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ORIGINAL ARTICLE

Efficient transduction of neurons using Ross River glycoprotein-pseudotyped lentiviral vectors

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Lentiviral vectors are promising tools for CNS gene transfer since they efficiently transduce the cells of the nervous system in vivo. *In this study, we have investigated the transduction efficiency of lentiviral vectors pseudotyped with Ross River virus glycoprotein (RRV-G) (RRV-G-pseudotyped lentiviral vectors (RRV-LV)). The RRV is an alphavirus with an extremely broad host range, including the cells of the central nervous system. Previous studies have shown that lentiviral vectors can be efficiently pseudotyped with this envelope protein and have demonstrated promising features of such vectors, including the possibility to establish stable producer cell lines. After injection of RRV-LV expressing green fluorescent protein into different structures in the rat brain we found efficient transduction of both neurons and glial cells. By using two cell-type-specific promoters, neuron-specific enolase and human glial fibrillary acidic protein, we demonstrated cell-specific transgene expression in the desired cell type. Ross River virus glycoprotein-pseudotyped lentiviral vectors also transduced human neural progenitor cells* in vitro, *showing that receptors for the RRV-G are present on human neural cells.*
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Introduction

Lentiviral vectors, such as those derived from the human immunodeficiency virus type I (HIV-I), are useful tools for CNS gene transfer since they allow transduction of both proliferating and postmitotic cells.[1] Previous studies have demonstrated efficient and long-term expression of both marker and functional transgenes in the rodent and primate brain.[1–3] In the majority of these studies, the lentiviral vectors have been pseudotyped with the glycoprotein of vesicular stomatitis virus (VSV-G). Pseudotyping with this envelope allows concentration of lentiviral vectors using ultracentrifugation and confers a broad host range. We and others have previously shown that VSV-G-pseudotyped lentiviral vectors transduce both neuronal and glial cells upon injection into the brain and that transgene expression can be directed to the appropriate cell-type depending on the promoter used.[1,4,5] However, the VSV-G envelope comes with a number of shortcomings that may limit its clinical use. In particular, the establishment of stable producer cell lines is problematic due to toxicity when VSV-G is constitutively expressed.[6–8]

In a number of recent reports, glycoproteins from alphaviruses, in particular Ross River virus (RRV), have been used to pseudotype retroviral and lentiviral vectors and has been shown to be a promising alternative to VSV-G.[9–13] Ross River virus glycoprotein (RRV-G) can efficiently pseudotype retroviral and lentiviral vectors and withstand concentration using ultracentrifugation, leading to viral vector preparations with comparable titers to VSV-G-pseudotyped vectors. These vectors are capable of transducing a wide variety of cell types *in vitro* and *in vivo*. Furthermore, RRV-G can be used to establish stable retroviral and lentiviral vector producer cell lines and the viral particles also appear to be more stable and less toxic than VSV-G-pseudotyped virions.[9–13]

The alphaviruses, including Ross River, Similiki Forest and Sindbis viruses, have an extremely broad host range, both in terms of species that they infect, as well as in regards to the different cell types the virus can replicate.[14] Alphaviruses are single-stranded RNA viruses with a core capsid surrounded by a host-derived lipid bilayer. In this lipid envelope, the glycoproteins are organized into 80 trimers. The glycoprotein of RRV is initially synthesized as a polyprotein (C-E3-E2-6K-E1), which is then proteolytically processed into three subunits (E1, E2 and E3). The E1 and E2 are the main components of the glycoprotein, where E2 is responsible for binding to host cell receptors, while E1 mediates fusion with the host cells. The cellular receptors for RRV-G are still unknown but a protein receptor or combinations of such have been proposed.[14] In *in vitro* experiments, using recombinant viral vectors it has been demonstrated that RRV-G and VSV-G use different receptors.[10]

The aim of the present study was to characterize the transduction efficiency of RRV-G-pseudotyped lentiviral

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vectors (RRV-LV) when injected into the rat brain. We found that injection of these vectors into different structures of the brain led to stable and efficient transduction of both glial and, in particular, neuronal cell types. These data suggest that RRV-LV can be useful tools for CNS gene delivery and gene therapy.

Results

We produced Ross River-pseudotyped lentiviral vectors using transient transfection of 293T cells according to the protocol established by Kahl *et al*.[11] Presence of RRV-G in lentiviral vector preparations was confirmed by performing Western blot analysis on concentrated viral vectors using a monoclonal antibody against the RRV E1 glycoprotein (kindly provided by Richard Kuhn, Purdue University). The blot revealed strong immunoreactivity of RRV-pseudotyped vectors with a number of positive bands in the range of 20–60 kDa (Figure 1b). A weak crossreactivity of the VSV-G-pseudotyped vectors was observed and revealed a band of a smaller size (data not shown). This band was not found in RRV-pseudotyped vectors.

The functional titer of Ross River-pseudotyped vectors, when titrated on 293T cells, appeared to be about 10-fold lower than the VSV-G vectors we usually produce in the laboratory (see Materials and methods). This is in line with what has been published previously in regards to RRV vectors and is probably, at least to some extent, due to reduced capacity of these vectors to transduce the target cells rather than an actual lower physical titer.[11] By performing fluorescence-activated cell sorter (FACS) analysis we confirmed that transduction of 293T cells was stable for at least 7 days with similar levels of green fluorescent protein (GFP)-expressing cells, thus ruling out pseudotransduction (data not shown).

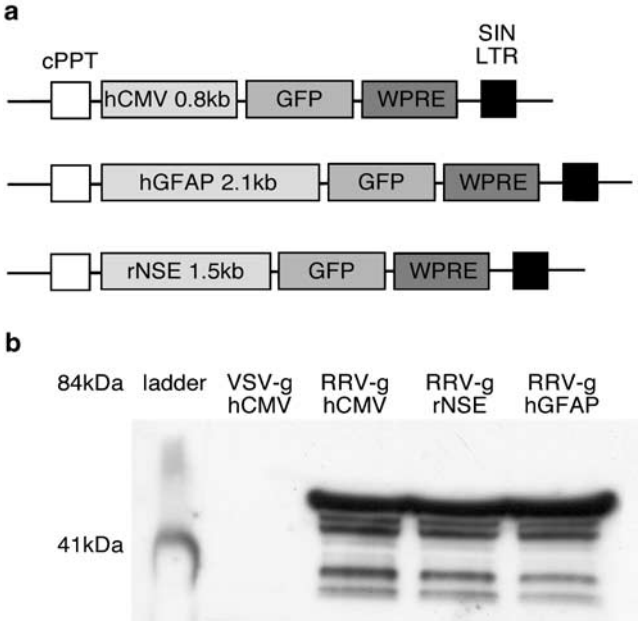

Figure 1 (**a**) Design of the lentiviral vectors used in this study. Transgene expression was driven by three promoters; a human cytomegalovirus promoter (hCMV), a rat neuron-specific enolase (rNSE) promoter and a human glial fibrillary acidic protein (hGFAP) promoter. (**b**) Western blot showing the presence of Ross River virus (RRV) glycoprotein in such vector batches.

Injection of Ross River virus-pseudotyped lentiviral vectors into the striatum

In order to accurately analyze the tropism of RRV-LV we pseudotyped three transfer constructs that were identical, with the exception for the promoters driving expression of GFP (Figure 1a). The promoters we used were; (1) the strong ubiquitous human cytomegalovirus promoter (hCMV); (2) a 1.5 kb neuron-specific promoter from the rat neuron-specific enolase gene (rNSE) and; (3) a 2.1 kb glial-specific promoter from the human glial fibrillary acidic protein gene (hGFAP).[15,16] We have previously shown that intrastriatal injections of VSV-G-pseudotyped lentiviral vectors with a hCMV-promoter confer transgene expression mainly to neurons, while VSV-LV with the rNSE and hGFAP promoters lead to highly cell-type-specific transduction.[4,5]

A total of 1 μl of the RRV-LVs was injected bilaterally into the striatum (STR) of adult rats and resulted in high levels of GFP expression in all animals ($n=9$). The hCMV promoter conferred GFP expression to cell types with either neuronal or glial phenotype. Co-labelling of GFP with antibodies for neuronal (NeuN) and glial markers (GFAP) revealed double-labelling with both antibodies (Figure 2a–f). However, the vast majority of the GFP-expressing cells in the STR displayed a morphology characteristic for adult neurons and co-labelled with NeuN in a manner that greatly resembled injections with VSV-LV (Figure 2d–f). When using the rNSE promoter, all GFP-expressing cells had a neuronal morphology and co-labelled with NeuN (Figure 2g–i), as well as with DARPP-32 (a marker for striatal projection neurons, data not shown). Conversely, GFAP promoter directed GFP expression exclusively to cells with a glial morphology. These cells were located in close proximity to the injection site and colocalized with the upregulated GFAP expression in the area where the injection needle had been inserted (Figure 2j–k). In addition, from the hCMV and rNSE vectors GFP-positive fibers were found in the globus pallidus and the substantia nigra (SN), demonstrating axonal transport of the transgene product (data not shown). The GFP expression was found throughout 2.1 ± 0.6 mm of the rostral–caudal axis covering the majority of the STR. There was no evidence for retrograde transport of the vectors as indicated by the lack of GFP-positive cell bodies in striatal output areas such as the SN and the globus pallidus (data not shown). There was no apparent upregulation of GFAP expression due to the injection of the vector, apart from where the injection needle had been inserted, suggesting that the RRV-G in itself does not cause an inflammatory response (Figure 2a–c). These results are similar to what we have found with VSV-G-pseudotyped vectors using these constructs and suggests that the receptors for RRV-G are present on both glial and neuronal cells in the rat brain.[5]

We quantified the number of GFP-expressing cells using unbiased stereology. We found 4320 ± 2470 GFP-expressing cells when using the hCMV promoter and 4133 ± 1970 (rNSE) and 450 ± 280 (hGFAP) cells when using the cell-specific promoters. Although these num-

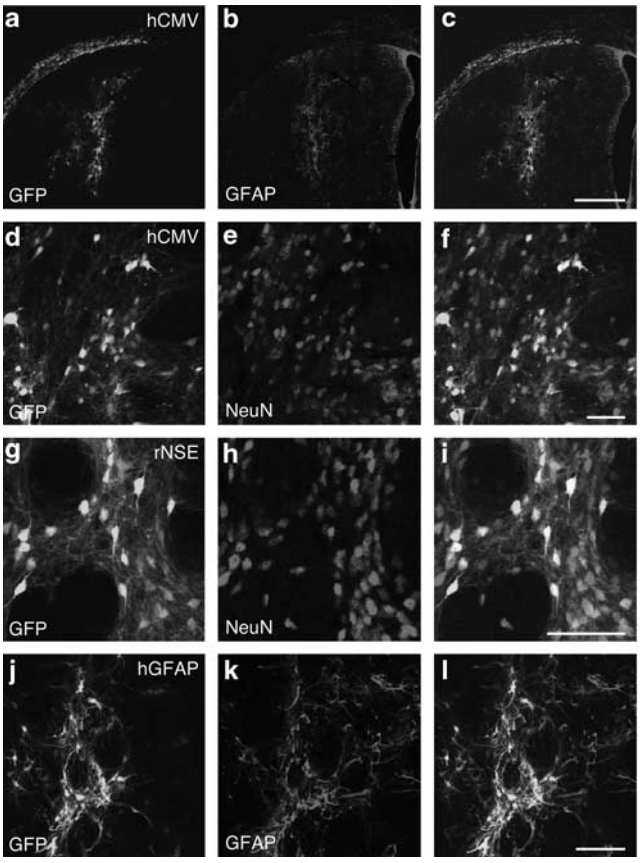

Figure 2 Transgene expression in rat brain after injection of Ross River virus glycoprotein (RRV-G)-pseudotyped lentiviral vectors (RRV-LV) into striatum (STR). (**a–f**) Green fluorescent protein (GFP) expression was found in both glial and neuronal cells when using an human cytomegalovirus promoter (hCMV) promoter. Note that upregulation of glial fibrillary acidic protein (GFAP) expression is only found just around the injection site suggesting that RRV-G does not cause an inflammatory response. (**g–i**) The neuron-specific enolase (NSE) promoter directed transgene expression specifically to neurons. (**j–l**) The human glial fibrillary acidic protein (hGFAP) promoter led to GFP expression in glial cells just around the injection area where the endogenous GFAP expression is upregulated as a consequence of the injection. Scale: (**a–c**): 800 μm and (**d–l**): 80 μm.

bers are about twofold lower to what we have previously reported when using VSV-LVs, these data still demonstrate that RRV-LVs transduce neural cells at a similar efficiency when similar vector production methods are used.

Injection of Ross River virus-pseudotyped lentiviral vectors into the substantia nigra

Previous studies have shown that transduction of the dopaminergic neurons of the SN is inefficient when using VSV-LVs.[17,18] In particular, the hCMV promoter has been reported to drive transgene expression poorly in this cell population. It is possible to transduce at least a portion of these cells using a different promoter, such as the PGK promoter, suggesting that a combination of poor vector uptake and low promoter activity is responsible for the low lentiviral transduction.[19,20] Injection of RRV-LV into the SN resulted in poor GFP expression in this structure (Figure 3a–c). Similar to the

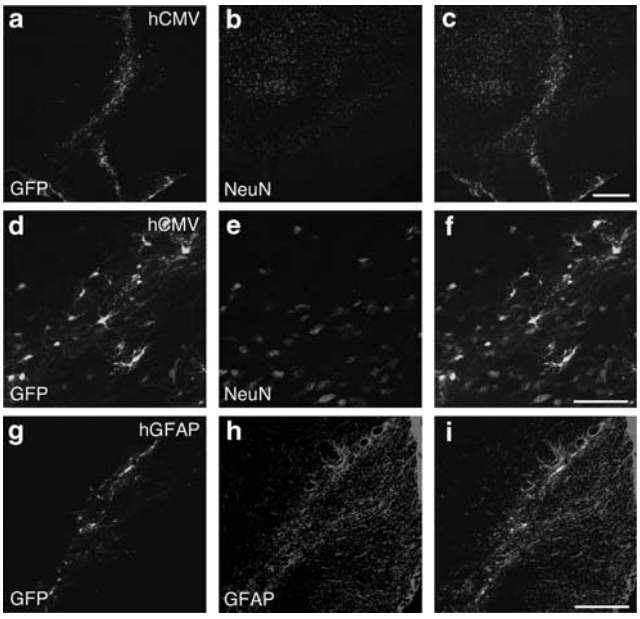

Figure 3 Transgene expression in rat midbrain after injection of Ross River virus glycoprotein-pseudotyped lentiviral vectors (RRV-LV). (**a–f**) The human cytomegalovirus promoter (hCMV) promoter mainly directed transgene expression to cells with a glial morphology in the substantia nigra (SN). (**g–i**) As expected the human glial fibrillary acidic protein (hGFAP) promoter led to green fluorescent protein (GFP) expression in GFAP-positive cells in the SN. Scale: (**a–c**): 40 μm, (**d–f**): 80 μm and (**g–i**): 200 μm.

VSV-LV, the hCMV-containing RRV-LV conferred expression to cells with mainly a glial morphology (Figure 3d–f). The hGFAP construct directed GFP expression to glial cells within the SN (Figure 3g–i). The rNSE construct did lead to GFP expression in cells with a neuronal morphology in the SN, but only at very low numbers (data not shown).

Injection of Ross River virus-pseudotyped lentiviral vectors into the hippocampus

We also injected the RRV vectors into the hippocampus (HPC). The results of these injections were similar to what we found after injection into the STR. Following injection, the hCMV promoter conferred expression to both glial and neuronal cells, but with an apparent preference for neurons (Figure 4a–c). As in the case of the STR, the rNSE and the hGFAP-vector conferred GFP expression to the appropriate cell type (Figure 4d–l). Green fluorescent protein expression was found throughout the rostral–caudal axis, covering a large part of the HPC.

Injection of Ross River virus-pseudotyped lentiviral vectors into the subventricular zone

Recent reports suggest that lentiviral vectors pseudotyped with VSV-G or lymphocytic choriomeningitis virus glycoprotein injected into the subventricular zone (SVZ) are able to target the adult neuronal stem cells present in this region.[21,22] Hence, we injected RRV-LV (hCMV) into the SVZ of adult rats and killed the animals at 2 and 8 weeks after injection. Green fluorescent protein-expressing cells were detected in the subventricular space at the site of injection. These cells had a morphology indicating

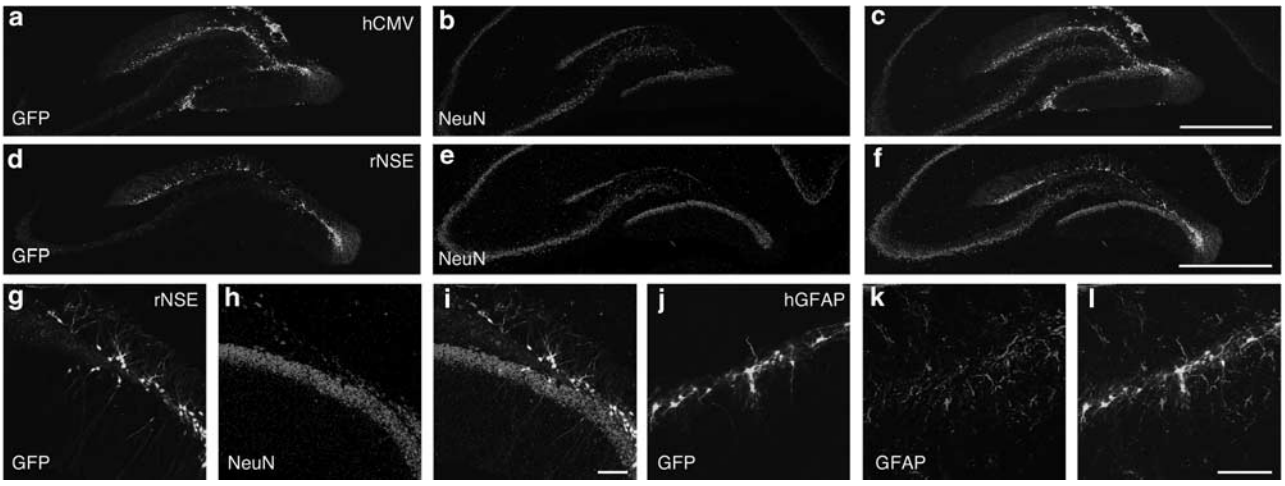

Figure 4 Transgene expression in rat hippocampus (HPC) after injection of Ross River virus glycoprotein-pseudotyped lentiviral vectors (RRV-LV). The use of the human cytomegalovirus promoter (hCMV) promoter (**a–c**) and neuron-specific enolase (NSE) promoter (**d–i**) led to widespread GFP expression mainly in neuronal cells. (**j–l**) The human glial fibrillary acidic protein (hGFAP) promoter-directed transgene expression to a glial cell population in the HPC. Scale: (**a–f**): 800 μm and (**g–l**): 80 μm.

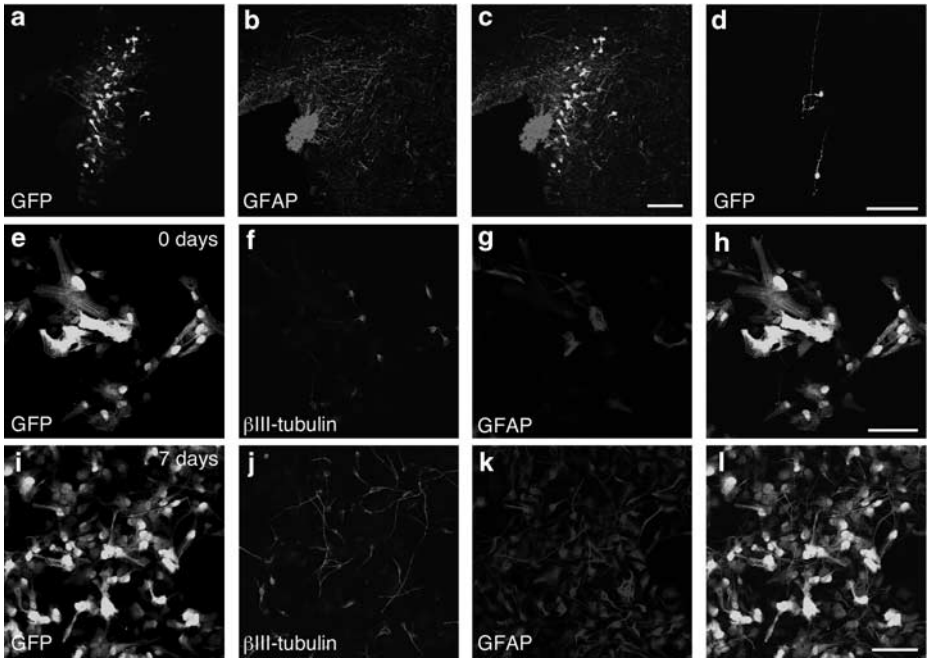

Figure 5 (**a–c**) At 2 weeks after injection of the human cytomegalovirus promoter (hCMV) (RRV-LV) into the subventricular zone (SVZ), neuronal progenitors expressing green fluorescent protein (GFP) expression but not glial fibrillary acidic protein (GFAP) was found. (**d**) At 8 weeks after injection GFP-expressing neurons could be found in the olfactory bulb (OB). (**e–l**) GFP expression in human neural progenitor cell transduced at an multiplicity of infection (MOI) of 1 either at the start of differentiation (**e–h**) or after 7 days of differentiation (**i–l**). Approximately 80% of the cells expressed GFP. Most GFP-expressing cells co-labelled with GFAP at both time points of transduction. The cells were fixed 7 days post-transduction. Scale: (**a–d**): 80 μm and (**e–l**): 40 μm.

of a glial or progenitor phenotype, but did not colocalize with GFAP, which in the SVZ is a marker for the neuronal stem cells (Figure 5a–c).[23] At this time point we could also detect a sparse number of GFP-expressing cells in the olfactory bulb (OB) and a fraction of these cells colocalized with the neuronal marker NeuN. At 8 weeks after injection, no GFP expressing cells could be detected in the SVZ. There were however, GFP expressing cells in the OB in these animals. Many of these cells had a neuronal morphology, extended processes and colocalized with NeuN (Figure 5d). These results suggest that the RRV envelope targets the neuronal progenitor cells, but not the adult neuronal stem cells in the SVZ.

Transduction of human neuronal progenitor cells using Ross River virus-lentiviral vectors

In order to confirm that the receptors for RRV-G are present on human neural cells we transduced human neural progenitor cells *in vitro*. These cells proliferate and

can be expanded when grown as neurospheres in the presence of growth factors.[24] However, if these cells are dissociated and grown in a non-conditioned media they attach and differentiate into neurons, astrocytes and oligodendrocytes.[24] We transduced the progenitor cells with RRV-LV (hCMV) at an multiplicity of infection (MOI) of 1 at the day of dissociation or after 7 days of differentiation. This period allows the cells to differentiate into phenotypes expressing neuronal and glial markers. Transduction at either time point revealed GFP expression in the majority of the cells (>80%), when using the hCMV promoter. The GFP-expressing cells mostly colocalized with the glial marker GFAP. The proportion of cells that differentiated into neuronal profiles (judged by βIII-tubulin staining, ~10%) did rarely express GFP and if so only at low levels (Figure 5e–l). The pattern and level of transduction was similar if cells were transduced either at the day of dissociation or after 7 days of differentiation, suggesting that differentiation of neuronal cells into more mature phenotypes did not down or upregulate the abundance of the receptors for RRV-G.

Discussion

In the present study, we have demonstrated that RRV-LV efficiently transduce both neurons and glial cells in the rat brain. Injections into the STR or HPC led to efficient transduction of neurons when using either an hCMV promoter or a neuron-specific rNSE promoter. The hCMV promoter as well as a glial-specific hGFAP promoter also conferred GFP expression in glial cell types. The cellular receptor for entry by RRV is currently unknown. However, our results demonstrate that these receptors are present on both neurons and glial cells in the rat brain, which is consistent with the notion that wild-type RRVs infect such cell types.[14] However, in a study by Kang *et al*.,[12] RRV pseudotyped feline immunodeficiency virus (FIV) lentivectors injected into the mouse brain mainly transduced glial cells. This is in contrast to the results obtained in our study and may be explained by differences in vector type (FIV vs HIV-I) and species (mouse vs rat). These factors may affect vector entry into the target cell, as well as the activity of the hCMV promoter.

A number of studies report the use of alternative envelopes to VSV-G for lentiviral-mediated gene transfer to the brain. These envelopes include murine leukemia virus, mokola virus, lymphocytic choriomeningitis virus and various strains of rabies virus among others.[25,26] These studies describe clear differences when using various envelopes, both in regards to transduction pattern and axonal transport. However, the majority of these studies rely on the use of a single transfer construct where a strong ubiquitous promoter, such as the hCMV promoter, drives transgene expression. This may complicate the interpretation of such results, since the activity of the so-called ubiquitous promoters varies in different cell types in the brain. For instance, the hCMV promoter is capable of driving high transgene expression in striatal projection neurons, but expresses the same transgene poorly in the resting glial cells of the STR as well as in the dopaminergic neurons of the SN, despite the fact that the vector clearly enters these cells since other promoters confer lentiviral-mediated transgene expression in these cell populations.[18–20] Hence, we used three different promoters to analyze the tropism of RRV vectors and our results clearly demonstrate that RRV vectors are capable of transducing both glial and neuronal cells in the rat brain, probably at a similar rate. The fact that we find a lower number of GFP-expressing cells in the STR when using the hGFAP promoter is probably due to the low endogenous levels of GFAP in the non-injured STR, thereby only allowing transgene expression in close proximity to the injection site where GFAP is upregulated as a consequence of the injury.

The cellular receptors that bind RRV-G are not identified but it is clear that it is not the same receptor as is utilized by VSV-G.[10] The quite widespread diffusion of the vector after injection into STR or HPC (but not SVZ) suggests that there is a redundancy of receptors on these cells in comparison to the receptors for VSV-G. This may be particularly useful for CNS gene transfer, since injection into the brain parenchyma with VSV-G-seudotyped vector results in an injection core, containing cells with multiple integrations leading to a limited spread of the vector.

When the RRV-LV was injected into the SVZ, GFP-expressing cells were detected in the area of injection after 1 week. However, at 2 and 8 weeks these cells were no longer present, although there were GFP-expressing cells in the OB of these animals. These results are consistent with a preferential transduction of neural progenitor cells in the SVZ, rather than transduction of the adult neuronal stem cells, which have been reported to be targeted by both VSV-G- and LCMV-pseudotyped vectors.[21,22] However, our study was performed in rats, while the above-mentioned studies were done in mice. Furthermore, these studies relied on vectors with very high titer (10^{10} TU/ml). We are currently investigating whether it is possible to target rat SVZ stem cells with high titer VSV-LV in order to see whether there is a species difference between mouse and rats regarding this issue.

Our results also demonstrate the presence of receptors for RRV-G on human neural cells. The fact that the majority of the progenitor cells that were transduced were of glial origin is intriguing. Most likely this is due to low activity of the hCMV promoter in the cultured neurons rather than lack of RRV-G-receptors on these cells, since similar results were found when immature non-differentiated cells or cells that had been differentiated for 7 days were transduced. Nevertheless, these results confirm data in a number of human cell lines where RRV-LV-mediated transduction is efficient and show that the receptors for RRV-G is present on human neural cells.

Ross River virus glycoprotein-pseudotyped lentiviral vectors possess many characteristics that make them an attractive alternative to other psuedotypes, including VSV-G. These features include heat resistance, reduced toxicity, protection against inactivation in human serum and, as recently described by Strang *et al*.,[9] the possibility to establish producer cell lines capable of producing high-titer RRV-LV. These features are all relevant to the development of clinical protocols utilizing lentiviral vectors. Although the titer achieved for RRV-LV is lower than what can be obtained with VSV-LV (this study and Kahl *et al*.[10]), the transduction level in the brain is still

at such a high level that it encourages further investigations. It is likely that by optimizing production parameters, as has been carried out for VSV-LV, it will be possible to obtain RRV-LV with higher titer.[27]

In conclusion, our results demonstrate that the RRV-G is a good candidate to serve as an alternative envelope to VSV-G when using lentiviral vectors for CNS gene therapy. We demonstrate efficient gene delivery to rat and human neuronal and glial cells using relatively low titer batches. Development of protocols enabling production of high titer RRV-LV utilizing stable producer cell lines will lead to lentiviral vectors for CNS gene therapy that are acceptable for clinical use.

Materials and methods

Vectors

The transfer vectors pHR′-cPPT.hCMV.W.SIN, pHR′-cPPT.rNSE.W.SIN and pHR′-cPPT.hGFAP.W.SIN (Figure 1a) have been described elsewhere.[5] Ross River-pseudotyped lentiviral vectors (RRV-LV) were produced as previously reported by Kahl *et al*.[11] using transient transfection of 293T cells. About 2×10^6 cells were seeded into Petri dishes (10 cm in diameter) and co-transfected with transfer plasmid (20 μg), helper plasmid pBR8.91 (15 μg) and envelope plasmid pRRV-E2E1A encoding Ross River glycoprotein (7.5 μg). The pRRV-E2E1A vector encodes the full-length RRV envelope glycoprotein, E3-E2-6K-E1, which is then processed proteolytically into individual subunits. The supernatant was collected on days 2 and 3 after transfection and concentrated by two rounds of ultracentrifugation at 141 000 *g* twice for 1.5 h. Glycoprotein of vesicular stomatitis virus-G-pseudotyped lentiviral vectors (VSV-LV) were produced using a similar protocol replacing pRRV-E2E1A with the pMD.G plasmid (5 μg) encoding VSV-G.[28]

Determination of vector titers

To determine the functional titer, 100 000 293T cells were seeded in six-well dishes and 16 h later, a serial dilution of the viral vector was added to the culture media. After 48 h, the GFP-positive cells in each well were counted in relation to GFP-negative cells and the percentage of GFP-positive cells was calculated. The dilution that resulted in approximately 10% GFP-positive cells was used for calculation of functional titer. In addition, cells were kept in culture for 5 more days and then analyzed by FACS in order to confirm stable transgene expression and rule out pseudotransduction.

To determine DNA titer, 50 000 cells (293T) were seeded in six-well dishes and cells were transduced with a serial dilution of the viral vector 16 h later. At 4 days post-transduction, the cells were trypsinized and collected. The cells were then lysated in 18 μl 50 mM Tris-HCl, pH 8.2 (Amresco Inc., Solon, OH, USA), 100 mM NaCl (Merck, Darmstadt, Germany), 5 mM EDTA (Chemicon, Hampshire, UK), and 0.5% SDS (Chemicon). After addition of 2 μl Proteinase K (10 mg/ml, Invitrogen, Paisley, UK), samples were incubated for 30 min at 55°C. Water (180 μl) was then added and samples were incubated at 100°C for 10 min to inactivate proteinase K. Taqman polymerase chain reaction was carried out on these samples using one set of primers for lentiviral vector sequence, as well as one primer set for a human reference gene (Albumin). Proviral load was estimated using C_t quantification as described in Userbulletin#2, http://www.appliedbiosystems.com and using a VSV-G control lentiviral vector with a known functional titer as a relative standard. The titer of the RRV-LV used in this study was estimated to: hCMV: 7.6×10^7; rNSE 1.4×10^7; hGFAP 1.8×10^7. These titers are about 10-fold lower when compared to the VSV-G-pseudotyped lentiviral vectors we produce in the lab using a similar protocol.

Surgical procedures

Young adult female Spruage–Dawley rats were housed 2–3 per cage with free access to food and water under 12 h light:dark cycle. All animal procedures were approved and carried out according to the guidelines of the Ethical Committee for Use of Laboratory Animals at Lund University. The animals were anesthetized with isofluorane (2% in air) and placed into a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). The skull was exposed and holes drilled. A total volume of 1 μl of viral stock was injected into the brain at different sites using a 5 μl Hamilton syringe. The viral vectors were injected at a rate of 0.5 μl/min and leaving the syringe in place for at least 3 min after the injection. For injections into the SVZ, the HPC or the SN a thin glass capillary was attached to the syringe. The following stereotaxic coordinates were used according to the atlas of Paxinos and Watson;[29] STR, anterior-posterior (AP): +1.2 mm, medial-lateral (ML): $\pm$2.7 and dorsoventral (DL): −4.5 (Paxinos) with the tooth bar set at 0 mm; SN, AP: −5.3, ML: −2.0, DV: −7.2, TB: −2.3; HPC, AP: −3,6, ML: −2.0, DV: −3.0, −2.2, TB: −3.3; SVZ, AP: +0.7, ML: −1.5, DV: −3.0, TB: −3.3.

Immunohistochemistry

At 2, 3 and 8 weeks after viral vector injection the rats were deeply anesthetized with pentobarbital and perfused through the ascending aorta with isotonic saline followed by 250 ml of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed and postfixed for a few hours in the same solution and then transferred to 25% sucrose before being sectioned on a freezing-stage microtome at 40 μm. Light-field and fluorescent immunohistochemical staining was performed as previously described.[5] The following antibodies and dilutions were used: anti-GFP (Chemicon, AB16901, 1:5000), anti-DARPP32 (Chemcion, AB1656, 1:1000), anti-GFAP (Dako, Z0334, 1:1000) and anti-NeuN (Chemicon MAB377, 1:100).

Culturing human neural progenitor cells

Embryonic-derived (9 weeks embryo) human neural progenitor cells were grown as neurospheres and differentiated as previously described.[24] Cells were transduced for 24 h at a MOI of 1. Transduction was performed either at the start of differentiation or after 7 days of differentiation. At 7 days post-transduction, the cells were fixed using 4% PFA and stained using immunohistochemical methods as previously described.[30] The following antibodies were used: anti-GFAP (Dako, Z0334, 1:1000) and anti βIII-tubulin (Sigma. T8660, 1:250).

Western blot

Immunoblotting was performed using standard procedures. In brief, 1 or 5 μl concentrated viral vector was run on a SDS-PAGE gel and blotted on a PVDF membrane. The membrane were blocked in 5% fat-free milk and subsequently incubated with a rabbit polyclonal anti-RRV E1 antibody (1:20 000 dilution, gift from Richard Kuhn, Purdue University). After three rinses, the membrane was incubated with a secondary antibody conjugated to horseradish peroxidase (donkey-anti-rabbit IgG, Amersham Pharmacia Biotech, Uppsala, Sweden). The signal was detected with ECL plus detection reagents (Amersham Pharmacia Biotech, Uppsala, Sweden). Ponceau red staining was performed in order to confirm similar load of protein.

Stereological analysis

To quantify the number of transduced cells, the sections were analyzed throughout the whole transduced area. Unbiased stereological quantification was carried out using the CAST-Grid system (Olympus, Denmark) according to West *et al*.[31]

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