

Differences in transductional tropism of adenoviral and lentiviral vectors in the rat brainstem

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Adenoviral vectors (AVVs) and lentiviral vectors (LVVs) are highly useful research tools which can be used to investigate the function of specific cell phenotypes in the brain. The transductional tropism of viral vectors has a critical impact upon the transgene expression in different brain areas. This largely depends on the properties of the viral particles, which for AVVs are most commonly analogous to the serotype 5 adenovirus and, in the case of LVVs, are determined by the envelope used for pseudotyping, for example the vesicular stomatitis virus coat (VSVG). We have created a matching set of shuttle plasmids that allow a one-step transfer of an entire expression cassette between the backbones of AVVs and LVVs. This has permitted a fair assessment of the impact of the vector type on tropism for both AVVs and LVVs. Thus, the aims of this study were twofold: (i) to develop and demonstrate the validity of a transgene 'swap' system between AVVs and LVVs; and (ii) using this system, to assess the tropism of AVVs and LVVs for neuronal *versus* glial cell types. We have constructed AVVs and VSVG-coated LVVs to express monomeric red fluorescent protein (mRFP) driven by the human cytomegalovirus promoter (hCMV). Transgene expression in neurones and glia in the hypoglossal and dorsal vagal motor nuclei of the rat brainstem was compared by determining the colocalization with immunostaining for the neuronal marker NeuN (neuronal nuclear antigen) and the glial marker GFAP (glial fibrillary acidic protein). We found that 55% of mRFP-expressing cells transduced with AVVs were immunopositive for GFAP, while only 38% were NeuN-immunoreactive. In contrast, when the same expression cassette was delivered by VSVG-coated LVVs, the neurone/glia ratio of mRFP expression was reversed with 56% of mRFP-positive cells identified as neurones and 26% as glia. Thus, the present study provides compelling evidence that VSVG-coated LVVs significantly shift transgene expression towards neurones while transduction with AVVs favours glia.

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Viral gene transfer in the central nervous system is emerging as a powerful neurophysiological research tool. The outcome of a viral gene delivery experiment will depend on the ability of a vector to express sufficient quantities of the transgenic protein in the target cells. Two major rate-limiting steps determine this ability: (i) the efficacy of viral entry into the target cell (transductional tropism); and (ii) the activity of the promoter used for transgene expression (transcriptional tropism). Viral vectors differ vastly in their transductional and transcriptional properties. Therefore, their ability to express transgenes in a given cell type, for example

neurones or glial cells, must be scrutinized prior to their use in a particular brain area.

A range of viral vector types has been tested as gene delivery vehicles for brain cells. These include alpha viruses, adeno-associated viruses, adenoviral vectors (AVVs), and lentiviral vectors (LVVs) (Ehrenguber, 2002; Büning *et al.* 2003; Coleman *et al.* 2003; Kügler *et al.* 2003; Teschemacher *et al.* 2005). Of those, AVVs and LVVs have been used most extensively to date (Davidson & Bohn, 1997; Ehrenguber *et al.* 1998, 2001; Bensadoun *et al.* 2000; Kasparov & Paton, 2000; Hirooka *et al.* 2001; Paton *et al.* 2001, 2004; Kasparov *et al.* 2002; Coleman

et al. 2003; Waki *et al.* 2003; Teschemacher *et al.* 2004; Wong *et al.* 2004). While it is known that both AVVs and LVVs can introduce transgenes into a range of neurones in the brain, it is still not clear whether these two types of vectors are equally effective and what the proportion of neuronal to glial transduction is across brain areas. As research tools, AVVs and LVVs offer distinct but complementary advantages (see Teschemacher *et al.* 2004, this issue). In brief, AVVs can be amplified rapidly with high yields. They are very convenient tools for *in vitro* experiments and can also be used *in vivo*. On the other hand, their initial construction is comparatively slow. LVVs, in contrast, can be quickly generated but with relatively lower yields. They are non-immunogenic and allow long-term expression without adverse tissue reactions *in vivo*. In actuality it would be most helpful to be able to exchange transgene expression cassettes between AVVs and LVVs; this was a major consideration of the present study.

Because of the growing interest in viral gene transfer, we compared the transductional tropism of the vector types used in our laboratory: AVVs originate from serotype 5 human adenoviruses with deletions in *E1* and *E3* early genes (Bett *et al.* 1994; Graham & Prevec, 1995); LVVs are derived from HIV-1 pseudotyped with a vesicular stomatitis virus G glycoprotein (VSVG) coat (Coleman *et al.* 2003). In particular we wanted to test the hypothesis that placement of the same expression cassette into the adenoviral vector (AVV) or lentiviral vector (LVV) backbone may change its tropism for neurones and glia. For such a comparison it was essential to select areas of the brain where a chosen promoter has a documented high activity in both neurones and glial cells. We decided to use the human cytomegalovirus (hCMV) 760 bp promoter (Wilkinson & Akrigg, 1992) as one of the most frequently used promoters for gene expression in viral vectors. We chose to test our constructs in brainstem areas that contain cholinergic motor neurones (nucleus of the hypoglossal nerve: XIIMN) and vagal preganglionic neurones (dorsal vagal motor nucleus: DVM). Consistent with findings of other groups (Irnaten *et al.* 2002; Mohan *et al.* 2002), our preliminary experiments demonstrated that hCMV is highly active in both these motor neuronal populations.

For a fair assessment of the impact of the vector type on tropism it was essential to use the same sequence construct in both AVVs and LVVs backbone because even small sequence modifications due to different cloning strategies might have an unpredictable impact on the activity of a transgene. To this end, we have redesigned the respective shuttle plasmids to facilitate the transfer of an entire expression cassette between AVVs and LVVs backbones. This strategy allowed us to draw direct comparisons of neuronal and glial transgene expression induced by the two different vector systems.

Methods

Viral vector construction

pXCX-CMV-mRFP was created by insertion of the MluI-BamHI fragment (containing the hCMV promoter) of pXCXCMV (Geddes *et al.* 1996) into pXCX-Sw-linker (see Results). The gene encoding for monomeric red fluorescent protein (mRFP) (Campbell *et al.* 2002) was extracted by polymerase chain reaction from a template kindly donated by Professor R.Y. Tsien (La Jolla, CA, USA) with included restriction sites for NheI in the forward and BsrGI in the reverse primer. The mRFP sequence was inserted between SpeI and BsrGI sites. To compare expression of identical transgene cassettes delivered by AVVs and LVVs, the hCMV-mRFP expression cassette was excised by I-SceI digestion and ligated into I-SceI-digested pTYF-Sw-linker to generate pTYF-CMV-mRFP (see Results).

AVV-CMV-mRFP was constructed by homologous recombination of the pXCX-CMV-mRFP shuttle vector and the helper plasmid pBHG10 in HEK293 cells, followed by clone isolation, amplification and CsCl gradient concentration (Bett *et al.* 1994; Graham & Prevec, 1995). AVVs stocks were titred by an immunoreactivity 'spot' assay similar to previously published protocols (Bewig & Schmidt, 2000). Briefly, HEK293 cells were fixed 48 h after infection with serial AVVs dilutions, incubated with primary antibody (goat Anti-Hexon 1:1000 in PBS +1% bovine serum albumin; Biodesign, USA), followed by exposure to a secondary anti-goat horseradish peroxidase-conjugated antibody (Zymed, CA, USA) and diaminobenzidine reaction (Sigma). Hexon-positive cells were counted and titres were calculated in transducing units (TU) per ml.

The LVVs system used is an HIV-1-derived system, pseudotyped with VSVG envelope, as described earlier in detail (Coleman *et al.* 2003). LVV-CMV-mRFP was produced by cotransfection of HEK293FT cells (Invitrogen R700-07) with the shuttle vector pTYF-CMV-mRFP, the packaging vector pNHP and the envelope plasmid pHEF-VSVG, followed by concentration of LVV-containing media by centrifugation (Coleman *et al.* 2003).

For establishing LVVs titres, 10% of HEK293FT cells were cotransfected using a shuttle plasmid for expression of human placental alkaline phosphatase (PLAP). Harvested media were pooled with and processed under identical conditions as LVV-CMV-mRFP. Titres for LVV-PLAP were determined using a previously published protocol (Fekete & Cepko, 1993) and extrapolated to LVV-CMV-mRFP titres (in TU ml⁻¹).

In vivo gene transfer in the brainstem

Male Wistar rats (225–250 g) were used. All procedures were carried out according to the Home Office

animals Scientific Procedures Act 1986. Animals were anaesthetized with an intramuscular injection of ketamine (60 mg kg^{-1}) and medetomidine ($250 \mu\text{g kg}^{-1}$). They were placed in a stereotaxic head holder and the caudal dorsal medulla was exposed through a midline incision in the dorsal neck. A total of six microinjections of viral vector were made bilaterally at the level of the calamus scriptorius and $250 \mu\text{m}$ rostral and caudal to it, $300\text{--}500 \mu\text{m}$ from the midline and $400\text{--}500 \mu\text{m}$ ventral to the dorsal surface of the medulla. AVVs-CMV-mRFP ($2.9 \times 10^9 \text{ TU ml}^{-1}$; $n = 3$) or LVV-CMV-mRFP ($4 \times 10^9 \text{ TU ml}^{-1}$; $n = 3$) was injected (330 nl per site) at 500 nl min^{-1} . This protocol ensured delivery of viral vectors to DVM and XIIMN. The wound was sutured, cleaned and treated with antiseptic powder. Medetomidine anaesthesia was reversed with a subcutaneous injection of atipamezole (1 mg kg^{-1}). Animals were returned to individual cages for recovery, and kept with normal rat chow and drinking water *ad libitum* on a 12 h light–12 h dark cycle.

Immunohistochemistry

Seven days after surgery, rats were terminally anaesthetized (sodium pentobarbital, 100 mg kg^{-1} , i.m.) and perfused intracardially with 0.9% saline and 4% formaldehyde in 0.1 M phosphate buffer, at pH 7.4. Brainstems were removed, postfixed overnight and then placed in 30% sucrose for 24 h. Serial $40\text{-}\mu\text{m}$ sections were cut on a freezing microtome and then alternating sections were processed for neuronal specific marker (NeuN, mouse anti-NeuN, 1:500, Chemicon) or glial fibrillary acidic protein (GFAP, mouse anti-GFAP, 1:400, Chemicon) immunoreactivity (IR), respectively. Sections were washed for 30 min with 50% ethanol, followed by $3 \times 15 \text{ min}$ washes in Tris-phosphate-buffered saline (TPBS). Sections were then incubated in primary antibody and 10% normal horse serum (NHS) in TPBS containing 0.3% Triton X-100, for 72 h at 4°C . This was followed by overnight incubation in biotinylated donkey anti-mouse F(ab)2 fragments (1:500, Jackson Immunolabs, PA, USA) and 5% NHS in TPBS, then ExtrAvidin-fluorescein isothiocyanate (FITC) in TPBS (1:1000, Sigma). Washes were performed between incubations (TPBS, $3 \times 15 \text{ min}$), at room temperature. Sections were then mounted using Vectashield® (Vector labs, CA, USA).

Fluorescence imaging and assessment of colocalization

Confocal images were obtained using a Leica SP spectral confocal microscope. Scans were made at 2- to $3\text{-}\mu\text{m}$ intervals through the upper layers of the section (which contained a high density of immunolabelled elements. The two channels were scanned sequentially: for mRFP

(excitation, 543 nm; emission, 607 nm) and for FITC (excitation, 488 nm; emission, 500–530 nm). In order to compare viral transduction of AVVs and LVVs, five to seven sections containing a high density of mRFP-expressing cells at different rostro-caudal levels were evaluated. In some sections more than one field of view was evaluated. The total number of mRFP-positive cells visible per field of view was counted and cells showing colocalization with NeuN or GFAP IR were determined using colour overlay on individual confocal images or their projections. Results were represented by average percentage of colocalization with marker $\pm \text{S.E.M.}$

Results

One-step transfer of expression cassettes between AVVs and LVVs backbones

The shuttle vectors for AVVs and LVVs were redesigned to include two restriction sites for I-SceI (New England Biolabs UK Ltd) flanking multiple cloning sites. I-SceI cuts within an 18-bp recognition sequence, leaving a non-palindromic-3'-overhang. This allows for unidirectional insertion of transgene cassettes. Following I-SceI digestion, we reliably obtained recombinant clones with molecular ratios of insert to (non-de-phosphorylated) vector of 5:1 using a rapid ligation kit (Roche). For generation of pXCX-Sw-linker, two polylinkers (oligonucleotides from Eurogentec, Belgium) containing I-SceI-BglII-NheI-MluI-HinDIII and XbaI-BclI-Sall-I-SceI restriction sites were inserted between MluI and HindIII sites and XbaI and BclI sites of pXCXCMV, respectively (Fig. 1). To produce pTYF-Sw-linker (Fig. 1), polylinkers with I-SceI-EcoRV and SpeI-MluI-XhoI-NotI-ClaI-I-SceI-KpnI sites were inserted into the NotI site and between SpeI and KpnI sites of the pTYF-linker, respectively (Coleman *et al.* 2003). This system was used to generate AVV-CMV-mRFP and LVV-CMV-mRFP bearing identical expression cassettes.

Comparison of transductional tropism of AVVs and LVVs

We found that $55 \pm 3\%$ of mRFP-expressing cells transduced with AVVs were immunopositive for the glial marker GFAP (Figs 2 and 3A) while in a minority of $38 \pm 5\%$ of cells mRFP could be colocalized with the neuronal marker NeuN-IR (Figs 2 and 3B).

In contrast, when delivered by LVVs, the neurone/glia ratio of mRFP expression was reversed with $56 \pm 6\%$ of red fluorescent cells identified as neurones (Figs 2 and 3D) and a minority ($26 \pm 9\%$) as glial (Figs 2 and 3C). Thus, LVVs significantly shifted transgene expression towards neurones while AVVs transduction favoured glial expression ($P < 0.05$; one-tailed Student's *t* test). These

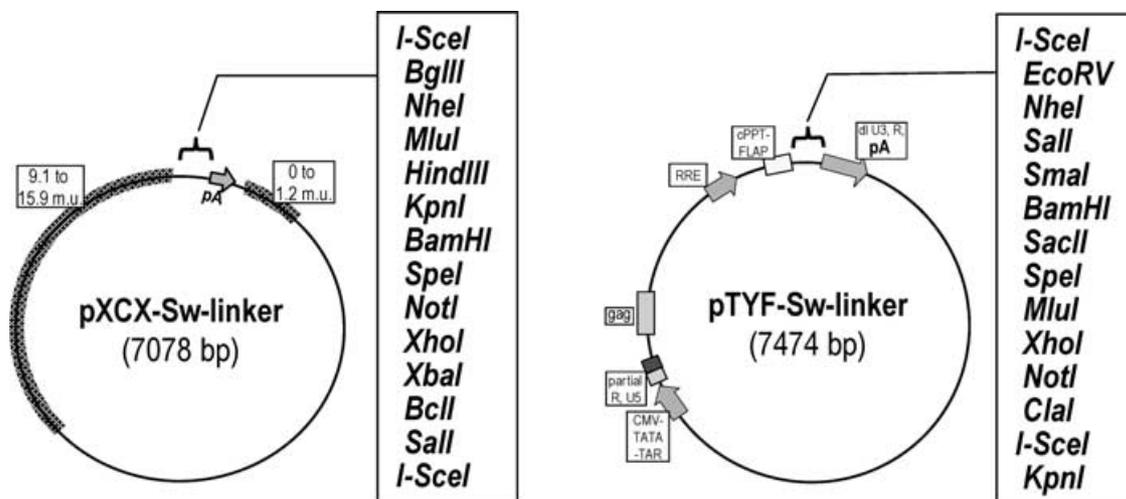


Figure 1. Improved AVVs and LVVs shuttle vectors for I-SceI-dependent transgene transfer

This set of plasmids was used to generate AVV-CMV-mRFP and LVV-CMV-mRFP bearing identical expression cassettes. The multiple cloning site is flanked by I-SceI sites, permitting one-step excision and insertion of transgene sequences.

findings were irrespective of whether DVM or XIIMN was analysed.

Comparable titres and volumes of AVVs and LVVs were used in this study. It has been noticed that, in some cases, small areas directly surrounding the focal point of AVV-CMV-mRFP injection showed signs of tissue damage and gliosis. Such areas were avoided when determining colocalization. Similar indicators of immune reactions were not apparent in any of the sections from the LVV-CMV-mRFP-transduced animals where LVVs allowed a very efficient and widespread neuronal transduction.

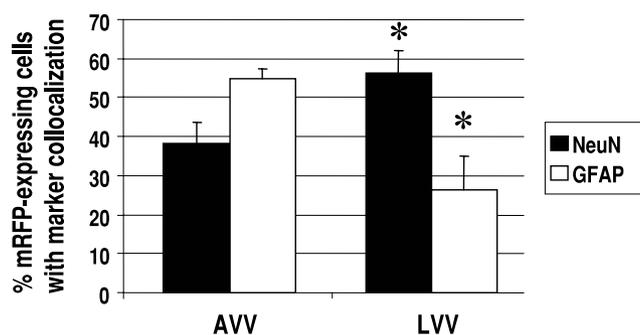


Figure 2. Colocalization of mRFP expression and IR for a neuronal and glial marker

Brainstem tissue sections were pretransduced with AVV-CMV-mRFP or LVV-CMV-mRFP and alternate sequential sections processed for immunocytochemical detection of NeuN or GFAP. In the hypoglossal and dorsal vagal motor nuclei, the percentages of mRFP-positive cells colocalizing with IR for either marker was determined. Error bars indicate s.e.m. Significant differences between AVVs and LVVs were found for neuronal and glial mRFP expression (* $P < 0.05$; one-tailed Student's t test).

Discussion

We provide compelling evidence that VSVG-pseudotyped LVVs preferentially transduce neurones as compared to AVVs in motornuclei of the brainstem in rat. In addition, we describe a novel shuttle plasmid system which allows a one-step transfer of an entire expression cassette between AVVs and LVVs backbones, thereby facilitating production of both types of vectors.

The current findings are highly relevant to ongoing gene transfer studies in different laboratories where the equivalent vector types are employed. Adenovirus fibre proteins interact with a range of ubiquitous cell surface proteins such as the major histocompatibility complex and the coxsackie-virus-adenovirus receptor. However, it is known that the serotype from which AVVs are derived may affect their neuronal tropism. While vectors originating from serotype 17 adenoviruses may be superior for targeting neurones (Chillon *et al.* 1999), we tested serotype 5-derived AVVs as it is the most widely used and commonly available type (Zolotukhin *et al.* 2002; Alisky & Davidson, 2004). In contrast to AVVs, the LVVs capsid is surrounded by an additional envelope, or coat, consisting of a protein encoded by a separate gene. The envelope is crucial for the binding of the viral particle to the cell membrane and modifications of the coat can therefore alter the tropism of the vector (Wong *et al.* 2004). Most currently used LVVs, irrespectively of the virus they have been derived from, are pseudotyped; that is, they employ a foreign envelope protein to define their transductional properties. We investigated the tropism of LVVs pseudotyped with the glycoprotein G of the vesicular stomatitis virus (VSVG) which is known to target many dividing and non-dividing cell types

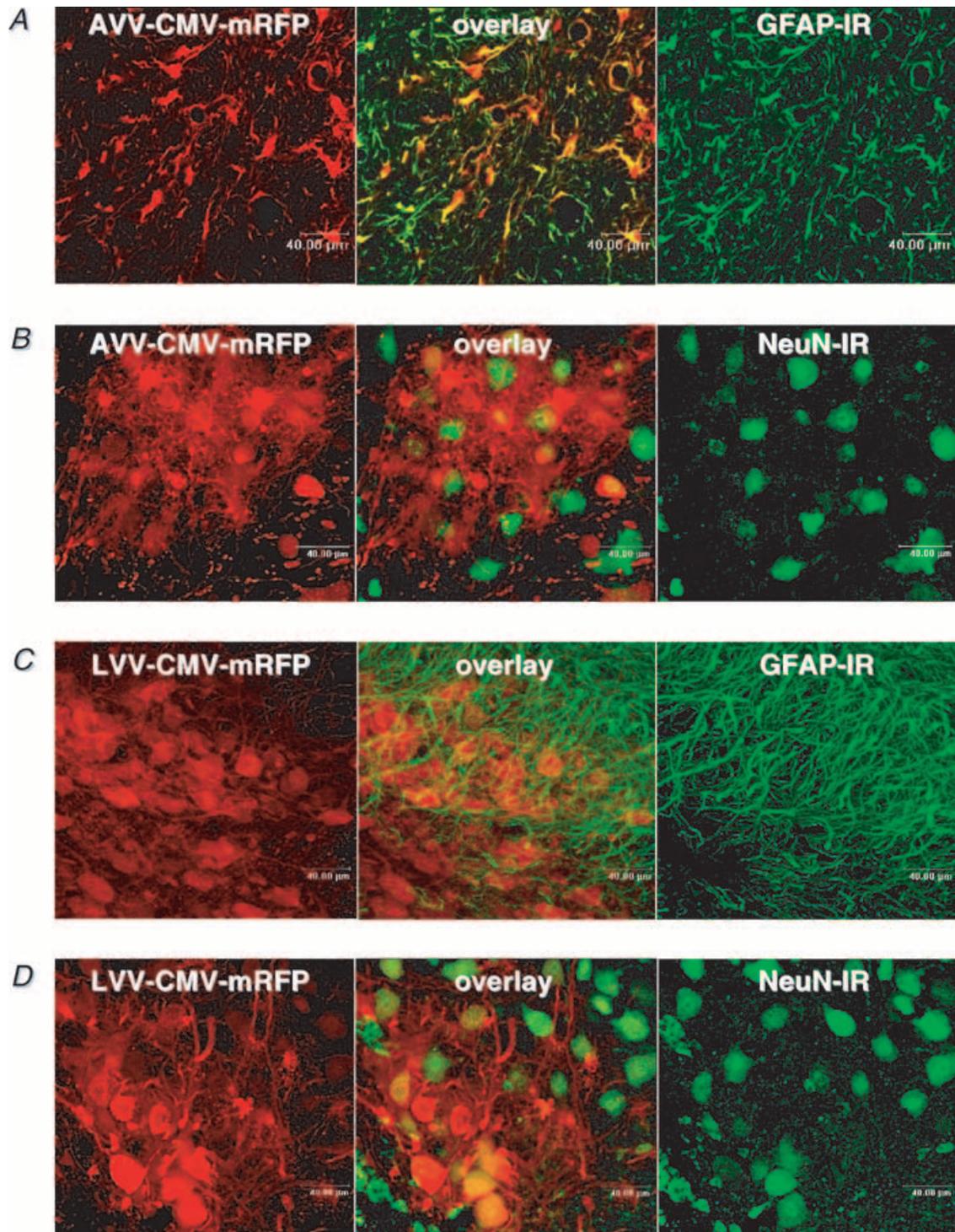


Figure 3. mRFP expression in neurones and glia in dorsal vagal and hypoglossal motor nuclei

Representative images are shown for the experimental conditions evaluated (see Fig. 2). mRFP-expressing cells transduced with either AVV- or LVV-CMV-mRFP are shown in the left hand panels; IR for NeuN or GFAP in the same sections is shown in green on the right. The central column represents the merged images of left and right columns. Yellow colour indicates colocalization which is particularly prominent in A and D. LVV shows preferential transduction for neurones *versus* glia compared with AVV.

(Iwakuma *et al.* 1999; Sinnayah *et al.* 2002; Coleman *et al.* 2003).

The central finding of this study was that the neurone/glia ratio of transgene expression was significantly higher when LVV than when AVVs were used as delivery vehicles. This indicates a higher neuronal tropism of VSVG-enveloped LVVs, resulting in a greater number of transgene copies, in neurones. The evidence was supported by complementary results from the two approaches used; that is, by identifying the proportion of both transduced glia as well as transduced neurones.

The total of mRFP-expressing cells, identified as either glial or neuronal, was close to 100% for AVVs (93%) as well as LVVs (82%) transduction. Assuming a low level of false-negative immunocytochemical signal, this suggests that the absolute majority of brain cells transduced were NeuN-expressing neurones and GFAP-positive glial cells. Other cell types present in this brain area, which may be transduced by LVVs, include vasculature-related cells and possibly some types of neurones and glia which cannot be labelled by either NeuN or GFAP.

To draw valid conclusions about vector tropism, we avoided variations in transcriptional activity of the transgene cassette or in detection level of a reporter gene by using an identical sequence in both vectors. Moreover, it was crucial to exclude any possible impact of variations in promoter activity between diverse neuronal populations present in some brain areas. In this respect, cholinergic motor nuclei of brainstem represent a good model as they contain a relatively homogenous population of cholinergic neurones in which hCMV is highly active (see Fig. 3B and T Lonergan & S Kasparov, unpublished observation; Irnaten *et al.* 2002). These nuclei also contain a substantial number of glial cells (see Fig. 3A and C) which are highly prone to hCMV-driven expression (Huang *et al.* 1999; Ehrenguber *et al.* 2001; Hermann *et al.* 2001).

It was noticed that some sections from the animals injected with AVV-CMV-mRFP contained patches of gliosis around the central spot of the injection. This is not entirely surprising given that the titre of the AVVs used was fairly high (2.9×10^9 TU ml⁻¹). Although AVVs lack a part of their genome and cannot replicate unless in certain specialized cell lines, they nevertheless can express low levels of their immunogenic capsid proteins in transduced cells. This feature makes E1–E3-deleted AVVs suboptimal for gene delivery into peripheral tissues but seems to be less problematic in the brain which is partially protected from the peripheral immune system by the blood–brain barrier. In addition, it has been shown that when very high numbers of viral particles bind to the astrocytes this can trigger c-fos expression followed by apoptosis (Rubio & Martin-Clemente, 2002). Yet another factor which might lead to adverse brain tissue reactions is the over-expression of a potentially toxic protein. Fluorescent

proteins derived from the jellyfish green fluorescent protein and from coral (such as mRFP used in this study) are generally well tolerated by various cells when expressed at low to moderate concentrations. However, very high concentrations of these (or other foreign) proteins that may be reached with large transgene copy numbers including a highly active promoter, may be damaging. At this point it is not entirely clear what proportion of these factors contributed to the signs of gliosis mentioned above. Nevertheless, these considerations substantiate the need for careful titre adjustment when AVVs are used *in vivo*.

Finally, apart from clear differences in transductional properties of AVVs and VSVG-coated LVVs, the two vector types differ in certain aspects of practical relevance. AVVs clones (once produced and isolated, a process which takes months) are easy to amplify to high yields. Besides their applications *in vivo*, they have proven particularly useful for high throughput *in vitro* experiments (see Teschemacher *et al.* 2004, this issue). LVVs, in contrast, can be quickly produced in small quantities which, at our scale of production, are sufficient for only a few *in vivo* experiments. To simplify production of either vector type from the same expression cassette we have generated a convenient system of ‘swap’ shuttle plasmids. This system circumvents duplicate cloning and offers the additional advantage that the transgene cassette can be assembled in the shuttle plasmid with more amenable cloning sites for a particular strategy. These vectors will be available upon request.

Conclusions

For transgene delivery into neurones within the dorsal vagal and hypoglossal motor nuclei of the rat brainstem *in vivo*, VSVG-coated LVVs are preferable to AVVs, for two main reasons. Firstly, neuronal transgene expression is increased and immune responses are avoided. Secondly, the modifications we have introduced into AVVs and LVVs shuttle plasmids aid a more flexible approach to transgene delivery, and enable the advantages of both vector systems to be exploited.

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