Deletion of the Virion Host Shut-off Gene Enhances Neuronal-Selective Transgene Expression from an HSV Vector Lacking Functional IE Genes

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The ability of herpes simplex virus (HSV) to establish lifelong latency in neurons suggests that HSV-derived vectors hold promise for gene delivery to the nervous system. However, vector toxicity and transgene silencing have created significant barriers to vector applications to the brain. Recently, we described a vector defective for all immediate-early gene expression and deleted for the joint region between the two unique genome segments that proved capable of extended transgene expression in non-neuronal cells. Sustained expression required the proximity of boundary elements from the latency locus. As confirmed here, we have also found that a transgene cassette introduced into the ICP4 locus is highly active in neurons but silent in primary fibroblasts. Remarkably, we observed that removal of the virion host shutoff (vhs) gene further improved transgene expression in neurons without inducing expression of viral genes. In rat hippocampus, the vhs-deleted vector showed robust transgene expression exclusively in neurons for at least 1 month without evidence of toxicity or inflammation. This HSV vector design holds promise for gene delivery to the brain, including durable expression of large or complex transgene cassettes.

INTRODUCTION

Replication-defective herpes simplex virus (HSV)-based vectors have the potential to provide an invaluable tool to both studies of brain function and treatment of CNS disorders. HSV genomes persisting in neurons do not integrate and can serve as a platform for therapeutic gene expression. Replication-defective HSV vectors have been tested for gene therapy approaches in the CNS and peripheral nervous system. However, CNS applications have been plagued by residual neuronal toxicity and progressive loss of transgene expression. Viral toxicity results from expression of the viral immediate early (IE) protein ICP0, a protein with functions related to viral chromatin modification and resistance to innate anti-viral responses. In the absence of ICP0, rapid genome silencing ensues, resulting in low transgene expression, while, in the presence of ICP0, significant toxicity results in loss of neuronal cell function and viability.

In sensory neurons, ICP0 fails to accumulate in the nucleus, and ICP0-mediated cytotoxicity is further avoided by silencing of the viral genome and the establishment of latency. The viral latency-associated transcript (LAT) locus remains transcriptionally active during latency, and the LAT promoter elements have been used to express transgenes in sensory neurons. However, expression was typically short-term or low, and promoter activity was not seen in all latently infected neurons. We have recently reported that, in the absence of IE gene activity, transgene expression from a non-viral promoter can be maintained in non-neuronal cells when the expression cassette is placed between two clusters of CTCF binding motifs referred to as CTRL1 and CTRL2 flanking the LAT promoter. This arrangement remained active when relocated to different intergenic regions unrelated to the latency locus. Interestingly, primary neurons isolated from fetal rat dorsal root ganglia (rDRG) also displayed expression of a second reporter cassette located in the deleted ICP4 locus. This finding prompted further examination of the ability of the ICP4 locus to support transgene expression in neurons in the brain.

Preliminary observations in our lab suggested that elimination of the virion host shut-off function (vhs) may further reduce the already minimal toxicity of IE gene-depleted vectors. Vhs functions as a virulence factor that reduces immune recognition of infected cells in vivo and helps evade the innate anti-viral interferon (IFN) responses; vhs-deficient viruses are attenuated for neurovirulence. Since vhs is an mRNA-specific RNase, we reasoned that it could potentially reduce transgene mRNA and corresponding protein expression. Thus we...
deleted the \( vhs \) (U\(_{41} \)) gene from our most recent vector, J\( \Delta N5 \),\(^{18} \) generating a new backbone that was named J\( \Delta N8 \), and compared the 2 vectors for transgene expression from the deleted ICP4 locus in rat brain.

The results of our analyses show that the human ubiquitin C (UbC) promoter placed between the end points of the ICP4 gene deletion allows expression of the linked reporter gene in vivo in rat hippocampal neurons. Furthermore, expression was enhanced by deletion of the \( vhs \) gene. No vector-associated toxicity was observed following rat brain injections and expression persisted in neurons of the CNS for at least 1 month, providing an effective platform for the delivery of therapeutic transgenes to the CNS, including multiple transgenes or transgenes that are too large for accommodation by other vector systems.

**RESULTS**

**Vector Construction and Characterization in Non-complementing Fibroblasts**

J\( \Delta N8 \) was derived from J\( \Delta N5 \) by deletion of the \( vhs \) locus (Figure 1A). J\( \Delta N5 \) and J\( \Delta N8 \) grew at comparable rates on ICP4- and ICP27-complementing U2OS cells [U2OS-ICP4/27]\(^{15} \) (Figure 1B), showing that \( vhs \) did not clearly contribute to virus yield in cultured cells. Infection of human dermal fibroblasts (HDFs) with J\( \Delta N5 \) and J\( \Delta N8 \) in complementing cells, U2OS-ICP4/27 cells were infected with J\( \Delta N5 \) and J\( \Delta N8 \) vectors at 1 gc/cell, and supernatant virus titers were determined in gc per milliliter every day for 5 days (averages of duplicate wells). (C) Relative nuclear viral DNA levels at 2 hr post-infection of HDFs with 5,000 gc of J\( \Delta N5 \) and J\( \Delta N8 \). Viral gc numbers were determined by qPCR for the gD gene normalized to the cellular 18S rRNA gene content (averages of duplicate wells). (D) mCherry protein and (E) mRNA expression in vector-infected HDFs without and with ICP0 complementation. Cells were infected at 25,000 gc/cell with J\( \Delta N5 \Delta B \) or J\( \Delta N8 \Delta B \) and superinfected 6 days later with mock or QOZH vector (10\(^8 \) gc/well). One day later, microscopy fields were imaged for mCherry fluorescence, DAPI-stained nuclei, and bright field. Separately, mRNA was collected 1 day after superinfection, and mCherry mRNA levels in duplicate samples were measured in triplicate, averaged, and normalized to viral gc in the same samples. Normalized values ±SD are presented relative to J\( \Delta N5 \Delta B \) infected cells at 7 dpi.

We previously demonstrated that removal of bacterial artificial chromosome (BAC) sequences from BAC-based viral vectors reduces vector toxicity for non-complementing cells.\(^{18} \) Cre recombination was used to remove the bacterial sequences from J\( \Delta N5 \) and
JDNI8, creating JDNI5AB and JDNI8AB, respectively. Following transduction of HDFs with JDNI5AB and JDNI8AB, neither vector showed noticeable mCherry transgene expression at 7 dpi (Figure 1D), consistent with our previous results for JDNI5.18 The presence of viral DNA in the nuclei of infected HDFs (Figure 1C) with little corresponding protein expression suggested that both viral genomes were epigenetically silenced. Restoration of the IE gene ICP0 to ICP0-deficient vectors has been shown to counteract epigenetic repression.22,23 As anticipated, when JDNI5AB- and JDNI8AB-infected HDFs were superinfected with QOZHG virus12 to supply ICP0 expression, mCherry expression was restored (Figures 1D and 1E). While the qRT-PCR data (Figure 1E) suggested that the vhs deletion caused a decrease in mCherry mRNA levels (~6-fold), these levels were extremely low compared to those in the presence of ICP0 (Figure 1E).

Characterization of Gene Expression in Neuronal Cells
We previously reported that mCherry expression from JDNI5-based vectors was markedly higher in rDRG neurons than in fibroblasts in vitro at 3 dpi. We therefore infected rDRG cultures with JDNI5AB and JDNI8AB to assess the effect of vhs deletion on transgene expression in these cells. At 7 and 14 dpi, rDRGs infected with either virus showed abundant mCherry fluorescence, but the signals were stronger in JDNI8AB- than in JDNI5AB-infected cultures (Figures 2A and S1). Analysis of reverse transcribed mRNA by qPCR revealed that mCherry gene expression in JDNI8AB-infected rDRGs was approximately 2.5- and 3-fold higher than in JDNI5AB-infected rDRGs at 7 and 14 dpi, respectively (Figure 2B). To eliminate the possibility that mCherry expression was affected by cytotoxicity, we assessed cell viability after transduction of rDRGs with JDNI5AB or JDNI8AB. No difference in cell viability was observed between vector- and mock-infected cells, indicating that neither vector displayed noticeable cytotoxicity (Figure 2C). Together, these results showed that in neuronal cells, both JDNI5 and
JDNI8 can mediate prolonged transgene expression from the UbC promoter at the ICP4 locus without apparent cytotoxicity and that increased transgene expression correlates with the vhs gene deletion in JDNI8.

To determine whether the vhs gene influences global viral gene expression, we measured the mRNA levels of the mCherry transgene and selected viral genes representing different kinetic classes and regions of the viral genome in both HDFs and rDRGs (Figure 2D). In HDFs, expression of most viral genes from JDNI5ΔB and JDNI8ΔB was comparable, regardless of kinetic class or genomic location, indicating that removal of vhs did not dramatically alter viral gene expression (Figure 2D, left side). In DRGs, expression of individual genes was also similar between JDNI5ΔB and JDNI8ΔB, but with a few notable exceptions. mCherry expression per genome copy was 50- to 100-fold higher than in HDFs, considerably higher than expression of any of the viral genes, and approximately 2-fold higher from JDNI8ΔB than from JDNI5ΔB (Figure 2D, right side). Of interest, the expression of ICP34.5 in DRGs, while similar between the two vectors, was also elevated compared to HDFs (~25-fold for JDNI8ΔB, ~17-fold for JDNI5ΔB). The ICP34.5 gene (γ134.5) and the mCherry transgene are both located in a terminal repeat, relatively close to one another in the circular genome. The kinetically modified promoter of the ICP22 gene is also located in a terminal repeat in our JDNI viruses and ICP22 was expressed more abundantly than the majority of viral genes in JDNI5ΔB-infected DRGs. However, this was not seen in JDNI8ΔB-infected DRGs. Together, these results indicated that the vhs deletion caused selective upregulation of mCherry expression in a cell-restricted manner without considerable changes in the global pattern of HSV gene expression.

Transgene Expression from the LAT Region in Neuronal and Non-neuronal Cells

We recently reported that GFP transgene expression from the CAG promoter (CMV immediate-early enhancer/chicken β-actin promoter/chimeric intron) was maintained in non-neuronal cells when the expression cassette was placed between two clusters of CTCF binding motifs flanking the LAT promoter in JDNI5; this vector was referred to as JDNI7GFP.18 We deleted the vhs gene from JDNI7GFP, creating JDNI8GFP (Figure 3A), to assess the effect on GFP expression. HDFs and rDRGs were infected with BAC-deleted ΔB JDNI7GFP or JDNI8GFP, GFP fluorescence from infected cells
was visualized at 7 and 14 dpi, and GFP mRNA levels were measured in the same cells. In HDFs, GFP fluorescence in \( \text{JAN}17\text{GFP}\text{ΔB} \)-infected cells was higher at both time points than in \( \text{JAN}18\text{GFP}\text{ΔB} \)-infected cells (Figure 3B), and mRNA levels were approximately 3-fold higher in \( \text{JAN}17\text{GFP}\text{ΔB} \)-than in \( \text{JAN}18\text{GFP}\text{ΔB} \)-infected cells at both 7 and 14 dpi, decreasing by ~25% (\( \text{JAN}17\text{GFP}\text{ΔB} \)) and ~35% (\( \text{JAN}18\text{GFP}\text{ΔB} \)) between the two time points (Figure 3C). In contrast, GFP fluorescence in rDRGs was higher at 7 and 14 days post-infection with \( \text{JAN}18\text{GFP}\text{ΔB} \) than with \( \text{JAN}17\text{GFP}\text{ΔB} \) (Figure 3D), and GFP mRNA abundance was 2.5-fold higher in \( \text{JAN}18\text{GFP}\text{ΔB} \)-than in \( \text{JAN}17\text{GFP}\text{ΔB} \)-infected cultures at 7 dpi, although a difference was no longer observed at 14 dpi (Figure 3E). While the increase in GFP expression in rDRGs caused by the \( \text{vhs} \) deletion was thus transient compared to the increase in mCherry expression in rDRGs noted earlier, these results indicated that the \( \text{vhs} \) deletion promoted transgene expression in neuronal cells independent of the promoter or location of the expression cassette within the viral genome. We confirmed that the disruption of the LAT locus and insertion of the GFP cassette did not noticeably alter the effect of the \( \text{vhs} \) deletion on mCherry expression (Figure S2). The reduced expression of GFP observed in HDFs highlights the cell-type dependence of the effect of \( \text{vhs} \) removal on transgene expression.

**Analysis of the \( \text{JAN}18\text{ΔB} \) Vector in Neurons In Vivo**

To determine whether our vectors were capable of non-cytotoxic transgene expression in neuronal cells in vivo, we injected \( \text{JAN}15\text{ΔB} \) and \( \text{JAN}18\text{ΔB} \) into rat hippocampus. We used \( \text{JAN}15\text{R0ΔB} \), an ICP0-rescued derivative of \( \text{JAN}15 \) (Figure S3), as a positive control for mCherry expression and vector-associated toxicity. At 7 days post-vector administration, \( \text{JAN}15\text{ΔB} \) showed relatively low mCherry expression and normal neuronal morphology (Figure 4B) that were maintained at 1 month (Figure 4E). In comparison, more robust mCherry expression was observed in \( \text{JAN}15\text{R0ΔB} \)-infected animals at 7 days (Figure 4A), but a significant loss of mCherry signal was observed after 1 month, and the morphology of the mCherry positive cells indicated substantial cytotoxicity (Figure 4D). \( \text{JAN}18\text{ΔB} \)-infected animals displayed enhanced mCherry expression in the hippocampus at 7 days compared to \( \text{JAN}15\text{ΔB} \) (Figure 4C), and only a modest reduction in mCherry signal was apparent at 1 month without overt change in cell morphology (Figure 4F). As proposed to \( \text{JAN}15\text{R0ΔB} \)-infected hippocampi, cells in \( \text{JAN}18\text{ΔB} \)-infected hippocampi maintained a clear neuronal morphology with many dendritic and axonal elongations. We confirmed that \( \text{JAN}18\text{ΔB} \) transgene expression was restricted to neurons by double-label immunofluorescence for mCherry and markers of neurons (Nissl, astrocytes (GFAP), and oligodendrocytes (O4) (Figure S4). Quantification of mCherry expression in the hippocampus (Figure 4G) demonstrated that, in \( \text{JAN}15\text{ΔB} \)-infected animals, only approximately 2% of the pixels were mCherry positive at both time points, whereas some 12% were positive in \( \text{JAN}18\text{ΔB} \)-infected animals at 7 days with only a modest reduction to approximately 7% by 1 month. The \( \text{JAN}15\text{R0ΔB} \) injection yielded about 15% mCherry positive pixels at 7 days, but this fell to less than 2% by 1 month. Both the loss of mCherry positive cells and the changes in cell morphology in the \( \text{JAN}15\text{R0ΔB} \)-infected animals were consistent with vector cytotoxicity that was not observed in animals injected with either of the ICP0-deficient vectors.

To further assess toxicity in rat brain, Fluoro-Jade C staining was performed on brain sections at 7 days post-vector administration. Fluoro-Jade C stains degenerating neurons, regardless of the cause of degeneration, and provides a clear indication of cytotoxicity. In \( \text{JAN}15\text{R0ΔB} \)-infected animals, many green, Fluoro-Jade C-positive cells were observed, whereas no signal was detected in either \( \text{JAN}15\text{ΔB} \) or \( \text{JAN}18\text{ΔB} \)-infected animals (Figures 5A–5C). We also examined the expression of CD45 as a marker for the accumulation of inflammatory cells in the injected area. Many CD45 positive cells accumulated near the mCherry positive cells in \( \text{JAN}15\text{R0ΔB} \)-infected animals, while only a few CD45 positive cells were observed in either \( \text{JAN}15\text{ΔB} \)– or \( \text{JAN}18\text{ΔB} \)-infected animals (Figures 5D–5F). Similarly, both caspase-3, an apoptotic marker (Figures 5G–5I), and NOS-2, an inducible nitric oxide synthase involved in the immune response (Figures 5J–5L), were induced by \( \text{JAN}15\text{R0ΔB} \) injection, but not by either \( \text{JAN}15\text{ΔB} \) or \( \text{JAN}18\text{B} \) injection. Quantification of these data is shown in Figure 5M. Taken together, these observations confirmed that the presence of ICP0 results in neuronal cytotoxicity and apoptosis in the hippocampus, along with activation of inflammatory cell recruitment. Deletion of ICP0 reduced these negative consequences although transgene expression was also reduced. Importantly, removal of the \( \text{vhs} \) gene from the ICP0-deficient vector resulted in enhanced transgene expression in the hippocampus without toxicity.

**DISCUSSION**

HSV vectors offer the distinct advantage that they can accommodate large payloads; for example, our current replication-defective backbones offer approximately 30 kb of usable space. This makes HSV an ideal vector for gene therapy requiring the expression of large or multiple transgenes. Given HSV’s ability to enter and establish latency in sensory ganglia, current gene therapy studies using HSV have focused on the peripheral nervous system, treating conditions such as chronic pain and neuropathy. Establishing a platform for replication-defective gene delivery to the CNS has been hampered by problems including early silencing of CMV-promoter driven transgenes and limited, low-level transgene expression from other promoters such as the viral latency promoter. The level of transgene expression is intricately tied to expression of the viral ICP0 protein: while ICP0 increases transgene expression, it also increases apoptosis in the hippocampus, along with activation of inflammatory cell recruitment. Deletion of ICP0 reduced these negative consequences although transgene expression was also reduced. Importantly, removal of the \( \text{vhs} \) gene from the ICP0-deficient vector resulted in enhanced transgene expression in the hippocampus without toxicity.

We recently generated an ICP0-deficient vector, \( \text{JAN}15 \), that is nontoxic and allowed transgene expression from an ectopic promoter in multiple non-neuronal cell lines and rDRGs in vitro when the transgene cassette was inserted between CTRLs associated with the viral latency locus. Consistent with an overall silencing of expression from the viral genome, a UbC-promoter-driven second transgene present in the deleted ICP4 locus was not expressed in non-neuronal cells. Surprisingly, however, it was expressed in DRGs. This
Figure 4. Rat Hippocampal Transduction by \( \Delta N I 5 R 0 A B, \Delta N I 5 A B, \) and \( \Delta N I 8 A B \)

2 \( \times \) 10^5 gc of either vector was injected in the rat hippocampus, and DAPI (blue) and mCherry fluorescence (red) were imaged at 7 days (A–C) or 1 month (D–F) after injection. Upper images for each time point show a section of the injected hippocampus; bottom images are enlargements of the boxed areas. Scale bars in (A) and (D) upper panels (for all upper panels), 500 \( \mu \)m; scale bars in (A) and (D) lower panels (for all lower panels), 100 \( \mu \)m. (G) Quantification of mCherry expression in the hippocampus of rats injected with \( \Delta N I 5 R 0 A B \) (red), \( \Delta N I 5 A B \) (orange), or \( \Delta N I 8 A B \) (green) at 1 week (1w) or 1 month (1m) after vector inoculation. Data are means \( \pm \) SEM (n = three to five animals/group, five sections/animal). *p < 0.05; **p < 0.01 (ANOVA and post hoc Kruskal-Wallis test).
Figure 5. Vector Toxicity in Rat Hippocampus

2 × 10^7 genome copies of JANi5ROΔB, JANi5ΔB, or JANi8ΔB were injected into rat hippocampi, and brain sections were collected and processed 7 days later. (A–C) Fluoro-Jade C (FJC)-stained hippocampal sections from JANi-injected animals. Representative merged images of FJC staining (green), DAPI staining (blue), and mCherry fluorescence (red) are shown. (D–L) Immunohistochemical staining of brain sections for CD45 (D–F), caspase-3 (G–I), and NOS-2 (J–L) (all in green) is shown as merged images with DAPI staining (blue) and mCherry fluorescence (red). (M) Quantification of FJC, CD45, caspase-3, and NOS-2 in the hippocampus of rats 1 week after injection of JANi5ROΔB, JANi5ΔB, or JANi8ΔB. Data are means ± SEM (n = three to five animals/group, five sections/animal). *p < 0.05; **p < 0.01 (ANOVA and post hoc Kruskal-Wallis test).
observation prompted our current efforts to examine the potential of this vector and its vhs-deficient counterpart, JΔNI8, for CNS applications. The results indicated that both vectors are safe in the brain and express their transgene at detectable levels from the ICP4 locus for at least 1 month but also that JΔNI8 provides the more robust expression profile of the two.

Vhs plays a key role in attenuating the host anti-viral response by mediating mRNA degradation and inhibiting host protein synthesis. After virus entry into the cell through membrane fusion, vhs is present in the virion tegument and released into the cytoplasm where it causes the shutdown of host protein synthesis. Available evidence suggests that vhs-induced host shutoff action contributes to the regulation of viral gene expression, in part by removing competition with cellular mRNAs for translation factors and sharpening the transition between the kinetic classes of viral genes, thereby supporting efficient virus replication during the lytic cycle. However, the functional significance of vhs in cell culture appears to vary between host cells. For example, U2OS cells were reported to be permissive for replication of a nonsense mutant of vhs. In contrast, other reports, a nonsense mutant of vhs was highly toxic to Vero and NIH3T3 mouse embryo cells, while a severe growth defect in human fibrosarcoma HT1080 cells was displayed in vhs mutant vhs113. These observations clearly indicate that additional deletion of vhs from the viral genome may further enhance the transcriptional activity of this genomic location in neuronal cells. While other repeat-associated genes were also expressed at higher levels in DRGs than in HDFs, than genes within the unique regions of the viral genome in DRGs, vhs deletion did not increase their expression (γ,34,5) and in fact reduced ICP22 mRNA abundance. The effect of vhs removal on transgene expression from the terminal repeat-based LAT locus was also not dramatic. Expression in neurons was modestly enhanced by vhs deletion, but this effect was not as robust, nor was it maintained as long as at the ICP4 locus. In HDFs, vhs removal led to reduced reporter gene expression from the LAT locus and the already low levels of viral mRNAs appeared to trend toward further reduction. Together, these observations suggest distinctly separate utilities for the JΔNI5 and JΔNI8 vectors with respect to the target cell for transgene expression; the ICP4 locus of JΔNI8 is favored for expression in neurons, while the LAT locus of JΔNI5 is preferable for expression in non-neuronal cell types.

The key event differentiating between JΔNI5 and JΔNI8 infection would appear to be the initial introduction of vhs protein into non-complementing cells as a component of the JΔNI5 virion as (1) vhs protein is synthesized and incorporated into infectious particles during JΔNI5, but not JΔNI8 virus production by ICP4/ICP27-complementing U2OS cells, and (2) de novo expression of viral genes is
extremely low in neurons infected with our vectors. However, we observed expression differences between the two vectors out to at least 14 days in vitro and 1 month in vivo, long after the vhs protein is degraded. As the incoming HSV genome is not epigenetically modified and its subsequent silencing is controlled by cellular and viral factors upon entry into the host cell, it is conceivable that the initial exposure of ΔN15-infected neurons to vhs indirectly affects the epigenetic status of the mCherry locus, causing persistent downregulation of the gene. While further studies are required to test this and other possibilities, our current results indicate that vhs deletion may be an attractive feature for highly defective HSV gene therapy vectors for nervous system disorders.

MATERIALS AND METHODS

Cells

HDFs (PCS-201-010; ATCC) were cultured in DMEM (Lonza) with 10% (v/v) fetal bovine serum (FBS; Sigma) and penicillin-streptomycin (P/S). Vero-based ICP4/ICP27-complementing 7b cells (Vero-7b) were cultured in DMEM with 5% FBS and P/S. Primary fetal rDRGs were isolated and cultured as described previously.18 U2OS-ICP4/27 cells were grown in DMEM with 10% FBS and P/S in the presence of puromycin (2 μg/mL) and blasticidin (10 μg/mL), and U2OS-ICP4/27/Cre cells were maintained in DMEM with 10% FBS and P/S in the presence of puromycin, blasticidin, and hygromycin (200 μg/mL), as described previously.18

HSV-BAC Engineering

All BAC engineering was performed in E. coli strain GS1783 as described.18 ΔN18 and ΔN18GFP BAC constructs were derived, respectively, from ΔN15 and ΔN17GFP BACs.18 To delete the vhs (U74) coding sequence (GenBank JQ673480, positions 91,088–92,557), the kanamycin selection marker, I-SceI-aphAI, was amplified from pEPkan-S2 by PCR with primers 5'-tataactgttgctgtggttg tgaagaacagccctgtatgatgtgtgtcagccgagagtcgacagcataagaggata-3' and 5'-aattctgaggggacccctctactaatttggtgcttttgaagggag-3'. The PCR product was gel-purified and recombined with the vhs gene of ΔN15 BAC or ΔN17GFP BAC, followed by removal of the aphA1 gene.

ΔN15R0 was constructed as follows: KOS-BAC DNA was digested with DraI and PstI to isolate a fragment containing the complete ICP0 gene. The ICP0 fragment was cloned into the pCR-Blunt vector using the Zero Blunt PCR cloning kit (Thermo Fisher Scientific), creating pCRBlunt-ICP0. The I-SceI-aphAI fragment was amplified by PCR with primers (5'-tataactgttgccacccgtcctgtcagccgagagtcgacagcataagaggata-3' and 5'-aattctgaggggacccctctactaatttggtgcttttgaagggag-3'). The product was inserted into the Mfi site of pCRBlunt-ICP0, creating pCRBlunt-ICP0-KAN. The pCRBlunt-ICP0-KAN plasmid was digested with EcoRI and PstI, and the isolated ICP0-KAN fragment was used for recombination with ΔN15 BAC DNA. All HSV-BAC modifications were confirmed by field inversion gel electrophoresis (FIGE) analysis of restriction enzyme digests (FIGE mapper; Bio-Rad), PCR analysis, and targeted DNA sequencing.

Viruses

Production of infectious virus from BAC recombinants, Cre-mediated BAC excision, and virus titration were performed as described previously.18 ΔN1 viruses were grown in U2OS-ICP4/27 cells, the KOS virus stock was produced on Vero cells, and QOZH virus were grown in Vero-7b cells.

Virus Growth Curves

Duplicate wells of 4 × 10^5 U2OS-ICP4/27 cells were infected with ΔN1 viruses at 1 gc/cell for 2 hr at 37°C, treated with 0.1 M glycine (pH 3.0) for 1 min to inactivate extracellular virus, and incubated at 37°C and 5% CO2. The supernatants were collected daily for viral DNA extraction and titration by qPCR for the gD gene, as described.18

Cytotoxicity Assay

Cell viability was measured essentially as described.45 Briefly, dissociated rDRGs were plated in a 24-well plate (9 × 10^4 cells/well) and infected with KOS or ΔN1 viruses at 3,000 gc/cell. Cell viability was determined 5 days later by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

qRT-PCR and Genomic qPCR

HDF and rDRG cells were plated in 24-well plates, and duplicate wells were infected 24 hr later with ΔN1 viruses at MOIs of 25,000 gc/cell or 3,000 gc/cell, respectively. Total RNA was extracted, and RT was carried out by Cells-to-cDNA II Kit (Ambion). Real-time PCR for each sample was performed in triplicate using the StepOnePlus Real-Time PCR System (Applied Biosystems). The data were normalized to viral gc in the same samples determined by qPCR for the gD gene or (Figure 2D) using separate ΔN15 BAC DNA standard curves generated for each gene with the gene-specific primer pairs used for cDNA qPCR to allow for mRNA(gc)/cell measurements between genes and across host cells. All primers for qPCR were as previously reported.18 The gc titers of virus stocks and nuclear viral DNA copy numbers were determined as described.18

In Vivo Experiments

Under ketamine (90 mg/kg i.p.) and xylazine (13 mg/kg i.p.) anesthesia, 2 × 10^9 gc of ΔN1 vectors were inoculated into the right dorsal hippocampus of male rats (Harlan Sprague Dawley, 300 g). The viral vectors (ΔN15R0, ΔN15, and ΔN18) were injected by stereotactic implantation of a borosilicate glass needle linked to a microperfusion pump in a volume of 3 μL at a flow rate of 200 nl/min. In order to facilitate the needle entry into the brain tissue and to reduce the mechanical damage, the needle tip had been laser-chamfered (inner diameter at tip = 60 μm) using Leica Laser Microdissector CTR6000 (Leica Microsystems). The stereotactic coordinates for the hippocampal inoculation, based on the Paxinos atlas, were 2.1 mm lateral and 3 mm posterior to bregma, 3.5 mm deep from dura. Experiments were performed at the University of Ferrara in compliance with the university’s guidelines for the ethical treatment of experimental animals.
Tissue Preparation
Animals were anesthetized with pentobarbital at 7 or 30 days post-virus injection and perfused with 0.1 M PBS followed by 4% paraformaldehyde. The brains were quickly removed and post-fixed in 4% paraformaldehyde for 1 hr, cryoprotected in 30% sucrose at 4°C until the tissue sank, and snap frozen in isopentane at ~80°C.

Immunofluorescence
20-μm coronal cryostat sections were cut at ~20°C on a freezing microtome, rinsed in 0.1 M PBS for free-floating immunostaining, and blocked with 10% normal goat serum/0.3% Triton X-100. Sections were then incubated overnight at 4°C with primary antibodies dissolved in blocking solution as follows: CD45 (Santa Cruz Biotechnology, sc-53047), 1:100; cleaved caspase-3 (Cell Signaling Technology, 9664), 1:100; nitric oxide synthase-2 (Santa Cruz, sc-651), 1:100; mCherry (Thermo Fisher Scientific), 1:100; GFAP (Sigma), 1:200; O4 (Sigma), 1:100. After washing in PBS, sections were incubated in blocking solution with 10 μM DAPI (Molecular Probes, D3571) and either Alexa Fluor 488 goat anti-rabbit (Molecular Probes, A11008, 1:1,000) or Alexa Fluor 488 goat anti-mouse (Molecular Probes, A11029, 1:1,000) for 1 hr at room temperature.

Fluoro-Jade C Staining
Brain sections were mounted on frosted microscope slides (Thermo Fisher Scientific) and pretreated for 5 min in 80% EtOH/1% NaOH, 2 min in 70% EtOH, and 2 min in distilled water. Sections were then incubated for 10 min in a 0.06% KMnO4 solution and rinsed in distilled water for 3 min before incubation in a 0.0001% solution of Fluoro-Jade C (Immunological Sciences, IS-0012) in 0.1% acetic acid for 10 min. After three washes in distilled water for 1 min, slides were dried, dehydrated with xylene, and coverslipped with DPX mountant (Sigma) for microscopy.

Hippocampal Image Quantifications
Brain sections were imaged using a DMRA2 Leica microscope (Leica Microsystems) and Hamamatsu C11440 camera. Confocal images were captured using a HAL 100 camera (Zeiss) mounted on a Zeiss LSM510 confocal microscope. Imaged mCherry expression was quantified with MetaMorph (Universal Imaging). The entire hippocampus was selected as the region of interest (ROI). mCherry-positive pixels were identified by thresholding at the gray level corresponding to the mean plus the difference between average and minimum. Using this approach, only those pixels that were significantly above background (i.e., mCherry-positive) were selected. The percentage of mCherry-positive pixels in the hippocampus was calculated as the ratio of pixels above threshold to total pixels in the ROI.

A similar procedure was employed for quantification of FJC, CD45, caspase-3, and NOS-2 signals. An outline was drawn around five positive cells, and mean gray values were measured and averaged; the resulting value was used to set the image threshold. Pixels above the threshold were identified, as described above, and the number of positive pixels was expressed as the percentage of total pixels.

For each injected animal, the number of mCherry-, FJC-, CD45-, caspase-3-, and NOS-2-positive pixels was quantified from five regularly interspaced sections spanning across the injection site (one of every five 20-μm sections cut across the injection site, i.e., one per 100 μm with the third at the site of injection), and the average per animal (three to five animals/group) was determined. These averages were used for statistical analysis.

Statistical Analyses
Data from in vitro experiments are presented as the mean ± SD. Data from in vivo experiments were analyzed by the Kruskal-Wallis test to determine significance.

SUPPLEMENTAL INFORMATION
Supplemental Information includes four figures and can be found with this article online at http://dx.doi.org/10.1016/j.omtm.2017.06.001.

AUTHOR CONTRIBUTIONS
Conceptualization, Y.M.; Investigation, Y.M., G.V., F.H., H.U., S.Z., and W.F.G.; Formal Analysis, Y.M., G.V., B.R., M.S., J.B.C., and J.C.G.; Supervision, M.S., J.B.C., and J.C.G.; Writing – Original Draft, Y.M.; Writing – Review & Editing, B.R., J.B.C., and J.C.G.; Funding Acquisition, W.F.G., M.S., J.B.C., and J.C.G.

CONFLICTS OF INTEREST
G.V., M.S., and J.C.G. are founders of NuvoVec srl. Y.M., J.B.C., and J.C.G. are co-inventors of intellectual property licensed to SwitchBio, Inc. H.U., J.B.C., and J.C.G. are co-inventors of intellectual property licensed to Oncorus, Inc. J.C.G. is a founder and consultant of SwitchBio, Inc. and Oncorus, Inc. B.R. and W.F.G. are consultants of Oncorus, Inc.

ACKNOWLEDGMENTS
We are grateful to Klaus Osterrieder (Free University of Berlin, Germany) for plasmid pEPkan-S2, Greg Smith (Northwestern University) for E. coli strain GS1783, David Leib (Dartmouth Medical School) for KOS-37 BAC DNA, Mingdi Zhang for rat DRG cultures, and Jim Smiley (University of Alberta, Canada) for helpful comments on the manuscript. This work was supported by grants to J.C.G. from the NIH (NS064988 and DK044935), the CHDI Foundation (A3777 and A8790), and the Commonwealth of Pennsylvania (SAP #4100061184) and to M.S. from the European Community (FP7-PEOPLE-2011-IAPP project 285827 [EPIXCHANGE]) and the Italian Ministry for Education, University and Research (PRIN project 2010N8PBAA [INBDNF]).

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