

Lentiviral Vectors for Use in the Central Nervous System

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Lentiviral vectors have been used extensively as gene transfer tools for the central nervous system throughout the past decade since they transduce most cell types in the brain, resulting in high-level and long-term transgene expression. This review discusses some of the recent progress in this field, including preclinical gene therapy experiments in disease models, development of regulated vectors, and the application of siRNA's using lentiviral vectors. We also describe some of the features that make lentiviral vectors a likely candidate for human gene therapy in the brain.

Key Words: lentivirus, gene therapy, brain, neurological disorders, Tet system, siRNA

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INTRODUCTION

Efficient transduction of neurons *in vivo* was demonstrated in the very first publication describing a vector system based on human immunodeficiency virus type I (HIV-I) [1]. Since then, hundreds of reports have described the use of lentiviral vectors (LVs) for therapeutic and experimental gene transfer purposes in the central nervous system (CNS). LVs are considered attractive CNS gene transfer tools due their capacity to transduce slowly or nondividing cells in the brain [2,3], the extremely low probability of occurrence of replication-competent retroviruses [4–6], the lack of expression of viral genes [1], the

relatively large cloning capacity [1], the ability to expand the host range by pseudotyping LVs with a variety of envelopes [7,8], and the possibility of incorporating complex expression cassettes [9]. LVs have been demonstrated to transduce most cell-types within the CNS *in vivo*, including neurons, astrocytes, adult neuronal stem cells, oligodendrocytes, and glioma cells [2,10–12]. The most widely used lentiviral vector system for CNS gene transfer is based on HIV-I [1,4,6] (Fig. 1). Naturally, the use of HIV as the origin of a vector system is controversial due to the pathogenic nature of the wild-type virus. To circumvent this issue, vector systems based on feline and

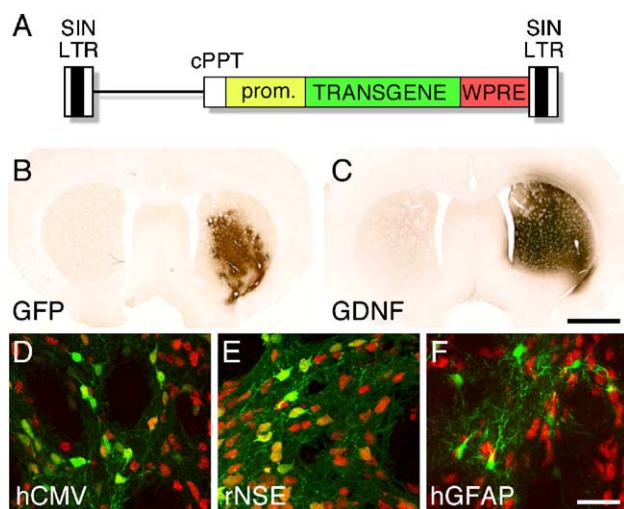


FIG. 1. (A) Schematic drawing of a standard HIV-based lentiviral vector in its integrated form. An internal promoter drives transgene expression. The 3' LTR carries a 400-bp self-inactivating (SIN) deletion, which is duplicated following reverse transcription and enhances vector performance and increases the safety of the vector [5]. Two *cis*-acting elements, the central polypurine tract (cPPT) [121,122] and the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) [123], have been inserted in the vector to improve transduction efficiency and transgene expression levels. (B and C) Widespread transgene expression of either a reporter gene (GFP) or a therapeutic gene (GDNF) detected after injection of a small volume (2 μ l) of VSV-G-pseudotyped LV into the striatum of adult rats. (D–F) Confocal images of sections that have been double immunostained for GFP (green) and the neuronal NeuN marker (red). (D) Injection of a VSV-G LV in which transgene expression is driven by the ubiquitous hCMV promoter reveals a preferential neuronal phenotype since most of the transduced cells are double labeled. (E and F) Interestingly, transgene expression can be specifically directed to the desired cell type when using the cell-type-specific promoter for rat neuron-specific enolase (rNSE) or human glial fibrillary acidic protein (hGFAP), with which all (rNSE) or no (hGFAP) transduced cells colabel with NeuN. Scale bar: B and C, 2 mm; D–F, 40 μ m.

equine (EIAV) lentiviruses have been developed [13,14]. Although these vector systems show the same general properties, including efficient transduction in the CNS, it has been speculated that HIV-based lentiviral vectors will be more efficient in human cells due to species-specific restrictions [9,15].

TRANSDUCTION IN THE CENTRAL NERVOUS SYSTEM

LVs have typically been pseudotyped with the glycoprotein of vesicular stomatitis virus (VSV-G) [16]. Injection of such vectors into various compartments of rodent and primate brains results in stable and long-term transgene expression [2,17] with more than 1,500,000 β -gal-expressing cells detected 3 months after injection of 40 μ l LV into primate striatum [17]. In the initial reports using VSV-G LVs a preference for neuronal transduction was found [1–3,17–19]. After injection into rat striatum almost 90% of transduced cells were found to be neurons [2], with similar results found in rat hippocampus and primate striatum and substantia nigra (SN) [2,17]. How-

ever, recent experiments have demonstrated that this neuronal preference is due to the “ubiquitous” internal promoters used in these studies (hCMV and mPGK) [11,20]. Apparently these promoters as well as other so-called ubiquitous promoters have a very low activity in glial cells *in vivo* [11]. Results from our group demonstrate that if the promoter is active in glial cells, high-level transgene expression in astrocytes can be achieved [11,20]. It is likely that VSV-G LVs enter all cells in the CNS with similar efficiency (unpublished results) and that the different transduction patterns that have been reported are due to the activity of the internal promoter in the various cell-types.

Two main approaches have been considered to achieve targeted LV transduction in the CNS. First, it is possible to direct expression to certain cell types by using promoter elements that are active only in the desired cells. Second, it is possible to use envelope proteins that bind to specific receptors found only on the desired cell type. Cell-type-specific gene transfer is important since it will allow genetic modification of only a subset of cells within an organ. This has consequences for both gene therapy and experimental gene transfer. More efficient gene therapy with reduced immune responses and more appropriate levels of transgene expression will be possible to develop. Cell-specific gene transfer may also prevent or facilitate anterograde transport of the transgene product, which may be crucial depending on the nature of the transgene. In terms of experimental gene transfer, it will allow *in vivo* studies of gene function in isolated populations of cells, an effort that today requires the time-consuming and expensive establishment of transgenic mice.

Both neuron-specific and glial-specific promoters have been shown to confer cell-type-specific transgene expression in the desired cell-type [11,20–22]. Promoter-based targeted transgene expression appears to be highly specific at least when reporter transgenes such as green fluorescent protein (GFP) are used. However, it should be noted that the expression from the glial-specific hGFAP promoter was not located exclusively to glial cells when using another transgene, glial cell line-derived neurotrophic factor (GDNF), rather than GFP. In this report, an elevated level of GDNF in the SN was detected, probably due to anterograde transport of the GDNF protein within neurons expressing the transgene [20]. Although this result can, at least to some extent, be explained by the high vector dose used in this experiment, it still highlights one of the problems when using a promoter-based strategy. If a high number of integration events take place within the transduced cells, a portion of the integrated transgenes may be nonspecifically activated due to positional effects from surrounding genes.

A solution to the problem of nonspecific expression from cell-type-specific promoters would be to combine them with a modification of the envelope protein so that uptake of the vector is restricted to a desired cell

population. This is in theory a more compelling idea than the promoter-based strategy since the actual genetic modification would be directed at a specific cell type, not only at the activity of the promoter. A large number of various viral envelopes have been used to pseudotype lentiviral vectors, including glycoproteins from various strains of VSV, various strains of rabies virus, Mokola virus, lymphocytic choriomeningitis virus (LCMV), Ross River virus, and others [7,8,23,24]. These experiments have revealed that different envelopes confer differences in vector uptake and vector processing (Table 1). Of special interest has been the rabies G-protein pseudotype, which confers retrograde transportation of the vector, thereby allowing it to be injected into the periphery [23]. This strategy has been successfully used in treating animal models of amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy, in which lentiviral vectors expressing either vascular endothelial growth factor or the human survival motor neuron gene were retrogradely transported to motor neurons after intramuscular injection [25,26]. However, in terms of targeting of specific cell populations the results have not been so encouraging. Although three recent reports describe targeting of glial cells, glioma cells, and adult neuronal stem cells when using the Ross River envelope or the LCMV envelope, the results of these studies are difficult to interpret since all have relied on a single promoter to drive transgene expression [12,24,27]. An attractive solution would be to use synthetic envelopes that are “tailor made” for certain cell-types. Despite the problems associated with this approach it is an area of active research and it is likely that in the near future sufficient progress will be made allowing efficient targeting with such envelopes [28].

GENE THERAPY AND DISEASE MODELS

The first use of lentiviral vectors to express a therapeutic transgene (Bcl-xL) in the CNS was reported by Blömer and colleagues in 1998 [29]. Since then, efficient therapeutic effects of LV-mediated transgene expression have been documented in animal models of lysosomal storage diseases (LSDs) [30–33], Alzheimer disease (AD) [34–37], ALS [26,38,39], Huntington disease (HD) [40–42], and Parkinson disease (PD) [43–52], among others. Further-

more, new genetic animal models of HD and PD have been developed using lentiviral vectors [53–55].

Huntington Disease

HD is a fatal autosomal dominant inherited neurodegenerative disease that is caused by an expansion of a CAG repeat in the huntingtin gene. HD is characterized by a loss of neurons within the striatum and to a lesser degree in the cortex [56]. Overexpression of ciliary neurotrophic factor and interleukin-6 has provided neuroprotection in various rodent models of HD by supporting dying cells [40–42]. Since HD is an autosomal dominant disorder an attractive gene therapy approach would be to use small interfering RNA (siRNA; discussed in more detail below) to knock down the mutated allele. Proof-of-principle of such an approach has been demonstrated using recombinant adeno-associated viral vectors but remains to be shown using LVs [57,58].

Furthermore, by overexpressing parts of the mutated huntingtin gene in the striatum using LVs it has been possible to induce pathological changes resembling those of HD [54,55,59]. This transgenic approach has certain advantages compared to classical genetic mouse models of neurodegenerative diseases. The use of viral vectors allows high-level expression of the disease-causing transgenes, which seems necessary for clear disease pathology, since classical transgenic mouse models overexpressing huntingtin at lower levels develop far less severe symptoms [59]. The use of viral vectors also allows for transgenic disease models in rats and primates, which is important for behavioral studies.

Parkinson Disease

PD is characterized by a selective loss of dopaminergic cells in the SN. In affected individuals these cells die in a progressive fashion with a typical disease onset at about 50–60 years of age [60]. There are both genetic and sporadic cases of PD, with sporadic cases constituting the vast majority [61]. The cause of sporadic PD is currently unknown. What is interesting with PD in regard to gene therapy is the fact that the affected cell population is small and restricted, i.e., the dopaminergic (DA) neurons in the SN. Hence, only a localized gene therapy is needed in PD. The loss of DA cells in the SN leads to a reduced dopamine level in the striatum, the projection target of these cells.

TABLE 1: Envelopes used to pseudotype lentiviral vectors for use in the CNS

Envelope	Host species	Transduction	Reference
VSV-G	Mouse, rat, primate	Efficient, ubiquitous, promoter-dependent	[2,7,8,11]
Rabies	Mouse, rat	Efficient, neuronal, retrograde transport	[8,23,25,26]
Mokola	Mouse, rat	Efficient, neuronal	[7,8]
LCMV	Mouse, rat	Targets glioma and neural stem cells	[12,27]
Ross River	Mouse	Targets glial cells	[24]
Ebola	Mouse	No transduction	[7]
MuLV	Mouse	Efficient, neuronal	[7]

This gives rise to motor and cognitive disturbances. The motor symptoms are typically characterized by difficulties in initiating movements, resting tremor, and rigidity. PD patients are typically treated pharmacologically with L-DOPA or a similar derivative replacing the dopamine in the striatum [62]. Such treatments are successful only for a limited time due to development of serious side effects such as involuntary movements. Hence, new treatment strategies are necessary.

Two main approaches for PD gene therapy have been focused on restoration of dopamine levels within the striatum and neuroprotection of the dying DA neurons using neurotrophic factors. Since PD leads to reduced levels of dopamine in the striatum, a strategy that restores dopamine levels to normal is an obvious choice. This could be achieved by modifying the cells in the striatum so that they become dopamine-producing cells by overexpressing essential enzymes for dopamine production. Overexpression of tyrosine hydroxylase (TH) and GTPchI, in combination, leads to production of dopamine in the transformed cells [63,64]. Despite the apparent simplicity, this approach presents problems. It has been shown that the number of transduced cells needs to be high in rodent models of PD if the levels of dopamine are to reach relevant levels [65]. Due to this issue this strategy has been exploited mainly using high-titer adeno-associated virus (AAV) vectors that appear to diffuse more than LVs following intracerebral injection. However, in a study in which a tricistronic EIAV vector expressing TH, AADC, and GTPchI was used, amelioration of drug-induced behavior in a model of PD could be detected [51].

The second approach that aims at halting the disease process in PD, rather than restoring the dopamine levels, is to overexpress trophic factors that provide support for the dying DA neurons in the SN. The most studied trophic factor in this paradigm is GDNF. In both rodent and primate models of PD, overexpression of GDNF using LVs has been shown to be neuroprotective [48,66]. However, it has also become clear that the level of GDNF expression is critical. In rodents, high levels of GDNF expression lead to unwanted side effects, such as sprouting of dopaminergic fibers in inappropriate areas and down-regulation of TH expression, as well as altered dopamine levels within the striatum [46,67,68]. In primate studies, elevated TH activity and DA turnover as well as negative behavioral effects have been reported following GDNF gene delivery [49,69,70]. Hence, it is necessary to develop gene transfer systems that allow for adjustable and reversible GDNF expression to ensure a safe therapy [71]. Furthermore, from previous studies it is not clear in what cell type GDNF should be expressed. It has been demonstrated that overexpression in neurons using LVs leads to secretion of GDNF in output areas to which these neurons project, thus leading to sprouting of dopaminergic fibers outside the striatum [46]. Hence, an ideal vector for GDNF gene therapy may be one that is

possible to regulate and is bona fide glial specific. No such vector exists today.

Similar to HD, new animal models of PD have been developed by overexpressing α -synuclein in the DA cells of rats and primates using viral vectors [53,72,73]. Interestingly, GDNF gene therapy, which is neuroprotective in toxin-induced models of PD, does not seem to provide neuroprotection in a LV-based α -synuclein model, demonstrating the added value of new genetic models to develop effective treatments [74]. However, the establishment of genetic models of PD has allowed evaluation of overexpression of intracellular target genes such as Parkin and β -synuclein that may prevent formation of intracellular aggregates [50,52]. These strategies hold great promise for future gene therapy in PD since modification of only the small pool of DA neurons should be necessary.

Lysosomal Storage Diseases

LSDs are a group of about 40 inherited disorders caused by mutations in genes for one or several enzymes involved in the cellular lysosomal machinery. Although the precise pathological mechanisms differ depending on the mutated gene, the presence of a dysfunctional enzyme generally leads to an accumulation of catabolites within lysosomes that progressively interferes with cell function and cell survival. Gene therapy has been considered for LSDs for several reasons. Since cells expressing a functional enzyme are capable of “cross-correcting” neighboring cells only a minority of the host cells need to be genetically modified to achieve therapeutic efficacy. Furthermore, in most LSDs the level of functional enzyme needs to be only a fraction of what is normal for phenotypic correction to occur. Still, since about 60% of the LSDs display neurological symptoms, either direct *in vivo* brain gene transfer or the use of strategies that target the CNS will be necessary. Such alternatives include direct intraparenchymal injection of viral vectors, intravenous injection of viral vectors, *ex vivo* transduction of hematopoietic stem cells, or transplantation of genetically modified neural progenitors [75].

Direct parenchymal injection of lentiviral vectors into the CNS have provided benefit in at least two LSDs, mucopolysaccharidosis type VII and metachromatic leukodystrophy [31–33]. Importantly, unilateral injection of LVs was sufficient to reverse pathology in the contralateral hemisphere providing *in vivo* evidence for cross-correction of cells that contact each other [33,76]. Still, the widespread pathology, in combination with the relatively poor diffusion of LVs after brain injection makes such a strategy using LVs an unlikely candidate for clinical use for most LSDs. However, the use of AAV vectors, which are capable of transducing a larger number of cells after parenchymal injection, has allowed remarkable improvement in a large-animal model of α -mannosidosis and also led to the initiation of clinical trials for

Canavan disease and late infantile neuronal ceroid lipofuscinosis using intracerebral injections of AVV vectors into affected children [77–79].

TECHNICAL IMPROVEMENTS OF LENTIVIRAL VECTORS

Tet-Regulated Expression

It would be preferable for many clinical gene transfer strategies, including GDNF for PD, if the level of transgene expression could be efficiently regulated. Most approaches for regulated gene transfer rely on drug-controlled molecular switches. A number of such systems have been developed and used in the context of viral vectors, including for example the tetracycline (Tet), rapamycin, ecdysone, and progesterone systems. This review will focus on the Tet system (Fig. 2), and for an update on the other systems, as well as a detailed review of the literature on the use of the Tet system in organs other than the brain, we refer the reader to an excellent recent review by Castro and colleagues [80].

The Tet system was developed in the lab of Hermann Bujard and is based on the tetracycline-resistance operon of *Escherichia coli* and operates via the interaction of two components, the Tet repressor protein (TetR) and the

tetracycline-response element (TRE) [81]. In bacteria, TetR inhibits expression of the genes on the tetracycline-resistance operon by docking with the Tet operator sequences (*tetO*) in the absence of tetracycline. Fusion of TetR to the activating domain of the VP16 protein from herpes simplex virus resulted in a transcriptional activator protein referred to as a tetracycline-controlled transactivator (tTA) [81]. The synthetic TRE is generally composed of seven tandem *tetO* sequences positioned just upstream of a minimal CMV promoter. In the absence of tetracycline the tTA binds with high affinity to the *tetO*, activating transcription from the minimal CMV promoter. Addition of tetracycline or doxycycline leads to a conformational change in the tTA, release from the TRE, and repression of transcription. This system is generally referred to as the Tet-Off system [81]. Introduction of random mutations in the tTA led to the subsequent development of the Tet-On system. A 4-amino-acid mutation in the TetR led to a reverse phenotype (rtTA) in which transcription was activated in the presence of tetracycline and vice versa [82].

The Tet system has many advantages compared to other regulatory systems. The inducer molecules (tetracycline or the widely used derivative doxycycline) have been used clinically as antibiotics for decades and are nontoxic at the doses needed for gene activation [80]. The pharmacological kinetics of tetracycline and doxycycline are well understood and the rapid turnover of the drugs makes them ideal for control of transgene expression. Furthermore, since the system is based on prokaryotic proteins and DNA sequences it does not interfere with the native cellular transcription. Despite this, no clinical gene therapy protocols using the Tet system have been initiated [79]. This is due mainly to two reasons. First, the prokaryotic origin of the Tet system suggests that it may be recognized by the immune system. Despite reports of such phenomena it is unclear to what extent the target organ or the vector of choice contributes to these problems [83,84]. In the brain, no immunological complications due to the use of the Tet system have been reported. Second, there have been numerous reports of gene transcription in the “off” state. This undesired transcription, often referred to as “leakiness,” is a problem that is crucial to solve if the Tet system is to reach clinical use and is currently a very active area of research.

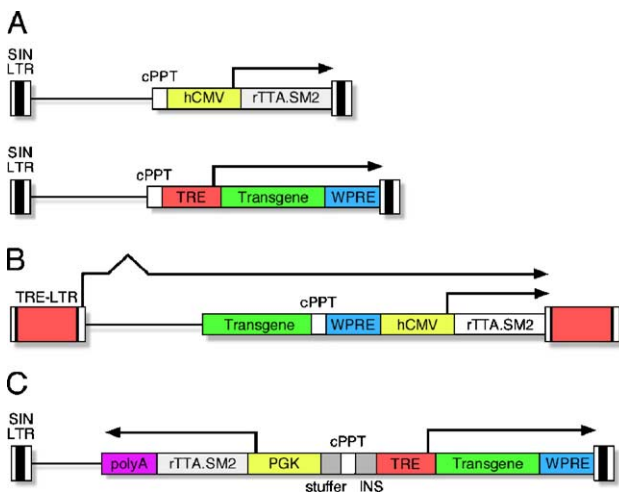


FIG. 2. Examples of recent Tet-regulated LV systems. All vectors incorporate a novel version of the rTA (rtTA2s-M2), which is an improved phenotype generated by directed mutagenesis in *Saccharomyces cerevisiae*. It displays lower basal activity and 10-fold more sensitivity to doxycycline [124]. The vectors are displayed in their integrated form. (A) Two-vector system described in Georgievska *et al.* [71] in which the transactivator and the Tet-responsive element (TRE) are separated onto individual LVs. The main drawback of such a system is the need for transduction of both vectors into the same cell. (B and C) Complex designs of LVs in which all Tet elements are included in a single LV. (B) The design in Vigna *et al.* [85] uses a modified LTR in which the TRE has been inserted. (C) Vogel *et al.* [88] separated the Tet elements with parts of the β -globin insulator (INS) and stuffer DNA. One-vector systems generally suffer from increased background expression due to cross talk between the different Tet elements when placed in the same vector.

Tet-Regulated LVs

The Tet-Off and Tet-On systems have, with success, been incorporated into lentiviral vectors. Since the system is composed of two parts (actually three or more parts in some newer and more elaborate designs) there are several options of how to design the vector. Lentiviral vectors in which all transcriptional elements are included in a single LV [84–87] as well as systems in which the elements are separated onto individual vectors have been described [37,40,54,71,89]. Regulated LVs have been used

to drive expression of therapeutic transgenes in disease models [37,40] and used for more experimental purposes [54,90,91]. What is clear is that these systems display substantial leakiness. This is a concern in particular in regard to the expression of trophic factors, such as GDNF, which are potent even at very low concentrations. The reasons behind the leakiness are likely to be multiple. First, the TRE promoter displays basal activity in certain cell types without the presence of a transactivator. Second, the tTA or rtTA binds the *tetO* in the absence of doxycycline to some extent. In the brain there is also the complication of a local injection site leading to multiple viral copies in the cells close to the injection site. This may lead to very high expression levels of the transactivator in some cells, contributing to nonspecific binding to the *tetO*. It may also increase positional effects on the TRE promoter from surrounding genes. Furthermore, it is preferable if all elements are included in a single LV. However, the development of such a vector is not straightforward. The placement of the different elements necessary for regulation in close vicinity to each other leads to cross talk between the different *cis*-acting elements and poor regulation of transgene expression. In adenoviral-vectors, in which the cloning capacity is much greater (40 kb compared to 8 kb for lentiviral vectors), it has been shown that it is possible to design effective one-vector systems if the different elements are separated by large pieces of stuffer DNA [92]. However, due to the small cloning capacity of lentiviral vectors, such a strategy is not feasible. There are reports of one-vector lentiviral systems in which the design of the expression cassette is highly advanced, suggesting that it may be actually possible to produce such systems without losing the regulation [84,87]. The efficiency of these vectors with regard to expression of therapeutic genes in the brain remains to be evaluated. Novel strategies aimed at solving these problems include the design of new TRE promoters that display reduced leakiness [93] and coexpression of transcriptional silencers such as tTSkid [94]. tTSkid is a fusion of the TetR with the transcriptional silencing domain of the Kid protein. Studies have shown that the use of such an element in combination with the Tet-On system reduces the basal activity substantially, without affecting the maximal expression level [80].

Autoregulated Transgene Expression

An alternative approach to regulated transgene expression, developed and used in our laboratory, is to use an endogenous promoter from a gene that is specifically up-regulated during a pathological state (Fig. 3). By using such a promoter to drive transgene expression, regulated gene delivery that is not dependent on a transactivator and its ligand would be possible. In a recent report we provided a proof-of-principle of lesion-induced transgene expression using LVs [20]. We injected a LV expressing GFP under the control of a hGFAP promoter bilaterally

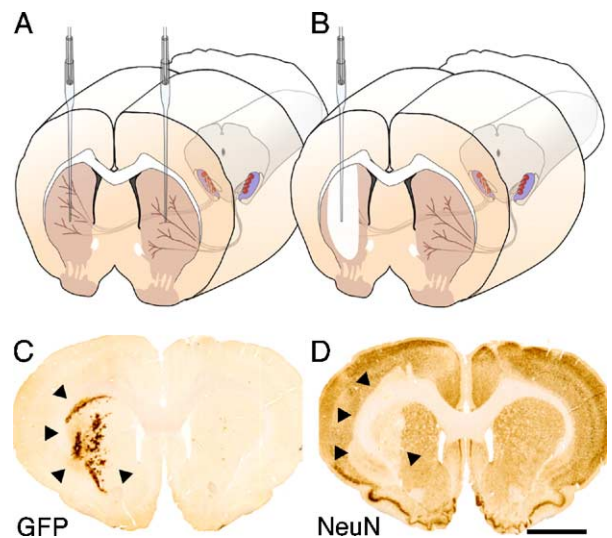


FIG. 3. Example of autoregulated transgene expression using an endogenous promoter from a gene that is up-regulated after a lesion. GFAP is a glial-specific filamentous protein expressed at low levels in the intact striatum. However, following injury or various lesions to the striatum, GFAP expression is up-regulated as a consequence of the gliotic reaction. This feature was exploited to demonstrate damage-induced transgene expression. (A) Similar amounts of a LV in which a GFAP-promoter drives GFP expression were injected bilaterally into the striatum of adult rats. (B) 3 weeks after injection of the LVs the rats received a unilateral excitotoxic ibotenic acid lesion. (C and D) 1 week after the lesion the animals were sacrificed and stained for GFP and the neuronal marker NeuN. In the intact striatum only a few GFP-expressing cells were detected, while in the lesioned hemisphere a large number of GFP-expressing astrocytes were found in the same area in which neuronal cell death could be detected due to the lesion. Modified, by permission of the publisher, from Jakobsson *et al.* [20]. Scale bar, 2 mm.

into the striatum of rats, and 3 weeks later the animals received a unilateral lesion. At 1 week after the lesion we found a large number of GFP-expressing cells on the lesioned side and only very few GFP cells on the intact side. The GFP-expressing cells colocalized with GFAP expression were up-regulated due to the gliotic reaction after the lesion, suggesting that silent lentiviral vectors had been activated by the same signals that activate the endogenous GFAP gene. The GFAP promoter used in our study may not be the optimal choice for this strategy but with the increasing knowledge of gene regulation during different pathological states that is emerging, due to the use of techniques such as microarrays and proteomics, it should be possible to identify optimal promoters for autoregulated gene therapy in disorders such as stroke and multiple sclerosis.

siRNA

A new exciting application for LVs is the expression of siRNA's. In recent years, RNA interference (RNAi) has been developed as a novel tool allowing for specific and efficient knockdown of gene expression [95]. RNAi is a naturally occurring gene-silencing mechanism that acts at the posttranscriptional level. Small synthetic double-

stranded RNA molecules (around 20–25 nucleotides in length, siRNA's) can be introduced into cells, where they will induce the down-regulation of specific genes. The siRNA's can also be generated from plasmid or viral vectors expressing short double-stranded RNAs within the context of an inverted repeat sequence containing a hairpin loop, as these short hairpin RNAs (shRNA's) are processed by the cell machinery into siRNA's. By using proper viral vectors, it is possible to obtain long-term siRNA delivery and persistent gene knockdown *in vivo* [95]. Lentiviral vectors can be engineered to produce shRNA's resulting in persistent genetic knockdown in the brain [96]. Lentiviral vector-mediated RNA interference can also be designed to be regulated by doxycycline or triggered by a recombination event, for instance, via the Cre/lox system [97,98]. The use of siRNA's in lentiviral vectors has already shown promising results in preclinical gene therapy in mouse models of ALS and AD [38,39,99]. Moreover, siRNA's delivered by LVs are currently used as experimental tools in basic research, allowing studies of gene function *in vivo* in the brain in a completely new way [100]. However, a crucial problem that remains to be solved is the development of cell-specific siRNA LVs.

CONSIDERATIONS FOR CLINICAL GENE THERAPY IN THE BRAIN USING VIRAL VECTORS

If lentiviral vectors are going to be used for clinical brain gene transfer, several aspects regarding the safety of the vectors must be completely resolved. There are multiple issues that need to be considered, including the emergence of replication-competent viruses, production of clinical-grade lentiviral vectors, potential immune responses, insertional mutagenesis, and germ-line transmission. Lentiviral vectors are not the only vectors capable of stable gene transfer in the brain. In particular, viral vector systems based on AAV and adenovirus have been demonstrated to confer high-level and long-term transgene expression in the CNS [101]. These vector systems differ in several important aspects and the choice of system should be based on a combination of safety and effectiveness of the vector in that particular gene therapy strategy. It is not likely that one vector system will solve all problems. It is possible to concentrate and purify AAV vectors to very high titers, resulting in widespread and stable transduction with low toxicity. The use of different serotypes of AAV allows targeting of various cell pools and a more widespread gene delivery [102,103]. The development of pure high-titer stocks of AAV has led to the initiation of clinical trials for PD and LSD using intraparenchymal injection of viral vectors [78,79,104]. Still, there are limitations to AAV vectors. The small cloning capacity (4–5 kb) excludes the use of long cDNAs and complex expression cassettes and there are immunological issues that need to be resolved in regard to the use of AAV vectors in human subjects (see below). The use of

adenoviral vectors for CNS gene therapy has been hampered by inflammatory responses. Although the development of the “gutless” helper-dependent adenoviral vectors that do not express any viral proteins allows stable long-term transgene expression in the brain, it is evident that when used in high doses these vectors still induce an acute inflammation [105,106]. Nevertheless, the high insert capacity of helper-dependent adenoviruses that allows delivery of several expression cassettes or stretches of genomic DNA warrants further development of this system.

Immune Responses to LVs

Since lentiviral vectors, as well as AAV and helper-dependent adenoviral vectors, do not encode any viral proteins after transduction, a potential immune response will be initiated either against the viral particle or the transgene product. Although the brain has been considered to be “immune privileged” it is clear that viral vectors injected into the brain can elicit an immune response that leads to diminishing transgene expression and detrimental effects to the host [107]. AAV and lentiviral vectors appear to induce a minimal inflammatory response when injected into the brain. However, human patients are not the same as research animals. The majority of humans have circulating antibodies against wild-type adenovirus and AAV [108]. Hence, the adaptive immune system has already been primed toward these viruses. Injection of viral vectors into such individuals may prime an immune response against the vector, in particular if vector leaks out into the cerebrospinal fluid or blood circulation, where it can be recognized by antigen-presenting cells. Studies trying to mimic a situation in which the animal has a primed immune system for AAV vectors have shown that transgene expression is severely diminished and have also shown that transduction by subsequent injections of the vectors is prevented by the immune response [109]. Lentiviral vectors have certain characteristics that make them suitable in regard to not eliciting an immune response. Only HIV patients will have circulating antibodies against the proteins in the vector. Such patients can be easily monitored and excluded from gene therapies based on lentiviral vectors. Furthermore, the HIV-I capsid proteins appear to have a short half-life, making the time span in which the immune system can recognize the transduced cells short. This is in contrast to the AAV capsid, which appears to be more stable and this may explain why preimmunization against AAV vectors, but not lentiviral vectors, induces immune responses that are detrimental to the transgene expression in the brain [110,111].

Insertional Mutagenesis

Another potential complication of lentiviral gene transfer that has been recently highlighted is insertional mutagenesis. The occurrence of leukemia in 3 of 11 children

with X-linked SCID following *ex vivo* gene therapy using a murine leukemia virus (MLV) vector encoding the common γ chain has emphasized that this is an actual risk [112]. However, lentiviral vectors and the oncoretroviral vector used in this trial differ in certain aspects that may influence the occurrence of insertional mutagenesis. Genome-wide mapping of integration sites of MLV and lentiviral vectors has shown interesting differences. MLV vectors predominantly integrate in promoter regions and CpG islands, while lentiviral vectors apparently integrate into transcriptionally active genes [113,114]. The integration of MLV vectors near the transcription start site may increase oncogenesis, either by influencing the activity of the host promoter or by giving rise to new full-length transcripts. In contrast, lentiviral vectors that integrate into the entire transcribed region are less likely to disturb the regulation and expression of host genes. These speculations are supported by new experimental data suggesting that lentiviral vectors are less oncogenic than MLV vectors in mouse models that are prone to insertional mutagenesis [115]. If insertional mutagenesis could lead to transformation of the cells of the brain is still unknown. However, as has been demonstrated in our laboratory [11,20], it is clear that lentiviral vectors infect and integrate their genomes into glial cells, a population that retains proliferative properties. This is something that has to be taken into account when estimating the possibilities of insertional mutagenesis after lentiviral brain gene transfer.

FUTURE DIRECTIONS

An advantage of LVs is the flexibility with which the expression cassette can be designed. LVs expressing multiple genes [51,116], siRNA's [96], the Cre recombinase in combination with loxP sites [97], and all parts of the Tet systems [85] have been designed and produced. Applications of LVs in basic research include development of animal models [59,117], preclinical gene therapy experiments [59,117], and visualization of neurons for electrophysiological studies [21] and as marker vectors in stem cell research [118]. By infecting single-cell embryos, early blastocysts, or embryonic stem cells, LVs have been used to generate transgenic mice, rats, and other mammals. LV transgenesis is far more efficient, less technically demanding, and less expensive and time consuming than the standard method of pronuclear injection of naked DNA. Interestingly, several research groups have shown that it is possible to combine lentivector-mediated transgenesis and siRNA technology to produce knockdown, rather than knockout, animals [119,120]. Use of lentiviral transgenesis will allow for the generation of a large number of genetic mouse and rat models that will increase knowledge of basic biology, have an impact on understanding disease processes, and contribute to drug

discovery. In conclusion, it is likely that lentiviral vectors will continue to be widely used as tools for basic research. Development of cell-type-specific and controllable vectors will have an impact on basic research, enabling experiments that were previously impossible to perform. If these techniques can be combined with siRNA-mediated knockdown it will be possible to study gene function *in vivo* in a fashion that has no precedent.

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