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ARTICLE

Transduction Patterns of Pseudotyped Lentiviral Vectors in the Nervous System

Liang-Fong Wong,* Mimoun Azzouz, Lucy E. Walmsley, Zoe Askham, Fraser J. Wilkes, Kyriacos A. Mitrophanous, Susan M. Kingsman, and Nicholas D. Mazarakis

Neurobiology Group, Oxford Biomedica (UK) Ltd., Medawar Centre, Robert Robinson Avenue, The Oxford Science Park, Oxford OX4 4GA, UK

**To whom correspondence and reprint requests should be addressed. Fax: 44-1865-783044. E-mail: l.wong@oxfordbiomedica.co.uk.*

We have developed a non-primate-based lentiviral vector based on the equine infectious anemia virus (EIAV) for efficient gene transfer to the central and peripheral nervous systems. Previously we have demonstrated that pseudotyping lentiviral vectors with the rabies virus glycoprotein confers retrograde axonal transport to these vectors. In the present study we have successfully produced high-titer EIAV vectors pseudotyped with envelope glycoproteins from *Rhabdovirus* vesicular stomatitis virus (VSV) serotypes (Indiana and Chandipura strains); rabies virus [various Evelyn–Rokitnicki–Abelseth ERA strains and challenge virus standard (CVS)]; *Lyssavirus* Mokola virus, a rabies-related virus; and *Arenavirus* lymphocytic choriomeningitis virus (LCMV). These vectors were delivered to the striatum or spinal cord of adult rats or muscle of neonatal mice by direct injection. We report that the lentiviral vectors pseudotyped with envelopes from the VSV Indiana strain, wild-type ERA, and CVS strains resulted in strong transduction in the striatum, while Mokola- and LCMV-pseudotyped vectors exhibited moderate and weak transduction, respectively. Furthermore ERA- and CVS-pseudotyped lentiviral vectors demonstrated retrograde transport and expression in distal neurons after injection in brain, spinal cord, and muscle. The differences in transduction efficiencies and retrograde transport conferred by these envelope glycoproteins present novel opportunities in designing therapeutic strategies for different neurological diseases.

Key Words: lentivirus, gene therapy, EIAV, retrograde transport, pseudotyping

INTRODUCTION

Gene therapy for neurological diseases will require the transfer of genetic material to the postmitotic neuron. Primate and nonprimate lentiviral vectors are ideal candidates for such gene delivery tools, as they can transduce both dividing and nondividing cells, resulting in stable integration and long-term expression of the transgene [1–4]. Targeting such vectors to specific cell populations *in vivo* can be achieved by using cell-specific promoters to drive expression of the transgene or by using or modifying natural viral tropisms. Viral tropism is determined by specific interactions between the envelope glycoprotein on the vector and the surface receptor molecules on the target cell, which may be expressed in specific populations in differentiated tissue. This interaction is the earliest event in the transduction process after gene transfer and establishes the tropism of the lentiviral vector. The replacement of the original envelope of the lentiviral vector system with a heterologous envelope—termed pseudotyping—can thus affect the transducing properties of the viral vector and alter the host range of the lentivirus.

Lentiviral vectors such as those derived from the human immunodeficiency virus (HIV), feline immunodeficiency virus (FIV), and equine infectious anemia virus (EIAV) have been pseudotyped with a wide range of envelope glycoproteins, for example, those from the vesicular stomatitis virus (VSV) [1,5,6], Ebola virus (Zaire and Reston subtypes) [5,7,8], Marburg virus [8], rabies virus [1], lymphocytic choriomeningitis virus (LCMV) [9], Mokola virus [5,10], human foamy virus [10], gibbon ape leukemia virus [11], murine leukemia virus (amphotropic envelope) [11,12], influenza virus [13], avian leukosis-sarcoma virus [14], respiratory syncytial virus (F and G envelope proteins) [13], Ross River virus glycoprotein [15], and Sendai virus [16]. Pseudotyping of lentiviral vector with certain envelopes (such as VSV-G) allows particle concentration by ultracentrifugation: however, different pseudotyped vectors vary in stability and efficiency of packaging and hence titers. Furthermore pseu-

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dotyped vectors exhibit different tissue tropisms *in vivo*. For example, an HIV-based lentiviral vector pseudotyped with Ebola-Zaire glycoprotein efficiently transduced lung epithelial cells from the apical surface [13], while the Ross River virus-pseudotyped FIV-based lentiviral vector transduced mainly hepatocytes and Kupffer cells in the liver after systemic administration and preferentially transduced neuroglial cells (astrocytes and oligodendrocytes) after injection into the brain [15].

We have previously reported the pseudotyping of the EIAV-based lentiviral vectors with the *Rhabdovirus* VSV and the rabies virus (Evelyn–Rokitnicki–Abelseth strain) envelope proteins [1–4]. The VSV-G-pseudotyped vectors have a broad capacity to transduce a wide variety of cell types *in vitro* but exhibit a preference for neuronal phenotypes *in vivo*. Transduction of neurons *in vivo* resulted in anterograde transport of the expressed protein throughout the cell bodies and axons [1]. The rabies-G-pseudotyped EIAV vectors also mediated strong transduction at the site of injection; in addition retrograde transport of the EIAV vector to distal neurons projecting to the site of injection was observed [1]. It was further demonstrated that the rabies-G-pseudotyped EIAV vectors allowed entry into the central nervous system after an intramuscular injection. In this paper, we have extended the study by investigating if pseudotyping EIAV vectors with other envelope proteins from the same or related viral families will confer similar transduction and retrograde transport characteristics. The expression patterns of EIAV vectors pseudotyped with various envelope proteins from the *Rhabdovirus,* VSV-G (Indiana and Chandipura serotypes), the wild-type and mutant Evelyn–Rokitnicki–Abelseth (ERA) strains as well as the challenge virus standard (CVS) strain of the rabies virus, the rabies-related *Lyssavirus* Mokola, and the *Arenavirus* LCMV were compared. We report that these envelope proteins can be used to pseudotype EIAV vectors successfully, producing concentrated viral preparations of high biological titers, but they differ in their abilities to transduce striatal cells *in vivo* and retrograde transport characteristics.

RESULTS

Viral Titers of Pseudotyped EIAV Vectors

We successfully pseudotyped EIAV vectors encoding the marker gene β-galactosidase with different envelope glycoproteins from various viral strains. The titers of the pseudotyped vectors, as determined by transduction efficiencies in canine osteosarcoma cells (D17), are illustrated in Table 1. We also estimated the copy numbers of viral RNA using quantitative PCR analysis (Table 1). For all pseudotyped vectors except for the ERA dm and LCMV pseudotypes, there is a good correlation between the biological titers determined on D17 cells and the number of viral RNA genomes. It has been previously demonstrated that the number of EIAV particles correlates with the number of RNA genomes [17]. For most viral preparations, the biological titers of unconcentrated stock solutions were in the range of 10^6 transducing units per milliliter (TU/ml). Concentration by ultracentrifugation enabled approximately 2000-fold increase in viral titers, yielding consistently high biological titers of about 10^8–10^9 TU/ml. In particular, viral vectors pseudotyped with VSV-G and Mokola envelopes gave high biological titers of $3–4 \times 10^9$ TU/ml, while EIAV vectors pseudotyped with Chandipura and CVS envelopes yielded titers of approximately 1×10^9 TU/ml. This is the first known report of lentiviral vectors that have been successfully pseudotyped with either the Chandipura or the CVS envelope. Furthermore the concentration of these viral preparations allowed the injection of EIAV vectors into

Table 1: Titers of EIAV vectors pseudotyped with various envelope proteins

Pseudotyped EIAV vector	Similarity (%)	Biological viral titer (TU/ml)	RNA titer[a] (TU/ml)	Number of transduced cells in striatum
Rhabdovirus				
VSV-G Indiana	100[b]	4×10^9	4×10^9	20,112 ± 1095
VSV-G Chandipura	38[b]	1×10^9	1×10^9	2,385 ± 75
Rabies ERA wt	100[c]	7×10^8	4×10^9	22,424 ± 985
Rabies ERA sm	99[c]	9×10^8	2×10^9	12,880 ± 815
Rabies ERA dm	99[c]	1×10^8	6×10^9	3,472 ± 176
Rabies CVS	88[c]	9×10^8	2×10^9	16,976 ± 990
Lyssavirus				
Mokola	57[c]	3×10^9	4×10^9	13,304 ± 630
Arenavirus				
LCMV	—	3×10^8	2×10^9	50 ± 3

[a] Titer based on RNA quantitation by quantitative PCR assay normalized to a known biological titer.
[b] Denotes % protein similarity to VSV-G Indiana envelope.
[c] Denotes % protein similarity to rabies ERA wt envelope.

the central nervous system in small volumes, thereby increasing gene transfer efficiencies.

Transduction Patterns of EIAV Vectors Pseudotyped with VSV-G Indiana and Chandipura Serotypes

To study the expression patterns of the various pseudotyped EIAV vectors, we injected a total of 2×10^6 TU, based on the biological titers, of each pseudotyped vector into rat striatum. Using the EIAV vector pseudotyped with the Indiana strain of the VSV-G envelope into rat caudate putamen, we observed strong 5-bromo-3-indolyl-β-*d*-galactosidase (X-gal) staining in the caudate putamen (Fig. 1A). This extended approximately 4.8 mm anteroposteriorly, 2 mm dorsoventrally, and 1.1 mm laterally around the site of injection; cell counts indicated that 20,112 ± 1095 cells were transduced (Table 1). This correlates with previous studies, in which anterograde transport of the vector was observed in the cortex, globus pallidus, and substantia nigra pars reticulata. We observed no evidence of retrograde transport of the viral vector. Confocal microscopy revealed that predominantly neuronal cells were transduced, as after cell counts 95% of the β-galactosidase-positive cells were estimated to colocalize with anti-NeuN immunostaining (Figs. 1E–1G), while 5% colocalized with glial fibrillary acidic protein (GFAP), a ubiquitous astrocytic marker (Figs. 1H–1J). In comparison, injection of EIAV vectors pseudotyped with the Chandipura strain of the VSV-G envelope showed minimal expression of the transgene in the striatum following 4 weeks postinjection. The area of β-galactosidase expression was limited to 1.1 mm anteroposteriorly, 1.3 mm dorsoventrally, and 0.9 mm laterally around the site of injection and only 2385 ± 75 cells in the striatum were transduced (Fig. 1B). Immunohistochemical colocalization studies and cell counting indicated that 70% of β-galactosidase expression colocalized with NeuN (Figs. 1K–1M), while 30% of β-galactosidase-positive cells were positive for the glial marker GFAP (Figs. 1N–1P), suggesting that the Chandipura-pseudotyped EIAV vector transduced a higher percentage of glial cells compared to the Indiana strain VSV-G-pseudotyped vector.

Transduction Patterns of EIAV Vectors Pseudotyped with *Lyssavirus* Mokola and *Arenavirus* LCMV

EIAV vector encoding β-galactosidase pseudotyped with the Mokola virus envelope, a rabies-related virus, demonstrated moderate β-galactosidase staining in the striatum after 4 weeks (Fig. 1C). Reporter gene expression was localized to 2.1 mm anteroposteriorly, 1.3 mm dorsoventrally, and 0.7 mm laterally around the site of injection and 13,304 ± 630 cells were transduced at the site of injection (Table 1). Furthermore, we observed no expression of the vector in cell bodies in areas that project to the striatum, indicating that the Mokola envelope could not confer transduction via nerve terminals or retrograde transport to distal neurons. Injection of EIAV vector pseudotyped with envelope protein from *Arenavirus* LCMV into the rat caudate putamen resulted in minimal transduction at the site of injection, with 50 ± 3 cells staining positive for β-galactosidase along the needle track (Fig. 1D).

Transduction Patterns of EIAV Vectors Pseudotyped with Rabies-G Serotypes

As demonstrated in Mazarakis *et al.* [1], injection of EIAV vectors pseudotyped with the envelope from the wild-type ERA strain, ERA wt, resulted in robust gene transfer to cells within the striatum (Fig. 2A). At the site of injection, the area of transduction extended 4.8 mm anteroposteriorly, 2 mm dorsoventrally, and 1.0 mm laterally and 22,424 ± 985 cells were transduced (Table 1). We observed retrograde transport of the vector to distal regions of the brain such as the thalamus (Fig. 2D). In addition we observed transgene expression in the cortex, globus pallidus, amygdala, hypothalamus, subthalamic nucleus, substantia nigra pars compacta, and pars reticulata, as reported in Mazarakis *et al.* [1]. However, an amino acid change from arginine to glutamine at residue 333 of the ERA wt envelope protein, termed ERA sm, resulted in weaker transduction of the caudate putamen after 4 weeks (Fig. 2B). β-Galactosidase expression was localized to an area of 3.0 mm anteroposteriorly, 1.5 mm dorsoventrally, and 0.8 mm laterally around the site of injection. Furthermore, only 12,880 ± 815 cells were transduced at the site of injection. Cell counting indicated that 90% of cells that were transduced colocalized with NeuN (Figs. 2G–2I), while 10% colocalized with GFAP marker (Figs. 2J–2L). Interestingly, we observed no retrograde transport of the vector to areas such as the thalamus (Fig. 2E). This suggests that this single mutation in the ERA viral envelope protein was sufficient to abolish the retrograde transport characteristics of the wild-type ERA envelope. A further amino acid change in the ERA envelope (from lysine to asparagine at residue 330), resulting in ERA dm, practically abolished vector transduction and expression in the caudate putamen. Detection of β-galactosidase in the striatum 4 weeks after stereotaxic injection was limited to 3472 ± 176 cells in the striatum (Fig. 2C). Moreover we observed no retrograde transport of the vector (Fig. 2F).

With vectors pseudotyped with envelope protein from another strain of the rabies-G virus, CVS, we observed strong transgene expression in the striatum (Figs. 3A and 3B) and in the globus pallidus (Figs. 3C and 3D), which was localized to 4.9 mm anteroposteriorly, 1.6 mm dorsoventrally, and 1.0 mm laterally around the site of injection, and we estimated 16,976 ± 990 cells to be transduced by the vector after cell counting (Table 1). We also detected β-galactosidase expression at sites distal to the area of injection such as the cortex (Fig. 3E); amygdala (Fig. 3B); anterodorsal,

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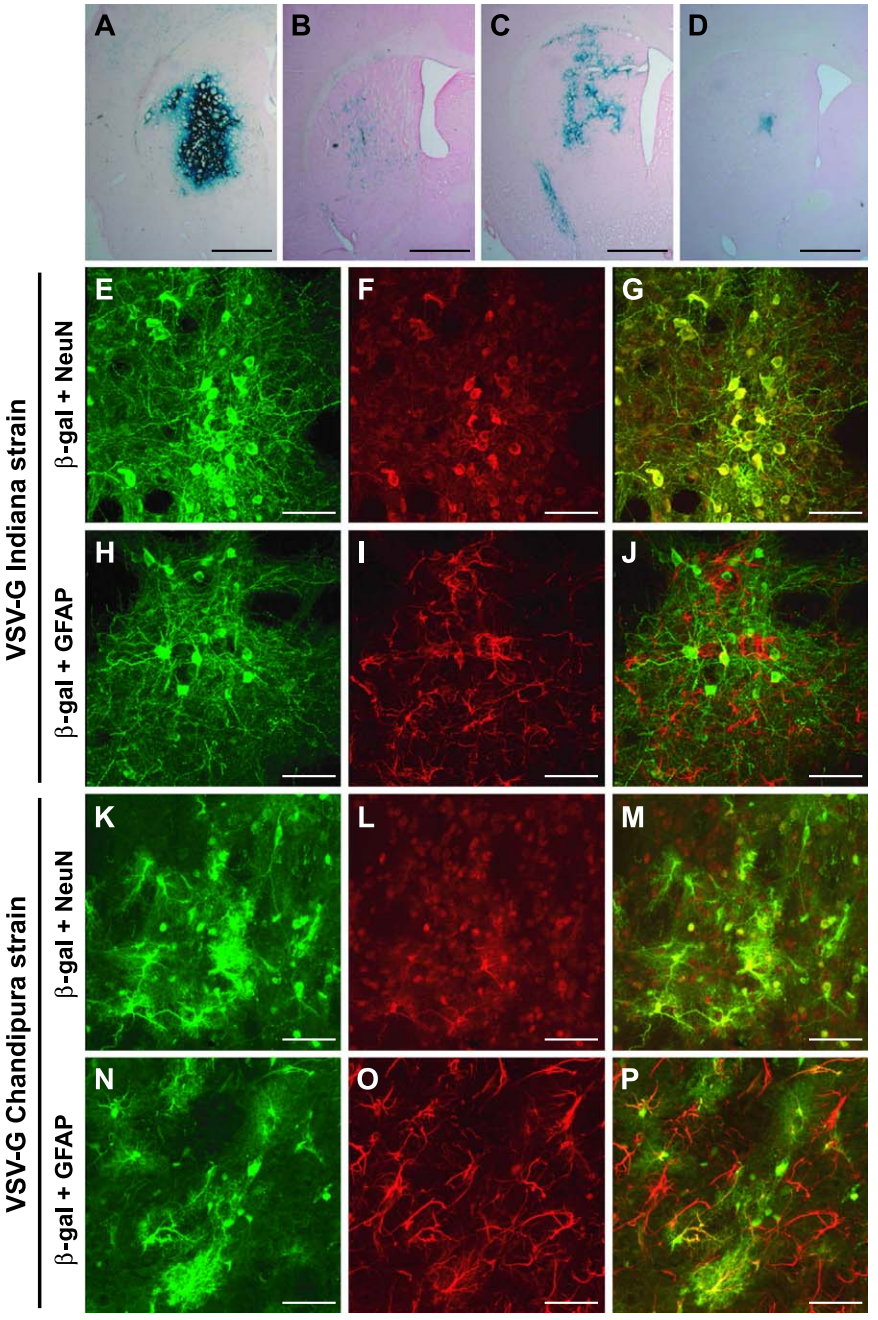

FIG. 1. Expression of pONY8.0Z vectors pseudotyped with Indiana and Chandipura strains of VSV-G serotypes and Mokola and LCMV envelopes in the rat striatum. (A) Extensive gene transfer of pONY8.0Z pseudotyped with the Indiana strain of the VSV-G glycoprotein was observed in the caudate putamen 4 weeks after unilateral intrastriatal injection. (B) In comparison, the expression of pONY8.0Z pseudotyped with the Chandipura envelope protein was significantly weaker. (C, D) Moderate and poor transgene expression was detected with the Mokola- and LCMV-pseudotyped vectors, respectively. In the pONY8.0Z VSV-G injected brains, immunohistochemistry revealed that 90% of the transduced cells at the site of injection expressed both β-galactosidase (in green) and NeuN (in red; E–G) while 10% of transduced cells (in green) were GFAP-expressing cells (in red; H–J). In Chandipura-pseudotyped EIAV vectors, 70% of the transduced cells (in green) were neuronal in nature and colocalized with NeuN antibody (in red; K–M), while 30% colocalized with GFAP antibody (in red; N–P). Scale bar represents 1 mm (A–D) or 50 μm (E–P).

ventrolateral, and ventroposterior thalamic nuclei (Figs. 3B and 3C); and substantia nigra pars compacta (Fig. 3F). Ninety percent of transduced cells were neurons, as revealed by confocal microscopy and cell counting (Figs. 3G–3I), with 10% of β-galactosidase-positive cells expressing GFAP. At the site of injection, 27% of choline acetyltransferase (ChAT)-expressing neurons colocalized with β-galactosidase (Figs. 3M–3O), suggesting that a proportion of cholinergic neurons were also transduced. Immunohistochemistry also revealed that β-galactosidase product was located in cells in the substantia nigra that express tyrosine hydroxylase (TH), indicating that retrograde transport of the pseudotyped vector was likely to have occurred in dopaminergic neurons that project from the substantia nigra pars compacta to the striatum (Figs. 3S–3U). By counting the number of TH-

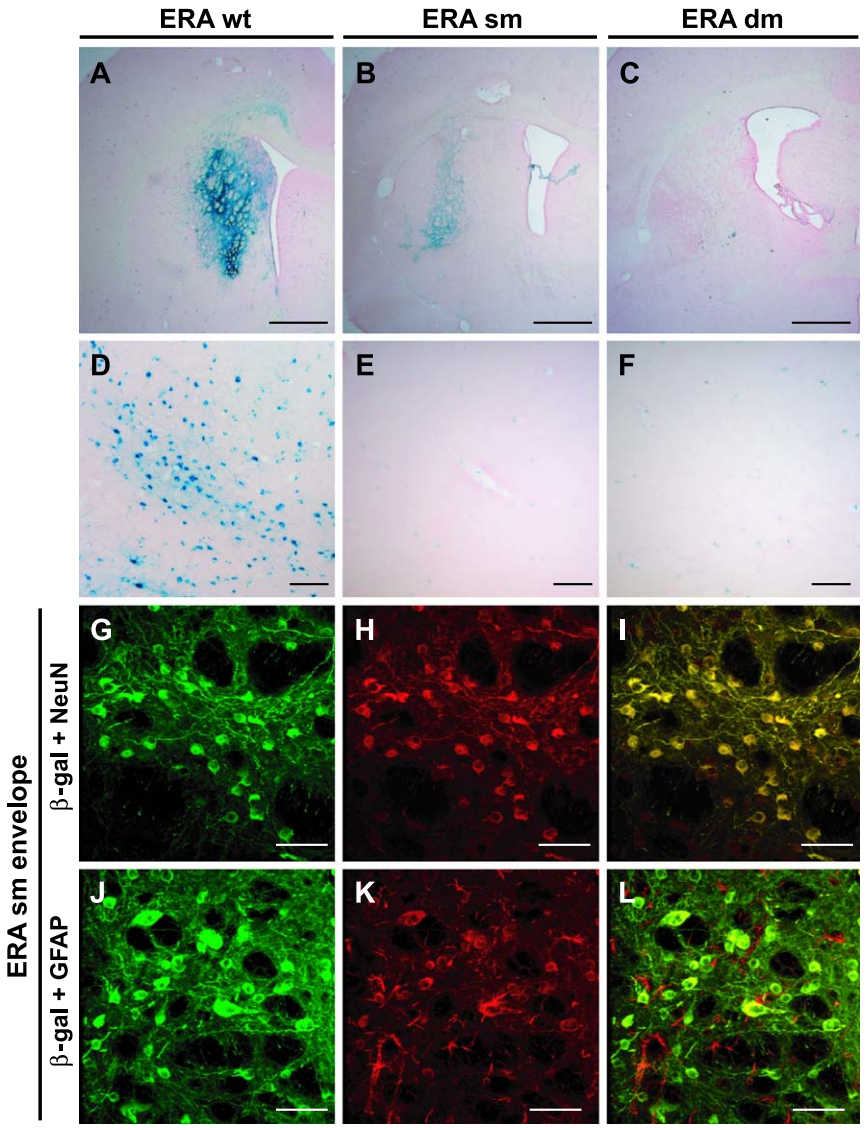

FIG. 2. Expression of pONY8.0Z vectors pseudotyped with ERA strains of rabies envelopes in the rat striatum. (A) Extensive gene transfer of wild-type ERA-pseudotyped EIAV vector was observed in the striatum 4 weeks after unilateral intrastriatal injection. (B) In comparison, the expression of ERA sm-pseudotyped EIAV vector was significantly weaker and (C) was completely abolished with the ERA dm envelope. Retrograde transport of the EIAV vector to the thalamus was observed with (D) the ERA wt envelope, but not (E) the ERA sm or (F) the ERA dm envelope. In the pONY8.0Z ERA sm-injected brains, immunohistochemistry revealed that 90% of the transduced cells at the site of injection expressed both β-galactosidase (in green) and NeuN (in red; G–I), while 10% colocalized with GFAP-expressing cells (in red; J–L). Scale bar represents 1 mm (A–C), 100 μm (D–F), or 50 μm (G–L).

positive cells that also expressed β-galactosidase, we estimated that 20% of TH neurons were retrogradely transduced. These transduction characteristics were similar to those of the ERA wt-pseudotyped vector, for which transgene expression was detected in 90% of the neurons in the striatum (Figs. 3J–3L), 25% ChAT-positive neurons (Figs. 3P–3R), and 22% of TH neurons in the substantia nigra (Figs. 3V–3X).

EIAV Vectors Pseudotyped with Rabies Envelope Proteins Are Retrogradely Transported into the CNS after Injection into the Spinal Cord and Muscle

Based on the data obtained with the CVS envelope in the brain, we decided to investigate further the retrograde transport properties of the EIAV vectors following intraspinal and intramuscular injections. We performed intraspinal injections of pONY8.0Z vector expressing β-galactosidase pseudotyped with the CVS strain of the rabies envelope in adult rats. Transduction of pONY8.0Z pseudotyped with the ERA strain of the rabies virus envelope in the spinal cord has been previously demonstrated, and intraspinal injection of the lentiviral vectors induced minimal inflammation at the site of injection, with no apparent cell damage [1]. Following injection of 8×10^5 TU (biological titer) of CVS-pseudotyped EIAV vector in a volume of 1 μl into the spinal cord, we observed strong reporter gene expression in cells located in the gray matter on one side of the spinal cord with retrograde transport to the contralateral side of the spinal cord (Fig. 4). Furthermore upon examination of the brain stem and cortex, we identified motor neurons in these areas to be expressing β-galactosidase, indicating retro-

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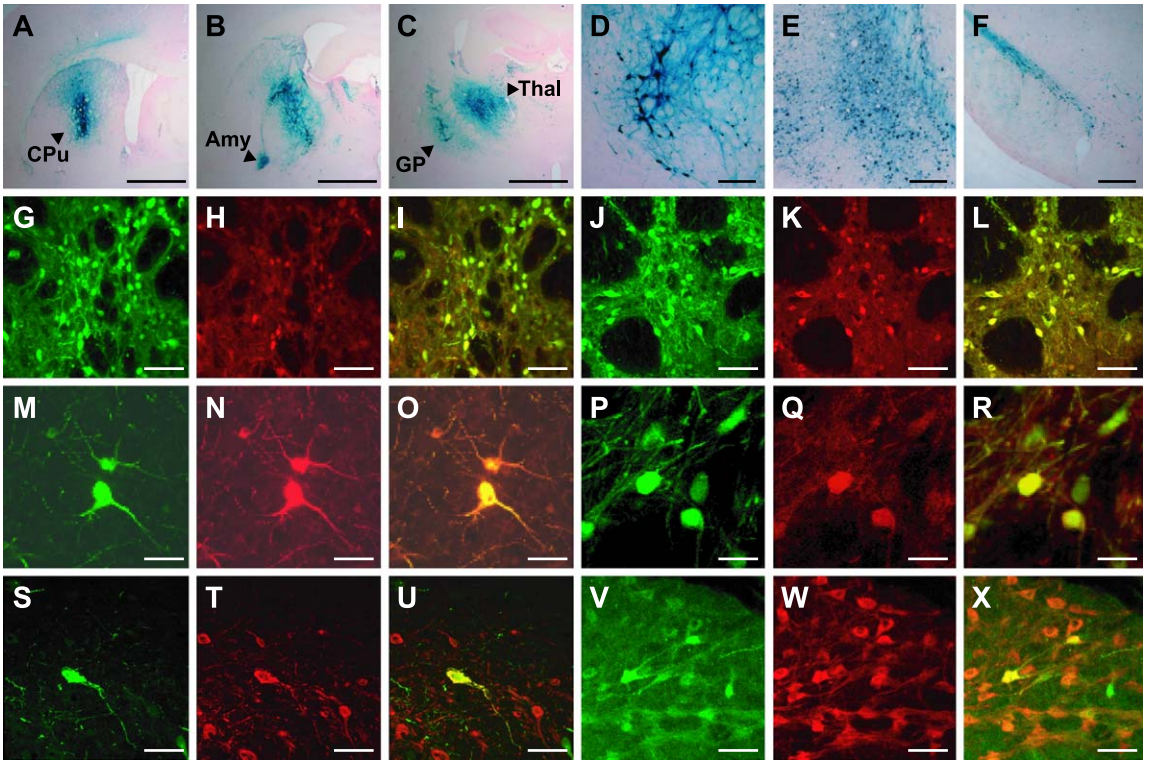

FIG. 3. Expression of pONY8.0Z vectors pseudotyped with CVS envelope in the rat striatum. (A) Strong transduction of pONY8.0Z pseudotyped with the CVS glycoprotein was observed in the striatum (or caudate putamen, CPu) at 4 weeks. (B, C) In more posterior sections, strong β-galactosidase expression was also detected in the caudal striatum and globus pallidus (GP), with retrograde transport to the amygdala (Amy) and to the anterodorsal, ventrolateral, and ventroposterior thalamic nuclei (Thal). (D–F) Higher magnification pictures indicating transgene expression in the globus pallidus, cortex, and substantia nigra, respectively. Comparison of NeuN (G–L), ChAT (M–R), and TH (S–X) immunohistochemistry of the CVS pseudotype (G–I, M–O, S–U) with the ERA wt pseudotype (J–L, P–R, V–X) is demonstrated in the following confocal pictures. (G–I) 90% of transduced cells in the striatum expressed both β-galactosidase in green (G) and NeuN in red (H); overlay picture shown in I. These transduction characteristics were similar to the ERA wt-pseudotyped vector, for which J–L show 90% β-galactosidase expression (J) colocalizing with NeuN (K); overlay picture shown in L. With both the CVS (M–O) and ERA wt (P–R) vectors, transduced cells (in green; M, P) located in the striatum also expressed ChAT (in red; N, Q); overlay pictures in O and R, respectively. Retrograde transport of CVS (S–U) and ERA wt (V–X) vectors was observed to the substantia nigra, where transduced cells (S, V) colocalized with tyrosine hydroxylase staining (T, W). Scale bar represents 1 mm (A–C), 100 μm (D–E), 50 μm (G–L), or 25 μm (M–X).

grade transport of the vector from the spinal cord in ascending pathways to the brain stem and motor cortex (Figs. 4B and 4C).

To determine if EIAV vectors pseudotyped with rabies envelope are retrogradely transported to the mouse spinal cord after transduction in the muscle, postnatal day 6 neonatal mice received an intramuscular injection of either ERA wt- or CVS-pseudotyped EIAV vectors. Following intramuscular injection of ERA wt-pseudotyped EIAV, we observed reporter gene expression in the gastrocnemius muscle, demonstrating that muscle tissue could be transduced by ERA wt-pseudotyped EIAV (Fig. 4D). We detected retrograde expression of β-galactosidase in neurons in the lumbar spinal cord with 10–12 transduced motor neurons per section (Figs. 4E and 4F). By quantifying the total number of large motor neurons with distinct nuclei and nucleoli, we estimated that ~50% of the motor neurons innervating the gastrocnemius muscle were transduced. The labeled neurons were localized to one side of the ventral horn, suggesting that the EIAV vectors were retrogradely transported from the ipsilateral muscle injection and expressed in the spinal cord. Similarly, intramuscular injection of CVS-pseudotyped EIAV also resulted in good transduction of muscle tissue (Fig. 4G) as well as retrograde expression of the reporter gene β-galactosidase in the spinal cord. We observed X-gal staining in 7–8 motor neurons per section and 33% of motor neurons were transduced (Figs. 4H and 4I). Interestingly, the morphology of X-gal staining was slightly different between the two vectors. Both the cell body and the neurites of ERA wt-transduced motor neurons were strongly stained for the vector product, while in CVS-transduced motor neurons axonal staining was weak with punctate X-gal staining within the cytoplasm of cell bodies. To confirm that it is indeed the viral genome, and not the β-

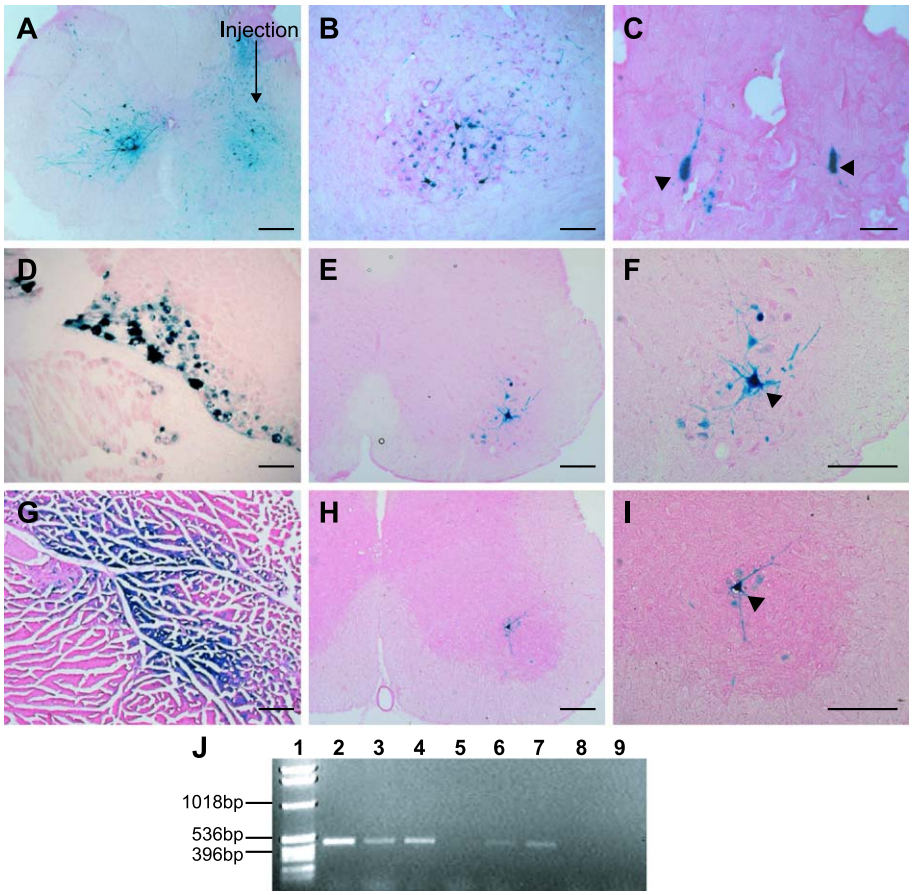

FIG. 4. Expression patterns of ERA wt- and CVS-pseudotyped EIAV vectors after intraspinal and intramuscular injection in adult rats and neonatal mice, respectively. (A) Following intraspinal injection of CVS-pseudotyped vector into the adult rat, strong transduction of neurons was observed in the gray matter on one side of the spinal cord, with retrograde transport to the contralateral side of the spinal cord (scale bar represents 200 μm). Motor neurons in the (B) brain stem and (C) cortex were also transduced by retrograde transport (scale bar represents 100 and 50 μm, respectively). (D) After intramuscular injection of ERA wt-pseudotyped vector into neonatal mice transgene expression of β-galactosidase was observed in the gastrocnemius muscle, with retrograde transport to motor neurons within the spinal cord (E and F, scale bars represent 100 μm). (G) Similar observations were also recorded when the CVS-pseudotyped vector was injected into neonatal muscle, with strong muscle expression and retrograde transport to motor neurons in the spinal cord (H and I, scale bars represent 100 μm). (J) PCR analysis showing detection of EIAV vector DNA in the gastrocnemius muscle and ipsilateral spinal cord. Lane 1, 1 kb ladder; lanes 2, 3, and 4 (gastrocnemius muscle), VSV-G-, ERA wt-, and CVS-pseudotyped vectors, respectively; lanes 5, 6, and 7 (ipsilateral spinal cord), VSV-G-, ERA wt-, and CVS-pseudotyped vectors, respectively; lane 8, kidney of CVS-injected animal; lane 9, water. A 450-bp product was detected in all lanes except for spinal cord of VSV-G-injected rat (lane 5), kidney of CVS-injected rat (lane 8), and water (lane 9).

galactosidase protein, that is retrogradely transported from the muscle to the spinal cord, we performed PCR to detect viral genome. PCR using primers specific for the β-galactosidase detected viral genome in the ipsilateral side of the spinal cord in mice that received intramuscular injections of ERA wt- and CVS-pseudotyped vectors, but not with the VSV-G-pseudotyped vectors (Fig. 4J).

DISCUSSION

The *in vivo* tropism of lentiviral vectors is dependent on the envelope glycoproteins that coat the viral particle. Previously, we have demonstrated that while EIAV vectors pseudotyped with the Indiana strain VSV-G and the wild-type ERA strain rabies envelope proteins both resulted in good expression in the brain at the site of injection, VSV-G-pseudotyped EIAV vectors could not confer retrograde transport properties to the vector, unlike those that had been pseudotyped with ERA wt [1]. In this study, we sought to pseudotype EIAV vectors with alternative envelope glycoproteins to determine if this could improve transduction and expression efficiencies in the central nervous system, as well as retrograde transport properties of the EIAV vectors. Of the envelopes that transduced only cells at the site of injection (both

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Indiana and Chandipura VSV strains, Mokola, LCMV envelopes), the Indiana strain of the VSV envelope had the highest transduction efficiency. Of the envelopes that were studied for their potential retrograde transport capabilities (Mokola, ERA wt, ERA sm, ERA dm, and CVS), both ERA wt and CVS envelopes demonstrated good expression at the site of injection and retrograde expression distal to the site of injection.

We have demonstrated that pseudotyping with the alternative Chandipura strain envelope did not improve the transduction efficiency of EIAV vectors compared to the Indiana strain of the VSV serotype, as the latter presented better expression in the striatum compared to the Chandipura strain. Furthermore pseudotyping with the Chandipura envelope did not confer retrograde transport properties to the vectors and appeared to transduce a higher percentage of glia. This suggests that EIAV vectors pseudotyped with the Indiana strain of VSV-G serotype have by far the most efficient transduction capacity in the striatum, the site of injection. Comparison between the two proteins revealed that the Chandipura glycoprotein had only 38% similarity to the Indiana strain envelope. It has been postulated that during VSV infection, glycoprotein binding to the target membranes depends on electrostatic interactions [18], and Masters *et al*. [19] reported that the Chandipura glycoprotein contained two unusual charged amino acids, aspartate and arginine, within the putative membrane anchor sequence and, unlike its Indiana serotype counterpart, did not contain a potential palmitate-accepting cysteine residue within its cytoplasmic domain. These amino acid differences may affect the transducing properties and tropism of Chandipura-pseudotyped EIAV vectors; however, this needs to be investigated further. Nevertheless, our results suggest that lentiviral vectors pseudotyped with the Chandipura glycoprotein had less efficient transducing capabilities than the Indiana serotype *in vivo*.

Pseudotyping with alternative envelopes from other viral families such as the Mokola or LCMV envelopes demonstrated moderate and poor reporter gene expression, respectively, in the rat striatum, compared to the Indiana strain of the VSV envelope. In addition no retrograde expression of vectors pseudotyped with either envelope was observed. Similar observations have been recorded with HIV-based vectors injected into mouse brain [6]. Watson *et al*. [5] have also reported decreased striatal transduction by LCMV-pseudotyped murine leukemia virus compared to the VSV pseudotype. In our studies the lack of *in vivo* transduction by LCMV-pseudotyped EIAV vectors is unlikely to be due to the low viral titers as the titers of the vector preparations, as determined by reporter gene expression in canine D17 cells and by quantitative PCR analysis of the number of RNA genomes, were as high as 3×10^8 TU/ml. However, it was noted that there was a log decrease in the biological titer of this vector compared to the RNA titer. This could be due to a differential ability of this pseudotype to transduce the D17 cell line, and perhaps a wider range of cell lines should be used to investigate the transducing characteristics of this pseudotype. Regardless, injection of at least 10^6 TU (based on the biological titer) of this vector into the brain *in vivo* did not result in good transduction.

EIAV vectors pseudotyped with the ERA wild-type and CVS glycoproteins of the rabies virus resulted in good transduction at the site of injection and conferred retrograde transport properties to the vectors. This was observed after injection of the vectors in rat striatum, rat spinal cord, and neonatal mouse muscle. For the first time, we have demonstrated significant transduction and high reporter gene expression in muscle after intramuscular injection of rabies-pseudotyped vector (both ERA wt and CVS envelopes) in the neonatal mouse, in contrast to the observations of Watson *et al*. [5]. Furthermore there was retrograde transport of EIAV vectors pseudotyped with both the ERA wt and the CVS envelopes to motor neurons within the spinal cord after intramuscular injection. A single mutation of the ERA envelope protein at amino acid residue 333 appeared to lower the efficiency with which the vector was able to transduce cells and express the marker gene at high levels. More importantly, the retrograde transduction with this vector was completely abolished, as was evident by the lack of expression at distal sites. This suggests that the arginine residue at position 333 may be crucial in mediating the uptake of the viral vector at axon terminals for the vector to be transported to the cell body located at a distal site. Indeed, this residue is conserved in the CVS envelope, which also demonstrated retrograde transport after intrastriatal, intraspinal, and intramuscular injections. In wild-type rabies CVS viruses, virulence is dependent on arginine residue 333 and mutants with arginine → glutamine change at residue 333 can infect olfactory receptor cells [20] and peripheral sensory and motor neurons [21,22] but do not propagate to the central nervous system. In our studies, mutations in the ERA envelope (ERA dm) at amino acid residues 330 and 333 markedly decreased striatal transduction, suggesting that these mutations greatly decreased the binding and entry of vector particles into the cell.

The retrograde expression observed with the rabies-pseudotyped EIAV vectors is due to the transport of the viral vector, and not of the β-galactosidase protein, as we have demonstrated the presence of viral DNA at distal sites by PCR analysis. Retrograde transport of the EIAV vectors is likely to be mediated by binding of the vectors to specific neuronal receptors at nerve terminals and axoplasmic transport to the cell body via endosomes or direct interaction with the microtubules. The rabies glycoprotein has been shown to interact with the p75 low-affinity neurotrophin receptor (p75NTR) [23],

the neural cell adhesion molecule [24], and the nicotinic acetylcholine receptor [25–27]. Langevin and Tuffereau [28] selected mutant rabies viruses that were incapable of interacting with p75NTR; these isolates had amino acid substitutions at positions 318 and 352. Our data suggest that mutation in the amino acid residue 333 abolished retrograde transport in the ERA sm-pseudotyped vector, and thus it will be interesting to determine if this mutation will affect binding of the ERA glycoprotein to p75NTR. Lalli and Schiavo [29] visualized directly the retrograde transport of a fluorescent fragment of tetanus toxin in living motor neurons by nonacidic endosomes that also carry NGF, as characterized by the presence of p75NTR; these nonacidic carriers lack markers of the classical endocytic pathway. In p75NTR has been shown to internalize into vesicles bound to differentiated PC12 cells, its ligand NGF [30]. This receptor-mediated mode of internalization, compared to the acidic endosomal pathway, which strips the viral envelope before transport, might explain the difference in retrograde transport characteristics between the VSV and the rabies envelopes. However, p75 receptors are sparsely expressed in the striatum; therefore the retrograde transport of rabies-pseudotyped vectors might be dependent on multiple interactions with different cell surface receptors.

The differences in the expression patterns of differently pseudotyped EIAV vectors in the central nervous system have obvious implications in the design of gene therapy vectors for neurological diseases. For strategies that require localized delivery of the therapeutic protein, EIAV vectors pseudotyped with the Indiana strain of the VSV-G envelope can provide efficient transduction and expression. For example, localized production of dopamine in the striatum by a multicistronic vector encoding DOPA decarboxylase, tyrosine hydroxylase, and GTP cyclohydrolase I was efficient in treating rat models of Parkinson disease [31]. For strategies that require disseminated release of the therapeutic protein, rabies-pseudotyped EIAV vectors can be utilized to deliver protein to large areas within the central nervous system. Furthermore the phenomenon of efficient retrograde transport as observed with the ERA wt- and CVS-pseudotyped vectors after intramuscular injection can be exploited as a new targeting approach for gene therapy for motor neuron diseases such as spinal muscular atrophy (SMA) or amyotrophic lateral sclerosis, chronic pain, and spinal injury. Indeed, the ability to transduce neonatal mouse muscle with these vectors can potentially provide therapeutic opportunities for SMA, as mouse models of SMA survive up to 28 days of age, and therefore any therapy to prevent the onset of disease has to be performed early at the neonate stage or even in utero. Retrograde transport has also been reported for a number of vector systems such as the adenoviral, adeno-associated viral, and herpes simplex viral systems [32–35]. The advantage of utilizing EIAV vectors as gene delivery tools include safety and long-term and stable expression of the therapeutic protein in postmitotic neurons with minimal inflammatory and immunological responses [1]. Furthermore the lack of pathogenicity of EIAV in humans allows EIAV-derived vectors to be used in gene therapy, compared to the safety issues surrounding the use of HIV-derived vectors. Indeed, long-term expression of EIAV vectors of up to 1 year and correction of diabetes insipidus has been demonstrated in the Brattleboro rat model [36].

We have shown that pseudotyping EIAV vectors with different envelopes from different viral families confers varying expression patterns within the central nervous system. Retrograde expression was demonstrated with the ERA wt and CVS envelopes, while a single mutation in the ERA wt envelope abolished retrograde transport. This has an impact on the design of EIAV vectors as gene therapy vehicles, and further applications of these pseudotyped vectors in animal models of diseases will prove to be useful in developing novel gene therapy strategies for neurological diseases.

Materials and Methods

Lentiviral vector construction and production. The pONY series of EIAV vectors and their pseudotyping with the different envelopes have been described previously [4]. Description of the pONY8.0 vector is given in Mazarakis *et al.* [1]. The Indiana strain of the VSV-G envelope and the ERA strain of the rabies-G envelope were kindly donated by Professor William Wunner of the Wistar Institute. The Chandipura strain of the VSV-G envelope was donated by John Rose (Yale University, New Haven, CT). A single mutant of the wild-type ERA strain (ERA wt) was generated by replacing arginine at amino acid 333 with glutamine. This new mutant, which is naturally occurring and apathogenic in adult mice, was termed ERA sm. An additional substitution at amino acid 330 from K to N resulted in a double mutant of ERA wt named ERA dm. Briefly a partial PCR fragment of ERA wt that incorporated the two amino acid changes was amplified using the primers 5′-CTACAACTCAGTCATGACTTGGAATGAGATCCTCCCCTCAAAAGGGTGTTTAAGAGTTGGGGGGAGG-3′ and 5′-CCTTTTGAGGGGAGGATCTCATTCCAAGTCATGACTGAGTTGTAGTGAGCATCGGCTTCCATCAAGGTC-3′. A full-length fragment of the ERA dm envelope was then amplified using the primers 5′-ACCGTCCTTGACACGAAGCT-3′ and 5′-GGGGGAGGTGTGGGAGGTTT-3′. CVS 2Bc envelope was obtained from ATCC. The Mokola and LCMV envelopes were kindly donated by Karl-Klaus Conzelmann (Ludwig–Maximilians-Universitat, Germany) and Dorothee von Laer (Heinrich-Pette-Institute, Germany), respectively. All genes encoding the envelope proteins were cloned into pRV67, pSA91, or pM108 before viral vector production [4,9].

Viral vector stocks were produced by transient transfection of human embryonic kidney 293T cells plated on 10-cm dishes (3.5×10^6 cells/dish) with FuGENE 6 (Roche, UK) according to the manufacturer's instructions. Three DNA components—2 μg vector plasmid encoding β-galactosidase (pONY8.0Z), 2 μg of gag/pol plasmid (pONY3.1), and 1–2 μg of plasmid encoding envelope glycoprotein—were added to a mix containing 17.8 μl FuGENE 6 (Roche) and 575 μl OptiMEM (Gibco BRL, UK). After transfection for 16 h, sodium butyrate was added to a final concentration of 10 mM. Supernatants were harvested 24–42 h after transfection and filtered through a 0.45-μm filter. Concentrated viral preparations were produced by an initial low-speed ultracentrifugation at 6000 *g* at 4°C for at least 18 h, followed by ultracentrifugation at 50,000 *g* at 4°C for 90 min. The virus was resuspended in formulation buffer containing 19.75 mM

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Tris–HCl, pH 7.0, 40 mg/ml lactose, 37.5 mM sodium chloride, 1 mg/ml human serum albumin, and 5 μl/ml protamine sulfate for 2–3 h at 4°C, aliquoted, and stored at −80°C. Biological titers of the viral preparations, expressed as number of transducing units per milliliter, were determined by transducing canine osteosarcoma (D17) cells in limiting dilutions in the presence of polybrene (8 μg/ml; sigma). After 2–3 days incubation, the cells were incubated in X-gal solution and blue colonies were counted. In addition, the titers of the viral preparations were also calculated by determining the number of viral RNA genomes per milliliter of viral stock solution using quantitative PCR analysis (ABI7700, PE Applied Biosystems) and comparing it to a bank viral preparation of known biological titer, as described in Rohll et al. [37] and Martin-Rendon *et al.* [38].

Delivery of EIAV vectors into the striatum. All surgical procedures were approved by the local veterinarian and ethical committee and were carried out according to UK Home Office Regulations. Stereotaxic administrations were performed on adult male Wistar rats under Hypnorm and Hypnovel anesthesia using either a finely drawn glass micropipette or a 10-μl Hamilton syringe with a 33-gauge blunt tip needle. Approximately 2×10^6 TU (based on biological titers) in a volume ranging from 1 to 4 μl of each vector was injected. As each pseudotyped vector varied slightly in biological titer, the volumes of vector were adjusted so that a total of 2×10^6 TU was slowly infused at a speed of 0.2 μl/min using an infusion pump (World Precision Instruments, Inc., Sarasota, FL), into the striatum using the stereotaxic coordinates anteroposterior 0 and +1 mm, mediolateral 3.5 mm, dorsoventral 4.75 mm (from the dura). At least four animals were injected with each envelope pseudotype. After viral vector injections, the needle was left in place for approximately 5 min before being withdrawn. The skin was closed using a 5-O Vicryl suture, and following surgery, animals were kept warm until recovery was complete. After 4 weeks, the rats were sacrificed by an ip injection of Euthatal (Rhone Merieux, USA) and transcardially perfused with 4% (w/v) paraformaldehyde containing 2 mM $MgCl_2$ and 5 mM ethylene glycol bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid. Following an overnight incubation in 4% paraformaldehyde, the brains were cryoprotected in 30% sucrose for at least 3 days at 4°C, after which they were frozen in Tissue-Tek OCT (Sakura Finetek). Forty-micrometer coronal sections were obtained on a CM3050 cryostat (Leica, UK) and collected into wells containing PBS. X-gal staining and immunohistochemistry were performed as described below.

Gene transfer to spinal cord in rats using pONY8.0Z CVS. Anesthetized 2-month old Wistar rats (n = 3) were placed in a stereotaxic frame and the spine was immobilized using a spinal adaptor (Stoelting Co., UK). Following laminectomy, pONY8.0Z CVS was injected into the lumbar spinal cord using a 10-μl Hamilton syringe with a 33-gauge blunt tip needle. The needle was left in place for 5 min before being retrieved. Approximately 8×10^5 TU of viral vector was injected in a volume of 1 μl at an injection rate of 0.1 μl per minute. Four weeks after injection, the rats were sacrificed by an ip injection of Euthatal (Rhone Merieux, USA) and transcardially perfused with 4% paraformaldehyde as described above. The lumbar spinal cord and rat brains were dissected out, cryoprotected in sucrose, and sectioned for further histological analysis.

Gene transfer to muscle using pONY8.0Z rabies-G and pONY8.0Z CVS. Postnatal day 6 neonatal mice were used in these experiments. pONY8.0Z rabies-G and pONY8.0Z CVS were injected into the gastrocnemius muscle through the skin with a 10-μl Hamilton syringe connected to a 33-gauge blunt tip needle. Mice received intramuscular injection of 30 μl of either pONY8.0Z rabies-G (n = 6) or pONY8.0Z CVS (n = 3). Three weeks after vector injection, the mice were sacrificed by an ip injection of Euthatal (Rhone Merieux) and transcardially perfused with 4% paraformaldehyde, and tissues from the muscle and spinal cord were dissected out. Sections from these tissues were analyzed by X-gal staining and immunohistochemistry.

Histological analysis. For X-gal staining, brain sections were incubated in X-gal solution made up in PBS for at least 3–5 h at 37°C. The sections were then washed with PBS, after which they were mounted onto APES-coated slides, dried, and counterstained with eosin. The dried sections were mounted in Permount (Fischer Scientific, UK). Sections were observed under the light microscope and images were captured (Axiovision Systems, UK). The defined area of transduction at the site of injection, as observed with positive X-gal staining, was measured in 1 in 5 consecutive sections (every 200 μm) using the Axiovision (UK) software. Thirty sections from each animal (n = 4) in each group were counted, and as each section was 40 μm thick, the total area covered 6 mm anterior posteriorly in the rat striatum. Double immunohistochemistry was also performed on the brain sections using rabbit anti-β-gal (1:300 made up in 10% normal goat serum; R&D Systems, UK) together with any of the following mouse antibodies: anti-NeuN (1:300; Chemicon, UK), anti-GFAP (1:200; Chemicon), anti-TH (1:400; Diasorin), or anti-CHAT (1:1000, gift from Dr. U. Hartmann, USA). Briefly, sections were washed twice in PBS and blocked in 10% normal goat serum for 1 h at room temperature. The sections were incubated in primary antibody at 4°C for at least 24 h, washed with PBS, and incubated in secondary antibodies anti-mouse Cy3 (Jackson Laboratories, 1:400) and anti-rabbit Alexa 488 (Molecular Probes, 1:200) for at least 2 h at room temperature. The sections were then washed in PBS and mounted on slides in FluorSave (Chemicon). In all pictures, the β-gal staining appeared green (Alexa 488) while the immunohistochemical markers are denoted in red (Cy3). All sections were examined under a fluorescence microscope and images were captured (Axiovision Systems). In addition, confocal microscopy was performed on Leica Systems TCS-SP microscope (UK). In all the quantifications, the numbers of immunoreactive cells against the respective antibodies (e.g., β-gal, NeuN, GFAP, ChAT, and TH) were counted in 1 in 5 consecutive 40-μm sections and at least 5 sections were counted from each animal (n = 4) in each group. The total number of cells transduced by the various vectors was estimated according to the method of Abercrombie [39]. The percentages of transduced cells were calculated based on the total number of NeuN-, GFAP-, ChAT-, or TH-positive cells. For quantification of motor neurons, consecutive spinal cord sections were stained for both X-gal and cresyl violet. In the ipsilateral spinal cord, large motor neurons with distinct nuclei and nucleoli that were X-gal positive were counted. The percentage of transduced cells was calculated based on the total number of motor neurons counted in cresyl violet-stained sections.

PCR. Thirty microliters of pONY8.0Z vector pseudotyped with VSV-G, ERA wt, or CVS (n = 3 in each group) was injected into the right gastrocnemius muscle of neonatal mice as described above. After 4 weeks, the animals were killed and the gastrocnemius muscle and ipsilateral lumbar spinal cord tissue were harvested and frozen in liquid nitrogen. The tissue was homogenized in 80 μl PBS and genomic DNA was isolated from samples using the DNeasy kit (Qiagen, UK) according to the manufacturer's instructions. PCRs were set up to detect the *Escherichia coli* LacZ gene (GenBank Accession No. V00296). The components of the 50-μl PCR were as follows (in final concentrations): 300 nM forward primer, 5′-CGTTGCTGCATAAACCGACTACAC-3′ (nucleotides 638–661); 300 nM reverse primer, 5′-TGCAGAGGATGATGCTCGTGAC-3′ (nucleotides 1088–1067); 0.3 mM dNTP (each); 1 mM $MgSO_4$; 1× *Pfx* amplification buffer; and 1 U Platinum *Pfx* DNA polymerase (Invitrogen, USA). Fifty nanograms of template DNA was used per reaction. PCR amplification was carried out on a PCR Express (Hybaid, Hercules, CA) using the following thermal cycling conditions: 95°C for 4 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 45 s, and extension at 72°C for 45 s, and one final cycle of extension at 72°C for 7 min. PCR products (20 μl/reaction) were resolved on a 1% TAE agarose gel.

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