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In vivo gene transfer to the brain cortex using a single injection of HSV-1 vector into the medial septum

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This study shows that an ICP4– replication-deficient herpes simplex virus containing the Moloney murine leukaemia virus LTR fused with the coding sequence for the β-galactosidase gene can be used as a very effective vector for delivering the β-galactosidase reporter gene into the rat brain septum. F344 rats received bilateral stereotaxic injections into the nucleus of the diagonal band and into the medial septum. The X-gal stain was used to detect the activity of the expressed β-galactosidase enzyme. The delivered reporter gene was expressed successfully not only in the neuronal cells of the injected areas but also in cells that project to the injection area such as cortex cells about 6mm away from the injection sites. Expression was visible at 1, 3 and 9 weeks following injection. We conclude that this vector can effectively deliver genes into different regions of the mature mammalian brain and also to areas distant from the injection site.

Key words: herpes simplex virus, gene therapy, β-galactosidase, Alzheimer's disease

INTRODUCTION

There are two general approaches to the transfection of genes, the *in vivo* and the *ex vivo* [2]. The *in vivo* approach is more direct and does not depend on the success of the cell culture or the subsequent survival of transplanted cells. However, it does require a highly efficient method for gene transfer, which is not an easy procedure, especially in the brain and other neuronal tissues. The presence of the blood-brain barrier tends to preclude vascular delivery and the principally post-mitotic neuronal population prevents the use of retroviruses [36]. The herpes simplex virus (HSV-1) has the ability to establish life-long latent infections within neurons and may therefore be considered as a vector for delivering biologically relevant peptides to the nervous system [11]. The herpes simplex virus does exhibit a neuronal specificity for establishing a latent infection and during acute infections it has the ability to infect a wide range of cell types. This fact, coupled with the ability of HSV-1 to make replication-deficient viruses (ICP4–), opens up the potential for other therapeutic applications. Therefore, the use of HSV-1, in particular, as a vector for delivering and expressing foreign genes within the nervous system is an attractive option because it is a neurotrophic virus possessing a sophisticated genetic program that allows it to spend most of its life cycle within the nervous system [30, 37].

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HSV-1 can enter neurons at the periphery and travel via axonal transport to the cell bodies of the sensory ganglia, where it becomes latent [30, 37]. In addition, normal HSV-1 that can replicate in cells has the capacity to travel transynaptically to anatomical locations distant from its original site of infection. During latency the 152 kb double-stranded DNA genome of the virus persists as circular episomes within the nuclei of neurons. The ability of this virus to co-exist naturally within the nervous system without causing disease or provoking the immune system makes it an ideal vector. Key advantages of HSV-1 vectors are that they establish life-long latent infections within neurons and the genomes exist as multiple episomal copies per neuron without integration being known to occur [27]. Because non-replicating HSV-1 recombinants can establish a latent infection efficiently, safe attenuated vectors for human gene therapy can be constructed [29]. There is a great deal of interest in these recombinants containing foreign genes because they can be constructed rapidly and the genome can accept large inserts of DNA, making the construction of vectors which express multiple therapeutic genes feasible [12, 14]. To date a number of recombinants have been generated expressing reporters such as b-galactosidase [1, 6, 7, 13], glucouronidase [38], tyrosine hydroxylase [8] and nerve growth factor (NGF) [9, 22].

An early experiment using stereotaxic injections of replication-competent HSV-1 with β-galactosidase-expressing lacZ reporter gene into the hippocampus and caudate of the rat brain resulted in limited and transient viral replication and the establishment of latency with transient lacZ expression detected in the dentate gyrus [13]. These early HSV-1 constructs showed transient high level expression, although this expression declined rapidly with time. This may be ideal for some applications. Other applications, on the other hand, may need long-term expression. This stable long term expression, however, has been elusive. Since, with the exception of the latency-associated transcript (LAT) [31], latent infections of HSV-1 are characterised by the absence of viral transcription, LAT promoter would be the ideal candidate for the expression of foreign genes during latency. However, recombinant viruses containing the genes for nerve growth factor and β-galactosidase driven by LAT promoter express β-galactosidase and NGF RNA at high levels initially but not during the latent infection [24]. Other promoters have been investigated. The LAP2 (a weaker promoter downstream of LAT promoter) was shown to be insufficient and caused a dramatic drop off of expression of some neuronal housekeeping genes [4]. However, the Moloney murine leukaemia virus (MoMuLV) LTR has been demonstrated to afford long-term expression of β-galactosidase [5, 7] in the context of LAT promoter [20]. This combination of LAT-core promoter and MoMuLV LTR allows extended expression of transgenes at high levels within the sensory neurons of the peripheral nervous system [22], yet only minimal levels of sustained expression within the CNS [5]. Recently the native region of the HSV-1 genome that may allow sustained expression has been further localised to an element downstream of LAT termed LTE (long-term expression) [3, 19]. In addition, there is evidence that a region upstream of the LAT core promoter may play a role in enhancing long-term expression properties [24], and this enhancing effect may be directed at maintaining the chromatin HSV-1 containing LAT region in a transcriptionally permissive conformation [18].

In the non-replicating virus the major viral transactivator gene (infected cell protein 4; ICP4 deleted) and the resulting virus must be propagated on a helper cell line that provides the ICP4 gene transfer. This vector contains the E. coli LacZ gene driven by LAT/MoMuLv LTR promoter [7].

The data presented from this project not only evaluated the *in vivo* expression of the reporter gene (lacZ) within the rat CNS using the replication-defective HSV-1 viral vector, but also showed that inoculation of the septal region with HSV-1 vector provided gene delivery to several regions of CNS using a single injection per hemisphere.

MATERIAL AND METHODS

Defective HSV-1 viral vector

The ICP4– replication-deficient virus recombined with the Moloney murine leukaemia virus LTR downstream of HSV LAT promoter were fused with the coding sequence for the β-gal gene to produce a ICP4--LAT/MoMuLVLTR/β-gal cassette. The viruses were prepared and propagated in E5 cells. The final viral preparation was determined by two-step titration analysis on E5 versus RS cells with less than 1 revertant per 5×10^6 plaque forming units (PFU)/ml [5]. The HSV 8117/43 construct utilised in this experiment was created by deleting the ICP4 gene from the non-replicating HSV vector and inserting the Moloney murine leukaemia virus (MoMuLV) long terminal region (LTR) promoter, a gene essential for replication, which is a transcription unit that replaces both

copies of the ICP4 gene [7, 21, 29]. Briefly, the construct 8117/43 contains the E. coli *lacZ* reporter gene driven by the MoMuLV LTR in place of the deleted ICP4. It also has a 2.3 kb BstEII deletion in the LAT (latency associated transcript) 5′ exon/intron region. The control construct, KD6, contains the same deletion but lacks the *lacZ* insert. Both KD6 and 8117/43 were propagated on E5 complementing cell line, a generous gift of N. DeLuca. Infected cells were harvested and concentrated by centrifugation at 10,000 × g (30 min at 4°C) and resuspended in 1/100 the original cell culture volume in MEM with 10% FBS. The vector was released from the infected cells and subjected to 2 rounds of freeze-thawing. The stocks were clarified by centrifugation for 2 minutes at 10,000 × g and the supernatant aliquoted and frozen at –80°C. Stocks were titrated on complementing E5 cells and the number of ICP4+ revertants determined by passage and titration on RS cells (non-permissive for ICP4–). All stocks used in this study had < 1 revertant per 1 × 10^6 PFU of ICP4– plaques (as determined on E5 cells).

Animal groups and stereotactic inoculation of HSV-1 vector to the rat septum (NDB and MS)

Fischer 344 rats (250 g) were randomly divided into two groups:

— I — HSV-1/β-gal experimental group, injected with 4 μl/site solution of ICP4–LAT/MoMuLVLTR/β-gal HSV-1 vector;

— II — control groups, injected with 4 μl/site solution consisting of either viral vector 4 × 10^6 PFU HSV-1 lacking the β-galactosidase reporter gene or glucose control.

All solutions were stereotactically injected (1 μl/min) to the septal region of the rat CNS using a 100 μl Hamilton syringe. Each rat was injected bilaterally in NDB first (2 μl). The needle was then pulled out 1 mm and the rest of the injection solution injected at MS. Adjustment off the bregma was as follows: MS and NDB: AP = +0.20, ML = ± 0.30, DV = –7.80 (NDB) –6.80 (MS). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC).

Tissue preparation

At different post-injection time points (1, 3, and 9 weeks) the rats were anaesthetised and then perfused intracardially with 100 ml of phosphate-buffered saline (PBS, 100 mM phosphate) pH 7.4, followed immediately with 150 ml fresh 4% paraformaldehyde. Brain tissues were cut into 1 mm sections, post-fixed for one hour at 4°C with β-gal tissue fixative (the pH of all the solutions used was above 7.2 to prevent detection of the acidic endogenous β-gal enzymes). Finally, the post-fixed 1mm sections were stained with X-gal staining solution for 7 hours. After being photographed the tissues were sectioned into 50 μm slices using a freezing stage microtome and then mounted on slides, counter-stained with Eosin Y, dehydrated with alcohol and cover slipped.

PCR detection of HSV-1 DNA

HSV-1 DNA was detected in dissected blocks of tissue (approx. 1–2 mm^2) as follows. After the removal of the brain from the skull, the brain was chilled to near freezing. With the use of different razor blades for each section the brain was sectioned into 1mm coronal slabs. Specific anatomical brain regions were identified using a brain atlas and were dissected. The frontal cortex sample (FCX) included both the parietal and occipital cortex. The BST sample included the pons and medulla but excluded the midbrain region. The tissue samples were snap frozen following dissection and the tissue was homogenised in 1.5 ml Eppendorf tubes using a mini bead-beater (Biospec Products, Bartlesville, OK) in 1.2 ml of Trizol (Life Technologies, Gaithersburg, MD) with 100 μl of 1.0 mm beads for 30 s (2 × with a 15 s pause between sets). Chloroform (240 μl) was then added, followed by vortexing. Samples were incubated for 5 min at room temperature and then microfuged (10,000 g) for 15 min at 4°C. The DNA was extracted from the interface with Tris + 0.1% Sarkosyl (3 × with 140–150 μl). Proteinase K (0.1% μg/μl) was then added and samples incubated overnight at 37°C. The next day DNA was extracted with phenol/chloroform/isoamyl followed by chloroform/ /isoamyl. The DNA was then ethanol precipitated (9 μl 5 M NaCl and 2.5 vol of ethanol) and resuspended in 25 μl of dH_20. For PCR analysis 5 μl of a 1:5 dilution of reaction buffer was used. The reaction buffer contained 5 μl of 10 × PCR buffer, 1 μl cold dNTPs (12.5 mol), 1 μl of primer 1, 1 μl of primer 2, 40.5 μl dH_20, 1 μl hot nucleotide (1:10 dilution of alpha ^{32}P in dH_20) and 0.5 μl Taq. The primers used were specific for HSV DNA polymerase gene (Pol): primer 1 = 5′ CAT-CAC-CGA-CCC-GGA-GAG--GGA-C and primer 2 = 5′ GGG-CCA-GGC-GCT-TGT--TGG-TGT-A. Conditions used for PCR were 1 × 94°C, 68°C, 72°C, 3 min each then 30 × 94°C, 68°C, 72°C, 1 min each. Following PCR samples were electrophoresed on a 7.5% acrylamide gel, stained and scanned. The DNA band was scanned with the

Molecular Dynamics Phosphorimager and quantified using ImageQuant software.

RESULTS AND DISCUSSION

The HSV-1 vector can clearly be used to introduce genes into different regions of the mature mammalian brain *in vivo* [20], with successful transfection of HSV-1 viral vector into the rat basal forebrain, hippocampus, *substantia nigra* and ovine corneal endothelium previously reported [5, 17, 21, 35]. Here we show transfection into various regions of the brain following injections into the septal region using ICP4– replication-deficient virus containing the Moloney murine leukaemia virus LTR located downstream of HSV latency-associated transcript (LAT) promoter fused with the coding sequence for lacZ gene (ICP4– LAT/MoMuLVLTR/β-gal cassette). *In vivo* transgene expression of β-gal in CNS following bilateral injection to the septal region using this virus was detected by the X-gal reaction (Figs. 1A, C). As a control for either non-transgenic endogenous β-gal or simply non-specific staining, glucose injections were tested and showed no staining (Figs. 1B, D). The specificity of the X-gal staining was confirmed by staining 50 μm thick sections with anti-β-gal antibody and reflected a similar pattern of staining (data not shown). We observed strong expression of the transferred lacZ gene at the injected septal areas at one week (Figs. 1A, C) and three weeks following injection (Fig. 2A). Expression at week 9 was reduced in comparison with that at the week 1 and week 3 time points but was still visible near the needle tracts (Fig. 2B).

In addition to the septal transfection, we detected the expression of β-gal throughout several other regions of the whole brain, as can be seen in the serial sections (Fig. 3). Clearly ICP4– replication-deficient protein was expressed at significant distances from the injection site (up to 6 mm away) in numerous regions that have bidirectional connections with the septum. β-gal activity was detected in cortical areas including the frontal, insular, orbital, piriform, olfactory, and retrosplenial regions. Some staining can also be seen in the dentate gyrus, hypothalamus and raphe. Higher power views

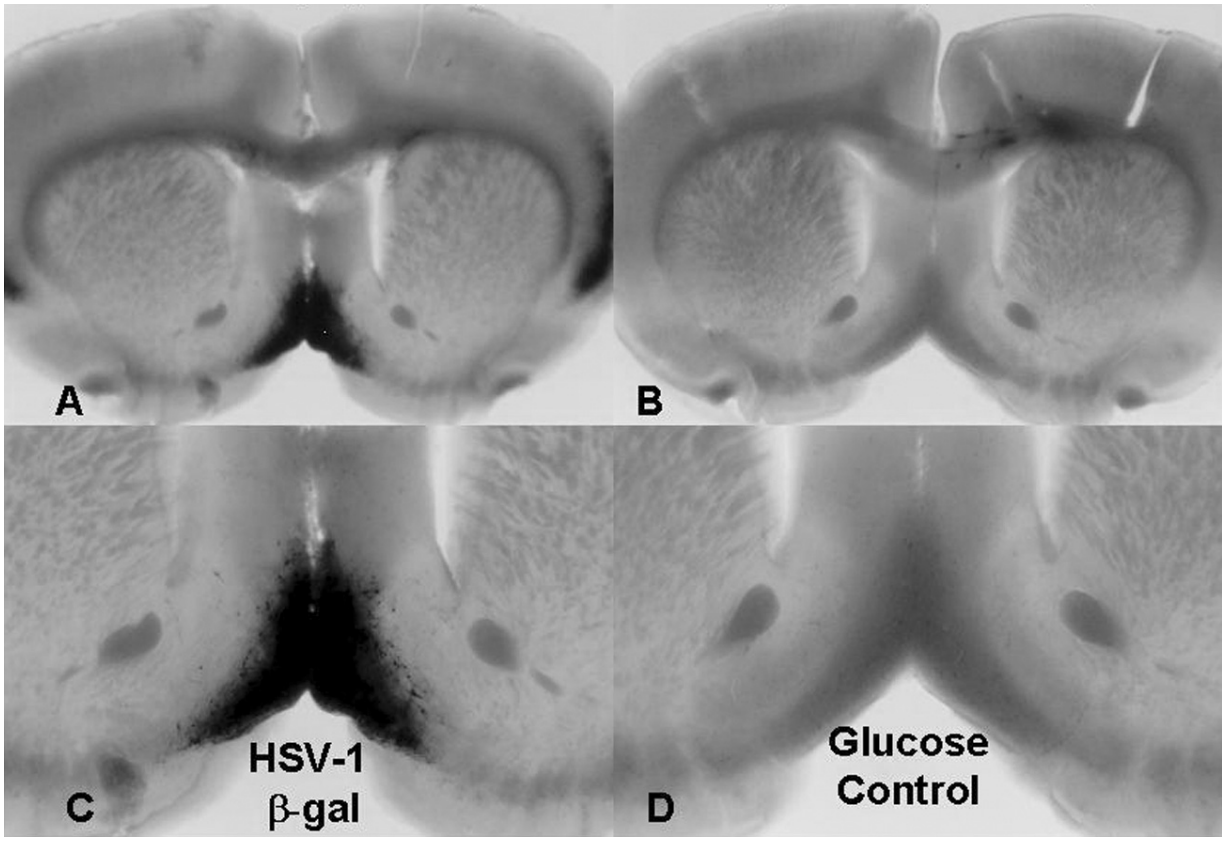

Figure 1. Coronal sections of X-gal stained rat brain following bilateral stereotaxic ICP4–LAT/MoMuL/LTR/β-gal HSV-1 vector injections (AP = +0.20, ML = ± 0.30, DV = –7.80 to –6.80 mm bregma), shown at the level of the septum. Lower panels (**C** and **D**) represent magnification of the septal region. Panel **A** and **C** show strong expression of β-gal activity detected at the injection sites in ventral portion of the septum. Panels **B** and **D** show no β-gal activity detected in the glucose injected control.

of the stained cells are shown in Figure 4, (20 × cortical areas), Figure 5 (20 × septal area) and Figure 6 (40 × septal area). The medial septum (MS) and the nucleus of the diagonal band (NDB) are major parts of the acetylcholinesterase/cholinergic projection system in CNS [25]. These project extensively to the hypothalamus and the hypothalamic innervation extends to the medial and lateral preoptic areas, the anterior, dorsomedial, and ventromedial nuclei, the lateral hypothalamic area, and the supramammillary and mammillary nuclei. The medial septum also projects to the ventral tegmental area and the raphe nuclei [33]. This could partially explain some of the pattern if transfection can jump to these cells trans-synaptically. However, this should not be the case since we are using a replication-defective virus. The staining pattern in the cortical areas is more likely to be the result of infection of the axon terminals of the cells projecting to the septum. The hypothalamus is known to have extensive projections to the septum [28] and the hippocampal areas, including CA3, also have projections to the caudal part of the lateral septal nucleus [26, 34]. The septum does receive glutaminergic input from the frontal cortex [15] and dopaminergic input from the raphe [32].

In order to verify whether the β-gal activity detected in the neuronal perikarya outside the septum was expressed from the virus harboured by those neurons,

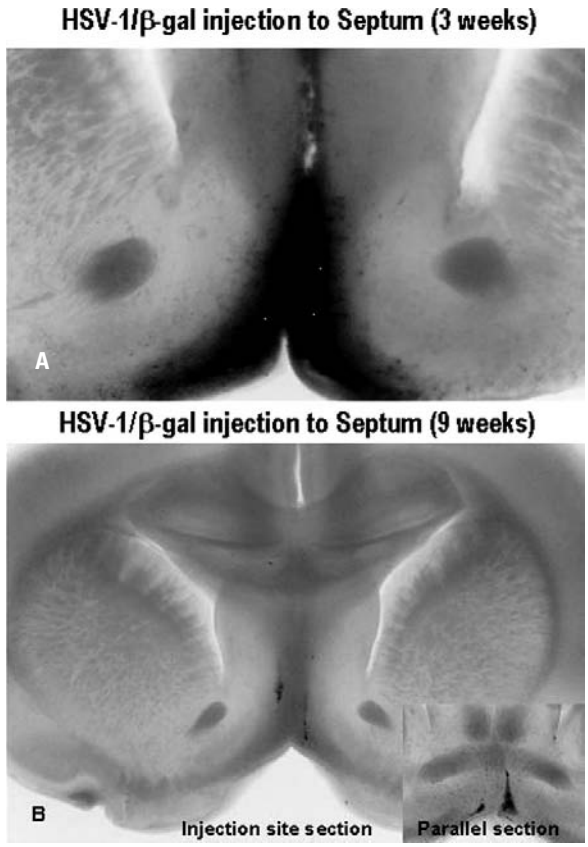

Figure 2. Coronal sections of X-gal stained rat brain following bilateral stereotaxic injections (AP = +0.20, ML = ± 0.30, DV = –7.80 to –6.80 mm bregma) shown at the level of the septum at 3 weeks following injection (panel **A**) and 9 weeks following injection (panel **B**). The staining is very robust at 3 weeks but is only visible in the immediate areas around the injection sites at 9 weeks following injection.

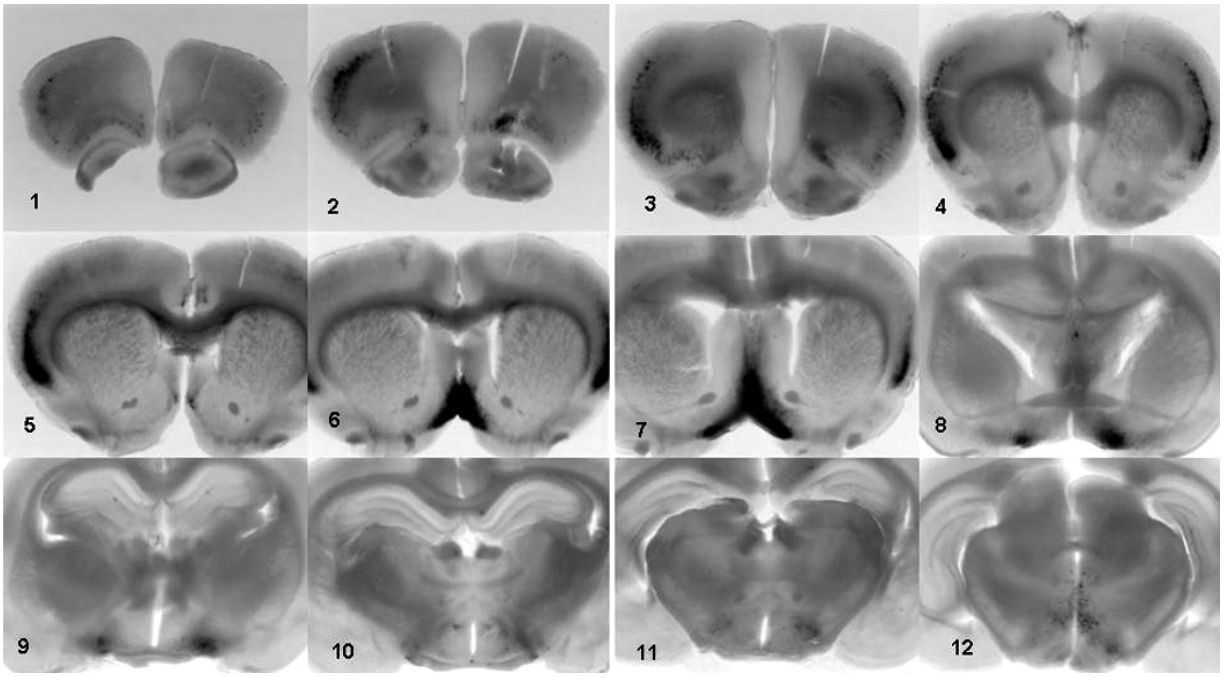

Figure 3. 12 consecutive coronal sections (1 mm apart) through the rat brain showing the extent of staining 1 week following bilateral septal region injections at the level shown in section No. 6. Note that significant staining is visible in the cortical regions in section Nos. 1–7 (+5 to –1 mm bregma).

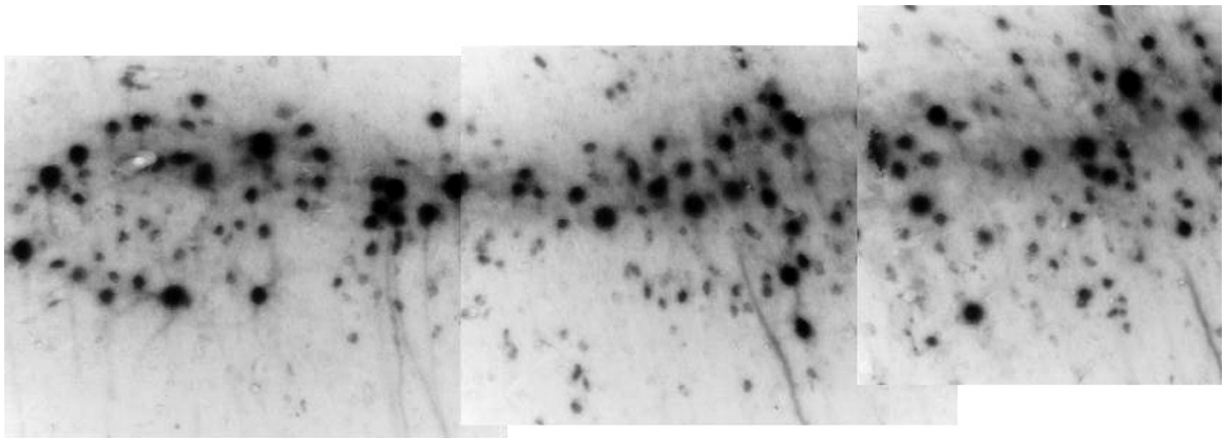

Figure 4. HSV-1/β-gal expression (1 week following injection) in cortex after injection to septum (20 $\times$).

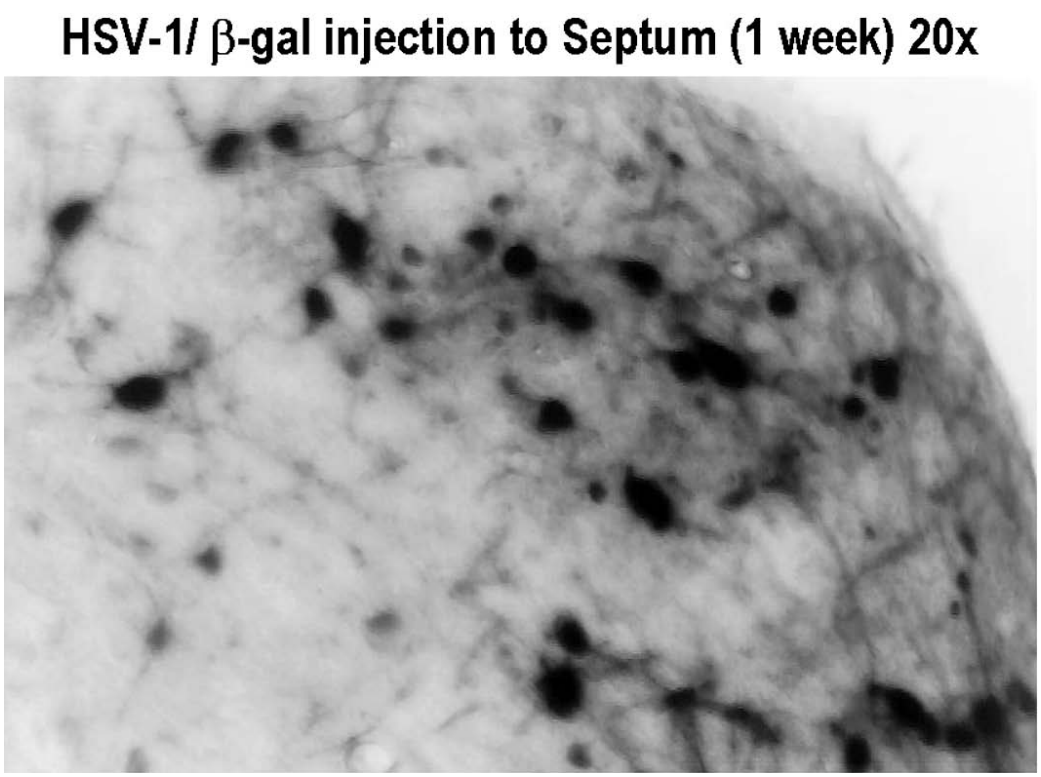

Figure 5. High power view of successful β-gal gene transfer into the septal neuronal cells using HSV-1 vector.

we conducted PCR analysis of the viral DNA in various brain regions. The detection of the HSV DNA by PCR was correlated with the histological observation, with most of the expression found near the injection site in the septum and significant retrograde migration to the cortex. Lesser but measurable HSV-1 vector DNA was detected in the hippocampus, striatum, thalamus, *substantia nigra*, brain stem, frontal cortex and tectum, consistent with the lower amounts of β-gal expression detected in these regions (Figs. 7A, B).

Successful methods for gene transfer require that the transgene is taken up by cells, bypass the normal pathways of lysosomal degradation, be expressed in the cells (Figs. 4–6), and finally produce long-term stable expression, either as an independent piece of DNA or by gaining access to the cell nucleus and incorporating into host genomic DNA [16]. Interestingly, our data showed that a single injection provided gene delivery to several regions of CNS (Fig. 3) and provided long-term gene expression at the injection site (Fig. 2).

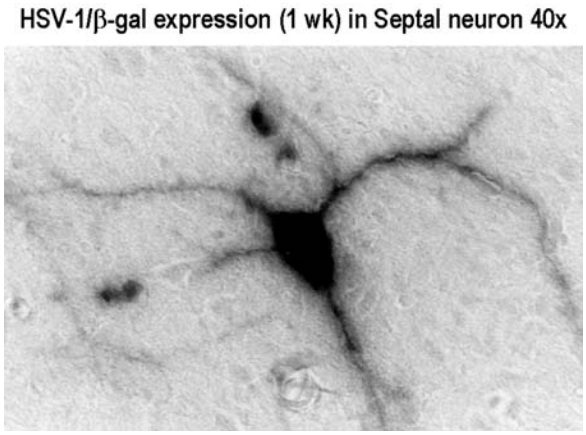

Figure 6. Successful expression of β-gal transgene revealed by X-gal reaction (blue) shown in an individual septal neuron (high magnification).

Therefore, it is possible to deliver genes *in vivo* to the cerebral cortex by injecting a single region of the brain with HSV-1 viral vector. This may reflect a retrograde uptake and transport of the non-replicating virus by the neurons which project to the inoculated area. This ability of even a replication-incompetent HSV-1 vector to be efficiently transported in a retrograde fashion highlights one of its most useful properties for use as a vector for delivering genes to CNS.

Future lines of study will focus on constructing viral vectors that express biologically active genes, such as β-endorphin to treat chronic pain [10], growth factors to treat degenerative and growth related neurological diseases [23], and defective growth factor receptors such as FGFR1(TK–), which we are studying

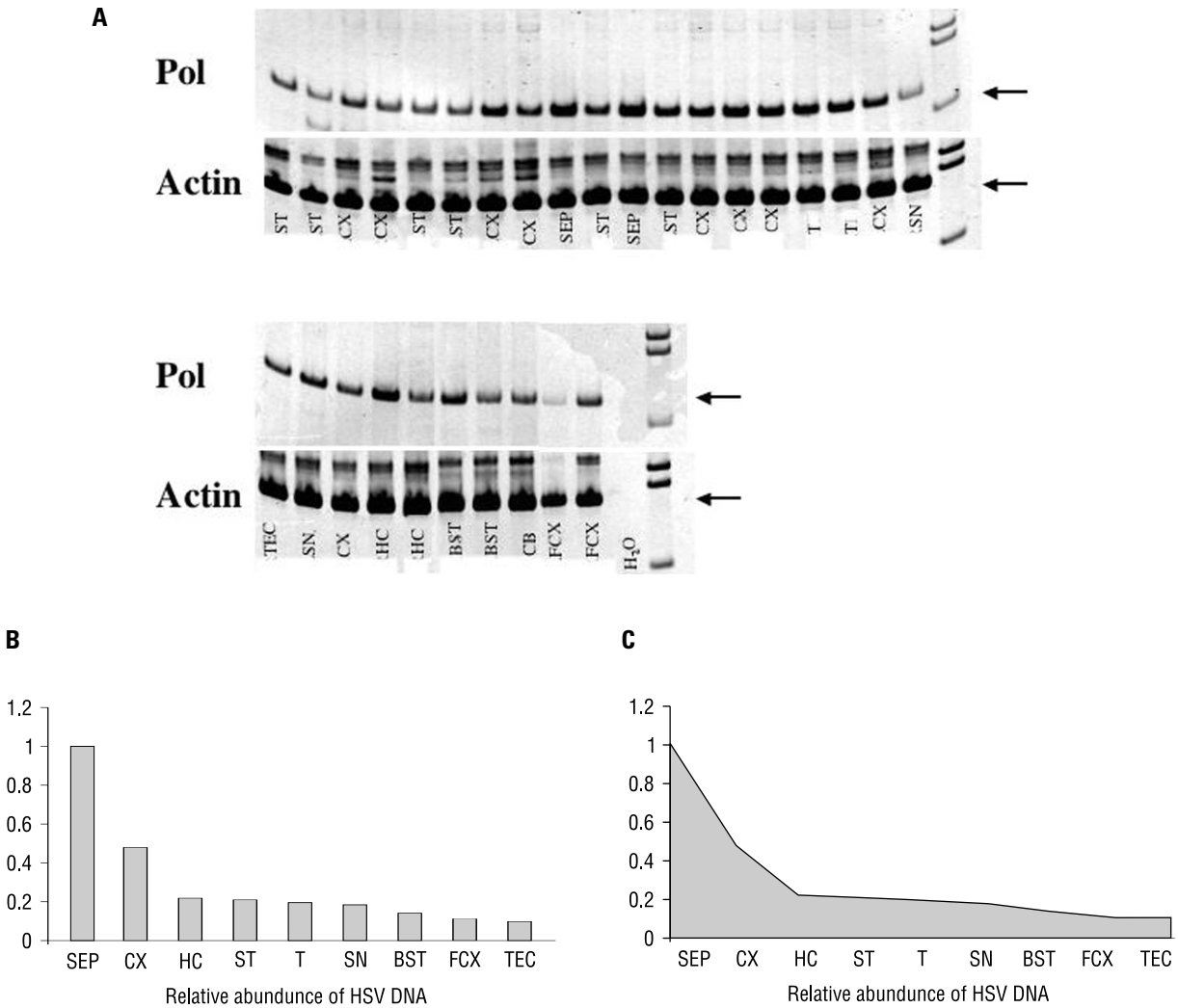

Figure 7. A. PCR analysis and detection of the viral HSV DNA in various brain regions correlate with the histological observations seen in Figure 3. **B.** Most of the expression was near the injection site in the septum (SEP). Significant retrograde migration to the cortex (CX) is observed. Lesser detection was also noted in the hippocampus (HC), striatum (ST), thalamus (T), *substantia nigra* (SN) and pons as well as the medulla parts of the brain stem (BST), frontal cortex (FCX) and tectum (TEC).

for animal models of human diseases. These studies will be aided by advances in HSV-1 vectors, with improvements in both the duration and control of transgene expression.

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