Journal of NeuroVirology, 12: 60–64, 2006

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280500516450

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Short Communication

Neuronal pathways of viral invasion in mice after intranasal inoculation of pseudorabies virus PrV-9112C2 expressing bovine herpesvirus 1 glycoprotein B

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In contrast to wild-type Pseudorabies virus (PrV), which infects the central nervous system mainly via fibres of the trigeminal and autonomous nerves, the PrV mutant PrV-9112C2, deleted in glycoprotein B but expressing its bovine herpesvirus 1 (BHV-1) homologue, was shown to infect the swine central nervous system (CNS) via the olfactory route. In this study application of PrV-9112C2 into the nose of mice resulted in CNS infection as described for wild-type PrV. These findings indicate that $gB_{(BHV-1)}$-dependent changes in PrV's capability to infect swine olfactory sensory neurons (OSNs) are not prominent in mice and give evidence for viral entry receptors present in swine but not mice OSNs.
Journal of NeuroVirology (2006) **12,** 60–64.

Keywords: mice; nasal mucosa; pseudorabies virus; trigeminal ganglion

Pseudorabies virus (PrV), also designated Suid herpesvirus 1, is the causative agent of Aujeszky's disease (AD), a serious illness in domestic and wild animals. PrV has a broad host range and productively infects birds and most mammals, except for horses and higher primates including humans, which are resistant to infection (reviewed in Mettenleiter, 1994b). Whereas pigs, considered to be the natural host of PrV, can survive a productive infection and remain latently infected for life, mortality in most infected species including rodents regularly amounts to 100%. Higher primates including man are resistant towards a PrV infection for unknown reasons. In contrast, bovine herpesvirus-1 (BHV-1) has a restricted natural host range, infecting only ruminants and producing relatively minor symptoms related primarily to infection of nasal and genital mucosae (Gibbs and Rweyemamu, 1977). Whereas PrV exhibits a pronounced neurovirulence, BHV-1 does not. However, both viruses can infect neurons and remain latent in them for the life of the animal (Beran *et al*, 1980; Gutekunst *et al*, 1980; Jones, 2003).

Natural infection with PrV normally occurs via the oronasopharyngeal route. The nasal cavity is innervated predominantly by four nerves: (i) the olfactory nerve (I cranial), (ii) the trigeminal nerve (V cranial), (iii) parasympathetic, and (iv) sympathetic efferents. Primary virus replication occurs in the mucosa, mostly in non-neuronal cells. Subsequently, the viral particles infect neurons via nerve endings innervating the mucosae and ascend toward the central nervous system (CNS), resulting in a nonsuppurative meningoencephylitis (Enquist, 1994; Pensaert and Kluge, 1989).

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This work was supported by the International Graduate School of Neuroscience (IGSN) and the Graduiertenkolleg "Development and Plasticity of the Nervous System: Molecular, Synaptic and Cellular Mechanisms," University of Bochum, Bochum, Germany. Monoclonal anti-$gB_{(BHV-1)}$ antibody was kindly provided by G. Keil (Riems).

Received 22 August 2005; revised 28 October 2005; accepted 25 November 2005.

Herpesvirus glycoproteins are involved in crucial steps during virus infection, particularly in attachment to and penetration into target cells and direct cell-to-cell spread (Mettenleiter, 1994a). Glycoprotein B (gB) is the most highly conserved herpesvirus glycoprotein, and homologues have been detected throughout members of the *Herpesviridae* (Pereira, 1994). It is essential for penetration, i.e., fusion between virion envelope and cellular cytoplasmic membrane, and transcellular infection by direct cell-to-cell spread (Spear, 1993). It is also required for neuroinvasion and transneuronal spread *in vivo* (Babic *et al*, 1993). Functional homology of gB proteins could be demonstrated by heterologous complementation. $gB_{(BHV\text{-}1)}$, which exhibits 63% amino acid identity to $gB_{(PrV)}$, was able to complement a lethal gB deletion in PrV (Rauh *et al*, 1991). Thus, a PrV recombinant lacking $gB_{(PrV)}$ but stably carrying the $gB_{(BHV\text{-}1)}$ gene in its genome was isolated and designated PrV-9112C2 (Kopp and Mettenleiter, 1992). Compared with wild-type virus, PrV-9112C2 was similar in its growth characteristics in cell culture (Gerdts *et al*, 2000) but showed a slight delay in cell penetration *in vitro* (Kopp and Mettenleiter, 1992). Interestingly, *in vivo* PrV-9112C2 showed a dramatic increase in neurovirulence in PrV's natural host, swine, combined with an alteration in the route of neuroinvasion and altered course of disease. Inoculation of wild-type PrV strain Kaplan (PrV-Ka) results in high fever, marked respiratory symptoms, minimal neurological disorders, and excretion of high amounts of virus. Animals usually survive the infection. In contrast, animals infected with PrV-9112C2 showed no respiratory symptoms and developed only mild fever. However, 5 days after infection all piglets developed severe CNS symptoms leading to death within 48 to 71 h. Histological analyses showed that PrV-9112C2 primarily infected the olfactory epithelium and spread via the olfactory route. In the CNS, more viral antigen and significantly more pronounced histological changes resulting in more severe encephalitis were found after PrV-9112C2 infection. These findings demonstrate that the essential gB is involved in determining neurotropism and neurovirulence of PrV. The present study was designed to clarify whether these consequences of the exchange of gB are restricted to pigs or whether the mutation also resulted in infection of the olfactory system in mice.

Adult swiss CD-1 mice were unilaterally intranasally inoculated with 5 μl of high titered PrV-9112C2 (containing $\sim 10^6$ plague-forming units [pfu]) grown on PK15 cells. Three days later animals were sacrificed and the nose, the base of the skull (including the trigeminal and autonomous ganglia), and the whole brain were removed and embedded in paraffin. Tissue was sectioned into 10 μm slices and used for immunohistological investigations. Antiserum (made in rabbits), raised against the major PrV capsid protein, UL19, was used to detect virus within neuronal and nasal tissue. A monoclonal antibody was used for detection of bovine $gB_{(BHV\text{-}1)}$. Antibody against the olfactory marker protein (OMP) was kindly provided by F. Margolis (University of Maryland Medical School, Maryland, USA). Calcitonin gene-related peptide (CGRP) antibody (US Biological) was used to identify trigeminal fibres within the nasal cavity. Primary antibodies were detected by fluorescence labelled secondary antibodies (Alexa Fluor 488, Alexa Fluor 546; Molecular Probes) or using biotinylated anti-rabbit antibody in combination with horseradish peroxidase (HRP)-coupled ABC reagent (Vector) and diamino benzidine (DAB) (after PrV-9112C2 infection) or AEC (after PrV wild type infection) staining. Fluorescence labeled sections were analyzed by means of confocal microscopy (Zeiss, LSM 510 Meta) using laser excitation at 488 nm and 543 nm. Light microscopy was used for identification of HRP-mediated labeling of viral antigen.

In our study, the average time to death after intranasal infection of mice with PrV-9112C2 was 76 ± 2.8 h p.i. ($n = 5$). Following infection with wild-type PrV, the animals died at 49 ± 1.58 h p.i. ($n = 10$). However, symptoms of PrV-9112C2 and wild-type PrV infection were the same: Mice became anorectic, apathetic, depressed, and cowered in a hunched position. Subsequently, infected mice showed frequent attacks characterized by extensive scratching of the skin of the face, nose, and maxillary region, thereby causing severe hemorrhagic dermal erosions and ulcerations with coinciding acute catarrhal conjunctivitis ("mad itch" syndrome). Hyperactivity with excitations and convulsions, heavy dyspnea with tachypnea, and a moderate swelling of the bridge of the snout were also observed.

Detection of the PrV major capsid protein encoded by the UL19 gene was used in paraffin sections as a marker for virus infection. Application of 5 μl of high titered ($\sim 2 \times 10^8$ pfu/ml) PrV-9112C2 to the right nostril of adult mice produced massive infection of the right sided respiratory (Figure 1A) and olfactory mucosae within 3 days (Figure 1D). PrV-mediated $gB_{(BHV\text{-}1)}$ expression in nasal epithelia was verified by double immunfluorescence directed against UL19 and $gB_{(BHV\text{-}1)}$ (Figure 1A to C). $gB_{(BHV\text{-}1)}$ was detected exclusively in structures that also expressed UL19 (Figure 1C). The olfactory epithelium was identified by counterstaining with OMP antibody, labeling exclusively mature olfactory receptor neurons (Margolis, 1982). Viral antigen could be localized primarily at the level of sustentacular cells (Figure 1D). Lesions within olfactory and respiratory epithelia were also identified but cofluorescence with OMP was not found, indicating that olfactory receptor neurons were not infected.

The nasal epithelium is richly invested with peptidergic (substance P and CGRP) trigeminal polymodal nociceptors (Finger *et al*, 1990). Within the trigeminal ganglia CGRP is localized in about 40% of small to medium-sized trigeminal neurons (Lee *et al*, 1985). Coimmunostaining of PrV antigen with

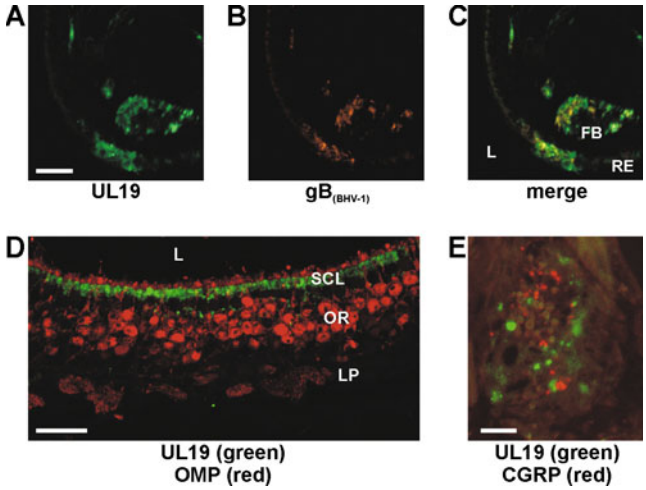

Figure 1 PrV infection in the nasal cavity. (**A–C**) UL19 (*green*) indicated infection of nasal tissue and was found in respiratory epithelia (RE) and subepithelial fibre bundles (FB). $gB_{(BHV-1)}$ (*red*) was coexpressed and restricted to UL19 expressing structures (L: lumen). (**D**) Viral antigen (*green*) was found in apical layers (sustentacular cell layer, SCL) of the olfactory epithelium identified by counterstaining of olfactory receptor neurons (OR) by OMP-antibody (*red*). No viral antigen was found in the filia olfactoria within the lamina propria (LP) (L: lumen). (**E**) CGRP immunostaining (*red*) identified subepithelial trigeminal fibre bundles where viral antigen (*green*) was found. Bars in **A** and **D**: 20 μm; E: 10 μm.

CGRP revealed infection of subepithelial fibre bundles (Figure 1E) presumably of trigeminal origin, indicating invasion of trigeminal afferents via the nasal mucosa. Interestingly, colabeling with CGRP was sparse, indicating infection of predominantly non-peptidergic fibers.

Three days after right sided inoculation of PrV-9112C2 viral antigen was found in the ipsilateral trigeminal ganglia (Figure 2A, B) as well as the ganglia of the autonomous nervous system, the parasympathetic pterigopalatine (Figure 2D, E) and sympathetic superior cervical ganglia (Figure 2G, H). CNS neurons of the spinotrigeminal nucleus caudalis (Sp5C) and paratrigeminal nucleus (Pa) were heavily infected (Figure 2J), indicating spread to second order neurons in anterograde direction. In contrast, no immunosignal was detected in the olfactory bulb (data not shown). PrV-9112C2 infection of ganglia and medullary structures was indistinguishable from wild type PrV infection after intranasal inoculation (Figure 2C, F, I, J, inlay).

Neuroinvasion of PrV occurs by microtubule associated transport within the axons in anterograde and retrograde direction (Mettenleiter, 2003; Smith *et al*, 2001). In this study we demonstrated that infection of mice with the $gB_{(BHV-1)}$-expressing recombinant PrV-9112C2 resulted in infection of the CNS via the trigeminal and autonomous neurons within the nasal cavity as described for wild type viruses. This is in contrast to infection of pigs where the olfactory route is described as the most important route of invasion for PrV-9112C2.

Within the nasal cavity of mice selectivity of PrV infection for the olfactory epithelium as described for pigs could not be shown (Gerdts *et al*, 2000). Instead we found infection of respiratory and olfactory mucosae. Viral antigen was detected neither in OMP expressing olfactory receptor neurons nor in subepithelial nerve fibres and axon bundles of the filia olfactoria. PrV did not reach the olfactory glomeruli of the olfactory bulb where olfactory receptor neurons exhibit their terminals and synapse with second order neurons. In swine, PrV-9112C2 was reported to effectively replicate in the olfactory bulb already at early stages after infection and the trigeminal ganglion appeared to be less infected.

The PrV mutant, PrV-Bartha, is widely used as a tract tracer after intracerebral (DeFalco *et al*, 2001; Jasmin *et al*, 1997; Krout *et al*, 2003) or peripheral (Billig *et al*, 2000; Cano *et al*, 2001; Horvath *et al*, 2003; Irnaten *et al*, 2001; Jansen *et al*, 1995, 1997; Smith *et al*, 2000) injection (for review see Song *et al*, 2005). This attenuated PrV variant provides a tool for transneuronal tracing, selectively in retrograde direction of signal processing. After peripheral injection, e.g., into the spleen or heart (Cano *et al*, 2001; Irnaten *et al*, 2001) PrV-Bartha invaded motor fibres and neurons of higher order within the CNS. Identification of CNS circuits involved in processing of sensory information requires transneuronal tracing from peripheral sensory organs in the anterograde direction towards the CNS. Both retrograde and anterograde spread can be accomplished by wild-type α-herpesviruses (Sabin, 1938), providing a potential tool for tracing sensory neuronal systems. However, transneuronal spread within the olfactory system of rodents cannot be achieved after intranasal application of PrV wild-type viruses (Babic *et al*, 1994; Sabin, 1938). Therefore the study at hand was also conducted to clarify whether tracing of olfactory neuronal pathways could be performed by a PrV-9112C2, described as PrV mutant with preferred invasion of the olfactory system in swine.

So far, a role for the essential glycoprotein gB in virulence and viral pathogenesis has been implicated by several groups. The absence of gB results in loss of infectivity in cell culture and *in vivo*, where no viral spread of phenotypically complemented mutant virus could be observed (Babic *et al*, 1993; Neubauer *et al*, 1997; Peeters *et al*, 1992; Rauh *et al*, 1991). In the present study we showed that the exchange of $gB_{(PrV)}$ by the homologous $gB_{(BHV-1)}$ resulted in pathways of neuroinvasion as shown for wild-type PrV in mice. This was unexpected because efficient replication of PrV-9112C2 in the olfactory system of piglets and successful viral invasion of the CNS resulted in a dramatically altered phenotype that was never observed before. The mechanism involved in differences of gB-selective neurovirulence between pigs and mice are not known. Future experiments will help to identify entry receptors accounting for $gB_{(BHV-1)}$–dependent PrV infection of swine OSNs.

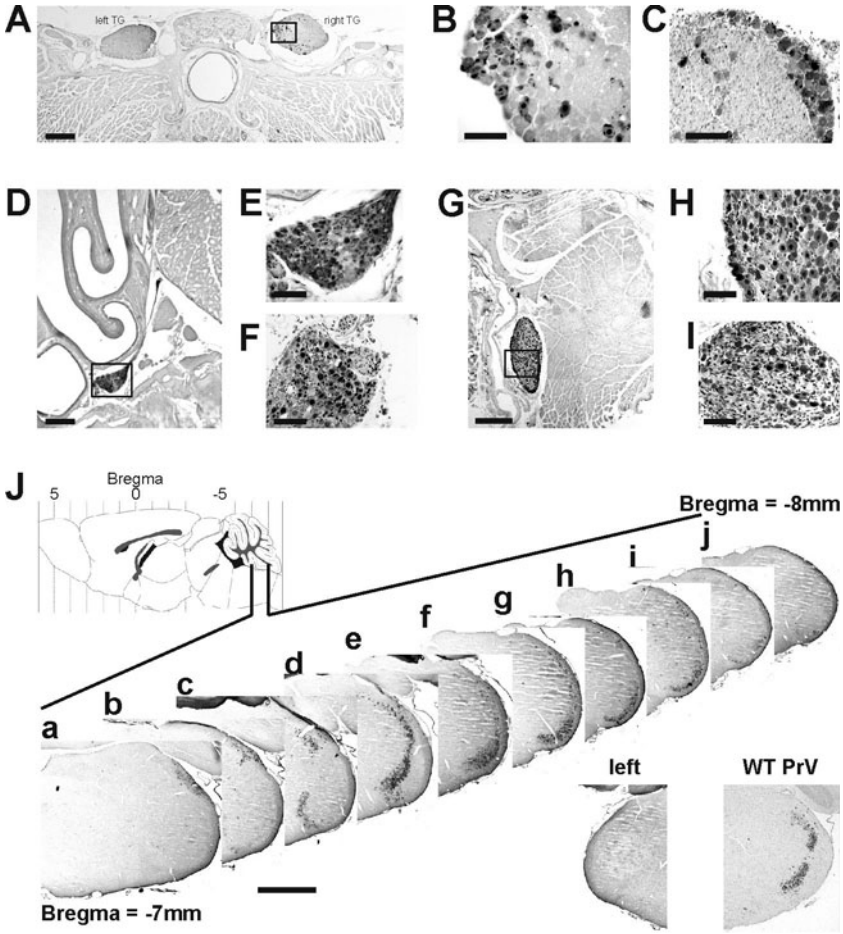

Figure 2 Detection of UL19 in coronal sections of the skull containing the (**A**) trigeminal, (**D**) right sided pterigopalatine, and (**G**) right sided superior cervical, ganglia. (**B**, **E**, **H**) Higher magnification of infected ganglia indicated by rectangles in **A, D**, and **G**. (**C**, **F**, **I**) Detection of UL19 in ganglia following infection with WT PrV. (**J**) Sequence of coronal sections of the brain stem revealed viral infection of the right sided Sp5C (c–i) and the paratrigeminal nucleus (a–c). The left Sp5C was free of viral antigen whereas infection of mice with WT PrV caused similar infection of the right sided Sp5 (**C**). TG: trigeminal ganglion. Bars in **A**: 500 μm; **B, C, E, F, H, I:** 90 μm; **D**, **G**: 300 μm; **J**: 750 μm.

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