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Rabies virus glycoprotein pseudotyping of lentiviral vectors enables retrograde axonal transport and access to the nervous system after peripheral delivery

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In this report it is demonstrated for the first time that rabies-G envelope of the rabies virus is sufficient to confer retrograde axonal transport to a heterologous virus/vector. After delivery of rabies-G pseudotyped equine infectious anaemia virus (EIAV) based vectors encoding a marker gene to the rat striatum, neurons in regions distal from but projecting to the injection site, such as the dopaminergic neurons of the substantia nigra pars compacta, become transduced. This retrograde transport to appropriate distal neurons was also demonstrated after delivery to substantia nigra, hippocampus and spinal cord and did not occur when vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped vectors were delivered to these sites. In addition, peripheral administration of rabies-G pseudotyped vectors to the rat gastrocnemius muscle leads to gene transfer in motoneurons of lumbar spinal cord. In contrast the same vector pseudotyped with VSV-G transduced muscle cells surrounding the injection site, but did not result in expression in any cells in the spinal cord. Long-term expression was observed after gene transfer in the nervous system and a minimal immune response which, together with the possibility of non-invasive administration, greatly extends the utility of lentiviral vectors for gene therapy of human neurological disease.

INTRODUCTION

Primate and non-primate lentiviral vectors have been developed as gene transfer tools that can mediate significant levels of gene transfer to the mammalian nervous system (1–3). The natural envelope of the lentivirus is generally replaced in the vector system with a heterologous envelope (referred to as pseudotyping). In particular the envelope glycoprotein from vesicular stomatitis virus (VSV-G), which confers on various vectors the ability to transfer genes to a broad range of different cell types, including mammalian and non-mammalian cells, has been used (4). A wide range of envelope glycoproteins, including those from rabies virus, Mokola virus, Moloney murine leukemia virus (MLV 4070A), gibbon ape leukemia virus, murine leukemia virus 10A1 and Ebola virus, have been shown to be capable of pseudotyping lentiviral vectors (2,5–8). After injection into the brain, lentiviral vectors, pseudotyped with VSV-G envelope, transduce neurons with consequent anterograde transport of the expressed protein throughout the cell bodies and axons (9). No retrograde axonal transport has been conclusively demonstrated for lentiviral vectors, although this has been seen with other vector systems including those derived from adenoviral and herpes simplex viruses (HSV) (10–12).

Retrograde axonal transport is believed to mediate the entry into the central nervous system (CNS) of a number of viruses such as polio, herpes and rabies (13–15). Neurotrophic factors (16) and molecules such as tetanus toxin (17) can also be transported to the CNS after binding to specific receptors at nerve endings, with subsequent internalization and fast axoplasmic transport (reviewed in 18). There have only been a few reports involving the delivery of foreign genes to the CNS via peripheral injection. One study involved injection of HSV vectors into the sciatic nerve and, less efficiently, in the footpad (19). Another study with a herpes viral vector encoding preproenkephalin reported gene transfer to dorsal root ganglia and antihyperalgesic effects after delivery to abraded skin of the dorsal hindpaw of mice (20). Others have used adenovirus with injection in the tongue or skeletal muscle (10,21–24) or direct injection into the sciatic nerve (25). In addition, naked DNA injection into the sciatic nerve or gastrocnemius muscle has also resulted in gene expression (26). However, most of these methods have proven very inefficient in achieving significant gene transfer or long-lasting therapeutic results.

In this paper we demonstrate that pseudotyping lentiviral vectors with rabies-G enhances gene transfer to neurons by facilitating retrograde axonal transport and infection of neurons at distal connected sites within the nervous system. Furthermore it allows their entry into the nervous system following peripheral intramuscular delivery.

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RESULTS

Transduction of primary neuronal cultures using VSV-G and rabies-G pseudotyped equine infectious anaemia virus (EIAV) vectors

In order to determine whether the EIAV vectors could transduce dopaminergic (TH-positive) neurons *in vitro*, rat or mouse embryonic day 14 (E14) ventral mesencephalic cultures were prepared and transduced after 7 days *in vitro*. It had been previously determined that most characteristic dopaminergic functions were established by this time point (27). Both VSV-G and rabies-G pseudotyped EIAV vectors were capable of transducing dopaminergic neurons *in vitro*, at about 10% efficiency at the highest multiplicity of infection (MOI) tried (Fig. 1A–D). Both vectors also transduced non-dopaminergic mesencephalic neurons and glial cells in these cultures as judged by morphological criteria (data not shown). In particular the VSV-G pseudotyped vector transduced ~80% of the estimated glia present, whereas the rabies-G pseudotyped vector transduced only 5–10%.

To determine whether viral infection altered dopaminergic properties in these neurons a ^{3}H-dopamine (^{3}H-DA) release assay was used. As dopamine transporters are localized exclusively on dopaminergic neurons in the midbrain (28), this approach allows for the selective analysis of dopaminergic function in the midst of a heterogeneous culture system. The data indicate that neither VSV-G nor rabies-G pseudotyped vectors affected ^{3}H-DA release (Fig. 1E) and, as such, these vectors do not cause an aberration in the dopaminergic function of these neurons after transduction.

Primary cultures of both hippocampal and striatal neurons could also be transduced *in vitro* by EIAV vectors pseudotyped with either VSV-G or rabies-G. This was demonstrated in hippocampal and striatal neurons by the colocalization of antibody staining for both the reporter protein β-gal and NeuN, a neuronal-specific marker (Fig. 1F–H and I–K, respectively). At MOIs of 1 and 10, there was no significant difference in transduction efficiency between the hippocampal and striatal neurons (MOI = 1, $P = 0.23$ and MOI = 10, $P = 0.81$, ANOVA; Fig. 1L and M), although an increase was observed compared with mesencephalic dopaminergic neurons. Similarly, there was no significant difference in transduction efficiency at MOI = 1 when vectors are pseudotyped with either VSV-G or rabies-G ($P = 0.14$, ANOVA). However, at an MOI of 10, the transduction efficiency of the rabies-G pseudotyped vector was significantly higher than that observed with the VSV-G pseudotyped vector ($P < 0.001$, ANOVA).

Gene transfer to striatum using VSV-G and rabies-G pseudotyped EIAV vectors

In order to compare the pattern of expression of the two different pseudotyped vectors in the adult rat brain, concentrated viral vector preparations were stereotactically injected into caudate putamen. At one month post-injection the VSV-G pseudotyped pONY8.0Z vectors gave strong gene transfer spanning an average area of 2.5 mm anteroposterior, 1 mm mediolateral and 5 mm dorsoventral around the site of injection. This equates to about 29 750 ± 1488 transduced cells (Fig. 2A and B). Over 90% of the transduced cells had neuronal morphology (striatal projection neurons and small spiny neurons) and this is further confirmed using confocal immunocolocalization of the neuronal marker NeuN and β-gal (Fig. 2M–O). A small number of transduced glia were seen in some rats in white matter tracts such as the corpus callosum. Transduction is localized to striatum with some anterograde

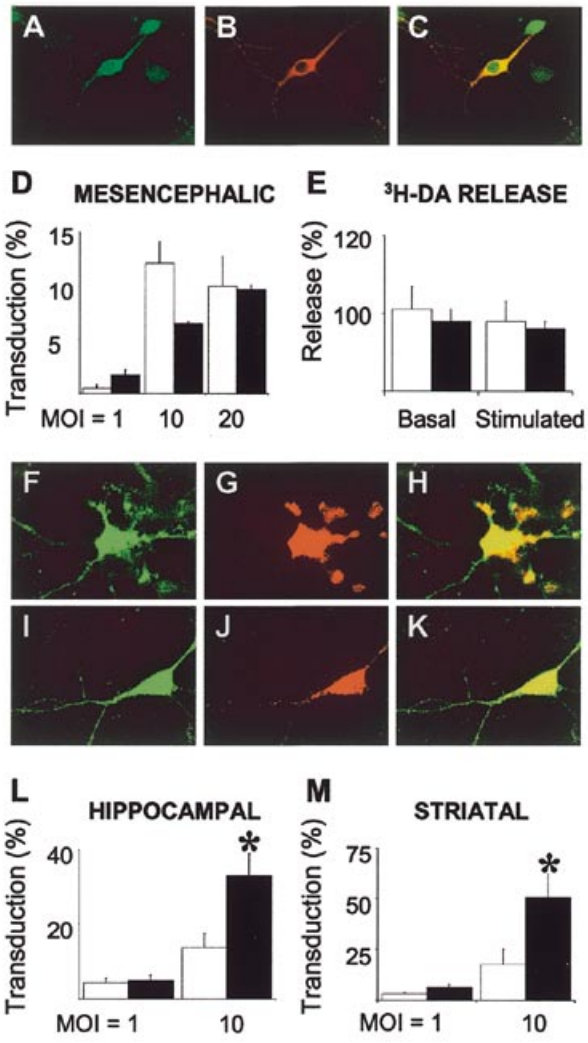

Figure 1. Gene transfer in primary neuronal cultures using EIAV lentiviral vectors. (**A**–**C**) Mouse E14 mesencephalic neurons infected with rabies-G pseudotyped pONY8.0G at a MOI of 10. A GFP expressing neuron from these cultures is shown in (A) labelled with an anti-GFP antibody (green) and an anti-TH antibody (red) (B). (C) GFP and TH colocalization in the merged confocal image in yellow. (**D**) Increasing the MOI leads to an increase in the number of neurons transduced but no significant differences between the two pseudotypes is observed. (**E**) No effect of transduction on ^{3}H-DA release by mesencephalic neurons after lentiviral gene transfer is observed compared with control neurons. In (D), (E), (**L**) and (**M**), clear bars indicate cells infected with VSV-G pseudotyped virus and black bars indicate cells infected with rabies-G pseudotyped virus. (**F**–**H**) Rat E17 hippocampal neurons and striatal neurons (**I**–**K**) infected with rabies pseudotyped EIAV vectors expressing β-gal at an MOI of 10. Cells are labelled with anti-β-gal (green) (F and I) and anti-NeuN antibodies (red). (G and J) Merged confocal images showing colocalization of the two antigens in yellow (H,K). As with the mesencephalic cultures, increasing MOI leads to an increase in the number of hippocampal (L) and striatal (M) neurons transduced. Asterisks indicate a significant increase in transduction efficiency with the rabies-G pseudotyped vector compared with the VSV-G pseudotype. Magnification: (A–C,F–K), ×60.

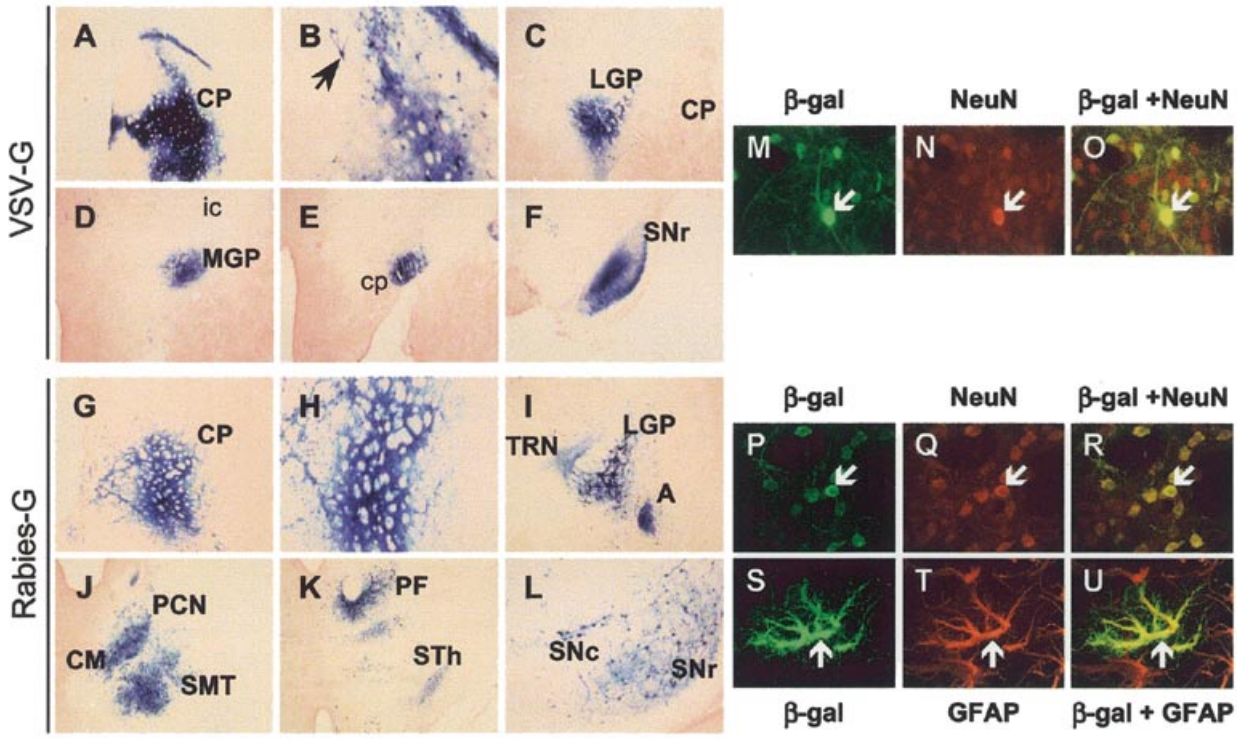

Figure 2. *In vivo* transduction of LacZ in the rat striatum with VSV-G (**A**–**F**) and rabies-G (**G**–**L**) pseudotyped pONY8.0Z vectors 1 month post-injection. (A) Extensive gene transfer at the site of injection in the caudate putamen is observed after VSV-G pseudotyped vector delivery, which is specific to the striatum and not to the fiber tracts transversing it. (B) Higher magnification image from (A), revealing cells with neuronal morphology close to the injection site (arrow). Anterograde transport of β-gal is observed in neuronal axons projecting from the injected striatum to anatomically linked projection sites such as the lateral and medial GP (C and D), the cerebral penduncle adjacent to the subthalamic nucleus (E) and to the SNr (F). The striatal projections to these sites are reviewed by Parent *et al.* (69). Some β-gal expressing cell bodies are observed only in the lateral GP, which implies that direct gene transfer has also occurred due to the proximity of this nucleus to the injection site. (G) Gene transfer with rabies-G pseudotyped vectors in striatum leads to extensive β-gal staining in caudate putamen (G and H) and also of the nearby GP (I). Pallidal transduction leads to anterograde labelling of projections to thalamic reticular nucleus (pale blue staining in I). Labelling of these afferents was observed when anterograde tracers were placed in the GP (70). Retrograde transport of rabies-G pseudotyped viral vectors results in transduction of cell bodies in distal neuronal nuclei at anatomically connected sites (71) including the amygdala (I), several thalamic nuclei (J and K), the subthalamic nucleus (K) and the substantia nigra (L). This phenomenon was not observed after similar delivery of VSV-G pseudotyped vectors. Confocal analysis of transduced cell types in the rat striatum following injection of VSV-G (**M**–**O**) and rabies-G (**P**–**U**) pseudotyped EIAV viral vectors. Transduction is mainly neuronal in both cases as demonstrated with β-gal (M and P) and NeuN antibody staining (N and Q) in the same sections. Colocalization of β-gal and NeuN expression can be seen in yellow in the merged images (O and R). In addition to neurons (arrow) rabies-G pseudotyped vector transduces astrocytes (S–U, arrow), as demonstrated by anti-β-gal (S) and anti-GFAP (T) colocalization seen in yellow (U). A, amygdala; CP, caudate putamen; cp, cerebral penduncle; CM, centromedial thalamic nucleus; ic, internal capsule; LGP, lateral globus pallidus; MGP, medial globus pallidus; PCN, pericentral thalamic nucleus; PF, perifasicular thalamic nucleus; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; SMT, submedial thalamic nucleus; STh, subthalamic nucleus; TRN, thalamic reticular nucleus. Magnification: (A,C–G,I–K), ×10; (B), ×40; (H), ×25; (M–O), ×90; (P–R), ×120; (S–U), ×160.

transport of β-gal observed in axons projecting to the lateral and medial globus pallidus (GP) (Fig. 2C and D), cerebral penduncle (Fig. 2E) and the substantia nigra pars reticulata (SNr) (Fig. 2F). In rats where the lateral GP was co-transduced, reticular thalamic nucleus was also strongly stained by anterograde transport of β-gal (data not shown).

Transduction of rat striatum with rabies-G pseudotyped pONY8.0Z vectors gave strong gene transfer to cells within the caudate putamen (Fig. 2G and H). In addition transduced neurons were observed in many other regions distal to the injection site including the cortex, GP, thalamus, amygdala, hypothalamus, subthalamic nucleus, substantia nigra compacta and reticulata (Fig. 2I–L). Average transduction was seen 7.5 mm anteroposterior to the injection site, spanning many basal ganglia nuclei from the striatum to substantia nigra. Since anatomical connections are known to exist between these areas (29), and since the expression is only confined to neurons, this can only be explained by retrograde axonal transport of rabies-G pseudotyped vector and transduction of these distal cells. Cell counts indicate that 32 650 ± 1630 cells were transduced in striatum, whereas 14 880 ± 744 neurons were transduced in thalamus and 3050 ± 150 neurons were transduced in substantia nigra. Staining in the caudate putamen with the rabies-G vector was paler and more punctate in comparison with that observed with VSV-G pseudotyped vector, with ~80% neurons and 20% glia transduced (Fig. 2P–U). Confocal colocalization studies at the injection site indicate that the glia transduced were astrocytes. No projection neurons were transduced in contrast with the VSV-G pseudotyped vectors. Anterograde transport of β-gal was also present in neurons transduced

with the rabies-G pseudotyped vectors as indicated by the pale staining of the thalamic reticular nucleus (from lateral globus pallidal neurons) and the SNr (from striatal neurons) (Fig. 2I and L). Confocal studies confirmed the neuronal nature of the cells transduced distally when rabies-G pseudotyped vectors were delivered to the caudate putamen, such as the NeuN positive pallidal neurons and the tyrosine hydroxylase (TH) positive dopaminergic neurons of the substantia nigra [Fig.3 (ii)D–I].

Retrograde transport of viral vector itself was confirmed by PCR experiments using punches taken from thalamus and substantia nigra areas since viral DNA in these areas could only be detected after rabies-G pseudotyped EIAV striatal transduction [Fig. 3(iii)]. Control experiments where integrase mutant viral preparations or vector preparations preheated at 50°C were injected in the brain failed to give any significant levels of transduction, thus excluding the possibility that pseudotransduction was responsible for the observed gene transfer (30).

Long-term expression was observed after delivery of both types of vectors to the caudate putamen up to 8 months post-injection in the present study (not all data shown). Expression of rabies-G pseudotyped vectors was observed both at the site of injection and at all the distal neurons that were transduced at 1 month post-injection [Fig. 3(i)A–C; only thalamus and substantia nigra are shown].

Gene transfer to substantia nigra using VSV-G and rabies-G pseudotyped EIAV vectors

In order to compare the ability of the two different pseudotyped vectors to transduce CNS dopaminergic neurons, concentrated viral vector preparations were stereotactically injected in the vicinity of substantia nigra pars compacta. Perinigral injections were preferable so as to avoid possible damage to substantia nigra. VSV-G pseudotyped pONY8.0Z vectors gave very efficient transduction of the substantia nigra pars compacta and of some thalamic nuclei caudal to it (Fig. 4A and B). Neuronal projections from the compacta to reticulata were stained, although there was no staining of the SNr. β-gal was transported anterogradely to axon terminals of the nigrostriatal neurons giving pale staining in the striatum (Fig. 4C).

Perinigral injections of rabies-G pseudotyped EIAV vector gave strong transduction of both the substantia nigra compacta and reticulata and of neighbouring thalamic and hypothalamic nuclei (Fig. 4D–F). The β-gal staining was observed with the VSV-G pseudotyped vectors and in addition many fibres within the thalamus were stained. Transduction of distal neurons in the lateral GP and amygdala, where stronger β-gal staining was observed, was due to retrograde transport of virus from efferent connections to the SNr and pars lateralis, respectively (Fig. 4G and H). These neuronal projections from nigra were previously established by the retrograde tracer studies of Bunney and Aghajanian (31). In addition, on the contralateral side, transduction was observed of several commissural nuclei and their projections (Fig. 4I), providing further evidence of retrograde transport operating with this vector.

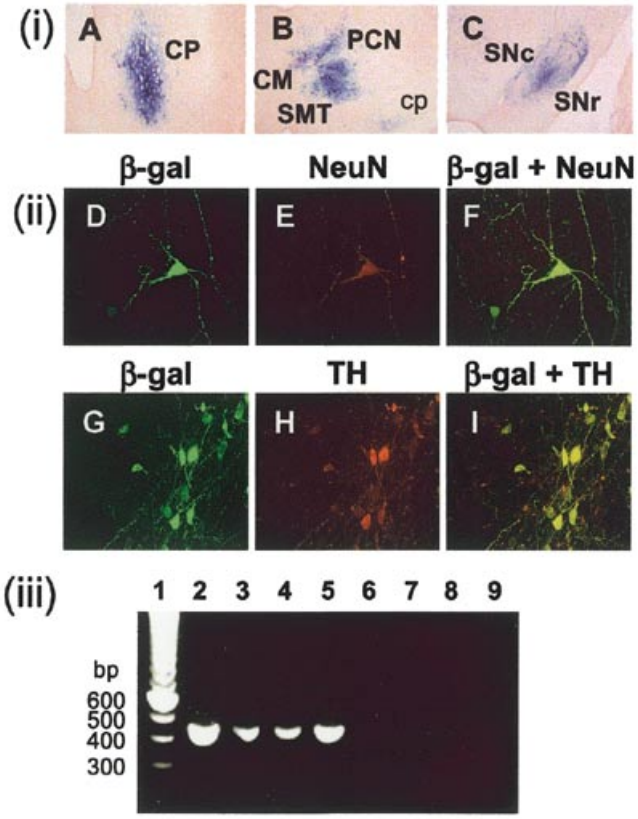

Figure 3. (i) Reporter gene expression at 8 months post-injection in the striatum and retrogradely transduced distal sites after striatal delivery of rabies-G pseudotyped pONY8.0Z vector. (**A**) Strong expression at the site of delivery in the caudate putamen. Expression also remains strong at distal sites projecting to caudate putamen, such as the medial thalalamic nuclei (**B**) and the substantia nigra (**C**), which are transduced by retrograde transport of the rabies-G pseudotyped pONY8.0Z vector. Pale staining is observed in cerebral penduncle and SNr from β-gal transported in axons of transduced striatal efferents. CM, centromedial thalamic nucleus; CP, caudate putamen; cp, cerebral penduncle; PCN, pericentral thalamic nucleus; SMT, submedial thalamic nucleus; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata. Magnification: (A,B), ×10; (C), ×15. (ii) Confocal analysis showing retrogradely transduced neurons in GP (**D–F**) and substantia nigra pars compacta (**G–I**), after injection of rabies-G pseudotyped virus into the striatum. Micrographs demonstrate immunofluorescent labelling of neurons with anti-β-gal (D and G), anti-NeuN (E) and anti-TH (H) antibodies. Expression of β-gal (green) colocalizes with the red immunofluorescence of NeuN in pallidal neurons (F) and TH in nigral dopaminergic neurons (I), producing yellow staining. Magnification: (D–I), ×50. (iii) PCR analysis showing detection of EIAV vector DNA in thalamus and substantia nigra ipsilateral to the site of injection of the rabies-G pseudotyped vector in the rat striatum. Lane 1, 100 bp ladder; lanes 2, 3 and 4, rat 1 (rabies-G pseudotyped vector) striatum, thalamus and substantia nigra; lanes 5, 6 and 7, rat 2 (VSV-G pseudotyped vector) striatum, thalamus and substantia nigra; lane 8, rat 5 uninjected; lane 9, water.

Gene transfer to hippocampus using VSV-G and rabies-G pseudotyped EIAV vectors

To test if VSV-G and rabies-G pseudotyped EIAV vectors exhibit similar transduction properties to those observed when injected into the basal ganglia, these vectors were stereotactically injected into the right anteriodorsal hippocampus of rats. In the case of the VSV-G pseudotyped vectors, this led to strong transduction of neurons in the subiculum and to a lesser extent

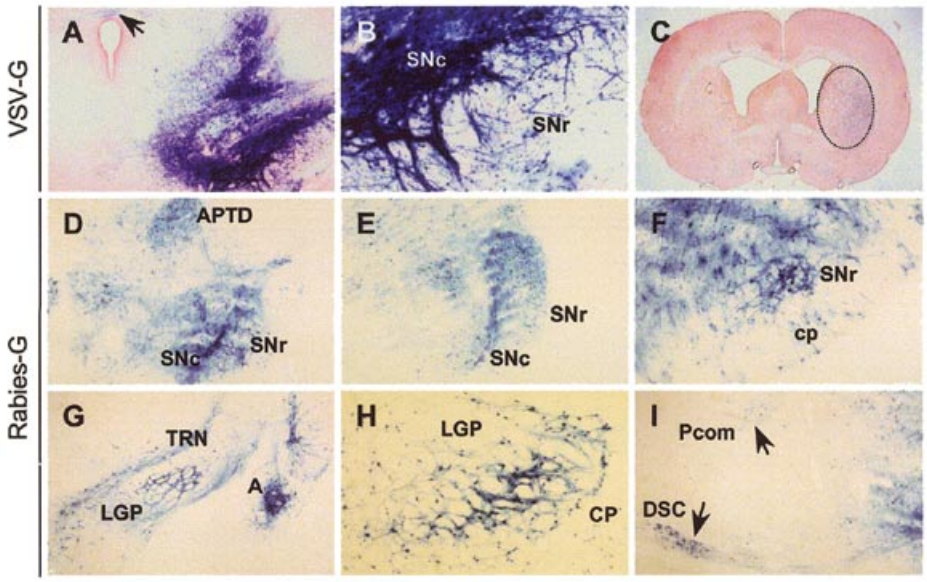

Figure 4. *In vivo* transduction in the rat substantia nigra with VSV-G (**A**–**C**) and rabies-G (**D**–**I**) pseudotyped pONY8.0Z vectors 1 month post-injection. (A) Extensive gene transfer is observed with the VSV-G pseudotyped vector in the substantia nigra pars compacta and thalamus. (B) Higher magnification of the substantia nigra showing extensive transduction of pars compacta neurons and their axons projecting to SNr. (C) β-gal protein is anterogradely transported to axon terminals of nigrostriatal neurons producing pale staining of ipsilateral striatum (circled). Arrow in (A) indicates anterograde transport of β-gal and staining of commisural axons projecting to contralateral side though no transduction of neuronal cell bodies was observed contralaterally. (D) Extensive transduction of both substantia nigra and different thalamic nuclei is observed after delivery of rabies-G pseudotyped EIAV vectors. In this case both substantia nigra pars compacta and SNr are transduced (E and F). Labelling of neurons in distal sites due to retrograde transport of this vector can be observed in lateral GP (G and H), amygdala (G) and commissural neurons projecting from contralateral thalamus (I, arrows). Anterograde transport of β-gal along axons is widespread, leading to staining of structures such as the thalamic reticular nucleus (G) (from lateral GP) and caudate putamen (G and H) (from substantia nigra pars compacta and lateral GP). A, amygdala; APTD, anterior pretectal thalamic nucleus; CP, caudate putamen; cp, cerebral penduncle; DSC, dorsal supraoptic decussation of the commissure of Maynert; LGP, lateral globus pallidus; Pcom, nucleus of posterior commissure; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; TRN, thalamic reticular nucleus. Magnification: (A,D,E,G,I), ×10; (B), ×40; (C), ×3.5; (F,H), ×25.

in the CA1 pyramidal cell layer (Fig. 5A and B). Cells with neuronal morphology within the stratum oriens were also stained while some glial transduction was observed within the corpus callosum. In addition, anterograde transport of β-gal was observed, resulting in weak staining of axon fibres projecting to stratum moleculare (Fig. 5B) and in few fibres projecting to septum (Fig. 5C).

In contrast, injections of rabies-G pseudotyped EIAV vectors into the hippocampal region led to strong β-gal staining of CA1 and CA3 pyramidal neurons within the stratum pyrimidale of the rostal hippocampus. This became restricted to the CA1 region in caudal aspects, and some staining was also observed in the CA4 pyramidal cell layer (Fig. 5D–F). Apical dendrites and axons of CA1 neurons were strongly stained. β-gal staining within the subiculum and corpus callosum was observed (Fig. 5F). Retrograde transport of the viral vector and transduction of distal neurons projecting to the area of viral delivery resulted in strong staining of the medial forebrain bundle nuclei in the lateral hypothalamus and in the vertical limb of the diagonal band of Broca (with axons projecting to the mediodorsal septal area and to the hippocampus via the fimbria of the fornix) (Fig. 5H), supramammillary hypothalamic nuclei and thalamic nuclei (laterodorsal, anterodorsal and anteroventral nuclei) (Fig. 5G) (32). Staining of the contralateral hippocampus was probably due to viral vector leakage during the injection along this folded structure, producing an identical but weaker pattern of staining on that side.

Gene transfer to spinal cord using VSV-G or rabies-G pseudotyped EIAV vectors

To determine the transduction efficiency of the EIAV vector, intraspinal and intramuscular injections of the β-gal-expressing vectors were performed in the rat. Intraspinal injection of the lentiviral vector was associated only with a mild degree of inflammation, with no significant cell damage (data not shown). All rats tolerated the surgery and lentiviral vector injections without complication. Moreover, coordination and movement of operated animals was unaffected, indicating the absence of functional deterioration following intraspinal injection of the viral vector. Examination of transverse sections of the spinal cord revealed robust reporter gene expression after delivery of both VSV-G and the rabies-G pseudotyped lentiviral vectors (Fig. 6A,B,H and I). Injection in the lumbar spinal cord led to β-gal expression in $10\ 260 \pm 513$ and $16\ 695 \pm 835$ cells with VSV-G and rabies-G pseudotyped vectors, respectively. The rabies-G pseudotyped lentiviral vectors produced a more extensive rostrocaudal spread of expressing cells within the area of viral delivery (lumbar spinal cord) and also in the adjoining thoracic spinal cord.

To identify the phenotype of the cells transduced after intraspinal injections, sections were double-labelled with

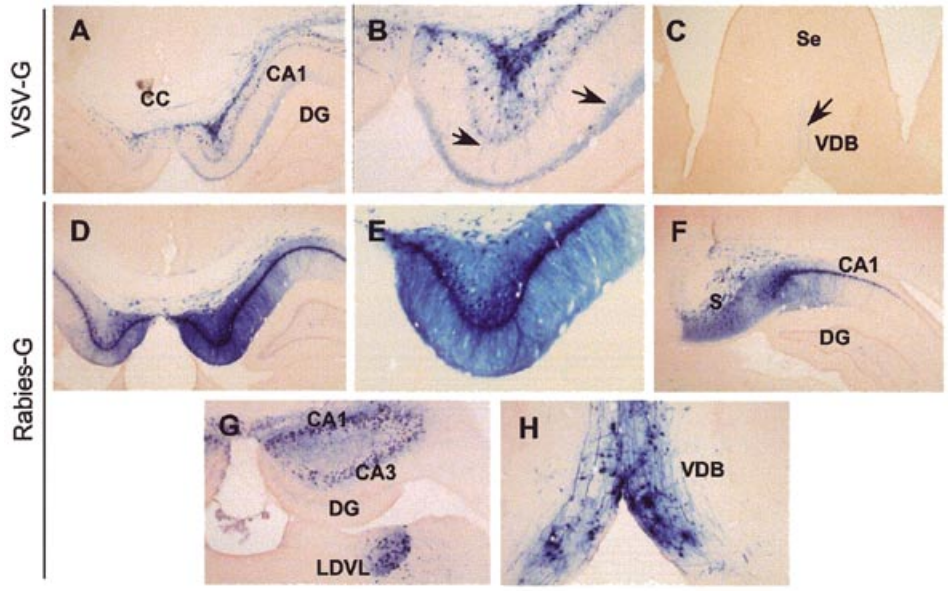

Figure 5. *In vivo* transduction in the rat hippocampus with VSV-G (**A–C**) and rabies-G (**D–H**) pseudotyped pONY8.0Z vectors 1 month post-injection. (A) Extensive gene transfer is observed with the VSV-G pseudotyped vector in the subiculum and to a lesser extent in the CA1 pyramidal cell layer and in the corpus callosum. Faint blue staining represents anterograde transport of β-gal staining of axon fibres projecting to the stratum moleculare (A and B, arrows) and a few fibres projecting to the septum and diagonal band of Broca (C, arrow). No cell body staining was observed in these regions. These neuronal projections are established from anterograde tracing experiments (72). (D) Strong transduction of CA1 cells with rabies-G compared with VSV-G pseudotyped vectors is observed. Some transduction of CA4 pyramidal cells is also present. (E) Higher magnification from the CA1 region depicted in (D) showing strong staining of apical dendrites and axons of pyramidal neurons. (F) β-gal staining of cells in the subiculum, CA1 pyramidal layer, corpus callosum and cortical fibres in the posterior hippocampus. (G) β-gal staining of CA1and CA3 pyramidal cells but not of dentate gyrus in the anterior hippocampus. Cortical fibres are stained and retrograde labelling of laterodorsal thalamic nucleus is also observed. (H) Strong transduction in neuronal nuclei and axons in the lateral hypothalamus and diagonal band of Broca due to retrograde transport of the rabies-G pseudotyped viral vector is observed. Afferents to the hippocampus from these sites have been described previously (31). DG, dentate gyrus; CA1 and CA3, hippocampal pyramidal neuronal cell layers; LDVL, vetrolateral aspect of laterodorsal thalamic nucleus; S, subiculum; Se, septum; VDB, vertical limb of the diagonal band of Broca. Magnification: (A,C,D,F), ×10; (B,H), ×25; (E), ×50; (G), ×15.

antibodies to β-gal and either NeuN or glial fibrillary acidic protein (GFAP). On average 90 and 80% of the transduced cells were double-labelled with NeuN after VSV-G and rabies-G pseudotyped vector delivery, respectively (Fig. 6E–G and L–N). To assess the percentage of motoneurons expressing the reporter gene, motoneurons were back-labelled with fluorogold (FG) (Fig. 6C,D and J,K). The number of FG-positive motoneurons expressing β-gal was evaluated in longitudinal sections of the lumbar spinal cord. Analysis of these sections showed that 52 and 67% of the FG-back-labelled motoneurons expressed β-gal after intraspinal injections of VSV-G and rabies-G pseudotyped EIAV vectors, respectively.

Interestingly, brainstem motoneurons of the tectospinal, vestibulospinal and reticulospinal tracts as well as corticospinal motoneurons located in the layer V of primary motor cortex were retrogradely transduced following intraspinal injection only of the rabies-G lentiviral pseudotyped vector (Fig. 6O and P). Some spinal commissural interneurons projecting from the contralateral side were also retrogradely transduced (Fig. 6H). Interestingly, retrograde transport of the rabies pseudotyped vector was also found in lumbar spinal motoneurons following injection into the gastrocnemius muscle (Fig. 6Q–S). Intramuscular injections of rabies-G pseudotyped lentiviral vector led to β-gal expression in 27% of the FG-back-labelled motoneurons (approximately 850 ± 90 transduced motoneurons). No muscle transduction was observed with this vector. By contrast, the VSV-G pseudotyped vector transduced muscle cells surrounding the injection site, at low efficiency, but did not label any cells in the spinal cord (data not shown).

Minimal immune response in CNS after EIAV vector injection

At different time points after gene transfer to the brain (striatum), specific antibody markers were used to detect immune responsive cells at the site of injection. In no cases after stereotactic delivery was any adverse brain pathology observed. Control injections with phosphate buffered saline (PBS) caused a negligible immune reaction that consisted of a small infiltration of $OX42^+/ED1^+$ activated macrophages/microglia around the needle tract in the cortex and striatum and also along white matter tracts such as corpus callosum (data not shown). No staining was observed with any of the other markers when PBS was injected. This $OX42^+/ED1^+$ immunoreactivity declined but was still detectable at 35 days. A similar response with OX42 was observed with both viral vector preparations and this probably represents the reaction to the injection procedure. In addition, the VSV-G pseudotyped vectors resulted in an infiltration of OX18+ MHC class I positive cells in the ipsilateral striatum, present at all time points, but no leucocytes or dendritic cells were observed at any time point (Fig. 7A–D). However, the rabies-G vector injection

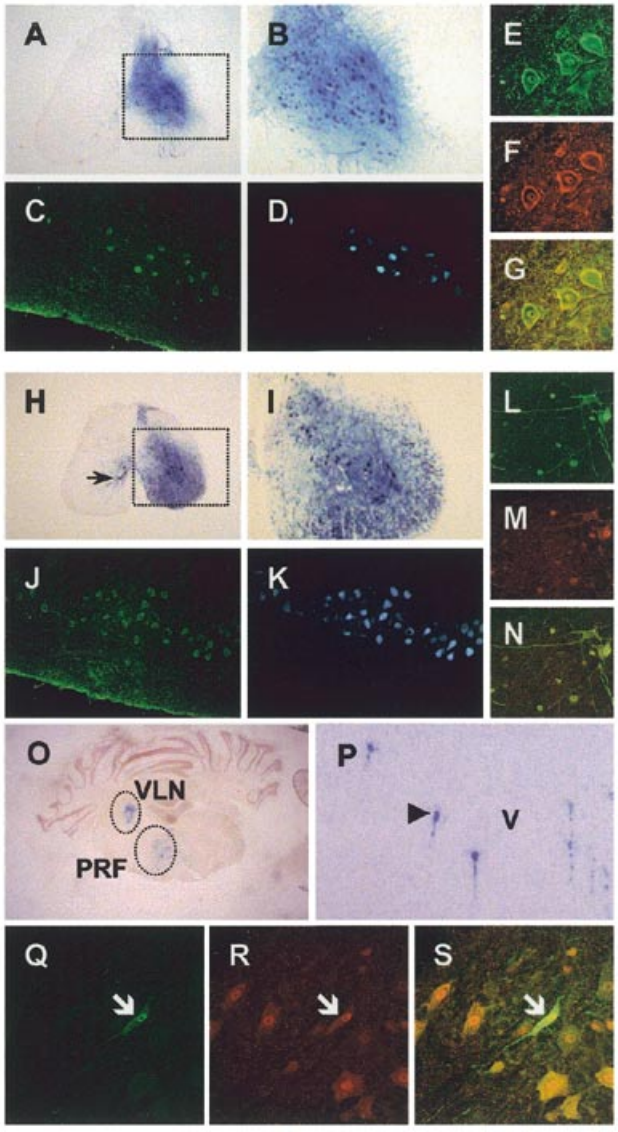

Figure 6. Reporter gene expression in the rat spinal cord 3 weeks following intraspinal or intramuscular delivery of pONY8.0Z lentiviral vectors. Micrographs of the ventral horn showing transduction after intraspinal injections with VSV-G (**A–G**) or rabies-G pseudotyped virus (**H–P**). Strong transduction with β-gal is observed with both types of vectors (A,B and H,I). (B) and (I) are higher magnifications from the area of transduction shown in (A) and (H). Longitudinal sections of the spinal cord showing retrogradely FG-labelled motoneurons (D and K) co-expressing β-gal (C and J). Transverse sections stained with anti-β-gal antibodies (E,L). The same sections were stained for the neuronal marker NeuN (F,M). Composite confocal images showing neuronal colocalization of NeuN and β-gal in yellow (G,N). Note the retrogradely transduced motoneurons in areas projecting to the site of injection such as brainstem (O) and layer V of the cerebral cortex (P) following intraspinal injection of rabies-G pseudotyped pONY8.0Z vectors (interconnections reviewed in 73). The arrow in (H) indicates retrogradely transduced commissural interneurons projecting from the contralateral side to the region of injection, along previously established anatomical connections (reviewed in 74). The arrowhead in (P) indicates a transduced layer V corticospinal motoneuron ipsilateral to the injection site. (**Q–S**) Transverse sections of the spinal cord showing retrograde transport of the viral particles and transduction of spinal cord motoneurons (arrow) after injection of rabies-G pseudotyped pONY8.0Z vector into the gastrocnemius muscle. (Q) Sections stained with anti-β-gal antibodies. (R) Same sections stained for the neuronal marker NeuN. Composite confocal images showing colocalization of NeuN and β-gal in yellow (S). Vln, vestibular lateral nucleus; Prf, pontine reticular formation. Magnification: (A,H) ×10; (B–D,I–K,O) ×25; (E–G,L–N,Q–S) ×60; (P) ×50.

initiated a more acute immune response with infiltrating leucocytes, dendritic cells and MHC class I immunopositive cells into striatum and cortex and also along white matter tracts, meninges and subventicular cell layers (Fig. 7E–H). Some perivascular cuffing and tightly packed inflammatory cells was observed within the striatum with the OX1 and OX18 markers (Fig. 7E and F). Reduced levels of response including the absence of dendritic cells were detected at 14 days and declined to background levels by 35 days.

DISCUSSION

This report describes gene transfer experiments to neural cells both *in vivo* and *in vitro* using minimal lentiviral vectors pseudotyped with two different viral envelope glycoproteins; VSV-G derived from vesicular stomatitis virus and rabies-G derived from rabies virus. Both types of vectors transduce neural cells efficiently, but the rabies-G pseudotyped vectors exhibit enhanced gene transfer to neurons due to retrograde axonal transport and transduction of distal neurons connected to the injection site. This retrograde transport is not restricted to specific neuronal populations, as it occurs after vector delivery to the striatum, substantia nigra, hippocampus, spinal cord and muscle.

An important feature of these vectors is that they transduce neural cells both *in vitro* and *in vivo*. For example, they can transduce mammalian primary hippocampal or striatal neuronal cultures with good efficiencies at low MOIs. In addition they can transduce TH^+ neurons from E14 ventral mesencephalic cultures (which typically contain 4% of TH^+ presumptive dopaminergic neurons, 80% TH^- mesencephalic neurons and 15% astrocytes) (33), at lower efficiencies but greater than those reported for other vector systems. Constantini *et al.* (34) have reported that such mesencephalic neuronal cultures are very refractory to transduction with vector systems including adeno-associated (AAV), adenoviral and HSV vectors. Although a similar gene transfer to EIAV vectors was achieved with a hybrid AAV/HSV amplicon vector, the percentage of TH^+ neurons transduced within these cultures was not determined (34). Significantly EIAV vectors did not cause cytotoxicity or alter the dopaminergic profile of these cells.

In vivo delivery of the EIAV VSV-G pseudotyped vectors led to transduction efficiencies comparable to those reported with primate HIV lentiviral vectors pseudotyped with VSV-G vectors in the brain (1,35). Injection into the spinal cord with VSV-G EIAV vector encoding the reporter gene LacZ led to β-gal expression in 67% of the FG-back-labelled motoneurons surrounding the injection site. In comparison, injection of an AAV vector encoding green fluorescent protein (GFP) into the mouse spinal cord leads to expression of the reporter gene in 34% of the motoneurons surrounding the injection site (36), and injection with HIV-based lentiviral vectors lead to 33% motoneuron transduction (M.Azzouz, unpublished data). The HIV vectors used contained the Woodchuck Hepatitis post-translational regulatory element, which has been shown to increase expression of transgenes in retroviral vectors (35,37) and was not used in the EIAV vectors in the present study.

The predominant transduction of neurons with the EIAV vectors *in vivo* has been observed with other types of vectors including primate lentiviral (1,9,38–40) and hybrid AAV/HSV amplicon vectors (34). The molecular basis for this remains

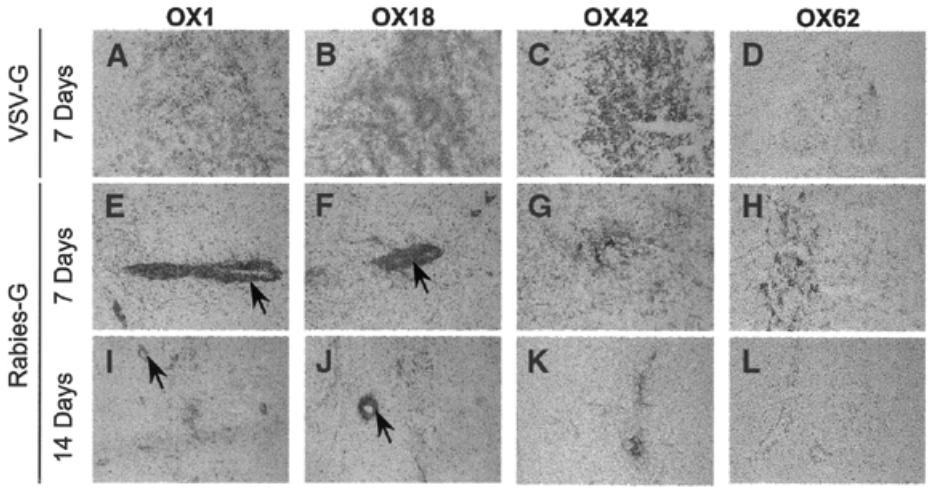

Figure 7. Immune response in the rat brain following pONY8.0Z vector delivery in the rat striatum. Antibodies used to detect components of the immune response in the injected area were as follows: OX1, leucocyte common antigen; OX18, MHC class I; OX42, complement receptor type 3 on microglia and macrophages; and OX62, dendritic cells. All animals (including PBS-injected controls, not shown) exhibited a minor infiltration of $OX42^+/ED1^+$ activated macrophages/microglia around the needle tract in the cortex and striatum (**C**,**G** and **K**). This response declined with time but was still partially evident at 35 days post-injection (not shown). Animals injected with VSV-G pseudotyped vectors (**A**–**D**) exhibited a minor immune response at 7 days post-injection, in addition to the microglial infiltration observed in controls. An infiltration of OX18 + MHC class I positive cells in ipsilateral striatum (B) was observed though neither leucocytes (A) nor dentritic cells (D) could be detected at any time after VSV-G pseudotyped virus injection in the brains of these animals. This response had declined by 14 days. Compared with VSV-G pseudotyped virus, a slightly stronger immune response was observed following injection of rabies-G pseudotyped virus. Infiltration of leucocytes (**E**), MHC class I immunopositive cells (**F**), dendritic cells (**H**) and the presence of perivascular cuffing (E and F) can be seen 7 days post-injection, decreasing in levels at 14 days after injection (**I**,**J** and K, respectively). Magnification: (A–D,F–L), ×25; (E), ×50.

unclear but is unlikely to be due to specific promoter usage, since it is also observed after delivery in the brain of lentiviral vectors expressing transgenes from cellular ubiquitous promoters (35). Cell-dependent differences in promoter efficiency have been previously demonstrated using adenoviral vectors (41) where the human cytomegalovirus immediate-early promoter was shown to be well expressed in hippocampal pyramidal neurons but poorly expressed in hippocampal dentate gyrus neurons compared with the same vectors driven by the Rous sarcoma virus (RSV) long terminal repeat. This could also account for the lack of expression in dentate gyrus after intra-hippocampal injections of the EIAV vectors.

Pseudotyping with rabies-G led to increased transduction efficiency (increased spread) both at the injection site and of distal neurons. Transduction of spinal motoneurons after intramuscular administration was achieved at about half the efficiency of that obtained after direct injection into lumbar spinal cord, implying that either alone or in combination, the two delivery methods of EIAV vectors should be more effective than those used previously (36).

Retrograde transport of viral particles was observed in our study exclusively with the rabies-G pseudotyped vectors. Only one study previously claimed the detection of a few TH-positive cells transduced in substantia nigra after injection of VSV-G pseudotyped HIV lentiviral vectors (42), implying retrograde transport, but that was not confirmed by subsequent studies by others using similar vectors (9). In contrast, anterograde transport of marker protein and labelling of axons at distal sites such as in the SNr after injection into the striatum of VSV-G pseudotyped HIV vectors was observed (9).

The demonstration that only the rabies-G pseudotyped vector could be retrogradely transported after transduction into different brain regions could be a function of the natural ability of wild-type rabies virus to travel along neuronal axons via fast axonal transport during infection and enter the CNS from peripheral nerve endings (43). Both VSV and rabies belong to the rhabdovirus family but their envelope glycoproteins only share 20% homology at the protein level, with homology increasing to 50% within domains (44). The tropism of these two viruses in the nervous system is very different. VSV has a strong tropism for olfactory receptor cells using them for entry to the CNS (45). Entry through the trigeminal ganglion nerve does not occur. Subsequent spread occurs transynaptically both by retrograde and anterograde transport as well as through non-neuronal pathways. Rabies virus is usually transmitted through broken skin by bite or scratch, spreading by retrograde axonal transport and replicating exclusively in neurons with the exception of acinar cells of salivary glands (46). Spread is through the transynaptic route by retrograde transport from adjacent axon terminals after viral budding from dendritic or perikaryal plasma membranes. Various studies using stereotactic injection of replication competent rabies virus in the rat brain have demonstrated that, prior to replication (within 24 h), the virus spreads to anatomically connected sites (47). Such retrograde transport could be blocked 2 days prior to viral delivery with colchicine, which leads to transient microtubule polymerization interrupting axoplasmic flow (48). In a subsequent study a G-deficient virus recombinant was used *in vivo* which lacked the ability to further infect but had retrograde transport, demonstrating that rabies-G is necessary for the transynaptic axonal transport (49). The present study

demonstrates for the first time that rabies-G alone is sufficient to confer retrograde axonal transport in neurons to a heterologous replication incompetent virus/vector. The difference in the ability of the two envelopes to mediate this type of transport might be the result of their interaction with different membrane receptors enabling both internalization and subsequent transport. The rabies-G has been shown to interact with many neuronal receptors such as p75 low affinity neurotrophin receptor (NTR) (50), neural cell adhesion molecule (51) and the nicotinic acetylcholine receptor, especially the α-bungarotoxin-sensitive nicotinic acetylcholine receptor at the neuromuscular junction (52–54), while VSV-G is thought to interact with phosphatidylserine (55). It is probable that some of these receptors not only mediate receptor binding and internalization but also enable fast axoplasmic transport via a microtubule-dependent process through some continued interaction with the virion. For example, it has been demonstrated that in sympathetic neurons the neurotrophin nerve growth factor complexed to its low affinity receptor p75 NTR and its high affinity tyrosine kinase receptor trkA is internalized into vesicles and is retrogradely transported to the nucleus where it activates expression through the transcription factor CREB (56). However, rabies virus is able to infect p75 deficient mice and spread through the brain, implying that this receptor is not essential for retrograde axonal transport to occur (57). Alternatively, the rabies-G might associate with cellular proteins during viral production which are attached to the capsid and, upon endosomal dissociation from the viral envelope after cell entry, may facilitate the attachment of the capsid to the microtubule motors and transport to the cytoplasm. Interestingly, it was recently demonstrated that for HSV the viral tegument and capsid, but not the envelope, are sufficient for recruitment of the squid giant axon retrograde transport machinery (58).

Our results demonstrate a minimal immune response associated with delivery of EIAV vectors in the brain. From both viral envelopes used, the rabies-G envelope appears to elicit the stronger acute inflammatory response with lymphocyte infiltration, perivascular cuffing of macrophages and local microglial activation that declines but is still apparent after 2 weeks and disappears by 35 days. In contrast, the immune response to VSV-G envelope is weaker from the start and declines more rapidly. At the 35 day time point microglial/macrophage infiltration for both envelopes was confined to the site of the needle injection and was no stronger than that elicited by injecting PBS alone. In the case of both envelopes, this short-lasting immune response does not seem to induce cell toxicity or mediate loss of long-term transgene expression. Similar results to those reported here have been observed in previous studies after stereotactic brain injections of VSV-G pseudotyped HIV-1 vectors (1,38). The results presented here for EIAV vectors are in stark contrast to those obtained after adenoviral (59,60) and HSV vector (61) delivery to the brain, where a significant chronic inflammatory immune response is observed that compromises tissue integrity and long-term expression. The long-term expression observed with our vectors is probably due to both integration into open chromatin regions in the genome and the minimal immune response exhibited against the transduced cells.

The efficient delivery and long-term expression of therapeutic genes in basal forebrain-hippocampal, nigrostriatal and lumbar motoneuron-neuromuscular pathways opens up the possibility for the treatment of incurable human neurodegenerative diseases such as Alzheimer's, Parkinson's and motoneuron diseases. Kordower *et al.* (62) recently demonstrated the utility of such an approach using lentiviral delivery of glial cell line neurotrophic factor in primate models of Parkinson's disease. The retrograde axonal transport of lentiviral vectors should provide a new targeting approach for gene therapy particularly in motoneuron diseases, chronic pain and spinal injury. Further experimentation in models of these diseases will establish the therapeutic utility of this vector system.

MATERIALS AND METHODS

Minimal lentiviral vector construction and production

The pONY series of EIAV vectors and their pseudotyping with the different envelopes have been described previously (2). Numbering used was as of Payne *et al.* (63). pONY8.0Z was derived from pONY4.0Z by introducing mutations that prevented expression of TAT by an 83 nucleotide deletion in exon 2 of tat, prevented S2 expression by a 51 nucleotide deletion, prevented REV expression by deletion of a single base within exon 1 of rev and prevented expression of the N-terminal portion of gag by insertion of T in the first two ATG codons, thereby changing the sequence to ATTG from ATG. With respect to the wild-type EIAV sequence (accession no. U01866), these correspond to deletions of nucleotides 5234–5316 inclusive, 5346–5396 inclusive and 5538. The insertion of T residues was after nucleotides 526 and 543. pONY8.0G was derived from pONY8.0Z by exchanging the LacZ reporter gene for the enhanced GFP gene. This was done by transferring the *Sac*II–*Kpn*I fragment corresponding to the GFP gene and flanking sequences from pONY4.0G into pONY8.0Z cut with the same enzymes.

Vector stocks were generated by calcium-phosphate transfection of human kidney 293T cells plated on 10 cm dishes with 16 μg of vector plasmid, 16 μg of gag/pol plasmid (pONY3.1) and 8 μg of VSV-G (pRSV67) or 16 μg of rabies-G (pSA91ERAwt) plasmid. Similar results were also obtained when vector was made in co-transfections in which EIAV gag/pol was expressed from plasmid pEsynGP, in which the gag/pol ORF was codon optimized for expression in human cells (64). This procedure renders expression of gag/pol independent of REV/RRE. A similiar approach for HIV gag/pol has been described previously (65). After transfection for 36–48 h, supernatants were filtered (0.45 μm), aliquoted and stored at –70°C. Concentrated vector preparations were made by initial low speed centrifugation at 6000 *g* and 4°C for 16 h followed by ultracentrifugation at 50 000 *g* and 4°C for 90 min. The virus was resuspended in PBS for 3–4 h, aliquoted and stored at –70°C. Transduction was carried out in the presence of polybrene (8 μg/ml).

Neuronal cultures

Primary neuronal cultures were established as follows: ventral mesencephalic cultures were prepared from embryonic mice (E14) exactly as described by Lotharius *et al.* (27) and striatal and hippocampal cultures were prepared from rats (E17) according to standard procedures (66). Briefly, after appropriate dissection, tissue was incubated in trypsin (0.1–0.25%) plus

DNase I (0.05%) for 5–30 min. Cells were plated in standard tissue culture media, either Dulbecco's modified Eagle's medium (DMEM) or DMEM/Hams F12 containing fetal calf serum (10%), glutamine (2 mM) and gentamicin (0.1 mg/ml). Cells were plated out onto either poly-D-lysine (50 µg/ml)/laminin (2.5 mg/ml) or poly-D-lysine (50 µg/ml)/fibronectin adhesion promoting peptide (10 µg/ml) coated dishes. Cultures were placed into a humidified 37°C incubator containing 5% CO_2 and 12 h after plating, the plating medium was replaced with neurobasal media supplemented with B27 (Life Technologies) and glutamine (0.5 mM). Cultures were fed every 2 days with supplemented neurobasal medium and were transduced on day 3–7 *in vitro*. Transduction was carried out in supplemented neurobasal media containing polybrene (2 µg/ml). Five hours after the onset of transduction, the virus was removed and was replaced with supplemented neurobasal medium. Neurons were maintained in culture for a further 48–72 h before fixation and analysis. Cells were fixed in 4% w/v paraformaldehyde (PFA) (20 min on ice) and were permeabilized with 0.1% v/v Triton-X100 in PBS (20 min on ice). Transduction was analysed by immunocytochemistry according to standard techniques (see below). Transduction efficiency was calculated either by cell counts or from random, equally processed digital images using the Aequitas image analysis software (Digital Data Ltd).

In order to measure dopamine release and content, mesencephalic neurons were plated at a density of 4×10^5 cells per 16 mm well (2×103 cells/mm^2), loaded with 2.4 Ci/ml ^{3}H-DA/KRS for 20 min at 37°C and washed three times for 3 min. Radioactive counts from a wash sample were measured using a Beckman scintillation counter and used as a control for basal levels of ^{3}H-DA release. Cells were then treated with 30 mM K^+ in KRS [adjusted as described by Dalman and O'Malley (67)] for 5 min and the amount of ^{3}H-DA released during this time period was collected. Subsequently, cultures were washed extensively and lysed in 0.1 N perchloric acid by freeze-thawing, and residual, intracellular ^{3}H-DA was measured. Total ^{3}H-DA uptake was calculated by summation of tritium content from all of the fractions collected, including the acid lysate.

Viral vector delivery to the nervous system

In order to examine virally encoded gene expression of pONY8.0Z pseudotyped with either VSV-G or rabies-G, viral preparations were stereotactically injected into the adult rat striatum as follows. Albino AO male adult rats were anaesthetized with Hypnorm and Hypnovel (11) and injected twice with 1 µl of viral stocks [titres were 6×10^8–3×10^9 T.U./ml based on transduction of the D17 dog osteosarcoma cell line and contained a similar number of viral particles to that determined by methods described previously (64,65)] into the striatum (n = 25), at coordinates Bregma 3.5 mm lateral, 4.75 mm vertical from dura, and 1 mm rostral, 3.5 mm lateral 4.75 mm vertical, using a fine drawn glass micropipette over a period of 2 min. The pipette was pulled up 1 mm and left for another 2 min before retracting slowly to the surface. For perinigral injections (n = 8), 2×1 µl of viral stocks were delivered at coordinates 4.7 mm caudal to Bregma, 2.2 mm lateral, 7 mm vertical from dura and 5.4 mm caudal, 2.2 mm lateral and 7.5 mm vertical. The pipette was pulled up 1 mm and left for another 2 min before retracting slowly to the surface. For intrahippocampal injections (n = 6), 3 µl of vector preparation were delivered in a slow extrusion manner (0.5 µl every 0.5 mm) at coordinates 3.5 mm caudal to Bregma, 2.5 mm lateral, starting at 3 mm and ending at 0.5 mm vertical from dura over 6 min with a subsequent waiting of 4 min before retracting the pipette to the surface.

Animals were analysed 1 or 4 weeks later and at subsequent monthly intervals for up to 8 months following striatal injections. Rats were perfused with 4% w/v PFA containing $MgCl_2$ (2 mM) and ethylene glycol bis (β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (5 mM). The brains were removed and placed in fixative overnight, cryoprotected in 30% w/v sucrose at 4°C for 2 days and frozen in Tissue-Tek OCT embedding compound (Sakura Finetek). Sections (50 µm) were cut on a CM3050 cryostat (Leica) and floated briefly in PBS-2 mM $MgCl_2$ at 4°C as a wash. Expression of β-gal was determined by placing the sections in 5-bromo-3-indolyl-β-D-galactosidase (X-gal) staining solution for 3–5 h at 37°C. Additionally, standard immunohistochemical techniques were applied in order to determine the cell types that were transduced after intrastriatal injections.

For intraspinal injection, anesthetized 2-month-old rats were placed in a stereotaxic frame and their spinal cords were immobilized using a spinal adaptor (Stoelting Co.). Following laminectomy, 1 µl of pONY8.0Z vector pseudotyped with rabies-G (n = 3) or VSV-G (n = 3) (6×10^8 T.U./ml) was injected at one site into the lumbar spinal cord. Injections, controlled by an infusion pump (World Precision Instruments Inc.), were at 0.1 µl per min through a 10 µl Hamilton syringe fitted with a 33 gauge needle. Following injection, the needle was left in place for 5 min before being retrieved. Two weeks following virus injection, rats received FG administration. The sciatic nerve was exposed at mid-thigh level and cut 5 mm proximal to the nerve trifurcation. A small cup containing a 4% w/v FG solution in saline was placed on the proximal segment of the transected nerve. Five days after application of FG the animals were perfused transcardially with 4% w/v PFA. The lumbar spinal cord was dissected out and analysed by immunohistochemistry and X-gal reaction. The number of FG and β-gal double-labelled motoneurons was counted 3 weeks after injection of the viral vector. In addition, brains from these animals were removed and 50 µm coronal sections were stained in X-gal solution as described above.

For intramuscular delivery, pONY8.0Z vectors were injected unilaterally in exposed gastrocnemius muscle with a microsyringe fitted with a 30-gauge needle (Hamilton). Two groups of rats were injected: the first group (n = 3) received pONY8.0Z pseudotyped with rabies-G and the second group (n = 3) received pONY8.0Z pseudotyped with VSV-G (titre of both types of vectors was 3×10^8 T.U./ml). Five sites per animal were injected with 10 µl per site. The solution was infused at a speed of ~1 µl/min. Two animals from each group were killed 3 weeks post-injection. The remaining two rats were anaesthetized by an intraperitoneal injection of Hypnorm/Hypnovel solution and FG administration was performed as described previously. Two days after application of FG the animals were killed. All animals were perfused transcardially with 4% w/v PFA. Subsequently, the muscles were excised and snap frozen in liquid nitrogen. Spinal cords were excised and cryoprotected in 30% w/v sucrose for 2 days. Transverse

and longitudinal sections (25 μm each) of both the muscle and spinal cords were analysed by immunohistochemistry and X-gal reaction. To evaluate the number of transduced neurons, motoneurons, lumbar and thoracic spinal cord were analysed. The number of β-gal-positive cells double-labelled with NeuN were examined in every third section. The proportion of infected motoneurons was expressed as the percentage of FG back-labelled cells expressing β-gal.

All cell counts were performed following immunolabelling of the reporter gene from sections 200 μm apart, according to the method of Abercrombie (68).

Immunohistochemistry

After cell and tissue preparation as described for each group of experiments, blocking was carried out in PBS containing 10% v/v normal goat or normal donkey serum as appropriate for the secondary antibodies. Primary antibodies were used as follows: anti-β-gal (1:5000, 1:100, 1:250 for primary cultures, brains and spinal cords, respectively, 5Prime3Prime Inc.), anti-TH (1:1000 DiaSorin for cultures, 1:50 Chemicon for sections), anti-NeuN (1:500 and 1:100 for cells and tissue, respectively, Chemicon), anti-GFAP (1:50 for tissue, Chemicon), anti-RIP (oligodendroglial marker, 1:1000, Chemicon) and anti-ED1 (microglial marker, 1:100 for tissue, Serotec). Antibodies were incubated overnight at 4°C for cells and 48 h for tissue in PBS-10% v/v serum and, for tissue, 0.5% v/v TritonX-100. Samples were washed three times with PBS and then incubated with secondary antibodies: Alexa 488-conjugated goat anti-rabbit IgG and Texas Red-X-conjugated goat anti-mouse IgG (both 1:100, Molecular Probes) or Texas Red-coupled donkey anti-mouse IgG and fluorescein isothiocyanate (FITC)-coupled donkey anti-rabbit IgG (1:100 and 1:50, respectively, Jackson Immunoresearch) or Alexa 488- conjugated anti-rabbit and a CY3-conjugated anti-mouse IgG (both 1:250 Molecular Probes and Jackson Immunoresearch) at room temperature for 2–3 h. After washing, samples were examined under a fluorescence microscope and analysed using a Leica TCS-SP confocal microscope.

Investigation of the immune response

Groups of rats received intrastriatal injections of pONY8.0Z vector pseudotyped either with VSV-G (n = 6) or rabies-G (n = 6) or an equivalent amount of PBS, using the stereotactic procedure described above. Following euthanasia at 7, 14 and 35 days post-injection brains were removed and snap frozen directly in OCT and analysed. Sections (15 μm) were cut onto APES (Sigma) coated slides using a Leica CM3500 cryostat. One in every 10 sections was stained with X-gal for 3 h at 37°C to identify areas of gene transfer and adjacent sections were selected and stained with monoclonal antibody tissue culture supernatant (TCS) against OX1 (leucocyte common antigen), OX18 (MHC class I), OX42 (complement receptor type 3 on microglia and macrophages) and OX62 (dendritic cells). These antibodies were a kind gift from the MRC Cellular Immunology Unit, Sir William Dunn School of Pathology, Oxford. Sections were incubated overnight in neat TCS and following several washes in PBS, incubated for 1 h with an HRP-conjugated rabbit anti-mouse antibody (Dako). Positive staining was then visualized to a brown colour using a diaminobenzidine (DAB) kit (Vector Laboratories). Sections were counterstained with haematoxylin, dehydrated, cleared and mounted using DePeX (BDH Merck). X-gal stained sections were counterstained using carminic acid (Sigma) and mounted using Permount (Fisher).

PCR

To detect viral DNA after injection of pONY8.0Z virus pseudotyped with VSV-G or rabies-G into rat striatum (n = 4) as described above, animals were killed 2 weeks post-transduction. Punches from striatum, thalamus and substantia nigra were quickly removed and frozen in liquid nitrogen. Genomic DNA was isolated from all samples using the Wizard Genomic DNA Purification kit (Promega, no. A1120). Thawed brain tissue (20 mg) was homogenized for 10 s using a disposable homogenizer in cooled nuclei lysis solution according to the manufacturer's protocol. PCRs were set to detect the *Escherichia coli* LacZ gene (GenBank accession no. V00296) expressed by injected vectors. Each reaction was set in 50 μl volume containing the following components (final concentration): 300 nM forward primer, 5′-CGT TGC TGC ATA AAC CGA CTA CAC-3′ (nucleotides 638–661); 300 nM reverse primer, 5′-TGC AGA GGA TGA TGC TCG TGA C-3′ (nucleotides 1088–1067), 200 μM dNTP (each), 2 mM $MgCl_2$, 1 × FastStart *Taq* DNA polymerase buffer and 2 U FastStart *Taq* DNA polymerase (Roche Diagnostics). 300 nanograms of template DNA was used per reaction. PCR amplification was carried out on a PCR Express (Hybaid, Hercules, USA) under the following thermal cycling conditions: initial denaturation and enzyme activation at 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 elongation at 72°C for 45 s and finally one cycle of extension at 72°C for 7 min. PCR products (10 μl/reaction) were resolved on 1.2% TBE agarose gel at 10 v/cm for 2 h.

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