An investigation of herpes simplex virus type 1 latency in a novel mouse dorsal root ganglion model suggests a role for ICP34.5 in reactivation

R. K. Mattila,1,2 K. Harila,1 S. M. Kangas,3 H. Paavilainen,2,4 A. M. Heape,3 I. J. Mohr5 and V. Hukkanen2

Correspondence
R. K. Mattila
riikka.mattila@oulu.fi

1Research Center for Biomedicine, Department of Medical Microbiology and Immunology, University of Oulu, Oulu, Finland
2Department of Virology, University of Turku, Turku, Finland
3Department of Anatomy and Cell Biology, Institute of Biomedicine, University of Oulu, Oulu, Finland
4Drug Research Doctoral Programme, University of Turku, Turku, Finland
5Department of Microbiology, NYU School of Medicine, New York, NY, USA

After a primary lytic infection at the epithelia, herpes simplex virus type 1 (HSV-1) enters the innervating sensory neurons and translocates to the nucleus, where it establishes a quiescent latent infection. Periodically, the virus can reactivate and the progeny viruses spread back to the epithelium. Here, we introduce an embryonic mouse dorsal root ganglion (DRG) culture system, which can be used to study the mechanisms that control the establishment, maintenance and reactivation from latency. Use of acyclovir is not necessary in our model. We examined different phases of the HSV-1 life cycle in DRG neurons, and showed that WT HSV-1 could establish both lytic and latent form of infection in the cells. After reactivating stimulus, the WT viruses showed all markers of true reactivation. In addition, we showed that deletion of the c1 34.5 gene rendered the virus incapable of reactivation, even though the virus was clearly able to replicate and persist in a quiescent form in the DRG neurons.

INTRODUCTION

The life cycle of herpes simplex virus type 1 (HSV-1) consists of two distinct phases: the productive (or lytic) phase in the epithelia and the latent phase in sensory neurons. The HSV genes are divided into three classes: α or immediate-early (IE), β or early (E) and γ or late (L). During the productive phase these lytic genes are expressed as a strictly ordered trans-acting cascade (Honess & Roizman, 1974). After replication in the mucosal epithelia, the virus enters the innervating neurons via the axonal termini. The virus is transported by retrograde axonal transport to the nucleus in the neuronal cell body, where it either initiates a productive infectious cycle or establishes latency (Roizman et al., 2007). During latency establishment in the nucleus, the virus genome is circularized to episomal form and associated with repressively modified histones on lytic gene promoters. Viral transcription occurs only from the latency-associated transcript (LAT) region of the viral genome, coding for an 8.3 kb minor LAT. The unstable long LAT is spliced to more stable 2.0 kb and further to 1.5 kb stable major LATs. There are also several microRNAs coded by the LAT region (Jurak et al., 2010; Umbach et al., 2008). Latency can last for decades, but in most cases the virus reactivates periodically (Perng & Jones, 2010; Wilson & Mohr, 2012).

The exact mechanisms of latency are currently poorly understood as it is difficult to study the virus–host interactions at the cellular and molecular level in available in vivo settings. The most widely used models for HSV-1 latency are certain strains of mice and rabbits (Webre et al., 2012). To study reactivation events at the ganglionic level, whole trigeminal ganglia from latently infected animals are often dissected and explanted on susceptible cells (Stevens & Cook, 1971). The dissected ganglion can also be cultured alone and reactivation events can be followed within it (Du et al., 2011). The early events and roles of other cell types during latency still cannot be distinguished using these methods.

A neuronal cell culture model would be a superior tool for the study of latency-related phenomena at the molecular level. The most widely used model (Wilcox & Johnson, 1988) utilizes embryonic or neonatal rat superior cervical ganglion neurons. Recently, an advanced superior cervical ganglion model has been developed and used successfully for research of HSV-1 latency and reactivation (Camarena et al., 2010; Kim et al., 2012, 2014; Kobayashi et al., 2012a, b).
HSV-1 latency has also been studied using neonatal rat dorsal root ganglion (DRG) neurons, adult mouse trigeminal ganglion neurons or whole embryonic trigeminal ganglia of chicken (Arthur et al., 2001; Bertke et al., 2011; Hafezi et al., 2012). Acyclovir (ACV) is often used to limit productive infection and neuronal death when studying HSV-1 in cell cultures (Svennerholm et al., 1981). Other means utilized in latency studies to limit the harmful effects of viral infection and spread include the use of replication-defective HSV mutants, human IgG-containing medium and compartmentalization by chamber systems (Arthur et al., 2001; Bertke et al., 2011; Hafezi et al., 2012).

The experimental models described above have been used to characterize the major steps of latency. A key factor in the process of latency establishment is the transactivating viral protein VP16. VP16 has to reach the nucleus in order to recruit cellular co-factors Oct-1 and HCF-1 (host cell factor 1) to a complex which derepresses the IE promoters in order to start the lytic cycle (Kristie et al., 1989). The IE infected cell protein ICP0 can then bring about the derepression of E and L genes (Roizman & Whitley, 2013). In the absence of VP16 in the nucleus, the HSV-1 genome remains silenced and the virus enters a non-productive phase (Hafezi et al., 2012). It is thought that similar mechanisms function during reactivation. After reactivating stimulus, all gene classes are first transcribed simultaneously. The resulting viral transactivator proteins modify repressed latent chromatin, which leads to the normal cascade of HSV-1 gene expression and virus production (Kim et al., 2012).

HSV neurovirulence relies strongly on the function of one gene, γ134.5. Deletion of the gene removes the ability of the virus to both invade and replicate in the central nervous system. In cultured human neuroblastoma cells infected with a Δγ134.5 virus, all HSV-1 mRNA classes are transcribed; however, all protein synthesis ceases after γ gene transcription (Chou & Roizman, 1992). These viruses are virulent in WT mice, but the neurovirulence is restored in γ134.5 mutant H1052, which also expresses GFP under the PmCMV promoter (Fig. 1). GFP had ceased by 11 days p.i. POST-INFECTION.

In this study, we describe the use of whole, non-dissociated mouse embryonic DRG three-dimensional cultures for HSV-1 latency studies. In our experimental setting, the use of ACV is not obligatory and the connective tissue capsule surrounding the ganglion provides a barrier for entry of the virus through the neuronal somas. We show how infection proceeds in the ganglion during a 3 week period of time and how the addition of the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 reactivates the virus within the neurons. Our results indicate that the mouse embryonic DRG model for HSV-1 latency mimics important aspects of in vivo infection. In addition, we provide evidence that Δγ134.5 viruses cannot reactivate; in particular, they do not yield infectious progeny as efficiently as WT HSV-1 virus strains even though they are able to establish a productive infection and display marker gene expression from the viral genome in the ganglia.

RESULTS

DRG cultures, infections and establishment of latency

DRGs of embryonic day 13.5 (E13.5) C57BL/6 mouse embryos were plated on three-dimensional Matrigel coated 96-well plates seeding one DRG per well. Dividing non-neuronal cells, such as Schwann cells and satellite glial cells, were eliminated with multiple cycles of treatment with 5′-fluoro-2′-deoxyuridine (as described in Methods). After the last elimination step, the cells were allowed to recover for 1 day before the infection. No cell proliferation was observed after this point. The DRG neurons were infected with the virus at m.o.i. 5 and the growth medium was replaced twice a week. The timing of the HSV-1 infection was designed to mimic the phases of in vivo latency experiments in mice and reactivation was induced at 21 days post-infection (p.i.), even though the bulk of viral shedding had ceased by 11 days p.i.

Progression of GFP expression in the ganglia

It has been estimated that a C57BL/6 mouse DRG contains ~10 000 neurons (Shi et al., 2001). The ganglia were infected using 50 000 p.f.u. per well (m.o.i. 5) either with a GFP-expressing WT virus HSV-1 (17+)Lox-PmCMV-GFP (in short, LoxGFP; Snijder et al., 2012; Fig. 1) or a HSV-1(17+)-derived Δγ134.5 mutant H1052, which also expresses GFP under the PmCMV promoter (Fig. 1).

GFP

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expression indicates that the HSV genome is present in the neurons and is derepressed. However, it does not necessarily prove production of infectious particles. GFP expression was followed throughout the experiments and images were captured at chosen time points (Fig. 2). At 2 days p.i., the expression of GFP could be observed mainly in the outmost neurons of the DRG (Fig. 2a). At 7 days p.i., 99±1% of the ganglia (140/142) contained GFP-positive neurons (Fig. 2c). In most of the ganglia all the neurons, at least in the plane of observation, appeared green, whereas in some of the ganglia only a few of the neurons expressed GFP even during the lytic phase of infection. This suggested that the ganglia differed in their susceptibility to HSV infection. GFP expression continued strong for nearly 2 weeks, after which a portion of DRGs remained GFP-positive (28±13%). GFP expression re-emerged sporadically in some individual neurons between 14 and 21 days p.i. Judged by GFP expression, the Δγ134.5 virus H1052 was also able to invade and express genes in the embryonic mouse DRG neurons (Fig. 2b). At 2 days p.i., GFP expression could be detected in 99±2% of the H1052-infected ganglia (142/144). The percentage of GFP-positive ganglia decreased to 29±9% by 21 days p.i. (Fig. 2c).

**GFP expression increases after reactivation**

On average, at 21 days p.i., GFP expression was either very low or completely absent in most of the ganglia (Figs 2 and 3a). In some cases, GFP could be detected in isolated cells within a ganglion, but this was likely due to residual fluorescence from earlier GFP expression. Still, these ganglia were included in the GFP-positive fraction.

At 21 days p.i., reactivation was induced by adding the PI3K inhibitor LY294002. Two days after the induction, 86% of the WT-like virus LoxGFP-infected ganglia were GFP-positive compared with 28±13% before reactivation. Similar enhancement in GFP expression could also be observed.

**Fig. 1.** Schematic representations of the recombinant viruses used in the study. In LoxGFP (see Methods), an EGFP cassette under a mouse cytomegalovirus (mCMV) promoter was inserted between UL55 and UL56. In H1052, both copies of the γ134.5 gene were deleted. A luciferase cassette (LUC) was inserted between UL55 and UL56, under the human CMV (hCMV) promoter, and EGFP in place of the left copy of γ134.5, under the mCMV promoter. KO, Knockout; UL, unique long; US, unique short.

**Fig. 2.** (a) Progress of HSV-1 infection in DRGs. GFP expression was detected in the outermost neurons of the DRG. Infection then proceeded further into the DRG and at 7 days p.i., almost all neurons were infected. Thereafter, GFP expression started to decline and at 21 days p.i. it was almost non-existent. After reactivating stimulus, GFP expression reappeared gradually. Upper panel, merged bright-field and GFP images; lower panel, GFP image only. (b) Progression of Δγ134.5 (H1052) infection in a DRG followed the same pattern as LoxGFP, showing that H1052 was able to invade and express genes in DRG neurons. (c) The percentage of GFP-positive ganglia was identical for both viruses.
LoxGFP (WT) and H1052 (Δγ134.5), during the course of the experiments. A ganglion was considered positive if GFP expression could be observed in at least one of the cells within the ganglion. During the first week after the infection almost every ganglia harboured GFP-expressing cells. Thereafter, GFP production started to decline and at 21 days p.i. ~30% of the ganglia, infected with either of the viruses, produced some GFP. Reactivation was carried out at 21 days p.i. with LY294002 and after 3 days, the percentage of GFP-producing ganglia had risen to 80%. Data represent mean ± SD of percentages calculated from a minimum of 30 DRGs per time point, originating from three separate experiments. p.RE, Post-reactivation.

detected with Δγ134.5 virus H1052: 29 ± 9% of H1052-infected ganglia were GFP-positive before reactivation and 82 ± 10% were GFP-positive 3 days after reactivation (Fig. 2c). However, the number of cells in a ganglion that expressed GFP after reactivation was low for both viruses in most cases (Fig. 3a). Hence, even though almost all the DRG cells were GFP-positive 7 days p.i., the virus usually reactivated only in a small fraction of these cells.

**GFP expression profiles differ between cells**

The GFP construct is under the control of a cytomegalovirus (CMV) IE promoter in the viruses we have used in this study, and GFP expression indicates the derepression of the promoter and gene expression from the HSV-1 genome. We observed that most of the cells expressed GFP at 2 days p.i., but did not reinitiate GFP expression after reactivation treatment. In a small subgroup of cells, GFP expression was first detectable, then absent and after reactivation detectable again (Fig. 3b). In some cells, GFP expression could not be observed at any time point before reactivation, but GFP expression was first launched after reactivating stimulus. This suggested that neurons differed in their susceptibility to HSV-1 latency and reactivation.

**Δγ134.5 mutant fails to restart virus production**

The ganglia were infected at m.o.i. 5 of each virus, and the medium was replaced and collected twice a week. After collection, the medium was applied on Vero cells on 24-well plates for virus culture. The plaques were allowed to develop for 4 days, fixed and the presence of infective viral particles was determined (Fig. 4). During 4–7 days p.i., plenty of infective virions were produced from ganglia infected with either of the viruses. This is in accordance with the GFP expression (Fig. 2c). At 14 days p.i., virus production had reached a stable level where ~20% of DRGs were producing virus. This level remained stable until 21 days p.i., when reactivation was induced. The majority of the WT-virus-infected DRGs started to produce virus again in 5 days. In contrast, the Δγ134.5 mutant failed to reinitiate virus production in the DRGs. The results were confirmed using HSV-1(F) based WT and Δγ134.5 viruses (data not shown).

**High viral genome load does not contribute to reactivation of the Δγ134.5 virus**

DNA samples were collected at 2, 7, 14 and 21 days p.i. Genome copy numbers were quantified by quantitative real-time (qRT)-PCR (Fig. 5). At 2 days p.i., amounts of genomes for ganglia infected with either of the viruses were equal (~1 million copies per ganglion). After this, the genome copy numbers for the WT virus LoxGFP increased to 7 days p.i. when virus production and lytic gene expression were at their highest. After this the genome copy numbers declined significantly to 0.1 million copies per ganglion at 21 days p.i. (P=0.001). In contrast, the amount of H1052 genomes did not decrease significantly by 21 days p.i.

**Amount of HSV-1 transcripts increases after reactivation**

RNA samples were collected at 2, 7, 14 and 21 days p.i., and at 20 h, 2 days, 3 days and 4 days post-reactivation. As the ganglia differed in their susceptibilities for infection, the deviation was high amongst the samples. The samples chosen for analysis were selected based on two criteria: (1) the number of mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA copies had to reach a
meaningful level of ~500 copies per sample and (ii) a HSV-1 transcript had to be detectable.

HSV-1 lytic transcripts (UL54, UL29 and VP16) could be detected at 2 days p.i. and they peaked at 7 days p.i., after which viral mRNA levels decreased significantly until 21 days p.i. VP16 gene expression normalized to GAPDH expression is presented in Fig. 6(a) as a representative transcript. After reactivation, all transcripts showed increased expression.

**Fig. 4.** Virus production from infected DRG cultures. The medium from infected DRGs was applied on Vero cells and the presence of infective virus particles in the medium was determined for each time point from media collected from at least 20 DRGs. If plaques could be observed, a ganglion was considered positive. Both the WT and the Δγ134.5 virus replicated well in the DRG cells during the first week. By 21 days p.i., the percentage of virus-producing DRGs dropped to <20%. After reactivation, it took 5 days until an increase in WT virus production could be detected. Even though H1052 exhibited a significant increase in GFP expression after reactivation, no significant increase in infective virus production could be observed. Data represent mean ± SD of percentages calculated from a minimum of 20 DRGs per time point, originating from two separate experiments. p.RE, Post-reactivation.

**Fig. 5.** Number of viral genomes in a DRG at different times during infection. The ganglia were infected at m.o.i. 5 and viral DNA was quantified at the indicated time points. The number of genome copies per DRG was quantified using qRT-PCR. The WT LoxGFP genomes peaked at 7 days p.i. and then decreased significantly by 21 days p.i. The amount of Δγ134.5 virus H1052 genomes, however, did not change significantly over time and the number of genomes still remained high at 21 days p.i. *P<0.05, **P<0.01, ***P<0.001. Six DRGs were included in the analysis for each time point.
Fig. 6. (a) Statistical comparison of GAPDH-normalized VP16 expression profiles of LoxGFP (WT) and H1052 (Δγ134.5) viruses at all studied time points. A minimum of 10 DRGs from three different experiments were used for the analysis. *P<0.05, **P<0.01. (b) Expression of HSV-1 lytic transcripts UL54 (IE), UL29 (E) and VP16 (L) in relation to LAT expression. The expression ratios are shown for both viruses at each time point. At 2 days p.i., no clear differences could be detected between the viruses. After this, the Δγ134.5 mutant H1052 expressed more LAT than the WT LoxGFP and thus the lytic:LAT ratios were lower. At day 21 p.i., no differences could be observed. After reactivation, a clear shift favouring lytic gene expression could be observed with the WT virus, whereas no change could be observed with the Δγ134.5 mutant. A minimum of six DRGs from three different experiments were used for the analysis. Data represent mean ± SEM of ratios calculated for each time point.
expression compared with 21 days p.i. for LoxGFP. When comparing 21 days p.i. and 3 days post-reactivation, a significant increase in VP16 expression could be observed ($P=0.025$). The higher mRNA expression at 3 days post-reactivation was in accordance with the GFP results. No significant changes between time points could be seen within H1052.

**Ratio of lytic transcripts compared with LAT expression**

When the copy numbers of U$_1$54, U$_1$29 and VP16 were each compared with the corresponding LAT copy numbers, it could be observed that the lytic:LAT ratios of the WT and the $\Delta$$\gamma_{134.5}$ mutant diverged from each other at most of the time points (Fig. 6b). At 2 days p.i., no considerable differences in the lytic:LAT expression ratios could be observed between the viruses. At 7 and 14 days p.i., the lytic:LAT ratios of LoxGFP were notably higher than those of H1052. At 21 days p.i., the ratios for both of the viruses were low, indicating low lytic gene expression and high LAT expression. After reactivation, the lytic:LAT ratios for H1052 remained stagnant. The LoxGFP-infected ganglia, however, started to express more lytic genes than LAT from 3 days post-reactivation onwards, which also resulted in shedding of infectious viruses (Fig. 4).

**DISCUSSION**

HSV latency has remained enigmatic since the 1930s and only during recent years have the underlying molecular mechanisms started to be unravelled. For a long time animal experiments have been the best and only way to study HSV latency. Several cell culture models have been applied to the study of HSV latency. In most of the experiments, either ACV or attenuated viruses have been used to artificially generate a quiescent form of infection. Here, we can study the relationship of the virus and the neuronal cell without the interference of other cell types. The DRGs are the dividing non-neuronal cells are eliminated by multiple rounds of mitotic inhibitors before infection, so we can study the relationship of the virus and the neuronal cell without the interference of other cell types. The DRGs are surrounded by a connective tissue capsule, so the somas are protected from direct entry of the virus, and the viruses enter via the axonal termini – the natural route of neural infection *in vivo*. This may be the reason why we are able to gain latent infection without the presence of ACV. We follow the course of infection for 21 days after which we reactivate the virus. The timing of the initial infection and reactivation can be followed using fluorescently labelled viruses, even though the capsule and spherical structure of the DRGs complicate the imaging of the innermost cells. Individual DRGs and DRG neurons seem to differ in their susceptibility to infection, which leads to high variance in the qRT-PCR results. Therefore, the number of parallel samples used for gene expression studies needed to be relatively high. In comparison with *in vivo* models, immunological cells are not present in the cultured DRGs, which facilitates studies of direct interaction of HSV with the neurons. However, the effects of the immune control on reactivation are not reproduced in our model.

In the viruses used in this study the GFP expression cassette is under the control of a cytomegalovirus promoter. It is an IE promoter that, during latent infection, is associated with repressive histones, just as are the HSV-1 lytic promoters (Stinski & Isomura, 2008). GFP expression in neuronal soma shows that the HSV-1 genome has entered the nucleus, and that at least some areas of the genome are derepressed and active.

We detected that GFP expression first starts at the peripheral part of the ganglion and then moves inwards. Nearly 100 % of the ganglia expressed GFP at 7 days p.i. and the number of GFP-expressing ganglia started to decline thereafter. At 21 days p.i., 30 % of the ganglia still contained some GFP-expressing cells, but in low numbers (1–20 cells per ganglion). After reactivating stimulus, the percentage of GFP-positive ganglia approached 80 %. Nevertheless, GFP expression could again be detected in a small fraction of cells. This also happens *in vivo* as reactivation occurs only in some latently infected cells at a time (Sawtell, 1998). One possible explanation for this is that different subtypes of neurons exhibit different microenvironments, which affects both establishment of latency and reactivation. There are, in fact, studies which support this view, as some sensory neuron subtypes seem to be non-permissive for productive infection (Bertke et al., 2011). In addition, nerve growth factor (NGF) was not removed from the medium after reactivation, which could be enough to promote the latent state of HSV in some cells.

In ganglion cultures, neurons with three GFP expression profiles were observed: (1) cells which expressed GFP within the first 2 weeks of infection, but not at 21 days p.i. or after reactivation, (2) cells expressing GFP for 2 weeks, after which the expression vanished to be reinitiated after reactivation, and (3) cells which did not express GFP before reactivation, but were able to launch GFP expression after reactivating stimulus. The first subgroup appeared as the most prevalent. These cells may either be capable of controlling the latent virus more efficiently or the virus may not be able to enter latency in these cells. The second subgroup seems to be able to terminate an ongoing lytic cycle and establish latency thereafter. Studies have shown that transient IE gene expression often precedes latency establishment (Proença et al., 2011). In the third subgroup of cells, the virus most likely enters latency directly without a preceding lytic infection. Defining the factors which separate these subgroups of neurons from each
other would provide important insights into different aspects of latency.

GFP expression seems to correlate well with virus production when examining the WT-like LoxGFP virus; however, even though an increase in GFP expression can be detected after reactivation, infective Δγ134.5 (H1052) viruses were not shed to the medium. The differences between the WT virus and Δγ134.5 mutants also included differences in genome copies per ganglion. It is notable that at 2 days p.i., the genomes of both of the viruses were present at equal numbers. However, at 21 days p.i., the number of WT virus genomes had decreased significantly, but the amount of Δγ134.5 virus genomes remained relatively constant. The WT-virus-infected cells exhibit a higher percentage of cell death, which might partly explain these observations; however, even though significantly higher numbers of Δγ134.5 virus genomes are present in the cells, only the WT virus seems to be able to reactivate fully and produce infective particles. γ134.5 is known as the neurovirulence gene and its removal renders the virus non-neuroinvasive. Neurovirulence regulation seems to be a multifactorial ability, including inhibition of both PKR signalling and autophagy. ICP34.5 is also involved in early immune evasion of the virus (Verpooten et al., 2009). It is possible that in the absence of the ICP34.5 protein, the elicited innate responses can keep the reactivating deletion viruses under control.

From the qRT-PCR data of RNA expression alone we could not detect many significant differences between the viruses; however, when the lytic mRNA:LAT transcript ratios were compared, it was clear that the Δγ134.5 virus was unable to reinitiate lytic gene expression after reactivation, when compared with WT virus after reactivation (Fig. 6b).

The Δγ134.5 viruses replicated in the DRG neurons and seemed to establish latency. Even though γ134.5 mutants are neuroattenuated in vivo, the deletion viruses have been shown to replicate in ependymal cells and in some neurons if inoculated in high amounts. In accordance with current data, Δγ134.5 viruses have also been shown to establish latency, but no reactivation events have been observed (Broberg & Hukkanen, 2005). Thus, ICP34.5 plays a crucial role during reactivation, both in vitro and in vivo.

We conclude that mouse DRGs can be used as a neuronal cell culture system to study different aspects of HSV-1 latency. Our data obtained from experiments using the system indicate that Δγ134.5 viruses cannot reactivate as efficiently as WT virus strains in mouse DRG cells, even though they are able to establish a productive infection in the ganglia. Future research will focus on the role of ICP34.5 during reactivation.

METHODS

Model for latency. The DRG cultures were established according to a previously published method (Päivälainen et al., 2008). In brief, DRGs from C57BL/6 mouse E13.5 embryos were dissected from the spinal cords. DRGs were incubated at 37 °C for 45 min in 2 ml L15 medium (Gibco) containing 2 μg dispase ml⁻¹ (Gibco), and were then washed three times by centrifugation (190 g for 5 min) and resuspension in 10 ml L15 medium containing 10 % horse serum (HS). The cleaned DRGs were then seeded in DRG Growth Medium [Eagle's minimum essential medium with Earle's salts (EMEM) with 4 g D-glucose l⁻¹, 50 ng NGF ml⁻¹, 10 % HS, 2 mM L-glutamine, 100 μ penicillin/streptomycin ml⁻¹] onto three-dimensional Matrigel (BD Biosciences)-coated 96-well plastic culture plates at one DRG per well. Proliferating non-neuronal cells were eliminated by growing the DRGs for 3 days in DRG Purification Medium (EMEM with 4 g D-glucose l⁻¹, 10 μm uridine, 10 μm 5'-fluoro-2'-deoxyuridine, 50 ng NGF ml⁻¹, 1× N2 supplement, 2 mM L-glutamine, 100 μ penicillin/streptomycin ml⁻¹) and then for 2 days in DRG Growth Medium. The purification step was repeated two more times over a period of 2 weeks. DRGs were then maintained in DRG Growth Medium for an additional 1 day before HSV-1 infections.

Viruses. The HSVs used in this study (Fig. 1) are derived from HSV-1 strain 17+. DRGs were infected using a WT-like bacterial artificial chromosome (BAC)-constructed virus HSV-1 (17+)Lox-PmCMVGFP (in short, LoxGFP; Snijder et al., 2012) or a Δγ134.5 mutant H1052 (below) that both express GFP.

Construction of viruses. The construction of WT-GFP expressing LoxGFP using BAC technology has been described previously (Nygaard et al., 2013; Snijder et al., 2012). The main difference between HSV-vector H1052 and LoxGFP is the lack of both copies of the γ134.5 neurovirulence gene in H1052. The addition of the PmCMVGFP cassette in H1052 was performed as described for LIF (leukaemia inhibitory factor) cassette insertion (Nygaard et al., 2013). The primers used to amplify the insertion were PMCMV-GFP γ134.5KO-L (5'-CCCGGGGGCCACGGGCGCCGTCCCAACCGCACAGGTCGCCAGTTAACCAACTCCGGGCGCTTGTATTAGC-3') and PMCMV-GFP γ134.5KO-R (5'-AGGCCGGCCTCGGGGTGTAACGTGTTA-CCGAGTTCGCGGCGCGTGCCCAACCGCACAGGTCGCCAGTTAACCAACTCCGGGCGCTTGTATTAGC-3'). In the place of the GFP cassette in LoxGFP, H1052 had a PmCMV-luciferase cassette. In addition to the previously described virus construction method, H1052 was plaque purified three times according to GFP expression. All the viruses were propagated in Vero (ATCC) cells and infection titres were quantified using a plaque assay (Nygaard et al., 2013).

Infection of DRGs. DRG cells were allowed to recover and were then infected with either recombinant HSV1 or WT-like virus. The medium was replaced with new DRG Growth Medium. The virus was reactivated after 21 days by adding the PI3K inhibitor LY294002 (Sigma-Aldrich) to cells (20 μM, 2.5 h). Monitoring GFP expression. Ganglia were monitored throughout the experiments and GFP expression was assessed. If one or more cells in a ganglion expressed GFP, the ganglion was considered positive. The number of GFP-positive DRGs was compared with the overall number of DRGs for each time point.

Total RNA isolation and cDNA preparation. RNA samples were collected at 2, 7, 14 and 21 days p.i., and at 20 h, 2 days, 3 days and 4 days post-reactivation, in TRI pure (Isogen Reagent (Roche), and RNA was extracted according to protocol. Total RNA was dissolved into 14 μl RNase-free water and treated with DNase I (New England Biolabs). cDNA was prepared with random hexamer primers using a High-Capacity cDNA Revertase Transcriptase kit (Applied Biosystems) with equal volumes of the total RNA as a template in all reactions.
Table 1. Primers for qRT-PCR detection of the HSV-1 transcripts and mouse GAPDH

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Total DNA extraction. Viral DNA was extracted using a ZR Viral DNA/RNA kit (Zymo Research) according to the manufacturer’s protocol (version 1.0.2) at 2, 7, 14 and 21 days p.i.

qRT-PCR. qRT-PCR was run on a Rotor-Gene 3000 (Corbett Life Science) using a Maxima SYBR Green qPCR Master mix (Thermo Scientific). The PCR protocol consisted of an initial incubation for 15 min at 95 °C followed by PCR cycling using a three-step cycle at 95 °C for 15 s, at 60 °C for 30 s and at 72 °C for 45 s for a total of 40 cycles. Cellular GAPDH mRNA expression was quantified by qRT-PCR as a control for changes in nucleic acid extraction and in cellular mRNA levels during the HSV infections. PCR primers used for amplification are presented in Table 1.

Quantification of virus shedding into medium. Fresh medium was added 2 days p.i. and later twice a week until reactivation. After collecting medium samples, the presence of infective virions (yes/no) was determined on 24-well plates of Vero cells.

Imaging of live cells. DRGs were infected with GFP-expressing viruses H1052 or LoxGFP. The progress of GFP expression was followed using an Axio Vert.A1 microscope (Zeiss). ZEN 2011 software was used for imaging.

Statistical analysis. The statistical analysis was done with SPSS Statistics 19 (IBM) using the Kruskal–Wallis non-parametric one-way ANOVA test.

ACKNOWLEDGEMENTS

We thank Professor Beate Sodeik for the HSV-1 (17+)Lox-P<sub>ΔmCMV</sub>GFP virus. This work was supported by the Academy of Finland (259725), the Tyyni Tani Fund, the Finnish Concordia Fund, the Oulu University Support Foundation, the Maud Kuistila Remembrance Foundation and the Orion Research Foundation.

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