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COMMENTARY

HSV-1 VECTOR MEDIATED NEURONAL GENE DELIVERY

STRATEGIES FOR MOLECULAR NEUROSCIENCE AND NEUROLOGY

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We recently developed a defective Herpes Simplex Virus One (HSV-1‖) vector system which, for the first time, provides a method to transfer genes into postmitotic cells, such as neurons [1–3]. As detailed below, we have demonstrated that HSV-1 vectors can transfer genes into peripheral [1] and CNS neurons [2] in culture, and into a chosen group of neurons in the adult rat brain [3, ¶]. In contrast, previously available gene transfer techniques, including DNA transfection, retrovirus vectors, and the construction of a transgenic animal, cannot deliver a gene directly into mature, postmitotic neurons.

The potential to transfer genes into neurons has a number of important consequences. In this article, we present a strategy to perturb the function of a selected region of the adult brain, with far greater precision than previously possible, by using HSV-1 vectors to express a gene in a particular type of neuron in a chosen area of the brain. We then summarize the properties of this HSV-1 vector system, compare HSV-1 vectors to other gene transfer systems, explore the potential to modify neuronal physiology with HSV-1 vectors expressing genes which encode components of second messenger systems or neurotransmitter release mechanisms, and raise the possibility of performing gene therapy to treat neurological disorders with HSV-1 vectors.

Perturbation of function in a chosen region of the adult brain through experimental manipulation of three parameters of defective HSV-1 vectors

The prototype HSV-1 vector, pHSVlac ([1–3, Fig. 1), contains the *Escherichia coli Lac Z* gene under the control of the HSV-1 IE 4/5 promoter. The *Lac Z* gene encodes an easily assayable protein, β-galactosidase, and the HSV-1 IE 4/5 promoter

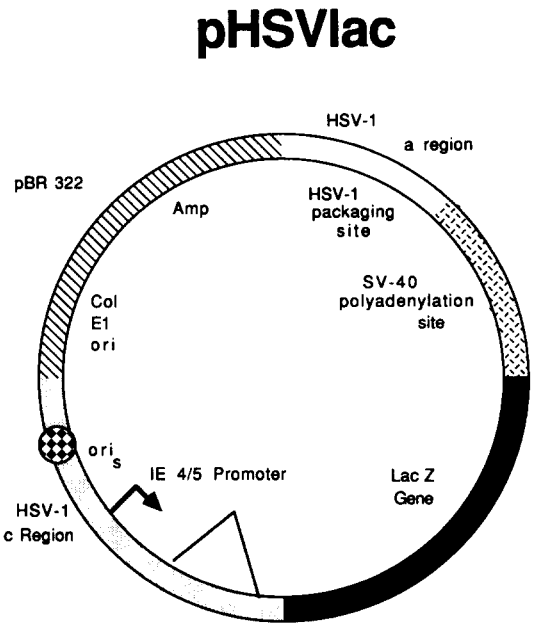

Fig. 1. Structure of the prototype defective HSV-1 vector, pHSVlac [1–3]. pHSVlac contains three kinds of genetic elements. (1) The transcription unit in pHSVlac is composed of the HSV-1 IE 4/5 promoter (arrow), the intervening sequence following that promoter (triangle), the *E. coli Lac Z* gene (black segment), and the SV-40 early region polyadenylation site (dotted segment). The IE 4/5 promoter and the *Lac Z* gene can be replaced with other genes or promoters respectively. (2) Two sequences are required for propagation of pHSVlac in a HSV-1 virus stock: The HSV-1 origin of DNA replication ori_s (circle on dotted segment) supports replication of pHSVlac DNA. The HSV-1 **a** sequence (clear segment) contains the packaging site which is responsible for subsequently packaging pHSVlac DNA into HSV-1 virus particles. (3) Sequences from pBR322 (diagonal line segment) support the growth of pHSVlac DNA in *E. coli*.

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‖ Abbreviations: HSV-1, Herpes Simplex Virus One; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; DEAE, diethylaminoethyldextran; L-DOPA, dihydroxyphenylethanolamine; IE, Immediate Early; IgG, immunoglobulin class G; kb, kilobase pair; MPTP, methylphenyltetrahydropyridine; NGF, nerve growth factor; *ori*, origin of DNA replication; pfu, plaque forming unit; *ts*, temperature sensitive; and VIP, vasoactive intestinal polypeptide.

¶ Geller AI, Freese A, Hemmendinger LH and Sabel B, Gene transfer into neurons in the adult rat brain. Manuscript submitted for publication.

functions in most cell types. Together, the IE 4/5 promoter and the *Lac Z* gene permitted the determination of which cells can be infected by HSV-1 vectors; however, the *Lac Z* gene and the IE 4/5 promoter can be replaced by virtually any other gene or promoter respectively. Stereotaxic injection of pHSVlac virus into the adult rat brain resulted in stable expression of β-galactosidase in cells around the injection site and in neurons projecting to the injection site; pHSVlac virus did not spread through the brain [3, *]. Consequently, in designing experiments with HSV-1 vectors there are three variables subject to experimental manipulation. These are: (1) the number and location of the cells infected; (2) the promoter in the vector; and (3) the gene in the vector.

The location of the infected cells is determined by several factors including the site of injection, the location of the neurons which project to the injection site, the number of virus particles in the inoculum, and the extent of diffusion of the virus in the extracellular space before infection [3, *]. Thus, the experimenter can control which cells are infected by choosing the site of injection and the number of virus particles injected.

The promoter in the vector is the second variable subject to experimental control. The fact that the HSV-1 IE 4/5 promoter in pHSVlac can be replaced with cell type specific promoters should allow one to restrict expression of a gene in the vector to a chosen cell type. For example, the vasoactive intestinal peptide (VIP) promoter [4] will restrict expression to VIP neurons; the VIP promoter is inactive in neurons which use neurotransmitters other than VIP.

The gene in the vector is the third variable subject to experimental manipulation; the *Lac Z* gene in pHSVlac may be replaced with virtually any gene. For example, introduction of the yeast adenylate cyclase gene [5] should result in elevated neuronal cAMP levels; in addition, expression of only the catalytic segment of the yeast adenylate cyclase gene should result in an unregulated adenylate cyclase enzyme which is always active [5]. Furthermore, by fusing the gene to subcellular targeting sequences, the expressed protein can be localized to a particular part of a neuron such as the cell body, nucleus, dendrites, or axons. For example, the first ten amino acids of the neuronal growth associated protein GAP 43 are thought to be involved in association of this protein with the membrane in the axonal growth cone or presynaptic terminal [6]. Therefore, fusion of this GAP 43 sequence to the coding sequence of another gene, such as the yeast adenylate cyclase gene, should target the resulting protein to axons.

An example of an experiment which might be performed with this technology is shown in Fig. 2. Consider a HSV-1 vector which contains the yeast adenylate cyclase gene [5] under the control of the VIP promoter [4]. Injection of virus containing this vector into the hippocampus would result in infection of hippocampal neurons. The VIP promoter functions only in VIP neurons in the CNS [4], thereby potentially restricting expression of the yeast adenylate cyclase gene to VIP neurons. The yeast adenylate cyclase [5] should raise cAMP levels in the neurons in which it is expressed. Therefore, this experiment may result in a selective increase in cAMP concentration in hippocampal VIP neurons. VIP neurons in other parts of the brain would not be infected with the vector and consequently would not be affected. Although other types of neurons in the hippocampus would contain the vector, because the VIP promoter would be inactive, the adenylate cyclase gene should not be expressed. Following the selective increase of cAMP concentration in hippocampal VIP neurons, other neurons in the hippocampus could be tested for altered electrophysiological responses. In addition, the performance of the animal in various learning paradigms may reveal a behavioral consequence of increasing the concentration of cAMP in hippocampal VIP neurons.

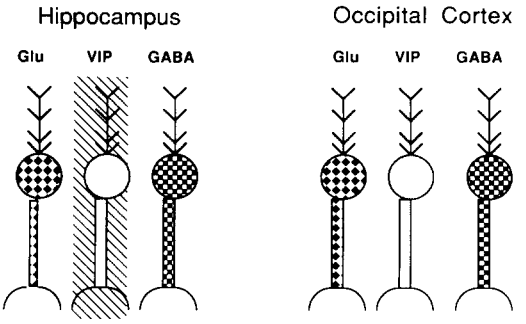

Fig. 2. Example of altering the function of a chosen region of the adult brain using HSV-1 vectors; elevation of the cAMP concentration in hippocampal VIP neurons. An HSV-1 vector is constructed which places the yeast adenylate cyclase gene [5] under the control of the VIP promoter [4]; this vector is packaged into HSV-1 virus particles [7]. Virus containing this vector is delivered by stereotaxic injection into the hippocampus, thereby infecting the various types of neurons around the injection site. The VIP promoter [4] should be active only in VIP neurons, resulting in expression of yeast adenylate cyclase [5], which should increase the cAMP concentration in VIP neurons (shown shaded). In surrounding neurons in the hippocampus which use a different neurotransmitter, such as glutamate or γ-aminobutyric acid, the VIP promoter should be silent, so the vector would have no effect. Neurons, including those expressing VIP, in other regions of the brain (such as the occipital cortex) are not infected with the vector, so they are not affected.

In summary, three variables can be manipulated when using HSV-1 vectors in the adult brain. First, the injection site determines which cells are infected. Second, the promoter determines in which of the infected cells the gene is expressed. Third, the chosen gene alters cellular physiology in a predetermined and predictable fashion. Thus, HSV-1 vectors have the potential to provide detailed information about the cellular locations and molecular mechanisms of information processing in the mammalian brain by making precise and reproducible modifications in the function of the

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brain on a level not presently possible. We now describe the properties of our HSV-1 vector system, and detail its capability to transfer genes into neurons, both in culture and in the adult rat brain. In the last two sections we will discuss how the tripartite experimental strategy presented above may be applied to help define determinants of neuronal physiology through the manipulation of second messenger enzymes, and to the understanding of a neurologic disease, Parkinson's disease.

The HSV-1 life cycle

To appreciate the properties of HSV-1 vectors, a brief detour through the HSV-1 life cycle is necessary. HSV-1 is a large double-stranded DNA virus of about 150 kb encoding approximately 75 genes [8]. A HSV-1 virus particle is a layered structure which consists of (from the inside out) DNA, an icosohedral protein capsid, a shell of proteins called the tegument, and a lipid bilayer derived from the nuclear membrane with viral encoded glycoproteins embedded in it [9]. These glycoproteins mediate a membrane–membrane fusion event between the lipid bilayer of the virus and the plasma membrane of the cell, depositing the remainder of the virus particle into the cytosol [9, 10]. This fusion event does not appear to require a specific protein receptor on the cell surface; rather its generality may account for the wide host range of HSV-1. Once inside the cell, the HSV-1 DNA is delivered into the nucleus where its genes are transcribed in a regulated cascade [9, 11]. The five IE genes, which encode the major regulatory proteins of the virus, are expressed first. The IE proteins induce expression of the early genes which primarily encode the biosynthetic enzymes responsible for DNA replication. Following DNA replication the late genes are induced; they encode most of the structural components of the virus particle and the enzymes reqired for virus particle assembly.

When wild type HSV-1 infects neurons, there is a presumed molecular switch early in the life cycle which allows HSV-1 to enter a latent state [12]. In the latent state the lytic cycle is suppressed and the virus persists indefinitely in the neuron in a benign state. The precise molecular mechanism of the lytic–latency switch remains to be elucidated [12–15]. When wild type HSV-1 is injected into the brain, the lytic cycle results in the production of progeny virus, thereby spreading the infection throughout the brain and subsequently killing the animal (in mice, the LD_{50} for wild type HSV-1 strain 17 is 10^3 pfu [16]).

In contrast, intracerebral injection of *ts* mutants results in a latent infection [16, 17]. These *ts* mutants [18] have a single base change in a gene essential for the lytic cycle, resulting in a single amino acid substitution in the encoded protein. The mutant protein is functional at 31°, but not at body temperature, 37–39°. Thus, the lytic cycle can proceed in tissue culture at 31° but not *in vivo* at 37°. By choosing an appropriate *ts* mutant which blocks the lytic cycle at the IE stage, it is possible to infect cells with HSV-1 at 37° with little if any cell damage. Furthermore, since these *ts* mutants do not grow *in vivo*, the infection is limited to cells around the injection site and neurons which project to the injection site; the infection does not spread throughout the brain [16, 17]. We exploited these *ts* mutants of HSV-1 to develop our HSV-1 vector system.

HSV-1 as a vector

HSV-1 has a number of advantages for gene transfer into neurons [19]: First, HSV-1 can infect postmitotic neurons in adult animals or in culture. Second, HSV-1 has a wide host range; HSV-1 can infect many different cell types such as fibroblasts, macrophages, glia, and neurons in many different organisms including humans, non-human primates, rodents, and birds. Third, HSV-1 can be maintained indefinitely in neurons in a latent state [9, 12]. Fourth, while in the latent state, HSV-1 is quiescent; expression of viral genes is limited to a latency associated transcript(s) and perhaps some IE genes [12–15], HSV-1 DNA replication does not occur, no progeny virus are produced [12], and electrophysiological properties of latently infected neurons are unaltered [20]. Fifth, HSV-1 gene regulation occurs in a complex, regulated cascade [9, 11] and HSV-1 genes are transcribed by the cellular RNA polymerase II [21], suggesting that cellular promoters in HSV-1 vectors could be appropriately regulated. Moreover, we have demonstrated recently the proper function of the mouse β-actin promoter in a HSV-1 vector (unpublished results). Sixth, the 150 kb genome of HSV-1 suggests that HSV-1 vectors could be designed to accommodate large genes.

The basic experimental procedure for using HSV-1 as a vector is diagrammed in Fig. 3. A recombinant vector is constructed in *E. coli* using standard molecular biological techniques [22]. Vector DNA is then packaged into HSV-1 virus particles [7]; vector DNA is delivered into CV1 monkey fibroblast cells by calcium phosphate mediated DNA transfection [23] and the cells are subsequently infected with helper virus, HSV-1 strain 17 *ts* K [24]. *ts* K has a mutation in the IE 3 gene, possesses an IE phenotype, and is not permissive for DNA replication [24]. The cells are then incubated at the permissive temperature of 31° and the resulting virus stock is used for expression experiments. Transformed cell lines or normal cells in primary culture are infected with virus stock containing the vector, and incubated at 37°; after an appropriate period of time, expression is analyzed. Alternatively, to infect a chosen group of neurons *in vivo*, virus is delivered directly into the brain of adult animals by stereotaxic injection.

Deletion mutants [25, 26] are being explored as an alternative helper virus to package a defective HSV-1 vector into HSV-1 virus particles. Deletion mutants contain a deletion in an essential gene of HSV-1, such as the IE3 gene. The IE3 gene is the major regulatory gene of HSV-1; deletion mutants in the IE3 gene express the four other IE genes and perhaps one or two early genes; they do not replicate their DNA or produce progeny virus [25]. The deletion mutant is grown in a cell line which contains the deleted gene in its genome [25, 26]. To package vector DNA using a deletion mutant as helper virus,

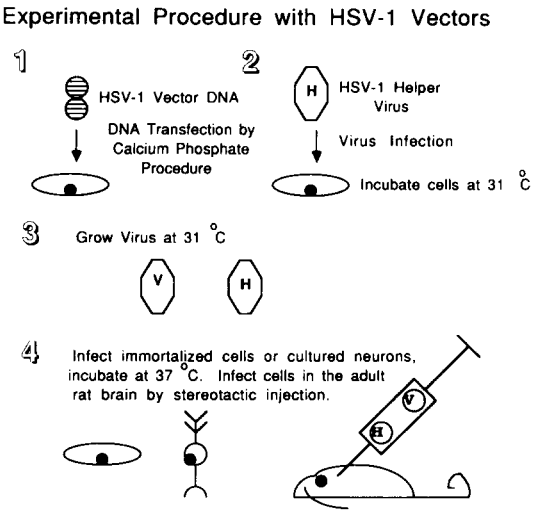

Fig. 3. Diagram of the experimental procedure followed with defective HSV-1 vectors. (**1**) The desired vector is constructed in *E. coli* using standard recombinant DNA techniques [22]. To package vector DNA into HSV-1 virus particles [7], CV1 monkey fibroblasts are transfected with vector DNA using the calcium phosphate procedure [23]. (**2**) The CV1 cells are superinfected with the helper virus, a *ts* mutant of HSV-1 [18, 24], and the cells are then incubated at the permissive temperature of 31°. (**3**) The resulting virus stock consists of identical HSV-1 particles which contain either the vector DNA or the helper virus, the *ts* mutant of HSV-1. (**4**) Virus containing the vector can then be used to infect cells and stably express the gene in the vector [1, 2]. Cells can be infected in culture, including immortalized cell lines or primary cultures of normal cells such as neurons. Alternatively, virus can be delivered into an animal by injection; for example, neurons in the adult rat brain can be infected following stereotaxic injection of virus into the desired site.

a cell line containing the IE3 gene would be transfected with vector DNA and then superinfected with deletion mutant virus. The resulting virus stock containing the vector could then be used to perform expression experiments in cells and animals which do not contain the IE3 gene. Deletion mutants essentially do not revert; therefore, they would be suitable for gene therapy in humans.

The prototype HSV-1 vector, pHSVlac

Our defective HSV-1 vector pHSVlac ([1–3]; Fig. 1) contains three kinds of genetic elements. First, it carries sequences for the propagation of pHSVlac in *E. coli*: the ampicillin resistance gene and the *Col EI* origin of DNA replication. Second, it includes two sequences from HSV-1 which support propagation of pHSVlac DNA in a HSV-1 virus stock: HSV-1 ori_s, a HSV-1 origin of DNA replication, which is required to replicate pHSVlac DNA, and the HSV-1 packaging site, contained in the *a* sequence, which is required for packaging pHSVlac DNA into HSV-1 virus particles. Third, it possesses a transcription unit which consists of the HSV-1 IE 4/5 promoter, the intervening sequence following that promoter, the *E. coli Lac Z* gene [27], and the SV40 early region polyadenylation site. Thus, the *Lac Z* gene is placed under the control of the HSV-1 IE 4/5 promoter, a constitutive promoter that functions in many cell types. Since the *Lac Z* gene encodes a bacterial β-galactosidase absent from mammalian cells, assays for expression of this gene product were readily available. Consequently, the IE 4/5 promoter and the *Lac Z* gene allowed us to use pHSVlac to define the properties of our HSV-1 vector system and, specifically, to determine which cells can be infected with HSV-1 vectors. pHSVlac DNA was packaged into HSV-1 virus particles [7] using HSV-1 strain 17 *ts* K [24] as a helper virus, as described above and in Fig. 3.

Stable expression of β-galactosidase in neural cell lines, cultured neurons, and in neurons in the adult rat brain from pHSVlac

We performed a series of studies using our prototype vector, pHSVlac, which demonstrated that pHSVlac can stably express β-galactosidase after infection of dividing and nonmitotic neural cell lines [28], cultured neurons [1, 2], and neurons in the adult rat brain [3]. In our protocol, cells in culture are infected with pHSVlac virus, and 1 day later expression of the *Lac Z* gene product, β-galactosidase, is detected with an *in situ* enzymatic assay using the chromogenic substrate X-Gal. We observed expression of β-galactosidase in a variety of mitotic neural cell lines including N1E-115 mouse adrenergic neuroblastoma cells, NS-20Y mouse cholinergic neuroblastoma cells, PC12 rat pheochromocytoma cells, AtT-20 mouse pituicytes, GH4 rat pituicytes, SK-N-BE(2) human neuroblastoma cells, U1 Mel human melanoma cells, and Hs 683 human glioma cells. Furthermore, expression of β-galactosidase was observed 1 day after infection with pHSVlac virus in two differentiated, nonmitotic neural cell lines: PC12 cells treated with NGF, and N1E-115 cells treated with dibutyryl cAMP [28, 29, *].

Expression of β-galactosidase from pHSVlac was also observed in cultured neurons from throughout the nervous system [1, 2]. Peripheral neurons derived from superior cervical ganglia and dorsal root ganglia were infected with pHSVlac virus, and 1 day later expression of β-galactosidase was detected with X-Gal, as shown in Fig. 4 [1]. Also, infection with pHSVlac virus of cultured neurons derived from various areas of the central nervous system resulted in expression of β-galactosidase, as shown with a double-immunofluorescent assay: β-galactosidase immunoreactivity was detected using a rabbit anti-*E. coli* β-galactosidase antibody and a rhodamine conjugated secondary antibody; neurofilament immunoreactivity was detected using a mouse anti-neurofilament antibody and a fluorescein conjugated secondary antibody. Using this assay, expression of β-galactosidase, 1 day after pHSVlac virus infection, was demonstrated in cultured neurons from spinal cord, cerebellum, thalamus, striatum, hippocampus, occipital cortex, temporal cortex, and frontal cortex [2].

* Geller AI, Expression of *E. coli* β-galactosidase from a defective HSV-1 vector in cultured mitotic and nonmitotic cell lines. Manuscript submitted for publication.

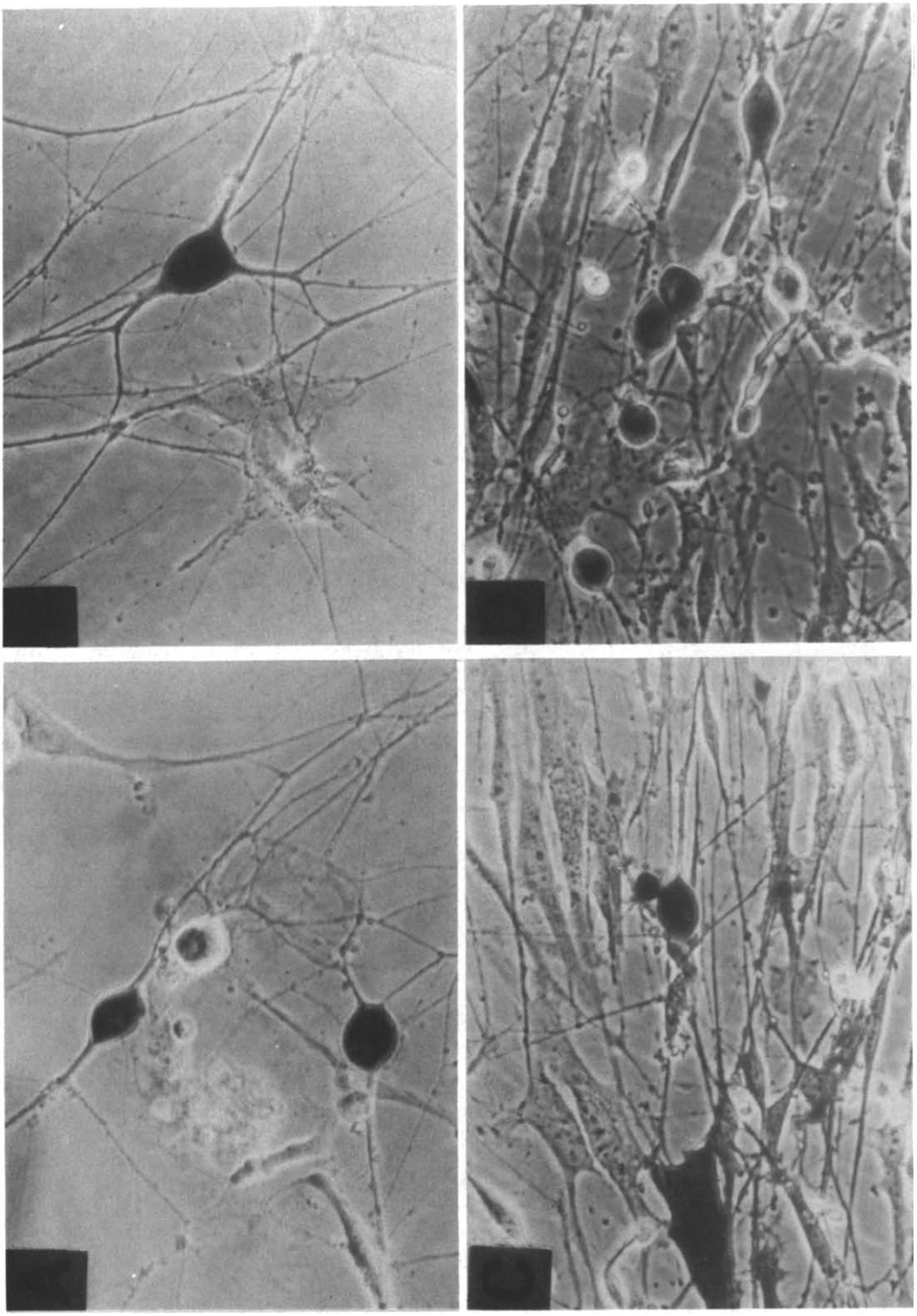

Fig. 4. β-Galactosidase expression in cultured cells from dorsal root ganglia (A and B) and superior cervical ganglia (C and D) 1 day after infection with pHSVlac virus. Long-term cultures of dorsal root ganglia and superior cervical ganglia were prepared from newborn rats, and the cultures were treated for 24 hr with 1×10^{-5} M cytosine arabinoside. Ten days after preparation, the cultures were infected with 0.1 mL pHSVlac virus grown using HSV-1 strain 17 *ts* K as helper virus. The titer of the virus stock was 1×10^6 pfu of *ts* K/mL and 8×10^5 infectious particles of pHSVlac/mL. One day after infection, the cells were fixed with 0.5% glutaraldehyde and stained for β-galactosidase activity with X-gal [30, 31].

To demonstrate that pHSVlac DNA persisted in neurons and stably expressed β-galactosidase, we infected differentiated PC12 cells, differentiated N1E-115 cells, and cultured neurons from sensory ganglia, striatum, total neocortex, and hippocampus with pHSVlac virus, maintained the infected cultures for at least 2 weeks, and demonstrated expression of β-galactosidase after this incubation. In addition, pHSVlac DNA persisted in these cells for at least 2 weeks and could be recovered following superinfection with HSV-1. Furthermore, pHSVlac DNA was stably maintained within the cells that were originally infected, and was not transmitted horizontally to other cells; its rate of horizontal transmission was negligible as shown by the low titers of both the helper virus, *ts* K, and pHSVlac virus in the culture medium and the presence of β-galactosidase negative cells in the cultures [1, 2, 28].

A series of *in vivo* experiments were performed which demonstrated that, following stereotaxic injection of pHSVlac virus into the brain of adult rats, β-galactosidase was expressed in neurons surrounding the injection site (hippocampus, occipital cortex, and superior colliculus), and in distant neurons whose axons project to the injection site [3, *]. Expression was stably maintained for at least 1.5 months, indicating that pHSVlac could escape immune surveillance. Furthermore, in contrast to wild type HSV-1, pHSVlac did not spread throughout the brain, demonstrating that transneuronal transport of pHSVlac virus, due to reactivation of persistent pHSVlac DNA, did not occur.

In addition to stereotaxic injection, there are several other well characterized methods to deliver molecules into the central nervous system which could be adapted to deliver HSV-1 vectors into a larger number of cells than possible with stereotaxic injection. Other possible modes of delivery include the recently developed intracerebral minipump [32]; packaging of HSV-1 particles into liposomes [33] or into polymers [34]; and transient breaching of the blood–brain barrier [35].

In summary, pHSVlac virus can infect neurons from throughout the nervous system, both *in vitro* and *in vivo*, and stably express a gene [1–3]. Therefore, HSV-1 vectors have a number of advantages over other systems for delivery of genes into cells, as detailed below. Furthermore, these results indicate that HSV-1 vectors might be used to transfer genes into neurons for studies on neuronal physiology or gene therapy. To illustrate the usefulness of HSV-1 vectors for altering the physiological activity of neurons, we will outline an approach to modulate the activity of enzymes involved in second messenger systems. Then, to explore the potential utility of HSV-1 vectors for performing gene therapy, we will discuss the possibility of treating Parkinson's disease by introducing the tyrosine hydroxylase gene into neurons.

* Geller AI, Freese A, Hemmendinger LH and Sabel B, Gene transfer into neurons in the adult rat brain. Manuscript submitted for publication.

Advantages of HSV-1 vectors for the delivery of genes into neurons

Until now, three approaches [19] have been used to deliver genes into cells; however, none is effective with postmitotic cells such as neurons. The three approaches are: (1) transfection of DNA into cells [23], (2) retrovirus vector infection of cells [36], and (3) construction of transgenic mice [37]. Transfection of DNA into cells [23, 38, 39] has been achieved using a number of different methods. These include coprecipitation of DNA with calcium phosphate [23], treatment of recipient cells with DEAE dextran [40], electroporation [41], and microinjection (performed predominantly with frog oocytes) [42]. Successful transfection of immortalized mitotic cell lines with many genes has been reported using these methods, but the low efficiency of DNA-mediated gene transfer and problems surrounding microinjection into somatic cells render these approaches essentially inapplicable to the delivery of genes into most normal cells or to cells *in vivo*. Despite these limitations, considerable information about gene function and regulation has been garnered by DNA-mediated gene transfer into cultured cells [19, 42].

The second frequently used method for gene transfer is retrovirus vectors [19, 43]. Advantages of retrovirus vectors are that gene transfer is efficient, approaching 100% in some cases; many vectors possess both selectable genes and convenient restriction sites for insertion of other genes; the vectors pose little biological hazard; and the host cell range is broad [36, 44, 45]. Retrovirus mediated gene transfer into embryos and neonates has yielded information about the development of the nervous system, especially in neuronal cell lineage studies [30, 31, 46]; and the use of retroviral vectors to infect immortalized neuronal cell lines in culture has provided information about the synthesis and processing of neuronal proteins [19, 47]. However, retroviruses require at least one mitotic cycle for integration and the resulting stability [48]; consequently, retrovirus vectors are ineffective for gene transfer into neurons.

The last method of gene transfer, the development of transgenic mice [37, 49], has been used in several types of experiments, including gene regulation studies [50], the understanding of oncogene function [51], the ablation of a cell type during development [52], and the correction of genetic disorders [53]. Transgenic mice are created by the microinjection of DNA into pronuclei of fertilized eggs, which are returned to, and allowed to develop in, pseudopregnant female mice. Frequently, the germ line cells of the resulting transgenic mice contain the foreign DNA which can then be passed on to subsequent generations by breeding [49]. Transgenic mice have provided much useful information; however, transgenic mice effect the delivery of a foreign gene into every cell in the animal; in contrast, a more localized delivery is desirable for many experiments.

To appreciate the different capabilities of the construction of a transgenic mouse and HSV-1 vectors, recall our tripartite strategy for probing the function of the brain using HSV-1 vectors, expression

of a gene in one type of neuron in a particular region of the adult brain. The example we presented (Fig. 2) would increase the cAMP concentration specifically in VIP neurons of the hippocampus, by delivery into the hippocampus of a HSV-1 vector which placed the yeast adenylate cyclase gene [5] under the control of the VIP promoter [4]. Contrast with this HSV-1 vector experiment the example of a transgenic mouse also expressing the yeast adenylate cyclase gene from the VIP promoter. In the latter case, every VIP containing cell in the animal, including VIP cells throughout the central nervous system and elsewhere, would contain elevated cAMP levels. It would be difficult to interpret the behavior of such a transgenic mouse in learning paradigms. HSV-1 vectors allow for a local specificity of recombinant gene expression that is not possible with transgenic mice. In summary, of the available gene transfer technologies, only HSV-1 vectors possess the required properties to alter the physiology of a particular type of neuron in a chosen region of the adult brain needed for most applications to gene therapy in neurological or psychiatric disease.

Modulation of neuronal physiology by expression of catalytic fragments of second messenger molecules

The ability of HSV-1 vectors to introduce and express a gene in neurons in a chosen area of the brain suggests possibilities for using these vectors to modify neuronal physiology in a specific manner *in vivo*. One can modify information transfer either between neurons or within a neuron. The primary mode of information transfer between neurons involves classical neurotransmitters and peptides; but the large number of neurotransmitters and neuromodulators, and the plurality of receptor subtypes that can bind many neurotransmitters or neuromodulators [54], makes it difficult to manipulate them in a meaningful manner. However, this multitude of neurotransmitter systems activates a much smaller number of intracellular processes, including several second messenger pathways [55] and, of course, action potentials [56], which result in neurotransmitter release [57]. Alteration of second messenger enzyme activity can sometimes result in modification of neuronal physiology; for example, microinjection of protein kinase C protein into hippocampal neurons elicits some of the features of long-term potentiation [58], and microinjection of calcium calmodulin dependent protein kinase II protein into presynaptic terminals enhances neurotransmitter release [59]. In summary, the ability to modulate the activity of approximately five to ten second messenger enzymes and to alter the amount of neurotransmitter released per action potential would permit the manipulation of the majority of the known functions of a neuron.

Our approach to manipulating second messenger physiology is to express altered second messenger enzymes which are no longer regulated and consequently are always active. The structure of many second messenger enzymes facilitates this approach; they are composed of a domain structure (Fig. 5) in which the regulatory and the catalytic regions of the enzyme are located on separate portions of the polypeptide [5, 60, 61, 65, 66].

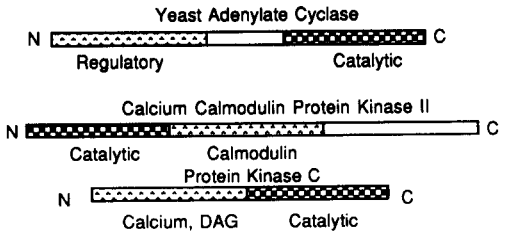

Fig. 5. Domain structure of the three second messenger enzymes: yeast adenylate cyclase, calcium calmodulin dependent protein kinase II, and protein kinase C. Yeast adenylate cyclase [5] contains its regulatory domain at the amino terminus, the catalytic domain at the carboxy terminus, and a region of unknown function between the two. In yeast, expression of the catalytic domain alone results in an unregulated adenylate cyclase enzyme [5]. Calcium calmodulin dependent protein kinase II [60] contains the catalytic domain at the amino terminus, followed by the regulatory domain which contains the calcium/calmodulin binding site, and the carboxy terminus contains a domain of unknown function which may play a role in subcellular localization of the enzyme. In contrast, protein kinase C [61] contains its regulatory domain at the amino terminus; the regulatory domain contains the calcium and diacylglycerol binding sites. The catalytic domain of protein kinase C is located in the carboxy terminus of the enzyme. All known serine/threonine protein kinases, including calcium calmodulin dependent protein kinase II and protein kinase C, contain homologous catalytic domains [62]. Limited proteolysis of either calmodulin dependent protein kinase II [63] or protein kinase C [64] results in a catalytic fragment which displays unregulated activity; the catalytic fragment of protein kinase C may be generated naturally in mammalian cells [61].

Therefore, the segment of the gene encoding the regulatory portion of the enzyme can be deleted, leaving an intact and unregulated catalytic segment. Theoretically, introduction of this unregulated, always active catalytic segment into neurons may change the physiological behavior of these neurons in a predictable and testable manner. Several examples will serve to illustrate our approach. At least four protein kinases are known to play important roles in information processing in neurons: protein kinase C, calcium calmodulin dependent protein kinase II, cAMP dependent protein kinase, and cGMP dependent protein kinase. Both protein kinase C [64] and calcium calmodulin dependent protein kinase II [63] have a domain structure in which the regulatory portion of the protein—that is, the calcium-diacylglycerol binding domain or the calcium-calmodulin binding domain, respectively—is a separate domain from the catalytic domain. Limited proteolytic cleavage of each enzyme *in vitro* [63, 64] or expression of the catalytic domain of protein kinase C in fibroblasts [67] results in unregulated activity; the enzyme is constitutively active. Thus, insertion of the portion of the protein kinase gene encoding the catalytic fragment of the enzyme into a HSV-1 vector should result in expression of an unregulated protein kinase, which might modify neuronal physiology. We have constructed four different recombinants of the catalytic domain of the alpha subunit of calcium

calmodulin protein kinase II in a HSV-1 vector that lack varying extents of the calmodulin binding domain. Preliminary studies indicate that calcium-dependent phosphorylation of intracellular proteins is, as predicted, altered in PC12 cells infected with some of the recombinants [68].

Additional strategies can be used to alter other second messenger systems. For example, cAMP-dependent protein kinase is activated by elevated cAMP levels [55], making the activity of this kinase controllable by the manipulation of cAMP levels. Yeast adenylate cyclase is also composed of a domain structure, in which the catalytic and regulatory segments are distinct. Expression of the catalytic domain of the yeast adenylate cyclase results in a 10-fold increase in cAMP levels in yeast [5]. Therefore, expression of the catalytic domain of the yeast adenylate cyclase in neurons may increase cAMP levels and result in activation of the cAMP-dependent protein kinase. We have shown in preliminary studies [68] that expression of the catalytic domain of yeast adenylate cyclase in PC12 cells results in approximately a 20-fold increase in levels of cAMP in an infected cell. Additional experiments are directed towards examining the effects of elevated cAMP levels on the regulation of genes which are responsive to cAMP, on the activity of cAMP-dependent protein kinases, and on the release of neurotransmitters from neurons. Activation of the cGMP-dependent protein kinase might be achieved in an analogous fashion. Phospholipases, including phospholipase C [65], and proteins involved in neurotransmitter release, such as synapsin I [66, 69], are also composed of a domain structure which is amenable to analogous manipulations.

Modification of neuronal physiology with recombinant genes in defective HSV-1 vectors may provide a powerful new approach to understanding the molecular interactions of second messenger systems, gene expression, neuronal physiological activity, and neurotransmitter release. Furthermore, the ability to modify neuronal physiology in the adult brain may yield new insights into the precise cellular locations and molecular mechanisms responsible for brain functions such as processing visual images and forming memories.

Parkinson's disease

Parkinson's disease is a neurodegenerative disorder resulting from the destruction of dopaminergic neurons in the substantia nigra pars compacta; these neurons project to the corpus striatum [70]. Therapy for Parkinson's disease has centered around compensating for the lowered dopamine levels in the striatum. Clinical and basic research efforts have used precursor loading (L-DOPA; [70–74]), dopamine agonists (such as bromocryptine; [70]), tissue transplants (fetal or autologous adrenal chromaffin; [75, 76], and implantable dopamine delivery systems (either polymeric or pump systems; [77–79]), with varying degrees of success. Ideally, however, replacement of lost dopaminergic function may be achieved by gene therapy: since tyrosine hydroxylase (L-tyrosine, tetrahydropteridine: oxygen oxidoreductase, EC 1.14.16.2) is the rate-limiting enzyme in dopamine biosynthesis, introduction of the tyrosine hydroxylase gene [80] into neurons in, or projecting to, the striatum may increase striatal dopamine levels. Moreover, gene therapy for Parkinson's disease will not require the use of human fetal tissue.

To explore the possibility of treating Parkinson's disease with recombinant HSV-1 vectors, we are now inserting the human tyrosine hydroxylase gene into pHSVlac. Infection of neurons in or projecting to the striatum with such a vector may cause increased localized conversion of L-tyrosine to L-DOPA, with a consequent increase in dopamine levels in the striatum. Two well-established animal models for Parkinson's disease could be used to test this approach; both models are produced by administration of a neurotoxin. Injection of 6-OH-dopamine directly into the substantia nigra of rats results in destruction of dopaminergic neurons which project to the striatum, eliciting a readily tested rotational model [81]. Alternatively, administration of MPTP to primates results in a Parkinsonian syndrome, which is characterized biochemically by dopamine depletion in the nigrostriatal system [82]. Both of these animal models provide a behavioral test for recovery of dopaminergic function. In addition, techniques for the measurement of catecholamines and their metabolites, such as intracerebral microdialysis coupled to HPLC [83], can be used to confirm localized dopaminergic functional restoration in the striatum *in vivo*. An initial attempt at gene therapy of Parkinson's disease has been reported. The tyrosine hydroxylase gene was transfected into fibroblasts, which in turn were transplanted adjacent to the striatum in animal models. Partial biochemical recovery of dopaminergic function was noted [84].

Previous cell transplantation studies have suggested that neural grafts may function by release of growth factors or neuromodulators, rather than by integration of the grafted cells into the host circuitry [85, 86]. Therefore, delivery of additional genes besides tyrosine hydroxylase may ameliorate dopamine-deficiency mediated behavior in animal models; among these are genes encoding dopamine receptors or growth factors.

Gene therapy of Parkinson's disease may prove to be a viable alternative to other, less optimal, therapeutic approaches to this disease. In addition, gene therapy of Parkinson's disease may serve as a prototype for treating other neurodegenerative disorders by the gene therapy approach. However, one of the salient features of Parkinson's disease is its restricted anatomical localization to the nigrostriatal system. For neurological disorders with a more global CNS effect, such as the lysosomal storage diseases [87], gene therapy using recombinant HSV-1 vectors is more problematic. Delivery of the vector throughout the brain becomes necessary, and alternative delivery technologies such as osmotic disruption of the blood–brain barrier [35] should be explored.

Summary and prospects

The advent of a technique for introducing genes into neurons *in vitro* and *in vivo* has a variety of

implications. Virtually any gene can be inserted into a defective HSV-1 vector, including genes missing or mutated in neurodegenerative diseases, and genes critical for the normal physiological function of the nervous system. Not only can a gene be delivered to a localized brain region, but also genetic elements regulating the expression of the gene can be included, adding a critical level of control. Thus, HSV-1 vectors may be capable of supporting a precise analysis of the cellular locations and molecular mechanisms of information processing in the neocortex of the mammalian brain. Ongoing experiments in basic neuroscience and applied restorative neurology are exploring the power and general utility of this approach.

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