

Pseudotyping Vesicular Stomatitis Virus with Lymphocytic Choriomeningitis Virus Glycoproteins Enhances Infectivity for Glioma Cells and Minimizes Neurotropism^{∇†}

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Vesicular stomatitis virus (VSV)-based oncolytic virotherapy has the potential to significantly improve the prognosis of aggressive malignancies such as brain cancer. However, VSV's inherent neurotoxicity has hindered clinical development so far. Given that this neurotropism is attributed to the glycoprotein VSV-G, VSV was pseudotyped with the nonneurotropic envelope glycoprotein of the lymphocytic choriomeningitis virus (LCMV-GP→VSV-GP). Compared to VSV, VSV-GP showed enhanced infectivity for brain cancer cells *in vitro* while sparing primary human and rat neurons *in vitro* and *in vivo*, respectively. In conclusion, VSV-GP has a much wider therapeutic window than VSV and is thus more suitable for clinical applications, especially in the brain.

Malignant gliomas are the most aggressive and lethal intracranial tumors, and despite advances in surgery, radiation, and chemotherapy, patient survival is still only 1 to 2 years (15, 40). Therefore, various innovative approaches are under investigation (25, 27). Several groups have developed oncolytic viruses (OV) for cancer therapy, but to date, most of these viruses are not sufficiently effective and safe for use in patient treatment (1, 2, 13, 46). The same holds true for the vesicular stomatitis virus (VSV), which is one of the most promising OV candidates, as it has a short, highly cytolytic, and productive replication cycle, a high susceptibility to interferon (IFN) responses, which mediates its cancer selectivity (38), and has virtually no preexisting immunity in humans (3, 32, 44). However, VSV is neurotoxic, which limits its application, especially in the brain (19, 22, 42).

Researchers have attempted to overcome this limitation by generating attenuated viruses (AV) (10, 23, 39). But the reduced toxicity comes at the expense of reduced replication competence and oncolytic activity, so that it is doubtful whether AVs would be effective in the patient (43). As the viral glycoprotein determines the tropism, the neurotropism is mainly attributed to the glycoprotein VSV-G (8, 41, 42). Hence, we aimed to detarget VSV from neurons at the entry level by pseudotyping. The glycoprotein GP of the viscer-

tropic lymphocytic choriomeningitis virus WE strain (LCMV-WE-HPI) was selected, based on previous findings with respect to the tropism of LCMV-GP (GP)-pseudotyped lentiviral vectors (4, 9). In contrast to VSV-G pseudotypes, GP-pseudotyped lentiviral vectors had little infectivity for neurons *in vitro* and in rat brains but were at least as infectious for malignant gliomas (28).

To reliably determine the cellular tropism of VSV-GP, we used two variants of a propagation-deficient, green fluorescent protein (GFP)-expressing VSV*ΔG vector (Indiana serotype) which lacks the G gene: (i) wild-type "cytopathic" VSV*M_{cp}ΔG and (ii) VSV*M_OΔG, which has reduced cytopathicity due to attenuating mutations in the M protein (16, 18). VSV*M_OΔG was included to ensure longer survival of infected cells to facilitate monitoring of infection. To produce the single-cycle infectious VSV-G or VSV-GP virus, the respective viral glycoprotein was provided in *trans* by transient expression of VSV-G or stable expression of LCMV-GP. The stable cell line was generated by transduction of BHK-21 cells with the lentiviral self-inactivating (SIN) vector pHR'SIN-GPopt, encoding codon-optimized LCMV-GP, as previously described (4, 5, 14).

The GP pseudotype (VSV-GP) is more infectious for brain tumor cells than VSV-G. A panel of human [G44, G62 (17, 28), Mz54, U-87 MG, U-373 MG, D283Med, Daoy, UKF-NB-3, UKF-NB-4, SK-N-BE(2)c, SH-SY5Y (6, 12), HuH7, and HepG2], murine (Tu-2449 and Tu-9648), and rat (9L and McA-RH7777) cancer cell lines from three different central nervous system (CNS) neoplasms (glioma, medulloblastoma, and neuroblastoma) as well as from hepatocellular carcinomas (HCC), as an unrelated control, were tested for their susceptibility to VSV*ΔG pseudotypes. Virus titers were determined by transduction of cells with serial 10-fold

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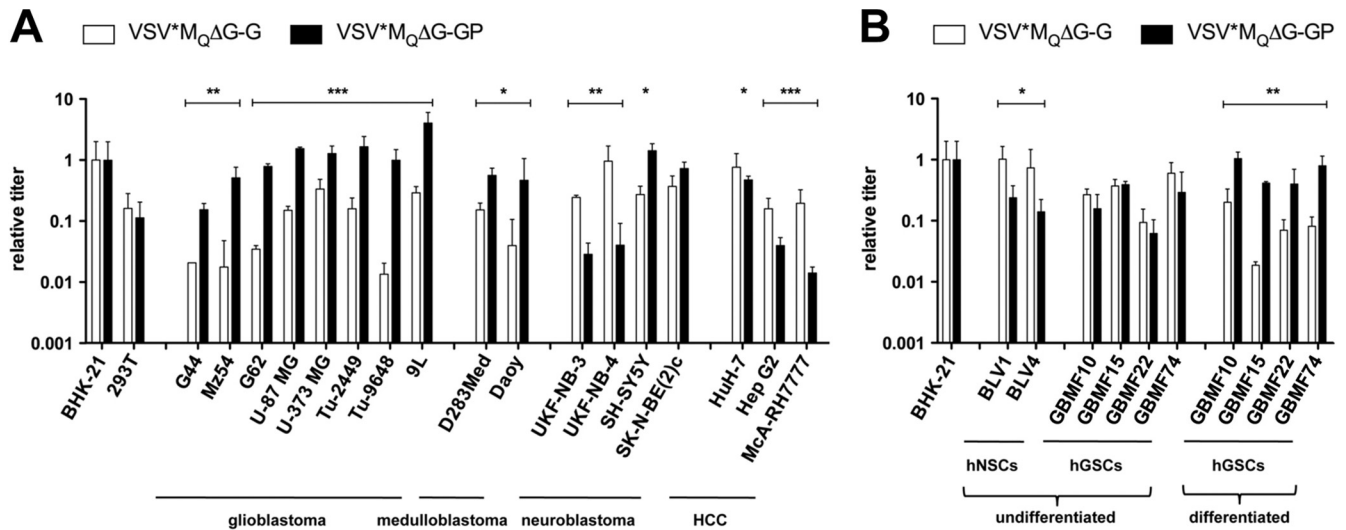


FIG. 1. Preferential tropism of VSV*ΔG-GP for malignant glioma cells. Different tumor cell lines (A) and “differentiated” as well as undifferentiated human neural stem cell (hNSC) and human glioma stem cell (hGSC) cultures (B) were infected with 10-fold serial dilutions of VSV* $M_{\Delta G}$ -G and VSV* $M_{\Delta G}$ -GP. Titers were analyzed 16 h posttransduction by flow cytometry based on GFP expression. Relative titers were determined in relation to BHK-21 as the reference cell line. The data shown represent the means and standard deviations of the results of at least three infection experiments. VSV* $M_{\Delta G}$ -G and VSV* $M_{\Delta G}$ -GP infection titers were compared. HCC, hepatocellular carcinoma; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

dilutions of the inoculum and flow cytometric analysis to determine GFP expression at 16 h posttransduction. Titers are given relative to the titer determined for BHK-21 as the reference cell line.

The LCMV-GP pseudotype VSV* $M_{\Delta G}$ -GP was significantly (5- to 80-fold) more infectious for all malignant glioma cell lines ($P < 0.01$ to $P < 0.0001$) and both medulloblastoma cell lines ($P < 0.05$) than VSV* $M_{\Delta G}$ -G (Fig. 1A). The neu-

roblastoma cell lines UKF-NB-3 and UKF-NB-4 were more susceptible to VSV* $M_{\Delta G}$ -G ($P < 0.01$), while SH-SY5Y cells were slightly more susceptible to VSV* $M_{\Delta G}$ -GP ($P < 0.05$). Titers of VSV* $M_{\Delta G}$ -G and VSV* $M_{\Delta G}$ -GP on SK-N-BE(2)c cells were not significantly different. Cell lines derived from HCC were all more susceptible to VSV* $M_{\Delta G}$ -G than to VSV* $M_{\Delta G}$ -GP ($P < 0.05$ to $P < 0.001$). The tropism of the cytopathic VSV* M_{cp} ΔG pseudotypes was consistent

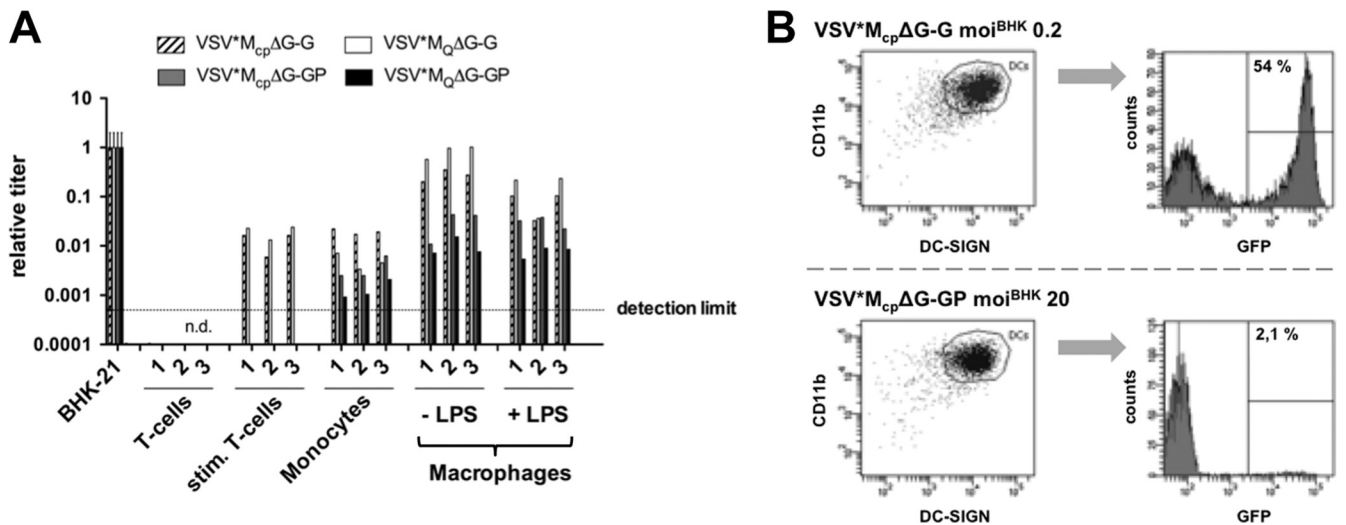


FIG. 2. VSV*ΔG-GP shows negligible transduction of PBMCs, macrophages, and dendritic cells. PBMCs were isolated from blood and T cells were stimulated as previously described (30). CD14⁺ monocytes were purified by magnetic sorting using anti-CD14 microbeads (Miltenyi Biotec). CD14⁺ monocyte-derived macrophages were differentiated *in vitro* by adding 50 ng/ml recombinant human macrophage colony stimulation factor (rhM-CSF) (Invitrogen) for 6 days. Monocyte-derived DCs were generated after culturing CD14⁺ cells with IL-4 and GM-CSF *in vitro* (35). Macrophages were activated overnight by LPS stimulation (100 ng/ml). Freshly isolated T cells and activated T cells as well as monocytes and macrophages (A) and DCs (B) were infected with 10-fold serial dilutions of VSV*ΔG-G and VSV*ΔG-GP. Titers were analyzed by flow cytometry based on the expression of GFP and normalized to the reference BHK-21 hamster cell line. GFP expression by DCs (gated on CD11b⁺/DC-SIGN⁺) was quantified 16 h postinfection by flow cytometry. n.d., not detectable.

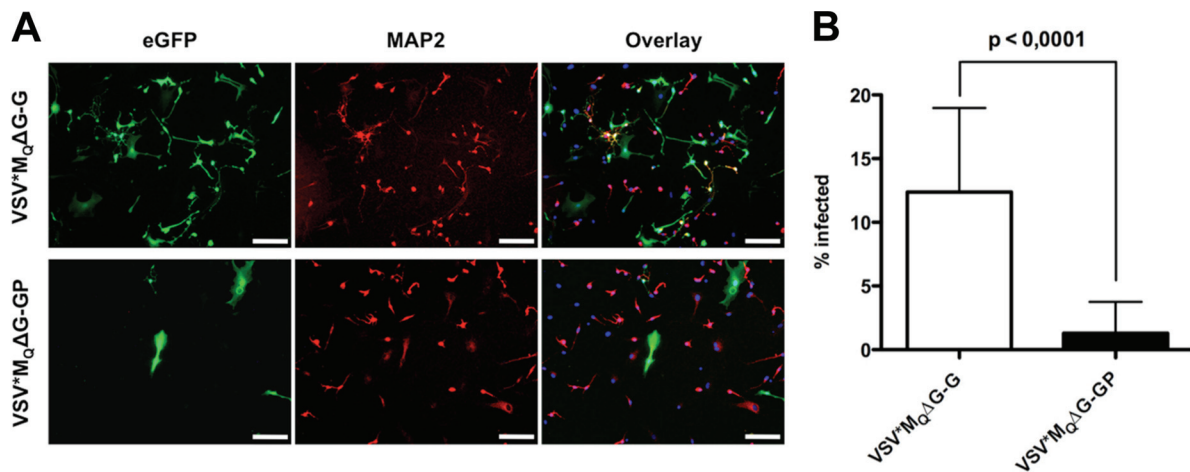


FIG. 3. Reduced neurotropism of LCMV-GP pseudotypes *in vitro*. Primary human neurons (ScienCell Research Laboratories) were grown on $2 \mu\text{g}/\text{cm}^2$ poly-L-lysine-coated culture vessels and infected with VSV $^*M_{\Delta}G-G$ and VSV $^*M_{\Delta}G-GP$ at an MOI^{BHK} of 4 (A) or 1 (B). At 16 h posttransduction, cultures were fixed in 4% paraformaldehyde. Neurons were stained with mouse anti-MAP2 (Sigma) primary antibody and goat anti-mouse Alexa Fluor 568-conjugated (Invitrogen) secondary antibody. Nuclei were counterstained with DAPI (Roche). (A) A representative microscopic field is shown. eGFP, enhanced GFP. Bars, 200 μm . (B) The percentages of GFP⁺/MAP2⁺ cells were determined by fluorescence microscopy, counting 30 microscopic fields per pseudotype. Values are expressed as means \pm standard deviations (SD) and were tested for significance by Student's *t* test ($P < 0.0001$).

with that of the VSV $^*M_{\Delta}G$ pseudotypes (see Fig. S1 in the supplemental material).

In addition, we tested infectivity by the use of primary human glioma-derived stem cell (hGSC) cultures, which have previously been shown to better reflect the genetic heterogeneity of the originating tumor (26). Human neural stem cells (hNSCs) were used as an internal control (Fig. 1B). Isolation and expansion of hNSCs (BLV1 and BLV4) and hGSCs (GBMF10, -15, -22, and -74) from primary tissues of different patients were essentially carried out as previously described for murine neural stem cells (21). While the hNSCs were more susceptible to VSV $^*M_{\Delta}G-G$ ($P < 0.05$), the levels of susceptibility of hGSCs for the two variants did not differ significantly. However, after induced "differentiation" of hGSCs by dissociation of neurospheres and replating in serum-supplemented Dulbecco's modified Eagle medium-F12 (DMEM-F12), the malignant cells became substantially (5- to 30-fold) more susceptible to VSV $^*M_{\Delta}G-GP$ than to VSV $^*M_{\Delta}G-G$ ($P < 0.01$ to $P < 0.001$), analogous to serum-grown glioma cell line results (Fig. 1A). Thus, these two types of brain cancer, malignant glioma and medulloblastoma, were especially susceptible to GP-pseudotyped VSV-complemented vectors relative to VSV-G-complemented vectors.

Low susceptibility of immune cells to VSV-GP. Peripheral blood mononuclear cells (PBMCs), as off-target cells, could mediate viral spread, thereby promoting systemic toxicity but possibly also enhancing therapeutic efficacy (20). Furthermore, infection of monocyte-derived macrophages and dendritic cells (DCs) could boost the antiviral immune response, restraining OV replication. Hence, the infectivity of VSV $^*\Delta G$ pseudotypes was analyzed using human T lymphocytes, monocytes, macrophages, and DCs of three healthy donors (Fig. 2).

No differences in infectivity between the reduced cytopathic and cytopathic VSV variants in T lymphocytes were observed (Fig. 2A). Freshly isolated T cells were more than 10^4 -fold less susceptible for both VSV pseudotypes than BHK-21 cells; even

after infection with 20 transducing units (TU)/cell, infection levels remained below the detection limit. After stimulation (30), detection of T lymphocyte infection required 100-fold-higher multiplicities of infection (MOIs) than BHK-21 infection for VSV $^*\Delta G-G$ and no infection of stimulated T cells was detected for VSV $^*\Delta G-GP$. In monocytes, the cytopathic variant tended to be more infectious, whereas unstimulated and lipopolysaccharide (LPS)-stimulated macrophages were slightly more susceptible to VSV $^*M_{\Delta}G-G$ than to VSV $^*M_{\Delta}G-GP$. Monocyte titers were around 100-fold and 1,000-fold lower than BHK-21 titers for VSV $^*\Delta G-G$ and VSV $^*\Delta G-GP$, respectively. After differentiation, the resulting macrophages became more susceptible to both pseudotypes but again slightly lost susceptibility after LPS-induced activation (Fig. 2A). In general, VSV $^*\Delta G-G$ infected monocytes and macrophages more efficiently than VSV $^*\Delta G-GP$. Monocyte-derived DCs were not at all susceptible to either VSV $^*M_{\Delta}G$ variant (see Fig. S2 in the supplemental material), presumably due to an induced interferon response. However, as shown in Fig. 2B, DCs are susceptible to infection with VSV $^*M_{cp}\Delta G-G$ but could not be efficiently infected with the GP pseudotype. Infection with VSV $^*M_{cp}\Delta G-G$ (MOI^{BHK} of 0.2) resulted in 54% GFP⁺/CD11b⁺/DC-SIGN⁺ DCs at 16 h postexposure, whereas 20 TU/cell of VSV $^*M_{cp}\Delta G-GP$ resulted in 2.1% infected DCs. In conclusion, all immune cells tested were considerably less susceptible to the GP pseudotype than to VSV-G-complemented vectors. Thus, VSV-GP may be less susceptible to viral clearance via the host immune system.

Reduced neurotropism of GP pseudotypes *in vitro* and *in vivo*. Neurotoxicity is dose limiting and has so far precluded VSV treatment of brain cancer. As we had determined that GP pseudotypes efficiently target brain tumor cells (Fig. 1), we next analyzed the tropism of VSV $^*\Delta G-GP$ relative to VSV $^*\Delta G-G$ for primary human neurons. Cells were infected with 4 TU/cell and stained with anti-microtubule-associated protein 2 (anti-MAP-2; for human neurons) and DAPI (4',6-diamidino-2-

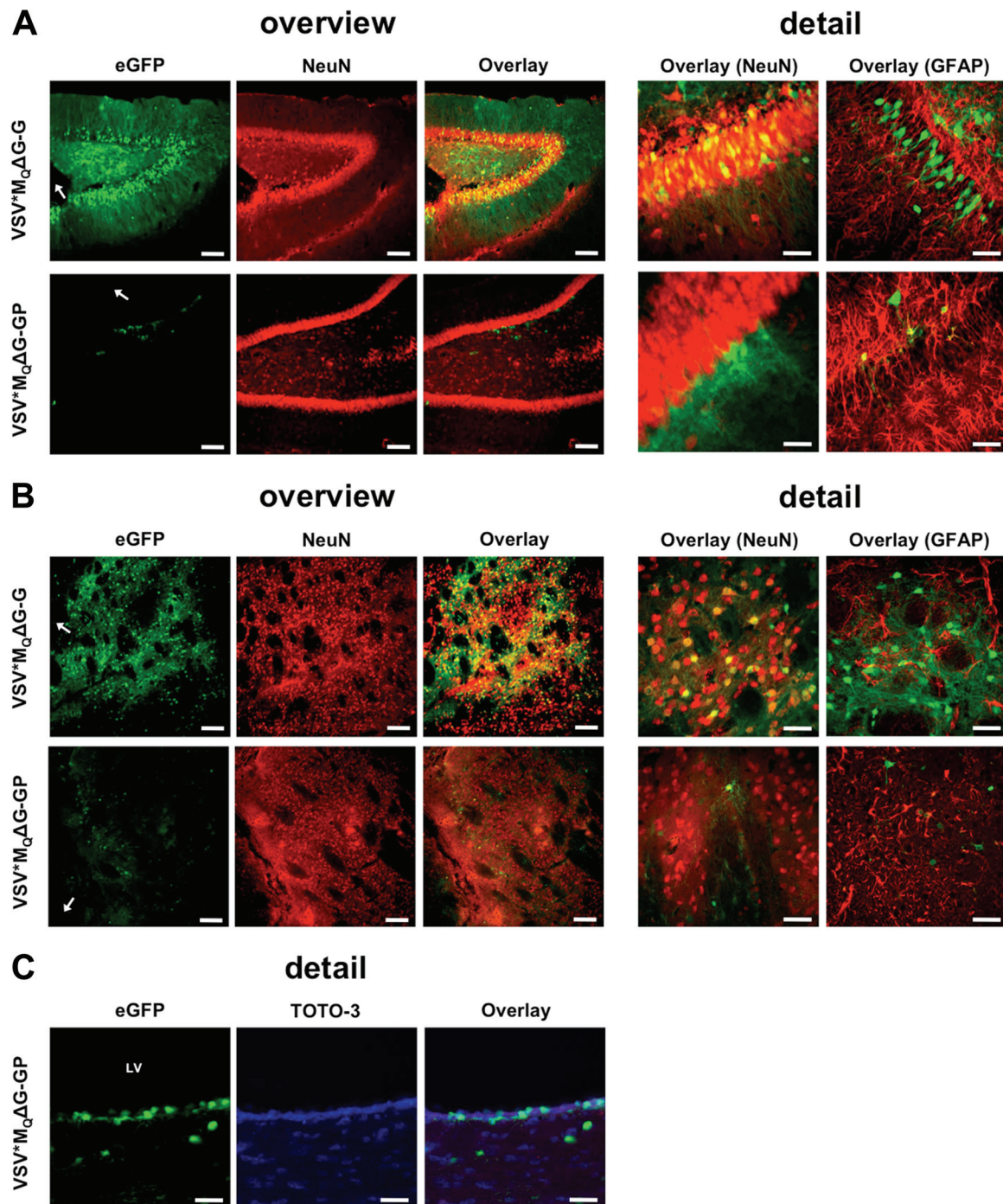


FIG. 4. Reduced neurotropism of LCMV-GP pseudotypes *in vivo*. Adult Fischer F344/NCrHsd rats were inoculated intracranially (hippocampus or striatum region) with 7×10^5 TU/ml of VSV-G-complemented or GP-pseudotyped VSV*M_QΔG vectors. At 24 h postinfection, brains were removed and suspended in phosphate-buffered saline (PBS)–4% paraformaldehyde. Coronal brain sections (40 μm thick) were prepared. Neurons were counterstained with mouse anti-NeuN (clone A60) and astrocytes with rabbit anti-GFAP (Dako) primary antibody. The respective Alexa Fluor 568-conjugated antibodies were used as secondary antibodies, and nuclear counterstaining was performed with DAPI and TOTO-3 iodide (Invitrogen). (A) Transduction of hippocampal neurons with VSV-G- and GP-pseudotyped vectors. (B) Transduction of striatal neurons by VSV-G- and GP-pseudotyped vectors. (C) Transduction of ependymal cells by VSV*M_QΔG-GP. Bars for overview, 400 μm; bars for detail, 50 μm; arrow, injection site; LV, lateral ventricle.

phenylindole) at 16 h postinfection. VSV*M_QΔG-G infection was much more efficient than VSV*M_QΔG-GP infection (Fig. 3A). For quantification, cultures were infected with 1 TU/cell and GFP⁺/MAP2⁺ cells were counted. The percentage of in-

fecting neurons was significantly ($P < 0.001$) higher in VSV*M_QΔG-G ($12.37 \pm 1.21\%$) infected cultures than in VSV*M_QΔG-GP infected cultures ($1.29 \pm 0.46\%$) (Fig. 3B).

Next, we injected 7×10^5 TU of each pseudotype (reduced

cytopathic variant) into the striatum and the hippocampus region of rat brains (28). On the following day, animals were sacrificed and microscopic slides were prepared. Neurons were stained with anti-NeuN and astrocytes with anti-gliofibrillary acidic protein (anti-GFAP), and expression of GFP was analyzed by confocal laser scanning microscopy. Massive infection, almost exclusively of neurons, was observed for VSV* $M_{O\Delta G}$ -G, as shown by GFP⁺/NeuN⁺ cells and neurite-localized GFP expression (Fig. 4). The virus was able to efficiently penetrate the examined brain region and infected cells as far as >2 mm from the injection site (Fig. 4A and B). These observations are in agreement with the previously described tropism of VSV and VSV-G-pseudotyped lentiviral vectors (29, 42). In contrast, VSV* $M_{O\Delta G}$ -GP infection rates were considerably lower, with only a few predominantly glial cells infected (Fig. 4A, detail). This is in accordance with our *in vitro* data on GP-pseudotyped VSV (Fig. 1B, Fig. 3) as well as with the tropism of lentiviral GP pseudotypes (28, 37). In particular, Stein et al. found a transduction preference of LCMV-WE-HPI-pseudotyped feline immunodeficiency virus for GFAP-positive astrocytes in the striatum and subventricular zone (SVZ) as well as for SVZ neural progenitor cells *in vivo* (37).

As the LCMV-WE-HPI envelope glycoprotein displays high-affinity binding to its α -dystroglycan cellular receptor (24), which is likewise expressed on neurons and astrocytes (36, 45), the observed scarce infection of neurons may be due to (i) different glycosylation patterns of α -dystroglycan in distinct cell types of the CNS (33), (ii) alternative, yet-unidentified receptor and/or coreceptor usage in the brain (24), or (iii) the use of propagation-deficient vectors, which, in contrast to wild-type LCMV vectors, cannot spread via direct cell-cell contact from glial cells to proximal neurons (7). Further studies to analyze whether replication-competent VSV-GP can spread from glial cells to neurons are ongoing. In accordance with previous studies on LCMV-WE tropism, VSV* $M_{O\Delta G}$ -GP was able to efficiently infect the ependymal cell layer of the lateral ventricle (Fig. 4C) (11, 34).

In summary, relative to VSV, LCMV-GP-pseudotyped VSV exhibits an increased infectivity for brain cancer cells, whereas its neurotropism is highly attenuated. Immune cells (as potential off-target cells) are also considerably less susceptible to VSV-GP than to VSV-G. Taken together, these consistent data indicate that GP-pseudotyped VSV has a beneficial toxicity as well as efficacy profile and may render VSV eligible for use in the brain for potential treatment of brain cancer. However, use of a propagation-incompetent VSV-GP would presumably result in minor antitumoral effectiveness, since pre-clinical and clinical trials have shown that incomplete viral transduction or distribution within the tumor is a major obstacle that eventually contributes to therapy failure (31). Thus, the use of carrier cell-assisted viral delivery as well as a replication-competent recombinant rVSV-GP is a promising strategy currently under evaluation.

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