Viral-based gene transfer to the mammalian CNS for functional genomic studies

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A fundamental problem in neuroscience has been the creation of suitable *in vivo* model systems to study basic neurological phenomena and pathology of the central nervous system (CNS). Somatic cell genetic engineering with viral vectors provides a versatile tool to model normal brain physiology and a variety of neurological diseases.

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Over a decade ago, herpes simplex viral (HSV) vector was proposed as a tool for brain functional genomics and for the molecular analysis of neuronal physiology[1]. At that time, it was considered the leading expression vector for introducing foreign genes to the mammalian CNS, with a view towards human gene therapy. The utility of viral vectors for the characterization of CNS gene function was confirmed when HSV was shown to promote biochemical and phenotypic changes in animals after neurosurgical delivery of genes for human enzymes[2,3]. Subsequently the genes for molecular transporters, neurotransmitter receptors, and transcription factors were introduced to the mammalian brain using HSV. Newer vectors such as recombinant adeno-associated virus (rAAV), which can direct much greater levels of sustained gene expression in the CNS, are better suited to *in vivo* analysis or clinical application. rAAV is a powerful tool for functional genomic analysis as a result of its key properties of neurotropism and non-pathogenicity, in addition to the high levels of expression that are possible, now over 10^5 transduced neurons per microliter of vector, with titers in excess of 10^{12} genomic particles per milliliter[4]. A growing viral repertoire including rAAV, lentivirus, and pseudotyped vectors with elements from two or more viruses, permits the efficient transfer of selected genes to specific groups of cells in the CNS in order to create and test models of brain development and disease.

The role for viral vectors in brain functional studies

The brain is the most complex mammalian organ, functionally as well as genetically, expressing a greater variety of genes compared with any other tissue[5,6]. Having the capability to rapidly and efficiently alter brain gene expression will be of benefit not only for gene therapy applications, the focus of most gene transfer studies to date, but it will be equally significant for defining the effects of genes throughout the many functional and anatomical regions of the CNS. In this context brain 'functional genomics' involves *in vivo* study and characterization of genes with unknown or inadequately defined properties. Until recently, with the tandem development of viral-based and antisense technologies, molecular genetic study of the brain was hampered by a lack of tools for fast targeting and modification of subcellular brain function in mammals. In this review we describe how viral vectors, in conjunction with other methods, can play a pivotal role for studying the function of genes in the mammalian CNS. Three emergent areas involving manipulation of the brain transcriptome will particularly benefit from more systematic and widespread use of neurotropic viral vectors.

(1) Large-scale expression studies of poorly characterized genes throughout different brain regions, to gain a better understanding of their physiological effects or to recapitulate developmental processes.

(2) Expression of selected genes to create novel animal models of disease.

(3) Expression of selected genes to ameliorate existing models of disease and to better define the actions of genes under pathological conditions.

We propose that viral vectors such as rAAV should be incorporated as part of a comprehensive approach to functional genomics in the brain, rather than merely as another isolated technique.

Why use viral vectors?

The basic premise underlying any viral-based functional genomics platform is that viral vectors can 'turn on' genes by overexpression of a gene of interest or associated transcription factors, or alternatively can 'turn off' genes through expression of antisense, protein transcriptional repressors[7], or dominant-negative isoforms. Viral vectors have become increasingly popular for *in vivo* expression, because of their efficiency, versatility, and relative ease of use. In theory any neurotropic virus can be modified so that its genome is replaced with other genes, together with associated promoters and regulatory sequences that are necessary to drive expression. This substitution requires the provision of deleted replicative genes in *trans* during the viral manufacturing or 'packaging'

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Box 1. Viral vectors: structure and mechanisms of action

In general, the process of viral cell entry involves binding and internalization, partial or complete disassembly of the capsid and core proteins, and finally interactions between the host and vector genome, which, depending on the virus might include insertion (i.e. stable incorporation into the host genome), episomal persistence, and intermediate steps such as replication or conversion of the nucleic acid. To become a gene transfer vector, the viral genome is partially or fully deleted in order to accommodate foreign genes and to render the virus incapable of replication or pathogenicity in the host. One key feature of any viral vector is the promoter/enhancer and regulatory elements chosen for a given application, which can be selected to maximize gene expression within subsets of neurons or glia, commonly known as promoter targeting. Although compatible with strong constitutive and cell type-specific promoters, rAAV transduces neurons to a much greater extent than glia, and some types of neuron are preferentially affected. Therefore, inherent limitations exist on promoter targeting, and capsid modifications have been investigated in order to re-direct rAAV to cells outside its natural tropism or to optimize expression in different subtypes of neurons.

The goal of capsid engineering is to retain structural integrity and infectiousness while eliminating immunogenic or toxic proteins. Because it is non-pathogenic, the rAAV capsid is modified mainly to alter the tropism, while pathogenic viruses such as adenovirus, lentivirus, or herpesvirus are modified primarily to decrease adverse effects on the host. Another reason to change the capsid proteins is to alter the topology and thereby increase the potential gene insert size for small viruses, such as rAAV, although this possibility has not been well-studied to date. Altering surface epitopes could create new immunogenicity problems, and structural modifications, particularly those that change subunit stoichiometry, could result in non-infectious virus. Nevertheless, the addition or deletion of peptides holds promise for more-selective targeting of viral particles. Several cellular receptors have been identified for viral vectors, and changes in the capsids have been shown to alter the receptor tropism, not only of AAV (Refs a–c) but also of lentivirus[d,e] and adenovirus[f–h].

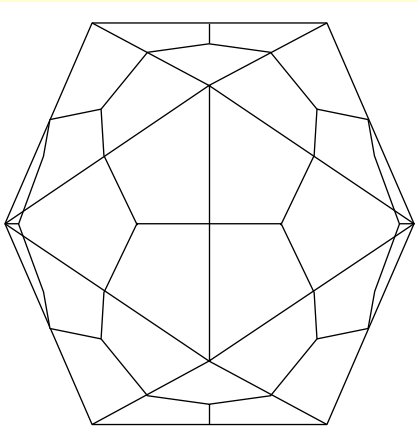

Fig. I. Schematic diagram of AAV. The AAV capsid is ~20 nm in diameter and consists of three proteins (VP-1,2,3) in a molar ratio of 1:1:8, which are non-toxic to neurons and glia, even at high concentrations. Although the precise crystal structure of AAV capsid has not been solved as of this writing, it is believed to be very similar to other parvoviruses such as human B19 virus and canine parvovirus. There are six naturally occurring human serotypes of AAV (AAV1-6), which are thought to share a similar structure. In terms of its nucleic acid component, the rAAV vector is totally devoid of all viral DNA elements except for the 145 bp long terminal repeats (LTRs), which provide promoter-enhancer activity. Hence the rAAV vector can be conceptualized as a recombinant DNA core encapsidated by three viral structural proteins.

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process, which was originally carried out with helper virus co-infection of cultured cells but now is often carried out with stably transfected packaging cell lines[8–10]. Although numerous viruses represent potential tools for gene transfer, only a handful are commonly in use, mainly because they are well-characterized or require less modification.

An ideal viral vector for gene transfer should be capable of efficiently infecting cells, yet nontoxic to the host organism; able to accommodate large gene inserts (or 'expression cassettes'); easily modified in its nucleic acid and protein structure; fully regulatable in its expression; and rapidly produced and purified in great quantities. Currently no single viral vector meets all these criteria and those in use vary in stability and strength of expression, immunogenicity, cytotoxicity, maximum gene insert size, cell binding and internalization properties ('tropism'), axonal transport, and technical challenges of manufacturing. Although a detailed comparison of viral vectors is beyond the scope of this article, which will focus on the single-stranded DNA (ssDNA) adeno-associated virus (AAV), some other vectors in common use include double-stranded DNA (dsDNA) viruses such as herpes simplex virus or adenovirus[11,12]; plus-strand RNA viruses such as picornavirus, alphavirus, and lentivirus, including a variety of modified zootropic viruses[13–16]; and minus-strand RNA viruses such as rhabdovirus[17,18].

Neurotropic viral vectors such as rAAV present many advantages for brain functional studies: they can efficiently express either single or multiple foreign genes; they can incorporate a wide variety of regulatory elements; they can be engineered at the

Box 2. Alternative approaches to somatic cell transgenesis

The main alternative to viral vectors for CNS somatic gene transfer has been liposomal-plasmid complexes. Though limited in their transport and expression levels in the brain, they are chronically less toxic compared with many viral vectors. Certain formulations and delivery protocols have provided high levels of mammalian brain expression *in vivo*[a,b], yet lipid-based gene transfer remains a relatively crude method and as a rule, genes cannot be directed to specific subsets of brain cells (e.g. neurons versus glia) for functional genomics applications, although expression can be restricted through promoter targeting. For this reason, lipid-based systems might be better suited for CNS delivery of antisense, or for the special requirements of regions such as the spinal cord. More-specific 'non-viral' techniques of gene transfer have been developed in the past few years, including peptide-based dendrimers and other protein compounds that ironically often share close homology with viral or bacterial surface proteins. For example, peptide fragments from HIV and SV40 have demonstrated promising neuronal gene transfer potential, as well as peptides derived from *Clostridium* bacteria[c–f]. Some investigators have introduced plasmid vectors to the brain via linkage to endothelial receptor-targeted antibodies[g] or antigen-coupled nucleic acid clamps[h] in order to traverse the blood–brain barrier, but levels and distribution of expression are lower than with viral vectors. Although interchangeable gene 'cassettes' in viral vectors have the potential for rapid and versatile gene expression, other approaches might be better for preliminary *in vivo* screening of gene function. One useful approach involves linear expression elements[i], which can drive short-term expression of genes in animals, although the levels of expression are not high and these vectors have no specificity for neuronal cells.

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capsid and promoter level to preferentially target either neurons or glia; they can be administered at any developmental stage, from *in utero* to adult or senescent animals; they are not host-specific when administered directly to the brain, or can be modified for infecting different species, and thus can be used in virtually any mammalian system as well as in many lower vertebrates and invertebrates; they permit either short-term or long-term CNS gene expression and temporal-spatial targeting of genes to different regions of the brain through stereotaxic delivery; they are inexpensive and rapid to generate compared with classical transgenics; they can be used in conjunction with other transgenic methods; and most importantly, they appear innocuous to normal brain functioning[19–21] and even at high doses can be used without fear of causing non-physiological artifacts (Box 1).

Limitations of viral vectors

An important limiting factor for functional genomics is viral packaging, both in terms of the time required and the maximum insert size. With rAAV, packaging is generally limited to ~5 kb, although some transgenes are thought to adopt unique conformations and might package differently, perhaps allowing for an insert of up to 5.7 kb (Ref. 4). In the future, technical advancements involving *in vivo* splicing of transcripts might expand the maximum size for gene inserts[22], although the reliability and general applicability of this technique is uncertain. Another general problem with viral vectors is the possibility of harmful recombination events such as insertional mutagenesis or reversion to wild-type, particularly when pathogenic viruses are re-designed for gene transfer. Because all viral genes are deleted in rAAV, reversion to wild-type is not a serious concern. Even if it were to occur, the wild-type virus (wtAAV) is non-pathogenic and incapable of autonomous replication; it is called a 'dependovirus,' because it ordinarily depends on other viruses such as adenovirus for replicative function. wtAAV is capable of stable incorporation into the genome and this insertion, mediated in part by the rep protein, is site-specific on human chromosome 19. Because *rep* is deleted in rAAV, the vector DNA is believed to exist mainly in episomal form, with variable levels of integration in non-dividing cells. Although rAAV integration is not site-specific, some investigators have suggested that it is not entirely random in brain cells[23,24]. Integrated rAAV has not been reported to have any mutagenic or deleterious effects, yet high-dose vascular delivery could be tumorigenic and further long-term studies need to be done[25]. Another issue is non-specific host effects on the activity of the virus, such as promoter silencing or immunological reaction. With rAAV a variety of strong promoters allow stable, high-level expression[4,26], and *in vivo* promoter silencing does not appear to be a problem, nor has immune reaction been reported upon repeat administration to the brain.

In the past, viral vectors were not considered useful for downregulation of genes. However, as the interactions among genes are better understood this limitation no longer applies. For example, viral vectors can be engineered to express transcriptional repressors, intracellular antibodies ('intrabodies')[27–29], antibody-stimulating antigens[30], or chemokines[31] and other cellular transduction factors, in order to specifically downregulate gene expression. The converse has been true with antisense technology – it is ideal for downregulating genes in the brain[32–34], but very few methods appear to upregulate genes as effectively as viral vectors[35,36]. In the future, delivery of antisense to the brain will remain vital for

Table 1. Methods of transgenesis for CNS functional studies

Method	Advantages	Disadvantages	Special features
ES Transgenic	Consistently reproducible, targeted mutagenesis possible.	Slow and expensive, currently limited to mouse, maintenance of breeding animals.	Germline transgenesis, up- or downregulation of genes, small-molecule and cre-lox control possible, useful in conjunction with viral vectors.
Nuclear transfer	Effective on any mammal, banks of clonal ES cells possible, possible therapeutic cloning.	Inefficient, technically demanding, not widely used at present.	Germline transgenesis, up- or downregulation of genes, small-molecule and cre-lox control possible, useful in conjunction with viral vectors.
Viral (rAAV)	Effective on any mammal, fast, specific, non-toxic, precise anatomical targeting, easy temporal control, many therapeutic applications.	Limited gene packaging size, variable long-term expression, invasive surgical delivery, global expression might be difficult.	Somatic or germline transgenesis, up- or downregulation of genes, small-molecule and cre-lox control available, fully compatible with all other methods, enormous variety of constructs available.
Antisense	Effective on any mammal, fast, specific, precise anatomical targeting, easy temporal control, many therapeutic applications.	Some constructs toxic, not consistently reproducible, effects often transient and require redosing.	Somatic transgenesis, mainly downregulation of genes, fully compatible with all other methods.

'knockdown' genomic analysis and *in situ* gene modification or repair[37–39], but viral vectors are likely to be the best method for targeted gene overexpression (Box 2). Several viral-based methods can be effectively combined with antisense, for example rAAV and adenovirus have been used to deliver ribozyme genes[40,41] in order to study gene function or correct a phenotype, and rAAV has been used to introduce antisense RNA with functional effects[42]. In addition, it might be possible to use derivatives of capsid proteins to introduce antisense constructs or plasmids to the brain[43]. By co-administering viral and antisense vectors (Table 1, Box 3) while developing animal models of disease, one can target multiple pathways simultaneously in a selective fashion.

For circumscribed regions of the brain, viral vectors can provide highly targeted and cell-specific gene transfer, and this has been an advantage for research on brain regions such as the basal ganglia. However, many brain diseases or developmental phenomena have widespread effects. Only a few studies have reported the maximum extent of gene expression using rAAV (Ref. 4) or other vectors, and a key issue is whether truly global expression can be achieved for modeling or treatment of diffuse cortical processes such as Alzheimer's disease or brain cancers. The evidence to date suggests that it is possible: for example, convection-enhanced delivery[44,45] has been shown to afford widespread expression after a single injection, and the use of multiple-site injections can significantly increase the extent of vector transduction and associated phenotypic effects[46]. Applications aimed at global effects using rAAV probably will incorporate a pan-neuronal promoter and will use high doses of virus; for example, human subjects that were recently enrolled in a US government-sponsored Phase I trial using rAAV in the brain received over 900 billion genomic rAAV particles spread over six separate subcortical delivery sites[47]. Fusion protein constructs have also shown promise for increasing the extent of global expression[48]. With the improvement of stereotactic guidance systems and injection devices to administer viral vectors, the potential to deliver viral vectors throughout the brain will also increase.

Development, modification, and testing of genetic models using viral vectors

Viral vectors such as rAAV are complementary to transgenic mouse 'knock-ins' or 'knockouts,' but perhaps the greatest advantage is that they are not limited to mice alone. Viral vectors can be used in animals in which embryonic stem cell (ES) technology has not been developed, and are faster and more universally applicable compared with ES-blastocyst fusion, oocyte injection, or nuclear transfer. Somatic cell gene transfer using rAAV has been particularly valuable in rats and primates, in which the physiology is well-defined and behavioral paradigms are well-established[49] but transgenic animals are not available. All mammals can be transduced with rAAV, including monkeys, sheep, and pigs, which might also benefit from advances in germ-line cloning in the coming years. In theory, neurotropic viral vectors make it possible to transfer genes to the brain of any mammal to create a disease model, assuming that suitable candidate genes can be isolated and packaged into the vector. Moreover, viral vectors can be introduced to existing transgenics to test specific gene functions, even genes not directly related to the original knock-in or knockout. Because some

Box 3. Combined use of viral, antisense, and germline transgenic methods

- Target multiple genes simultaneously for activation or downregulation.
- Affect regulation of genes in different neuroanatomical regions.
- Activate or suppress intersecting metabolic and signalling pathways.
- Observe further potentiation or downregulation of gene effects.
- Confirm gene specificity with transgenic knockout/viral vector rescue.

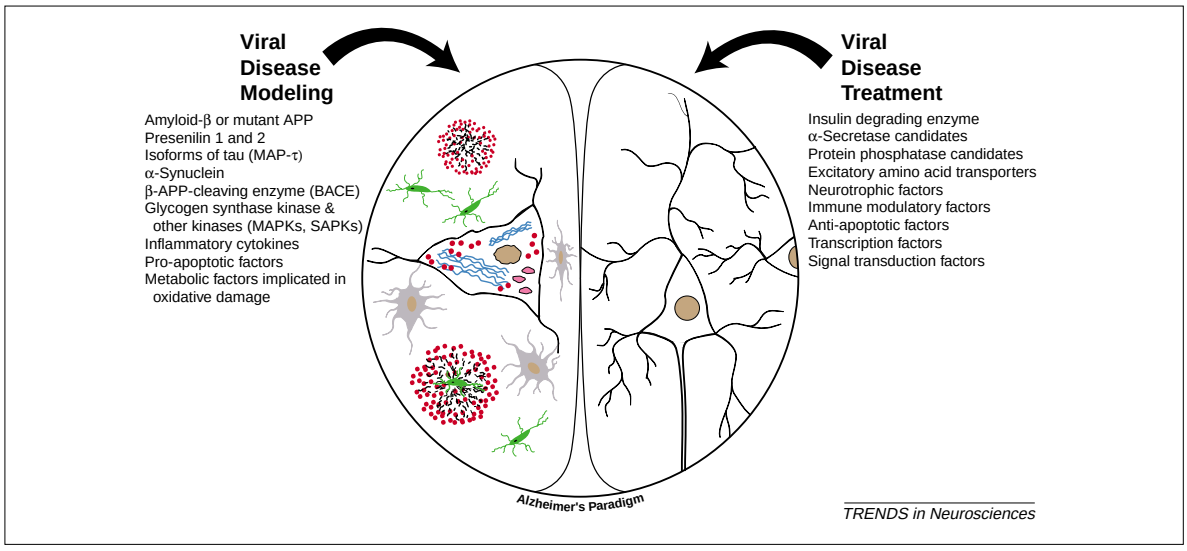

Fig. 1. Section through the primate hippocampus, showing possibilities for viral disease modeling and treatment. Neurons contain pathological Hirano bodies (pink), irregular nuclear membranes, and dystrophic neurites. Intracellular strands (blue) represent neurofibrillary tangles resulting from overexpression of hyperphosphorylated tau. Punctate lesions (red) represent intracellular Aβ, or amyloid plaques outside the neurons. One such plaque incorporates a microglial cell (green). Reactive astrocytes (gray) might also respond to local cues and interact with the diseased neurons. Several gene products are listed which might be amenable to viral gene transfer for the purpose of modeling and/or correcting neurodegenerative changes.

human diseases are not reproducible in a transgenic mouse model[50], a large-animal model is often crucial if treatments are to be rigorously evaluated. For such diseases, an ovine, porcine, or primate somatic-cell 'transgenic' can be generated through the use of neurotropic viral vectors. Viral-based gene transfer can be used to demonstrate the function of a particular gene in naive animals, to rescue the phenotype of an existing transgenic animal, to elucidate related pathways involving different genes in transgenics, and to design new somatic-cell models for use in corroborating gene function and testing therapeutics.

Viral vectors provide a targeted method for introducing multiple genes to the mammalian brain, including combinations of potentially neuroprotective and deleterious genes to assess their relative effects. Several neurological disease models are likely to benefit from these techniques, in particular Alzheimer's disease (AD). In addition to mutant amyloid precursor protein, candidates for amyloidogenic enzymes (β- and γ-secretases) have been isolated[51,52], and genes encoding microtubule-associated protein tau, α-synuclein, ApoE, presenilins, and nicastrin have been implicated in AD pathology. Other deleterious candidate genes might include pro-inflammatory cytokines that act locally to promote neurodegeneration, oxidative enzymes, and pro-apoptotic factors, such as caspases. On the protective side, several metalloproteinase enzymes (e.g. insulin degrading enzyme, neprilysin, ECE-1) capable of degrading amyloid-β have been described[53–59], in addition to non-amyloidogenic amyloid precursor protein (APP) processing enzymes known as α-secretases[60–62], and clearance pathways involving candidates such as α-macroglobulin and LRP (Ref. 63). There is great promise for defining relevant cellular pathways in AD through delivery of these genes to the mammalian brain using vectors such as rAAV (Fig. 1). A key advantage is that specific brain regions implicated in AD, such as the hippocampus and basal forebrain, can be independently targeted with disease-causative genes in animal models; these regions can then be targeted for testing the effects of potentially therapeutic genes (e.g. anti-amyloidogenic enzymes, growth factors, transcription factors). One recent *in vivo* study described the introduction of *Notch-1*, implicated in neural development and presenilin function, to the fetal mouse brain using a retroviral vector[64], and future studies with viral vectors will help to clarify the complex signaling pathways involving related gene clusters such as *PS-1/2*, *ADAM*, *NUMB*, *MASH*, among others.

In the case of AD a variety of transgenic mice have been generated, overexpressing amyloid-β (Refs 65–69) or mutant presenilin[70,71], and a transgenic presenilin rat has also been described[72], for which more sensitive behavioral paradigms are available. Unfortunately, viral-based gene transfer has not been widely used to study relevant Alzheimer genes *in vivo*. Some work involving growth factors in AD models has been reported using rAAV (Ref. 73), but it does not address the unique AD pathophysiology. Only one group has reported the expression of amyloid-β in the rat brain using a viral vector[74]. The animals in question exhibited neurodegenerative changes, yet the inherent cytotoxicity of the adenoviral vector chosen for gene transfer makes it non-ideal for this type of study. Given the probable interaction of different disease genes in AD, it will be useful to generate models with more than one genetic alteration. With existing transgenic methods, it has been difficult and costly to generate multi-factorial animal models for AD, for example, animal models incorporating tauopathies and synucleinopathies have been lacking[75]. Double transgenics (amyloid-β and either PS-1 or tau) have indeed been generated in

mice[76,77], but the mouse is not the ideal model system for testing subtle behavioral changes, and the pathophysiology might be quite different in other animals. For example, although amyloid-β loading causes behavioral impairment in mice, other distinctive pathology of AD such as paired helical filaments has not been convincingly described[78], although these same changes have been described in senescent primates[79–81] and sheep[82,83]. Given the similarity in senescent pathology between humans and monkeys, it would be instructive to introduce a variety of Alzheimer-related genes to the primate brain using rAAV; primate and human hippocampal slice cultures can also be treated with rAAV (Ref. 84).

Many other CNS disorders are being actively studied using viral vectors. rAAV has been used to express genes involved in Parkinson's disease (PD) (e.g. tyrosine hydroxylase, aromatic acid decarboxylase, brain-derived neurotrophic factor, and glutamic acid decarboxylase). Animal models of PD have long been available in rodents and primates through lesioning of the basal ganglia, and therapeutic genes could be systematically tested with viral vectors in the existing models. These studies helped to clarify the role that specific genes play in modifying the PD phenotype, although differences in vector design and experimental procedures often made it difficult to separate vector effects from disease effects. More recently, novel animal models of PD are being investigated with rAAV via gene delivery of parkin, α-synuclein, and other genes. In other neurological diseases, transgenic, naturally occurring, or chemically induced animal models do not exist, and accordingly viral vectors have been used to model the pathology. For example, although there are models of Huntington's disease (HD) involving chemical lesioning of the striatum, these models do not closely mimic the HD pathology. Because HD and other brain disorders are thought to be directly related to polyglutamine repeats, investigators recently created the first somatic-cell Huntington's rat using rAAV to express polyglutamine tracts in the striatum[85]. Stereotactic neurosurgical delivery of rAAV (Ref. 86) has been used to create a rat model of epilepsy by overexpression of GluR6 receptors in the hippocampus, and others have studied the functional effects of overexpressing the EAAT2 glutamate transporter gene [implicated in neurodegenerative diseases including AD and PD (Refs 87,88)], in standard mouse knockout models of amyotrophic lateral sclerosis (ALS) using the rAAV vector.

In summary, using viral vectors such as rAAV and complimentary transgenic techniques, it is possible to create diverse *in vivo* experimental models. As more molecular pathways are mapped, it appears that brain functional systems are linked to a limited number of canonical gene interactions, which can be modeled and tested with the help of viral vectors.

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