Journal of NeuroVirology, 16: 457–465, 2010

ISSN 1355-0284 print / 1538-2443 online
DOI: 10.3109/13550284.2010.530639

A bovine herpesvirus type 1 mutant virus with truncated glycoprotein E cytoplasmic tail has defective anterograde neuronal transport in rabbit dorsal root ganglia primary neuronal cultures in a microfluidic chamber system

S I Chowdhury,[1] J Coats,[1] R A Neis,[1] S M Navarro,[1] D B Paulsen,[1] and J-M Feng[2]

[1]Department of Pathobiological Sciences; and [2]Department of Comparative Biomedical Sciences (CBS), School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana, USA

Bovine herpesvirus type 1 (BHV-1) is an important component of the bovine respiratory disease complex (BRDC) in cattle. Following primary intranasal and ocular infection of cattle, BHV-1 establishes lifelong latent infection in trigeminal ganglia (TG). Upon reactivation from latency, the virus is transported from neuronal cell bodies in the TG to projected nerve endings in nose and cornea of latently infected cattle where the virus shedding occurs. This property of BHV-1 plays a significant role in the pathogenesis of BRDC and maintenance of BHV-1 in the cattle population. Recently, we have reported that a glycoprotein E (gE) cytoplasmic tail–truncated BHV-1 (BHV-1 gEAm453) did not reactivate from latency and was not shed in the nasal and ocular secretions of calves and rabbits. Here we describe the methods to establish rabbit primary dorsal root ganglia (DRG) neuron cultures in a microfluidic chamber system and to characterize *in vitro* anterograde and retrograde axonal transport properties of BHV-1 gE-deleted and BHV-1 cytoplasmic tail–truncated gEAm453 mutant viruses relative to BHV-1 gEAm453-rescued/wild-type viruses. The results clearly demonstrated that whereas the BHV-1 gE-deleted, BHV-1 gEAm453, and BHV-1 gEAm453-rescued/wild-type viruses were transported equally efficiently in the retrograde direction, only the BHV-1 gEAm453-rescued/wild-type virus was transported anterogradely. Therefore, we have concluded that sequences within the BHV-1 gE cytoplasmic tail are essential for anterograde axonal transport and that primary rabbit DRG neuronal cultures in the microfluidic chambers are suitable for BHV-1 neuronal transport studies. *Journal of NeuroVirology* (2010) **16,** 457–465.

Keywords: BHV-1 enveope glycoprotein gE; compartmentalized primary neuron culture; defective anterograde neuronal transport; gE cytoplasmic tail–truncated virus

Address correspondence to S. I. Chowdhury, Department of Pathobiological Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803, USA. E-mail: Chowdh@lsu.edu

This work was supported by USDA grants (2009-35204-05200) and 2004-35204-14657) to S. I. Chowdhury.

Received 9 July 2010; revised 17 September 2010; accepted 28 September 2010.

Introduction

Bovine herpesvirus type 1 (BHV-1) is an alphaherpesvirus that causes abortion, respiratory, and genital infections in cattle (Jones and Chowdhury, 2007; Tikoo *et al*, 1995; Wyler *et al*, 1989). Following primary infection of respiratory and ocular epithelium, BHV-1 replicates in the nasal and ocular epithelium. Subsequently, capsids together with tegument enter sensory nerve endings of the ophthalmic and maxillary branches of the trigeminal nerve located in the nasopharynx and eye (Jones, 1998; Jones and Chowdhury, 2007; Rock *et al*, 1986). These viral particles are transported retrogradely to cell bodies in trigeminal ganglia (TG) where the virus establishes a lifelong latent infection (Jones, 1998; Jones and Chowdhury, 2007; Rock *et al*, 1986). The episomal form of viral genomic DNA, transcripts originating from the latency-related (LR) gene, and proteins encoded by LR gene can be detected in TG during latency (Jiang *et al*, 1998; Jones, 1998; Jones and Chowdhury, 2007; Rock *et al*, 1986). However, there is no evidence of viral DNA replication and productive infection during latency (Jones, 1998; Rock *et al*, 1986). Periodic reactivation from BHV-1 latency usually results in nasal and ocular viral shedding. In this case, infectious virus particles are transported anterogradely to axon termini in the nasopharynx and eye where they infect epithelial cells, resulting in viral replication and shedding (Brum *et al*, 2009; Butchi *et al*; Liu *et al*, 2008b).

Results from our recent studies show that like the glycoprotein E (gE)-deleted BHV-1, a gE cytoplasmic tail–truncated BHV-1 (BHV-1 gEAm453) virus fails to reactivate from latency and is not shed in the nose and/or eye secretions following dexamethasone-induced reactivation (Brum *et al*, 2009; Liu *et al*, 2008b). Prior to this study, neuronal transport studies were only possible *in vivo* in calves or in rabbits because a suitable compartmentalized primary neuron culture system for BHV-1 was lacking. Based on *in vitro* neuronal transport properties of gE-deleted pseudorabies virus (PRV) and herpes simplex virus type 1 (HSV-1) (Ch'ng and Enquist, 2005b; Tirabassi and Enquist, 1999; Tirabassi *et al*, 1997; Snyder *et al*, 2008; Wang *et al*, 2005; Yang *et al*, 1999), we have earlier concluded that BHV-1 gE-deleted and BHV-1 gE cytoplasmic tail–truncated mutant viruses also should have defective anterograde axonal transport (Brum *et al*, 2009; Liu *et al*, 2008b). As a consequence, transport of the BHV-1 gE mutant viruses from neuronal cell bodies in TG to nerve endings in the nasal and ocular primary infection sites is defective.

Here we describe the methods of culturing primary dorsal root ganglia (DRG) neurons from fetal rabbits in a microfluidic chamber system. In addition, we demonstrate the utility of DRG primary neurons in microfluidic chamber systems for assessment of anterograde and retrograde neuronal transport of BHV-1 wild-type and BHV-1 gE mutant viruses. In these culture devices, DRG neuronal cell bodies located in the soma compartment were partitioned from non-neuronal Madin-Darby bovine kidney (MDBK) cells, located in the axonal compartment, and were linked only via the axons, which extended through microcapillary grooves to the opposing axonal compartment. By initiating virus infection either in the soma compartment or in the axonal compartment, we determined that relative to BHV-1 gEAm453-rescued or wild-type BHV-1, BHV-1 gE-deleted and BHV-1 cytoplasmic–tail truncated (BHV-1 gEAm453) mutant viruses have defective anterograde axonal transport, whereas their retrograde axonal transport properties are unaffected.

Results

Rabbit dorsal root ganglionic neurons in microfluidic chamber system

Neurite growth from DRG neuron cell bodies in the soma compartment were readily visible after one day of culture and by 3 to 4 days, axons extended across the grooves to the axonal compartments. Based on the immunoflurescence results shown in Figure 1A, by 2 weeks, the axonal compartments had dense neurite outgrowth in the microfluidic chambers and the soma and their axons were readily labeled with neurofilament-specific antibodies. After the neurons were approximately 19 to 20 days in culture, MDBK cells were plated in the axonal compartment, such that on the next day the MDBK cells formed a semiconfluent monolayer (Figure 1B). Under these conditions, neuronal cell bodies and MDBK cells were separated physically and are connected only via the axons, which extended through the microcapillary grooves to the opposing axonal compartment containing the MDBK cells.

Both BHV-1 gE-deleted and BHV-1gE cytoplasmic tail–truncated viruses had defective anterograde axonal transport but their retrograde axonal transport was unaffected

Both the vBHV-1 BAC gEAm453 and vBHV-1 BAC gEAm453R (rescued) Au: In the manuscript, "BAC" is sometimes placed after gEAm453/gEAm453R, with a hyphen, sometimes between vBHV-1 and gEAm453/gEAm453R, with word spaces flanking. The latter format is now used throughout for consistency and to match Figures 2 and 3. Also in the text, you have BHV-1 gEΔ (with some inconsistencies that have been edited) and vBHV-1 BAC gEAm453/gEAm453R, whereas the lowercase v (vBHV-1) is not used in Figures 2 and 3. Please see if the text should be edited to match. viruses contain one copy of BAC, respectively, and express diffusible green fluorescent protein (GFP), whereas the BHV-1 gEΔ virus contain a GFP expression cassette

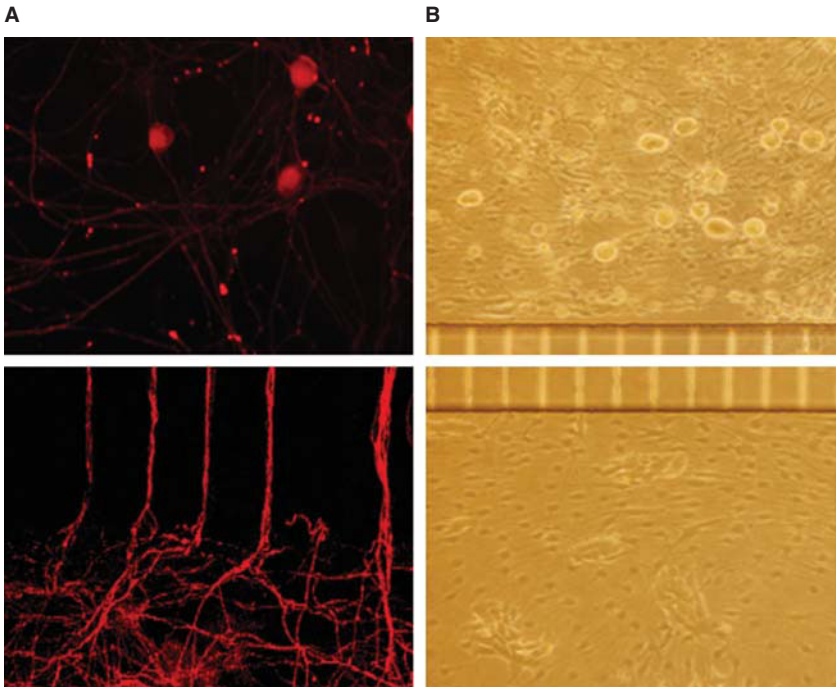

Figure 1 (**A**) Neurofilament-specific immunofluorescence images by epifluorescence microscopy. The DRG neurons cultured for 14 days in the microfluidic chambers are expressing neurofilament-specific proteins. The cell bodies are located in the soma chamber and the axon growth extended across the microgroove into the axonal chamber. Note that there is an extensive network of neurites in the axonal chamber. (**B**) Live phase-contrast images of DRG neurons and MDBK cells in the microfluidic chamber 24 h after MDBK cells were plated in the axonal compartment.

(Brum *et al*, 2009; Liu *et al*, 2008b). Therefore, infection of neuronal cell bodies and MDBK cells in the soma and axonal compartments, respectively, were visible by emission of green fluorescence under a fluorescent microscope.

To determine the retrograde axonal transport property of BHV-1 gEΔ, vBHV-1 BAC gEAm453, and vBHV-1 BAC gEAm453R (rescued) viruses, MDBK cells in the axonal compartments were infected at 24 h after the cells were plated. By 12 h post infection (h.p.i.), the earliest time we have observed, numerous neuronal cell bodies located in the soma compartments were emitting green fluorescence indicating that they were productively infected. As shown in Figure 2, at 24 h.p.i., numerous neuronal cell bodies with intense green fluorescence were readily detectable for all the viruses tested: vBHV-1 gEΔ, vBHV-1 BAC gEAm453, and vBHV-1 BAC gEAm453R viruses. Virus titers of samples at 48 h.p.i. from the soma compartments ranged between 1×10^3 and 2×10^3 PFU for all the three viruses (data not shown). These results indicated that all three viruses entered the axons within the axonal chamber and traveled in retrograde direction along the axons, crossed the grooves, and replicated further in the corresponding cell bodies at the soma chambers. Therefore, as expected from our earlier calf and rabbit experiments (Brum *et al*, 2009; Liu *et al*, 2008b), gE was not necessary for retrograde axonal transport.

To determine the anterograde axonal transport property of BHV-1gEΔ, vBHV-1 BAC gEAm453, and vBHV-1 BAC gEAm453R viruses, soma compartments were similarly infected with the respective viruses. At 48 h.p.i. (Figure 3) and 72 h.p.i. (data not shown), neuronal cell bodies infected with each virus had similar intensity of green fluorescence. Viral titers of 48 h.p.i. samples from the respective soma compartments yielded very similar titers (4×10^6 to 9×10^6 PFU) for all three viruses (data not shown). These results indicated that all three viruses, including the mutants, infected the neuronal cell body and replicated in them when the viruses were introduced directly into the soma compartment. However, MDBK cells, across the grooves, in the axonal compartment, were productively infected only in the case of the vBHV-1 BAC gEAm453R virus but not for the two BHV-1 gE mutants (BHV-1 gE-deleted and vBHV-1 BAC gEAm453) (Figure 3). Consistent with these results, virus titers in the axonal chambers of the vBHV-1 BAC gEAm453R (rescue virus) ranged from 1×10^5 to 1×10^6 PFU, whereas the axonal chambers from the two gE mutant viruses had no detectable infectious particles in the axonal compartments (data not shown). Taken together, our data demonstrated

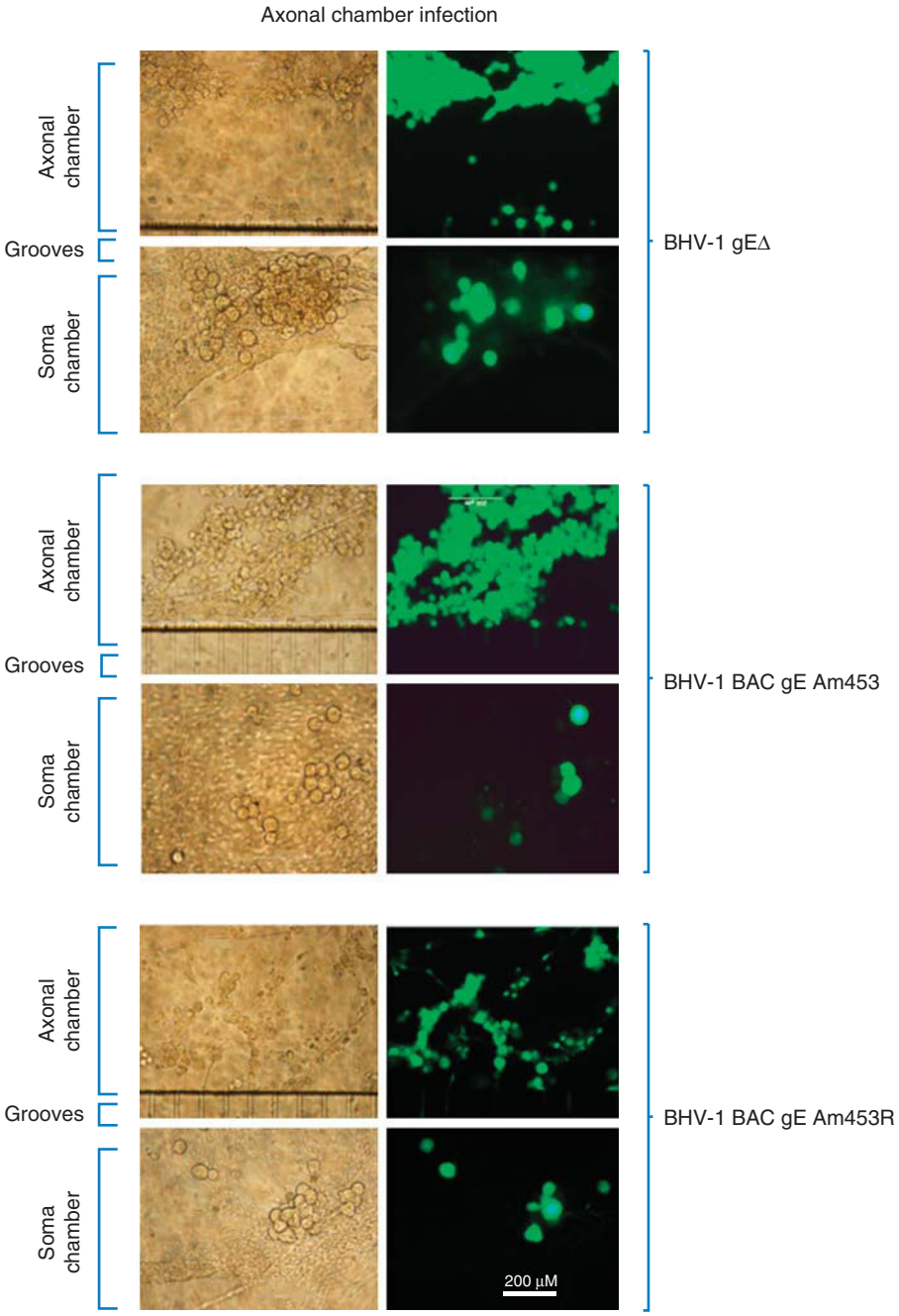

Figure 2 Cell-to-neuron retrograde spread (axonal chamber infection of BHV-1 gE-deleted (BHV-1 gEΔ) and reconstituted vBHV-1 BAC gEAm453 and vBHV-1 BAC gEAm453R (rescue)/vBHV-1 BAC (WT; data not shown) viruses. Left panels show live phase-contrast images and the right panels show the green fluorescent images (GFP) of the respective viruses in the soma and axonal chambers. MDBK cells were plated in the axonal chamber. The following day MDBK cells were infected with 1×10^7 PFU respective viruses/reservoir (approximately MOI of 50). Representative pictures shown were obtained at 24 h.p.i.

that gE cytoplasmic tail residues are essential for BHV-1 anterograde axonal transport.

Therefore, the results confirmed our earlier presumption that failure to isolate virus from the nasal ocular swabs *in vivo* in calves after dexamethasone (DEX)-induced reactivation was due the anterograde spread defect of BHV-1 gEΔ and vBHV-1 gEAm453lox viruses (see below).

Discussion

Earlier we reported that BHV-1 gE-deleted and gE cytoplasmic tail–truncated mutant viruses establish latent infection in the TG of calves following intranasal infection but fail to reactivate from latency (Brum *et al*, 2009; Liu *et al*, 2008b). Additionally, we determined that in both cases, virus replicated in

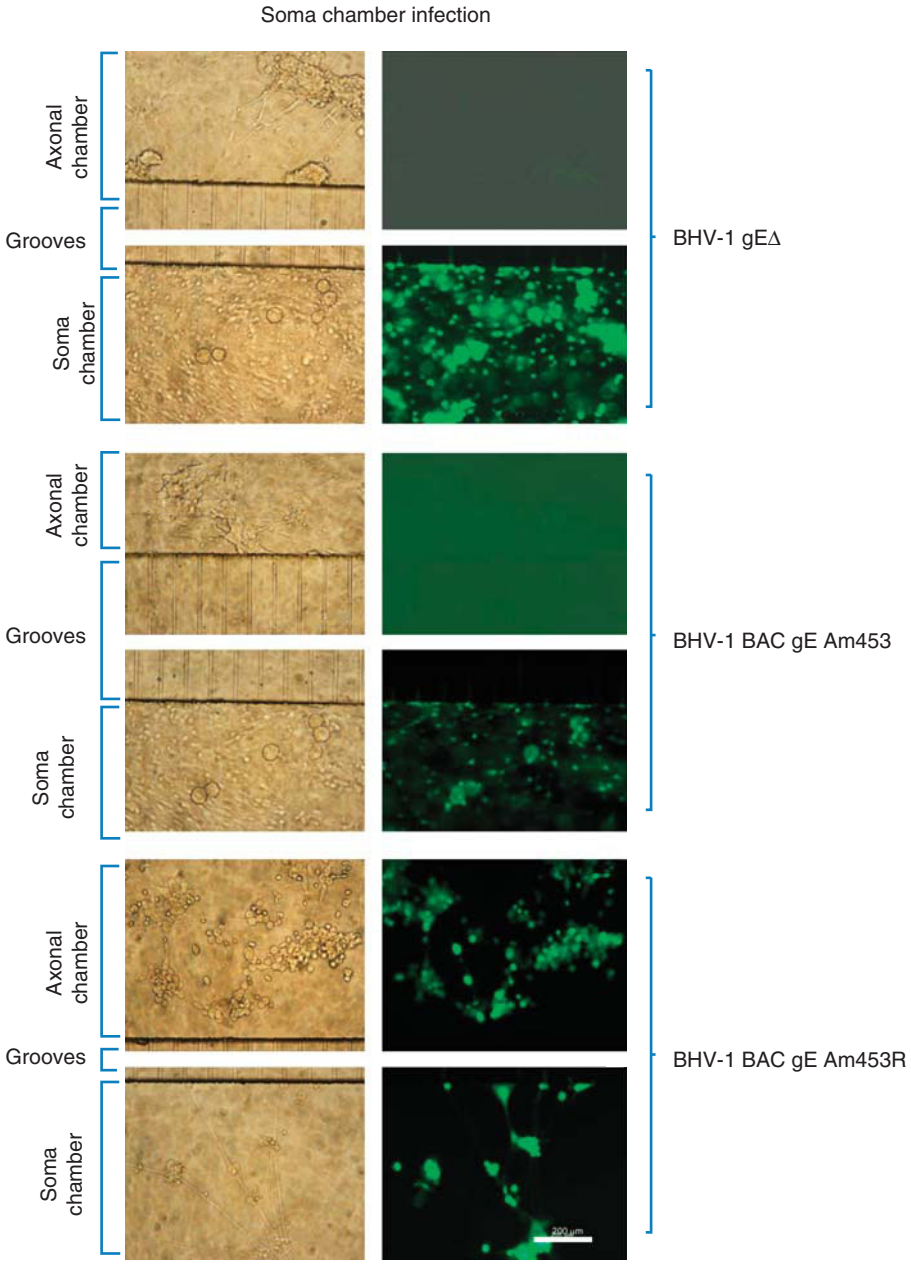

Figure 3 Neuron-to-cell anterograde spread (soma chamber infection) of BHV-1 gEΔ, vBHV-1 BAC gEAm453, and vBHV-1 BAC gEAm453R (rescue)/vBHV-1 BAC (WT; data not shown) viruses. Following MDBK cell plating, respective virus infection was performed as described in Figure 2, except the neuron cell bodies in the soma chamber were infected. Representative pictures shown were obtained at 48 h.p.i.

the TG following dexamethasone (DEX)-induced reactivation of the latent virus but was not isolated from nasal and ocular swabs (Brum *et al*, 2009; Liu *et al*, 2008b). From these results we inferred that gE was not required for retrograde axonal transport of the virus from sensory nerve ending in the nose or eye to neuronal cell bodies in TG but it was required for the anterograde transport of the virus from neuronal cell bodies in the TG to their nerve endings in the nose or eye. Because the neuronal transport of BHV-1, *in vivo*, in calves and rabbits, was not directly traceable, one of the objectives of this study was to establish primary neuronal cultures in a compartmentalized system and directly determine anterograde and retrograde transport properties of the gE mutant viruses. Results presented in this study demonstrated that relative to BHV-1 wild-type virus, the BHV-1 gE-deleted, and BHV-1 gE cytoplasmic tail–deleted viruses have defective anterograde neuron to cell transport in a compartmentalized

rabbit DRG primary neuronal culture. However, their retrograde transport from cells to neurons was unaffected.

Following intranasal and ocular infection of calves and/or rabbits, both BHV-1 gE-deleted and BHV-1 gEAm453 viruses established latent infection in the TG ((Brum *et al*, 2009; Liu *et al*, 2008b). Therefore, their retrograde transport from the sensory nerve endings to the neuronal cell bodies in the TG was not affected. As expected, when MDBK cells in the axonal chambers were infected with the BHV-1 gE-deleted, BHV-1 cytoplasmic tail–truncated (BHV-1 gEAm453), or BHV-1 gEAm453-rescued/wild-type viruses, all the viruses, including the mutants, were transported retrogradely from cells in axonal compartments to the neuronal cell bodies in the soma compartments and they replicated productively in the neuronal cell bodies.

Earlier, we had additionally determined that both BHV-1 gE-deleted and BHV-1 gEAm453 viruses replicated in the TG of latently infected calves and/or rabbits after DEX-induced reactivation of the latent viruses. However, they were not isolated from the nasal and ocular swabs (Brum *et al*, 2009; Liu *et al*, 2008b). These results implied that both BHV-1 gE-deleted and BHV-1 gEAm453 viruses have defective anterograde transport and are not transported from the TG neuron cell bodies to their nerve endings in the eye and nose. Therefore, we expected that in the microfluidic chamber system these two viruses would not be transported from the DRG neurons to MDBK cells in the axonal chamber. As expected, when the neuronal cell bodies in the soma compartments were infected with the respective viruses, gE-deleted and gE cytoplasmic tail–truncated viruses failed to transport anterogradely across the groove to the axonal chambers. In contrast, BHV-1 wild-type and BHV-1 gEAm453R viruses were transported anterogradely and replicated in the MDBK cells. Therefore, like PRV and HSV-1, gE and specifically the gE cytoplasmic tail residues are essential for the anterograde axonal transport of BHV-1 (Ch'ng and Enquist, 2005b; Tirabassi and Enquist, 1999; Tirabassi *et al*, 1997; Snyder *et al*, 2008; Wang *et al*, 2005; Yang *et al*, 1999). In conclusion, even though direct infection of the DRG neurons does not represent reactivation of the virus from a latently infected neurons in the TG, the neuronal spread results obtained from the microfluidic chamber system provided direct evidence that the lack of nasal and ocular virus shedding in calves and rabbits following reactivation was most likely due to a defect in the anterograde transport of the virus. These results further validated that the rabbit DRG primary neuronal culture in the microfluidic chambers is suitable for the study of axonal transport of BHV-1.

Recent reports in PRV demonstrated that fully enveloped viral particles are transported anterogradely within the axons in primary neurons and that membrane acquisition is required for anterograde axonal transport (Antinone and Smith, 2006; Maresch *et al*, 2010). Previously, it was reported that in PRV, cytoplasmic tails of gE and gM share redundant function in secondary envelopment because the defect in secondary envelopement is observed when both the cytoplasmic tails are deleted but not when one of them is deleted (Brack *et al*, 2000; Nixdorf *et al*, 2001). Since the gE cytoplasmic tail–truncated BHV-1 had the wild-type gM (data not shown), the anterograde spread defect of the gE cytoplasmic tail–truncated virus is not due to a defect in secondary envelopment, which is the case when both gE and gM cytoplasmic tails are deleted (Brack *et al*, 2000; Nixdorf *et al*, 2001).

In PRV, axonal targeting of capsids, viral glycoproteins, and tegument protein VP22 requires gE cytoplasmic tail residues (Ch'ng and Enquist, 2005b). Taken together, we predict that, like in PRV, the defective anterograde transport property of the BHV-1 cytoplasmic tail–truncated virus is most likely due to the defective axonal targeting of capsids and glycoproteins. Future studies in our laboratory will focus on using the primary rabbit DRG neurons in microfluidic chambers to identify the minimal gE cytoplasmic tail subdomains required for the anterograde axonal spread of BHV-1 and to identify novel neuronal protein(s) that is/are contributing to the axonal anterograde spread of BHV-1.

Materials and methods

Virus strains and cell line

BHV-1 Cooper (Colorado-1) strain, obtained from the American Type Culture Collection (Rockville, MD), was propagated and titrated in Madin-Darby bovine kidney (MDBK) cells as previously described (Brum *et al*, 2009: Butchi *et al*, 2007; Liu *et al*, 2008b). Construction and characterization of BHV-1 gE-deleted, BHV-1 gEAm453 (gE cytoplasmic tail–truncated), and their respective rescue viruses have been reported earlier (Brum *et al*, 2009; Liu *et al*, 2008b).

Neuronal cultures in microfluidic chamber system

Microfluidic chambers with microgroove length of 450 μm and a width of 10 μm (Xona SND450) were assembled, with minor modifications, as described earlier (Ch'ng and Enquist, 2005a & 2006; Liu *et al*, 2008b). Briefly, glass coverslips and microfluidic chambers were sterilized with alcohol. After air drying, coverslips were placed individually into 6-cm^2 cell culture Petri dishes and coated overnight (at 37°C in a humidified chamber) with 500 μg/ml of poly-DL-ornithine (Sigma, catalog no. P8638) in 0.1 M borate buffer. The following day, coated coverslips were washed 4 to 5 times with sterile nanopure water and air dried. Microfluidic chambers were then placed on the coverslips until it sealed to the

glass surface around the chamber. The compartments and the grooves in the microfluidic chambers were then coated overnight with 200 µl of 10 µg/ml laminin (Invitrogen catalog no. 23017-015) in calcium- and magnesium-free Hanks' buffered saline solution (HBSS-CMF) in a humidified chamber at 37°C. The following morning, prior to dissection of fetal rabbit, the microfluidic chambers were washed twice with HBSS-CMF and then neurobasal medium (100 µl/well; Gibco, catalog no. 21103) supplemented with 1% of penicillin and streptomycin was added into each well of both compartments.

A pregnant New Zealand white rabbits (Charles River), at 27 days of gestation, was then euthanized. Approximately 80 cervical dorsal root ganglia (DRG) were dissected out from six to eight fetuses and collected in 2.0-ml screw cap Eppendorf centrifuge tubes containing 1 ml of Hanks' buffered saline solution with calcium and magnesium (HBS) on ice. To remove contaminating red blood cells (RBCs), the ganglia were rinsed with HBSS-CMF by removing the supernatant carefully after the ganglia settled at the bottom of the tube. This rinsing procedure was repeated several times. After the final rinse, the ganglia were pelleted by centrifugation at 4°C (2000 rpm or 300 × *g*), resuspended in 1 ml of 5000 U/ml of prewarmed collagenase class I (Worthington, catalog no. LS004216) and incubated for 20 min at 37°C. During collagenase treatment, the ganglia were triturated by pipetting back and forth several times with a 1000-µl Eppendorf pipette attached to a cut pipette tip (wide mouth) that was precoated with sterile filtered 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) solution. Following centrifugation (2,300 rpm or 400 × *g* for 2 min), supernatant was removed and the collagenase-treated ganglia were incubated further (15 min at 37°C) in 1 ml of prewarmed trypsin (250 µg/ml; Worthington Biochemicals). After inactivation of trypsin with 100 µl of fetal bovine serum (10% final concentration), the ganglia were pelleted by centrifugation and rinsed as above. Trypsinized ganglia were resuspended in 1 to 1.5 ml of freshly prepared neuron culture medium containing neurobasal medium supplemented with 100 ng/ml of nerve growth factor 2.5 S (Invitrogen, catalog no. 13257-019), 1× B27 (Gibco, catalog no. 175204), and 1% penicillin and streptomycin. To dissociate the neurons, the medium and the trypsinized ganglia were pipetted, repeatedly, up and down using a flame polished Pasteur pipette coated with sterile 2% BSA in PBS solution. Following trituration, the dissociated neurons were loaded in the respective soma reservoirs of microfluidic chambers (see below).

Immediately before plating the dissociated neurons, neurobasal medium from the soma compartments was removed and approximately 30 to 40 µl of neuronal suspension (from above) was loaded into each of the two wells of the somal compartments and allowed to settle in the CO_2 incubator at 37°C for an hour. Then the medium in the axonal compartments was replaced with 170 µl of neuron culture medium per well. An additional 120 µl of neuron culture medium was then added into each well of the soma reservoirs and the chambers were then incubated further in a CO_2 incubator.

To inhibit and eliminate growth of non-neuronal cells, neuronal culture medium was replaced after 48 hours with neuron culture medium supplemented with 1 µM of cytosine β-D-arabinofuranoside (Ara C; Sigma-Aldrich). Twenty four hours after Ara C treatment, the medium in the soma chamber was replaced with 150 µl of neuron culture medium and the medium in the axonal chamber was replaced with freshly prepared 150 µl neurite-promoting medium (neuron culture plus neurite medium), which contained the following additional neurite growth-promoting factors: Recombinant human brain derived neurotropic factor (BDNF) 10 ng/ml (Invitrogen), recombinant rat ciliary neurotropic factor (CNTF) 10 ng/ml (Invitrogen), and recombinant human glial-derived neurotropic factor (GDNF) 10 ng/ml (invitrogen). The neuron culture medium and neurite-promoting medium were replaced every 3 days.

Indirect Immunofluorescence assay (IFA)

To determine that neuronal cell bodies and axons are expressing neurofilament-specific antigen, indirect IFA was performed using rabbit anti-neurofilament 200–specific antibody (Sigma N4142). Briefly, after 14 days of culture in microfluidic chambers, neuron culture medium was removed from the soma and axonal compartments, neurons were then rinsed briefly (2 min) 2 to 3 times with sterile Tris-buffered saline (TBS; 150 mM NaCl, 20 mM Tris) and fixed with 4% paraformaldehyde in TBS for 2 to 3 min. Microfluidic chambers were then removed carefully and the fixation was continued for an additional 7 to 8 min. After fixation, the glass coverslips containing the neurons were rinsed with plain TBS (2×), followed by washing for 15 min in 0.1% Triton X-100. After blocking in 3% immunoglobulin G (IgG)-free bovine serum albumin (Jackson Immuno Research) in TBS for 1 h, glass coverslips containing the neurons were incubated for 2 h with rabbit anti-neurofilament 200–specifc antibody. Following three washes, in TBS (15 min each), neurons were incubated for 1 h with Alexa Flour 594–conjugated donkey anti-rabbit IgG (secondary antibody). After three washes in TBS, the coverslips were mounted on a slide using a drop of mounting medium (Gel mount; Sigma catalog no. G0918), air dried, and examined under a fluorescent microscope.

Virus infection for the anterograde and retrograde transport assays

Twenty-four hours prior to virus infection when the DRG cultures are 19 to 20 days old, MDBK cells were seeded in the axonal compartment. Viral infections of neuronal cell bodies (anterograde transport assay)

or MDBK cells (retrograde transport assay) were then performed. For the neuronal cell body infection, first, the medium in the axonal chamber was replaced with 170 µl neurite medium. Then media in the two wells of the soma compartments were replaced with 120 µl of viral inoculums containing approximately 1.0×10^7 plaque-forming units (PFU) (at a multiplicity of infection [MOI] of 50). After virus adsorption (usually after 90 min), the medium in the soma compartment was replaced with 140 µl of fresh neuron culture medium. The volume difference between the soma and axonal chambers maintained a hydrostatic pressure gradient, thereby preventing the diffusion of infectious virions from the soma chamber through the microgroove into the axonal chamber. The chambers were then incubated further in a CO_2 incubator until fluorescent microscopy or harvesting of infected neurons or MDBK cells for viral plaque assay.

Virus infection of MDBK cells in the axonal compartment (retrograde transport assay) was performed as above but in a reverse order. First the medium in the soma chamber was replaced with 170 µl of fresh neuron culture medium. Thereafter, medium in the axonal chamber, containing the MDBK cells, was replaced with 120 µl of virus suspension/well containing 1.0×10^7 PFU in the neurite medium. The chambers were then processed as above and incubated further until fluorescent microscopy or harvesting of infected neurons or MDBK cells for viral plaque assay. Viral plaque assay was performed as previously described (Butchi *et al*, 2007).

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Antinone SE, Smith GA (2006). Two modes of herpesvirus trafficking in neurons: membrane acquisition directs motion. *J Virol* **80:** 11235–11240.

Brack AR, Klupp BG, Granzow H, Tirabassi R, Enquist L, Mettenleiter TC (2000). Role of the cytoplasmic tail of pseudorabies virus glycoprotein E in virion formation. *J Virol* **74:** 4004–4016.

Brum M, Coats C, Sangena BR, Doster A, Jones C, Chowdhury SI (2009). Bovine herpesvirus type 1 (BHV-1) anterograde neuronal transport from trigeminal ganglia to nose and requires glycoprotein E. *J NeuroVirol* **15:** 196–201.

Butchi NB, Jones C, Perez S, Doster A, Chowdhury SI (2007). Role of envelope protein Us9 in the anterograde transport of bovine herpesvirus-1 (BHV-1) following reactivation in the trigeminal ganglia. *J NeuroVirol* **13:** 384–388.

Ch'ng TH, Enquist LW (2005a). Neuron-to-cell spread of pseudorabies virus in a compartmented neuronal culture system. *J Virol* **79:** 10875–10889.

Ch'ng TH, Enquist LW (2005b). Efficient axonal localization of alphaherpesvirus structural proteins in cultured sympathetic neurons requires viral glycoprotein E. *J Virol* **79:** 8835–8846.

Ch'ng TH, Enquist LW (2006). An in vitro system to study trans-neuronal spread of pseudorabies virus infection. *Vet Microbiol* **113:** 193–197.

Jiang Y, Hossain AM, Winkler T, Holt T, Doster A, Jones C (1998). A protein encoded by the latency-related gene of bovine herpesvirus 1 is expressed in trigeminal ganglionic neurons of latently infected cattle and interacts with cyclin-dependent kinase 2 during productive infection. *J Virol* **72:** 8133–8142.

Jones C (1998). Alphaherpesvirus latency: its role in disease and survival of the virus in nature. *Adv Virus Res* **51:** 81–133.

Jones C, Chowdhury SI (2007). A review of the biology of bovine herpesvirus type 1 (BHV-1), its role as a cofactor in the bovine respiratory disease complex, and development of improved vaccines. *Adv Anim Health* **8:** 187–195.

Liu WW, Goodhouse J, Jeon NL, Enquist LW (2008a). A microfluidic chamber for analysis of neuron-to-cell spread and axonal transport of an alpha-herpesvirus. *PLoS ONE* **3:** e2382.

Liu Z-F, Brum M, Doster A, Jones C, Chowdhury SI (2008b). A bovine herpesvirus type 1 mutant virus specifying a carboxyl-terminal truncation of glycoprotein E is defective in anterograde neuronal transport in rabbits and calves. *J Virol* **82:** 7432–7442.

Maresch C, Granzow H, Negatsch A, Klupp BG, Fuchs W, Teifke JP, Mettenleiter TC (2010). Ultrastructural analysis of virion formation and anterograde intraaxonal transport of the alphherpesvirus pseudorabies virus in primary neurons. *J Virol* **84:** 5528–5539.

Nixdorf R, Klupp BG, Mettenleiter TC (2001). Role of the cytoplasmic tails of pseudorabies virus glycoproteins B, E and M in intracellular localization and virion incorporation. *J Gen Virol* **82:** 215–226.

Rock DL, Hagemoser WA, Osorio FA, Reed DE (1986). Detection of bovine herpesvirus type 1 RNA in trigeminal ganglia of latently infected rabbits by in situ hybridization. *J Gen Virol* **67:** 2515–2520.

Snyder A, Polcicova K, Johnson DC (2008). Herpes simplex virus gE/gI and Us9 proteins promote transport of both capsids and virion glycoproteins in neuronal axons. *J NeuroVirol* **82:** 10613–10624.

Tikoo SK, Campos M, Babiuk LA (1995). Bovine herpesvirus 1 (BHV-1): biology, pathogenesis and control. *Adv Virus Res* **45:** 191–223.

Tirabassi RS, Enquist LW (1999). Mutation of the YXXL endocytosis motif in the cytoplasmic tail of pseudorabies virus gE tail of Psudorabies virus gE. *J Virol* **73:** 2717–2728.

Tirabassi RS, Townley RA, Eldridge MG, Enquist LW (1997). Characterization of pseudorabies virus mutants expressing carboxy-terminal truncations of gE: evidence of envelope incorporation, virulence, and neurotropism domains. *J Virol* **71:** 6455–6464.

Wang F, Tang W, McGraw HM, Bennett J, Enquist LW, Friedman HM (2005). Herpes simplex virus type 1 glycoprotein e is required for axonal localization of capsid, tegument, and membrane glycoproteins. *J Virol* **79:** 13362–13372.

Wyler R, Engels M, Schwyzer M (1989). Infectious bovine rhinotracheitis/vulvo-vaginitis (BHV-1). In: Herpesvirus disease of cattle horses and pigs. Wittman, G (ed). Hingham, MA: Kluwer Academic Publishers, pp. 1–72.

Yang M, Card JP, Tirabassi RS, Miselis RR, Enquist LW (1999). Retrograde, transneuronal spread of pseudorabies virus in defined neuronal circuitry of the rat brain is facilitated by gE mutations that reduce virulence. *J Virol* **73:** 4350–4359.

This paper was first published online on Early Online on 16 November 2010.