

Direct Gene Transfer Into Human Epileptogenic Hippocampal Tissue with an Adeno-Associated Virus Vector: Implications for a Gene Therapy Approach to Epilepsy

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Summary: Purpose: Virus vectors capable of transferring genetic information into human cells provide hope for improved therapy in several neurological diseases, including epilepsy. We evaluated the ability of an adeno-associated virus (AAV) vector to transfer and cause expression of a *lacZ* marker gene in brain slices obtained from patients undergoing temporal lobectomy for control of medically intractable seizures.

Methods: Human brain slices were injected with an AAV vector (AAV*lacZ*) encoding *Escherichia coli* β -galactosidase and incubated for as long as 24 h. The presence of *lacZ* mRNA, β -galactosidase protein and enzymatic activity were assayed by reverse transcriptase polymerase chain reaction (rtPCR), immunocytochemistry, and the X-Gal technique, respectively.

Results: AAV*lacZ* directed the expression in human epilep-

togenic brain of *E. coli* β -galactosidase that had functional activity. Expression was observed in ≤ 5 h and was sustained for as long as the slices were viable. Morphological analysis indicated that neurons were preferentially transfected, and there was no evidence of cytotoxicity.

Conclusions: Our results confirm the feasibility of using AAV vectors to transfer genes into the human CNS and in particular, into neurons. Replacement of the *lacZ* gene with a functional gene modulating hippocampal neuronal physiology, might allow a localized genetic intervention for focal seizures based on the stereotaxic or endovascular delivery of such a vector system into the appropriate brain region. **Key Words:** Gene therapy—Epilepsy—Adeno-associated virus—Brain slice—Hippocampus.

Newly developed methods for gene transfer into the CNS provide hope for improved therapy in several neurological diseases, including epilepsy (1-5). Certain forms of epilepsy, such as complex partial seizures (CPS) originating in the mesial temporal lobe (6-8), are most likely to be candidates for a gene therapy approach since methods are available for local delivery of vector systems (9-12). Stereotaxic introduction of vectors containing genes encoding inhibitory neuropeptides, neurotransmitters, or their biosynthetic enzymes into the location responsible for seizure onset, for example, might result in abatement of such seizure activity. In addition, introduction of genes encoding postsynaptic receptors responsible for synaptic inhibition or proteins mediating

intracellular signaling pathways may provide alternative approaches for genetic intervention.

Recent studies lend support to the development of gene therapy-based strategies for epilepsy therapy. Introducing genes encoding enzymes involved in neurotransmitter or neuromodulator biosynthesis increases CNS production and release of these substances, with expected biochemical and behavioral correlates in vitro and in vivo. We and other groups have developed viral vectors that contain sequences encoding enzymes responsible for dopamine biosynthesis (2,13-15). After introduction of these vectors into striatal cells in vitro and in vivo, L-DOPA and/or dopamine synthesis and release are stimulated, resulting in restoration of aberrant behavior in the 6-hydroxy-dopamine lesioned rat model and methyl phenyl tetrahydropyridine (MPTP) primate model of Parkinson's disease (2,13,14). Similarly, viral vectors have been used to deliver neurotrophic factors into localized brain regions, resulting in restoration or preservation of CNS function (16-19).

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Some CNS gene transfer studies have bypassed the presynaptic neuropeptide, neurotransmitter, or growth factor (GF) entirely, focusing instead on their receptors and coupled signal transduction systems. Using a modified HSV-1 vector containing the gene encoding the GluR6 excitatory amino acid receptor subtype, Bergold et al. (19) demonstrated a selective loss of CA3 pyramidal cells and hilar neurons in transfected organotypic rat hippocampal slices, paralleling some of the pathology observed in temporal lobe epilepsy. An HSV-1 vector containing the gene encoding the human p75 nerve GF (NGF) receptor induced the expression of this gene in cultured cortical neurons, leading to high-affinity NGF binding (20). In additional studies, we and other investigators have developed HSV-1 vectors containing genes encoding signal transduction enzymes, with physiological effects evident *in vitro* and *in vivo*. For example, the HSV-1 amplicon vector, pHSVcyr, placing the catalytic domain of adenylate cyclase under control of the HSV IE 4/5 promoter, resulted in significant increases in adenylate cyclase activity and cyclic AMP formation in cultured rat sympathetic neurons (21).

Until recently, most studies of viral-based gene transfer into the CNS focused on retroviruses, HSV-1, vectors and Adenovirus (22–28). Because of the inability of retrovirus vectors to transfect nondividing, postmitotic cells, such as neurons and most dormant glial cells in the CNS, this virus system has been largely devoted to developing a gene therapy approach to malignant CNS tumors containing rapidly dividing cells. In contrast, HSV-1 vectors have several features that are advantageous for gene transfer into the postmitotic CNS, yet application of such vectors to human disease is problematic because of their documented cytotoxicity, potential for reversion to wild-type, and unknown interactions with a host already harboring latent HSV-1 (1,2,5,22,23). Like retroviruses, Adenovirus appears to transfect mitotic glial cells preferentially *in vivo* and *in vitro*, and concerns have been raised about its cytotoxicity and immunogenicity, believed to be related in part to persistent expression of viral proteins (25,26,29–35). We have therefore initiated a series of studies examining the potential of an alternative viral vector system based on AAV to introduce and cause the expression of genes in the CNS. AAV has several desirable features: It is a DNA virus, not directly associated with any human disease; it can direct transgene expression in numerous cell types, including postmitotic neurons; and it may stably integrate into the host chromosome (2,27,36–39). Furthermore, helper virus-free vector stocks that do not express any viral proteins, rendering an immune response less likely, can be obtained.

Until now, there has been no report of AAV vector-mediated gene transfer directly into postmitotic human CNS tissue. However, before viral vectors are applied to

human neurological diseases, such as epilepsy, demonstration of effective gene transfer into the postmitotic human CNS is essential. Therefore, we examined the ability of an AAV vector, AAVlacZ, which contains the marker *lacZ* gene, encoding *Escherichia coli* β -galactosidase, to transfer and cause expression of this transgene in human hippocampal tissue. When introduced into slices of human hippocampus derived from patients undergoing surgical resection for medically intractable seizures, AAVlacZ directed the expression of *E. coli* β -galactosidase that had functional activity. Neurons were preferentially transfected, and there was no evidence of cytotoxicity. These results further validate the potential of viral-vector-based approaches for therapy in human neurological diseases, in particular in certain forms of epilepsy. Replacement of the *lacZ* gene with a therapeutically relevant gene, such as that encoding glutamic acid decarboxylase (GAD) or the adenosine A₁ receptor (40,41), may provide localized genetic intervention for focal seizures based on the stereotaxic or endovascular delivery of such a vector system into the appropriate brain region.

MATERIALS AND METHODS

Viral vectors

The construction of AAVlacZ has been described previously (27,36). The AAV plasmid encoding the *lacZ* gene was developed by subcloning the cytomegalovirus (CMV) immediate-early promoter, *lacZ*, and a simian virus 40 (SV40) polyadenylation signal between the terminal repeats of the AAV genome in plasmid psub201. These termini contain the recognition signals necessary for packaging into an AAV vector. Cells were cotransfected with pAAVlacZ and pAd8, which provides AAV structural proteins but lacks AAV termini and cannot package into virus. Cells were then infected with Adenovirus type 5 to provide remaining functions necessary for cleavage and packaging. The resulting stock consisted of packaged AAVlacZ vectors and progeny helper Adenovirus; helper virus was then eliminated by heating at 56°C for 30 min. The final titer of the stock solution of AAVlacZ was 10⁷ particles/ml.

Human hippocampal slices

With approval of the Graduate Hospital Institutional Review Board and with informed consent from patients obtained before surgery, human hippocampal tissue was obtained in the operating room immediately at the time of dissection and placed in ice-cold, low Ca²⁺, high Mg²⁺ artificial cerebrospinal fluid (ACSF) containing (in mM) 124.0 NaCl, 5.0 m KCl, 1.25 m NaH₂PO₄ · 2H₂O, 26.0 NaHCO₃, 0.2 CaCl₂, 5.0 Mg₂SO₄ · 7H₂O, and 10 glucose, saturated with 95% O₂/5% CO₂. In the laboratory, blood vessels and connective tissue were carefully removed. Tissue blocks ~2–3 mm deep were injected in

several locations with 3–10 μ l virus stock or control (phosphate-buffered saline, PBS) with a 28-gauge Hamilton syringe in 2–3 min, after which the syringe was slowly removed to prevent viral reflux. With the tissue bathing for several minutes in the same ice-cold ACSF solution, 500- μ m sections were cut on a vibratome (Ted Pella, Redding, CA, U.S.A.). These sections were then incubated at 34°–35°C for 5–24 h in biological ACSF (in mM: 124.0 NaCl, 5.0 KCl, 1.25 NaH₂PO₄ · 2H₂O, 26.0 NaHCO₃, 2.0 CaCl₂, 5.0 Mg₂SO₄ · 7H₂O, and 10 glucose, saturated with 95% O₂/5% CO₂). (Chemicals were obtained from VWR, West Chester, PA, U.S.A.). Because human hippocampal slices were not viable after 24 h, expression after that timepoint could not be assessed. For immunocytochemical analysis, sections were fixed in 4% paraformaldehyde/PBS for 24–48 h. For reverse transcriptase polymerase chain reaction (rtPCR) and fluorometric analysis, the tissue sections were snap-frozen in Eppendorf tubes with dry-ice/methanol and maintained at –70°C until use.

Immunocytochemical techniques

After fixation, brain slices derived from 5 patients were immersed in 30% sucrose/PBS as a cryoprotectant for 24 h; 500- μ m slices were further sectioned into 30- μ m slices on a freezing microtome, incubated for 40–60 min in 0.1% H₂O₂ in PBS, immersed in blocking solution containing 2% goat serum, 2% avidin D and 0.2% triton for 1 h, and treated for 24–48 h in solution with or without primary antibody (5'→3', Boulder, CO, U.S.A.) to β -galactosidase (rabbit, 1:2000 dilution in 1.5% goat serum and 2% biotin in PBS). Thereafter, addition of secondary antibody and the visualization step were achieved by the Vectastain ABC-diaminobenzidine (DAB) method (Vector Laboratories, Burlingame, CA, U.S.A.). Floating sections were mounted in a PBS bath on gelatin- or poly-L-lysine-treated glass slides. Secondary antibody (50 μ l anti-rabbit, 150 μ l normal goat serum (NGS), and 10 mL PBS) and ABC solution (50 μ l A, 50 μ l B, and 10 ml PBS) were added for 1 h each. The DAB solution consisted of 50 μ l DAB, 50 μ l H₂O₂, 200 μ l buffer, and 10 ml H₂O with an exposure time of 3–4 min. Each step was followed by two washes in PBS.

Chemiluminescent assay for β -galactosidase

The lumiGAL chemiluminescence assay (Clontech, Palo Alto, CA, U.S.A.) was used to quantitate β -galactosidase activity after transfection of slices derived from 3 patients. This assay has been demonstrated to be more sensitive than standard colorimetric and fluorometric assays and has been described previously (42). For application to our experiments, cell lysates were prepared by first pulverizing AAVlacZ-transfected and control frozen hippocampal specimens on a liquid N₂-cooled mortar and pestle. Equivalent 100-mg pulverized specimens were then added to 200 μ l cell lysis buffer diethylthreitol

(DTT) (1 mM), vigorously vortexed and centrifuged (14,000 rpm); 20- μ l aliquots of the lysates were then added to 200 μ l cell reaction buffer and 2 μ l Galacton, the chemiluminescent substrate. The reaction was then allowed to proceed at room temperature for 30 min. After this incubation period, 300 μ l reaction accelerant was added to each sample before immediate measurement was made of relative light units (RLU) with a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI, U.S.A.).

rtPCR

We extracted RNA from snap-frozen tissue samples derived from 2 patients using RNAzol B (Cinna/Biotech, Houston, TX, U.S.A.), a commercial mixture containing phenol and guanidinium isothiocyanate. After chloroform extraction and ethanol precipitation, RNA was re-suspended in diethylpyrocarbonate-treated water and OD₂₆₀ readings were taken to determine concentration. We then performed rt on each sample with 1 μ g RNA, 1 \times PCR buffer, 1 mM each dCTP, dGTP, dTTP, dATP (Cetus, Emeryville, CA, U.S.A.), 5 mM downstream PCR primer and 200 U Mo-MuLV rt (GIBCO BRL, Grand Island, NY, U.S.A.) in a final volume of 20 ml. A negative control containing all of the reaction components without RNA was added. A positive control contained AAVlacZ plasmid and all other reaction components without RNA. rt reaction was allowed to proceed at 42°C for 60 min, followed by heat inactivation of rt at 65°C for 15 min. Samples were then brought to 100 μ l by addition of 5 mM of both upstream and downstream primers, additional PCR buffer for a final concentration of 1 \times , and 2.5 U Taq polymerase (Cetus). Primers directed against the lacZ gene were lacZ 182 (5'CCGAC-TGATGCCTT CTGAACAA-3') and lacZ 560 (5'-GACGACAGTATCGGCCTC AGGA-3'), which have been described previously (43). Thirty cycles of PCR were performed, with each cycle consisting of 45 s at 94°C, 45 s at 60°C, and 45 s at 72°C; 10 μ l of each PCR reaction was then analyzed on a 3% NuSieve/1% Agarose gel (FMC, Chicago, IL, U.S.A.) in 1 \times TAE, and products were visualized by staining for 15 min in 1 mg/ml ethidium bromide (Sigma, St. Louis, MO, U.S.A.). The expected length of the amplified lacZ sequence was 380 base pairs (bp). Reference markers (FX174/HaeIII digest) were obtained from New England Biolabs (Beverly, MA, U.S.A.).

RESULTS

As shown in Fig. 1, the prototype vector, AAVlacZ contains several sequences. The functional domain has the lacZ gene placed under control of the CMV promoter, with a downstream SV40 polyadenylation sequence. rtPCR analysis of human hippocampus transfected with AAVlacZ disclosed the presence of

lacZ mRNA, as shown in Fig. 2. A *lacZ* fragment was detected between the 310- and 603-bp markers in AAV*lacZ*-transfected tissue. An identical fragment, was not observed in PBS controls, was observed in the positive control containing AAV*lacZ* plasmid. This finding demonstrated that the AAV vector resulted in successful transfer and transcription of the *lacZ* transgene into the expected messenger RNA.

Cryostat sections of human hippocampal tissue injected with AAV*lacZ* demonstrated significant immunoreactivity for β -galactosidase in cells surrounding the injection tract, as shown in Fig. 3a. Staining of cells was apparent as much as several millimeters from the site of injection. Because $\sim 5 \times 10^4$ viral particles were injected into each hippocampal tissue section and only several hundred cells could be counted in the summed cryostat sections for a hippocampal slice, the efficiency of in situ transfection by AAV*lacZ* appears to be relatively low, a problem that plagues all viral vector-mediated gene transfer approaches. Hippocampal tissue injected with buffer did not demonstrate immunoreactivity for β -galactosidase (Fig. 3b); neither did sections incubated without primary antibody exhibit staining (data not shown). High-power magnification of β -galactosidase-immunoreactive cells demonstrated evidence of neuronal morphology with pyramidal and flask-shaped cell bodies and extensive processes (Fig. 3c and d). More than 95% of the cells that stained were of neuronal morphology. Staining was demonstrated in perikaryonal and nuclear regions, as well as processes. The specific immunoreactivity for β -galactosidase observed in tissue transfected with AAV*lacZ*, and not control, provided evidence of

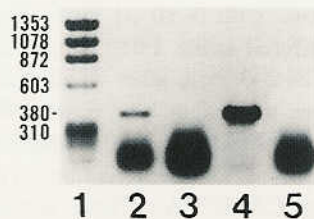


FIG. 2. Reverse transcriptase polymerase chain reaction (rtPCR) analysis of *lacZ* mRNA expression. *LacZ* mRNA was detected in hippocampal tissue transfected with AAV*lacZ*. rtPCR showed a partial *lacZ* sequence in AAV*lacZ*-transfected tissue, was not detected in the phosphate-buffered saline (PBS) control. This sequence was located between the 310-basepair (bp) and 603-bp markers; the expected size of the amplified sequence was 380 bp. Lane 1, Markers (bp); Lane 2, AAV*lacZ* transfection; Lane 3, PBS control transfection; Lane 4, positive control containing AAV*lacZ* plasmid; Lane 5, control without RNA.

transgene-directed transcription and translation into the expected protein.

Using an established chemiluminescent assay for β -galactosidase activity, we examined control and AAV*lacZ*-transfected tissues. Hippocampal slices from transfected tissue showed marked increases in β -galactosidase activity (in RLU) over control (Fig. 4), with a mean of 2.3×10^6 RLU for experimental slices and 1.2×10^4 RLU for control. These results demonstrate that not only did AAV*lacZ* direct synthesis of the bacterial protein in human hippocampal tissue, but that this protein had the expected functional enzymatic activity.

DISCUSSION

Significant advances in molecular biology made in the past decade have resulted in development of several approaches for delivery of genetic information into the CNS. One of the most promising of these approaches is the application of DNA-containing viral vectors that permit direct introduction of a cloned gene into neurons and/or glia, resulting in its expression into a functional protein. Three broad categories of DNA viruses are currently being used for CNS gene delivery; these are based on HSV-1, Adenovirus and, most recently, AAV (1-5,13-15,22-27,36,39).

As a gene therapy vector, AAV has several desirable features (2,27,36). Notably, unlike wild-type HSV-1 and Adenovirus, which can cause disseminated infections in humans, AAV has not been associated with any human disease, although more than three quarters of the United States population is seropositive for the AAV virus (36,39). In addition, AAV is a defective virus, unable to replicate in the absence of a helper virus, which commonly is Adenovirus. AAV is also unique in that it can integrate into a specific site in the host chromosome (37-39). AAV vectors can direct expression of genes in both mitotic and postmitotic, terminally differentiated cells, including neurons, whereas Adenovirus preferentially transfects dividing cells (14,26,29). A further ad-

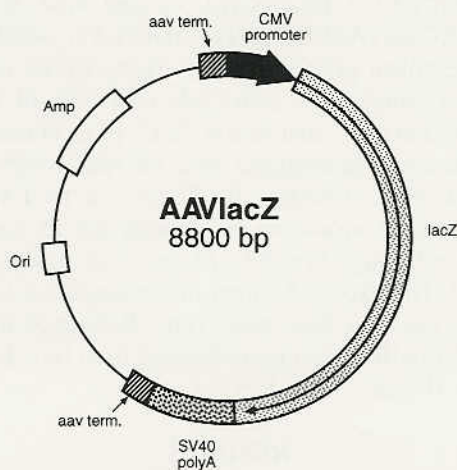


FIG. 1. Schematic of AAV*lacZ* plasmid. In the vector AAV*lacZ*, the *lacZ* gene encoding *Escherichia Coli* β -galactosidase has replaced all sequences encoding viral proteins between the AAV terminal repeats. The Cytomegalovirus (CMV) promoter drives *lacZ* transcription with a simian virus 40 (SV40) polyadenylation sequence downstream. The AAV plasmid also contains a bacterial origin of replication (Ori) and an ampicillin resistance sequence (Amp).

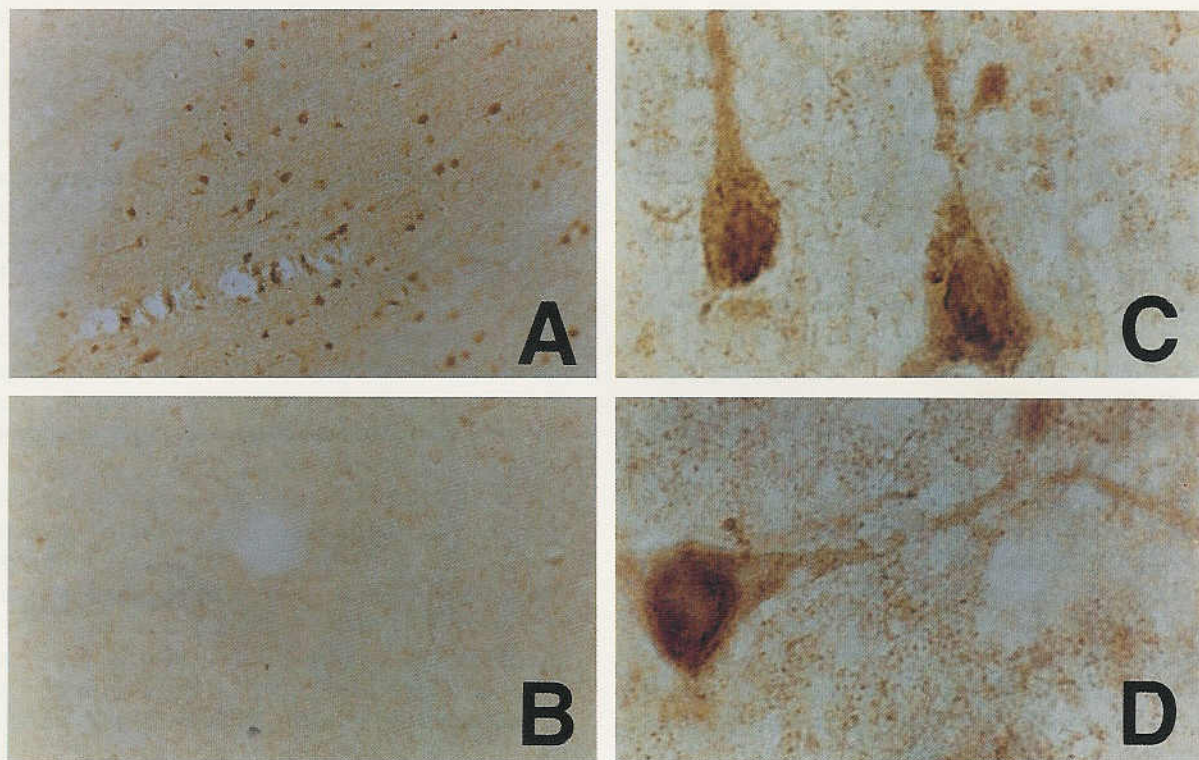


FIG. 3. Immunohistochemistry for β -galactosidase. β -Galactosidase immunoreactivity (IR) was demonstrated in hippocampal slices transfected with AAV/*lacZ* (A), which was not present in phosphate-buffered saline (PBS) control tissue (B). Higher magnification demonstrated that cells showing β -galactosidase-IR had neuronal morphology (C and D). Immunostained tissue sections in which primary antibody was omitted showed no β -galactosidase-IR (data not shown). Original magnifications: A and B $\times 300$, C and D $\times 650$.

vantage of AAV is that pure, helper-free stocks can readily be made with a novel packaging system that completely eliminates production of immunogenic viral proteins, a feature not currently available with either HSV-1 or Adenovirus (36). Finally, we recently observed that markers of neuronal cytotoxicity, such as lactate dehy-

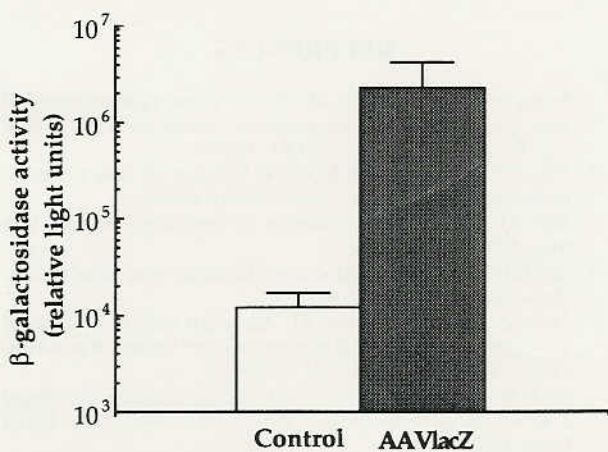


FIG. 4. LumiGal analysis of β -galactosidase activity. β -Galactosidase enzymatic activity was detected in AAV/*lacZ*-transfected hippocampus and was ~ 100 -fold higher than that in phosphate-buffered saline (PBS) controls. Results are mean \pm SEM of 8 experimental and 8 control tissue slices derived from 3 patients. Enzymatic activity is expressed in relative light units (RLU).

rogenase (LDH) release and heat shock protein-72 (hsp-72) expression are not induced by AAV, but are induced by some Adenovirus vectors (unpublished observations). These combined observations suggest that AAV vectors may prove to be safe and efficacious for gene therapy approaches to CNS disorders.

Disadvantages of AAV (2,27,36) include its small packaging size; inserts are limited to ~ 4.7 kilobases, making constructs with multiple genes or cell-specific promoters difficult to achieve. Unlike wild-type AAV, the vectors appear to have a lower frequency of targeted integration, probably reducing the stability of the transgene. The titers that can be obtained for AAV are significantly lower than those for Adenovirus. No studies using viral vectors in the CNS have yet demonstrated transgene expression beyond 1 year, although newer modifications of these vectors may soon permit long-term expression.

In the present study, we demonstrated for the first time the successful transfer and expression of a transgene directly in human CNS tissue, using an AAV vector. In human hippocampal slices obtained from patients undergoing temporal lobectomies for medically intractable CPS, we demonstrated that an AAV vector can transfer a transgene into this tissue, resulting in the proper transcription and translation of the transgene.

These studies confirm the feasibility of using AAV vectors to transfer genes into the human CNS, and neurons in particular, for genetic intervention in a variety of diseases. Recently, we demonstrated that AAV vectors could transfer the tyrosine hydroxylase gene, which encodes the rate-limiting enzyme in dopamine biosynthesis, into the rat striatum (27). In the 6-hydroxydopamine-lesioned rat model of Parkinson's disease, aberrant rotational behavior was reduced and striatal dopamine levels were restored for several months. Although gene transfer with such viral vector systems is of low efficiency, biochemical and behavioral function can be restored, particularly if modest augmentation of neurotransmitter, neuromodulator, GF, receptor, or second-messenger production is the goal. Unlike gene therapy for malignant tumors, in which virtually all cells must be either transfected or destroyed, a smaller percentage of cells must be transfected for neurotransmitter- or neuromodulator-based restorative genetic intervention.

Focal epilepsy disorders may prove an appropriate target for gene therapy intervention. Because of the focal nature and evidence of loss of inhibitory neurotransmission in such disorders, restoration of the inhibitory neurotransmitter milieu may successfully avert seizure onset (6-8,41,44-45); this could be accomplished by introducing genes encoding inhibitory neurotransmitters and/or their biosynthetic enzymes. One example is GAD, which is responsible for the conversion of glutamic acid into γ -amino butyric acid (GABA), the primary inhibitory neurotransmitter in the CNS (40). Recently, New et al. (46) and we (M. J. During et al., unpublished observations) developed viral vectors containing a GAD gene; in ongoing studies, this vector's effects on GABA biosynthesis and electrophysiological correlates in cultured hippocampal cells, rat epilepsy models, and human hippocampal slices are being evaluated.

Because AAV vectors can transfect neurons, gene therapy strategies based on introducing sequences encoding postsynaptic receptors, such as the α -subunit of the GABA_A receptor or the adenosine A₁-receptor, both believed to mediate inhibition of electrical activity in the hippocampus and in models of epilepsy, are feasible (27, 41,47,48). We recently developed an AAV vector containing the adenosine A₁-receptor, and studies in our laboratories are focused on evaluating the physiological effects of this vector in hippocampus (M. J. During et al., unpublished observations). Another strategy could use signal transduction systems coupled to postsynaptic receptors, bypassing the neurotransmitter machinery. Viral vectors have been developed that contain sequences encoding the catalytic domains of a variety of signal transduction enzymes, including adenylate cyclase, protein kinase C, and calcium/calmodulin-dependent protein kinase II (21,49,50).

AAV-mediated gene transfer into the human hippo-

campal slice preparation should also provide a useful model for investigation of the physiological and biochemical effects of potentially therapeutic genes and for elucidating the roles of other endogenous proteins (8,51-54). Introduction into the hippocampal slices of vectors directing production of antisense sequences to native mRNA species may also provide valuable information about the function of normally expressed genes by blocking their synthesis, essentially creating a 'knockout' hippocampal slice preparation. For example, for better understanding of the role of neuropeptide Y (NPY) in modulating seizure activity (55), introduction of a vector directing production of a NPY antisense sequence should provide a method to explore the role of this neuropeptide in human and rat hippocampal slices.

Current therapeutic approaches for epilepsy are often suboptimal, requiring either frequent dosing of pharmaceuticals that have significant side effects or disruptive surgical intervention. As many as one third of patients with epilepsy do not have adequate seizure control (56-58). For certain subsets of epilepsy, particularly those characterized by focal onset, genetic intervention may prove particularly effective. Methods for global CNS delivery (10,59) of viral vectors may allow application of such therapy to other forms of epilepsy, particularly because the genetic alterations responsible for a number of forms of epilepsy are being defined (60-64). In the present study, we demonstrated that AAV vectors may prove useful in delivering therapeutic genes into epileptogenic human brain. Further evaluation of the biochemical and electrophysiological correlates of such vectors in human brain slices may provide an incentive for eventual application to patients with epilepsy, although obstacles with regard to long-term expression, targeted delivery, cytotoxicity, and immunogenicity must first be overcome.

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