[14C]glucose uptake in adult rat CNS. Anterior/posterior analysis showing that 2-deoxy[14C]glucose uptake was enhanced nearest to the site of injection (arrow) of vIE1GT. Uptake in hippocampus (relative to the contralateral side) is shown as a function of distance from the injection site in the anterior/posterior plane, with data pooled for every three 20-μm section. Numbers in parentheses indicate the numbers of sections from the four rats analyzed; numbers varied because, in some cases, sections corresponding to the stated distance from the infusion site were not available.
approach with sensory neurons and hypoglossal motor neurons. In a recent advance in this approach, Fedroff et al. (31) demonstrated that the combination of vI1 promoter and a temperature-sensitive Neu-1 enhancer with the Nos promoter is sufficient to support cervical ganglion neurons and were able to alter neuronal survival after axotomy. In the present report, we have used a defective HSV vector, vIE1GT, to enhance glucose transport in the rat hippocampus. To our knowledge, this is the first report that such a system has been used to deliver a gene into the brain itself that alters physiology (rather than serving merely as a reporter gene).

The magnitude of vIE1GT’s effect upon glucose transport differed considerably between cell culture and intact host. In epithelial or mixed hippocampal cells in culture, our best vIE1GT stock (with a defective/helper ratio ≈ 1:1) increased 2-deoxyglucose uptake 200–300%. In contrast, in vivo, vIE1GT caused an ≈10% increase in 2-deoxyglucose uptake in a small sphere of tissue surrounding the injection site. However, the effect on uptake in individual cells might have been greater than that. The data regarding the number and distribution of β-Gal-positive cells demonstrated that ≈5% of cells within a sphere were positive. One can safely assume very roughly equivalent efficiencies of expression in vIE1GT and vIE1βgal. Thus, if the glucose uptake rate at the center of the sphere of tissue was increased 10% due to infection of only this small subset of cells, this implies that uptake was enhanced considerably in those individual cells. Such an increase might be physiologically meaningful within the tightly regulated realm of cerebral metabolism (32).

As noted, our studies utilized the HCMV iel promoter-enhancer and a temperature-sensitive mutant of HSV as helper. Although the strength of the HCMV promoter-enhancer is well established, its long-term pattern of expression in neurons is unclear. We observed β-Gal-expressing cells in rat hippocampus only for the first few days after infection, in agreement with a report (16) that also used the HCMV iel promoter to express lacZ. We (7) and others (10) have demonstrated that the latency-associated transcripts (LAT) promoter from HSV-1 can cause long-term gene expression in latently infected animals. Thus, the LAT promoter and other promoters from housekeeping genes may be more effective at causing sustained changes in CNS function. With respect to the helper virus, the internal body temperature of the rat is not completely nonpermissive for ts576 replication and we have observed some cytopathicity in the hippocampus by using ts576 (unpublished data). Viruses carrying deletion mutations in key regulatory genes such as IC P4 and IC P0 will potentially facilitate this technique (33).

These studies were initiated to find a genetic site to alter neuronal function and potentially protect neurons from insult. The choices of attempting to enhance glucose transport and using the hippocampus were not random. The brain is exquisitely dependent upon glucose; nervous tissue has extremely high metabolic rates, utilizes little other than glucose, and stores it poorly (34). Moreover, neurological insults such as hypoxia-ischemia, hypoglycemia, and sustained seizure disrupt energy charge in the brain and preferentially damage the hippocampus; in the case of sustained seizure, glucose transport can become the rate-limiting step determining whether there is a damaging mismatch between energy delivery and utilization (32). Furthermore, glucocorticoids damage hippocampal neurons and enhance the toxicity of these neurological diseases; this endangerance is energetic in nature, as it can be lessened by supplementation of the hippocampus with excessive glucose (35). Finally, neuron death emerges slowly over the 48–72 h after these neurological insults and a number of pharmacological interventions during this delayed period can be neuroprotective (35); thus, were the rat hippocampus exposed to vIE1GT immediately after onset of the insult, glucose transport would be enhanced during the time window of vulnerability. As noted, the enhancement of glucose transport in the small set of affected neurons was probably well in excess of 10% above control values; there is reason to believe that with optimization of the protocol described here, the percentage of neurons so affected and/or the magnitude of the effect can be improved. Our data suggest that this method of intervention might have neuroprotective potential.

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