Cryo Electron Tomography of Herpes Simplex Virus during Axonal Transport and Secondary Envelopment in Primary Neurons

Iosune Ibiricu1, Juha T. Huiskonen1,2,3, Katinka Döhner4, Frank Bradke5,6, Beate Sodeik4, Kay Grünewald1,3*

1 Department of Molecular Structural Biology, Max Planck Institute of Biochemistry, Martinsried, Germany, 2 Institute of Biotechnology, University of Helsinki, Helsinki, Finland, 3 Oxford Particle Imaging Centre, Division of Structural Biology, Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, United Kingdom, 4 Research Group Axonal Growth and Regeneration, Max Planck Institute of Neurobiology, Martinsried, Germany, 6 German Center for Neurodegenerative Diseases (DZNE), Bonn, Germany

Abstract

During herpes simplex virus 1 (HSV1) egress in neurons, viral particles travel from the neuronal cell body along the axon towards the synapse. Whether HSV1 particles are transported as enveloped virions as proposed by the ‘married’ model or as non-enveloped capsids suggested by the ‘separate’ model is controversial. Specific viral proteins may form a recruitment platform for microtubule motors that catalyze such transport. However, their subviral location has remained elusive. Here we established a system to analyze herpesvirus egress by cryo electron tomography. At 16 h post infection, we observed intra-axonal transport of progeny HSV1 viral particles in dissociated hippocampal neurons by live-cell fluorescence microscopy. Cryo electron tomography of frozen-hydrated neurons revealed that most egressing capsids were transported independently of the viral envelope. Unexpectedly, we found not only DNA-containing capsids (cytosolic C-capsids), but also capsids lacking DNA (cytosolic A-/B-capsids) in mid-axon regions. Subvolume averaging revealed lower amounts of tegument on cytosolic A-/B-capsids than on C-capsids. Nevertheless, all capsid types underwent active axonal transport. Therefore, even few tegument proteins on the capsid vertices seemed to suffice for transport. Secondary envelopment of capsids was observed at axon terminals. On their luminal face, the enveloping vesicles were studded with typical glycoprotein-like spikes. Furthermore, we noted an accretion of tegument density at the concave cytosolic face of the vesicle membrane in close proximity to the capsids. Three-dimensional analysis revealed that these assembly sites lacked cytoskeletal elements, but that filamentous actin surrounded them and formed an assembly compartment. Our data support the ‘separate model’ for HSV1 egress, i.e. progeny herpes viruses being transported along axons as subassemblies and not as complete virions within transport vesicles.

Introduction

Herpes simplex virus type 1 (HSV1) is the prototype of the Alphaherpesvirinae, a subfamily of the Herpesviridae. Viruses of this subfamily establish lifelong latent infections in the nervous system of the host organism. About 80 percent of the human population is infected with HSV1. The infection is typically manifested by cold sores near the oral cavity but can also provoke ocular lesions and in rare cases encephalitis. The pleomorphic HSV1 virion has a complex structure [1,2]: the viral capsid encloses the double-stranded DNA genome and is surrounded by an amorphous layer i.e. the viral envelope. The capsid is an icosahedrally symmetric protein shell with a diameter of 125 nm and composed of 162 capsomers [3–7]. Of these capsomers, 150 are hexons, 11 are pentons and one is the portal [8–10], responsible for DNA packaging into the capsid after procapsid assembly. The pentons and the portal are located at the 12 vertices. The structures connecting adjacent capsomers are termed triplexes.

HSV1 capsids assemble in the nucleus with the help of a scaffold protein [11]. The nuclear capsids have been classified into four types: round procapsids and angular A-, B- and C-capsids. Both A- and B-capsids are devoid of DNA, but B-capsids retain the scaffolding protein [12]. C-capsids are mature capsids containing the viral genome [11]. C-capsids have been reported to contain larger amounts of the minor capsid proteins pUL17 and pUL25 than A- and B-capsids [13–16]. When compared to virions, nuclear capsids are virtually devoid of tegument [16–20], although it has been suggested that association of the tegument proteins

* E-mail: kay@strubi.ox.ac.uk

Copyright: © 2011 Ibiricu et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: We acknowledge support by Deutsche Forschungsgemeinschaft Grants GR1990/1-2, 1-3, 1-4 and GR1990/2-1 (to KG) and So403/3 (to BS), Human Frontiers Science Programme grant RGY0079/2009-C (to KG), a Wellcome Trust Senior Research Fellowship (to KG), a NanoScienceE+ grant (to KG and BS, DFG GR1990/3-1 and So403/4-1), a European Commission New Emerging Science and Technologies Contract 012702 (to BS), the Academy of Finland grants 114649, 130750, 218080 (to JTH) and the Wellcome Trust core award 090532/Z/09/Z to the WTCHG. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.
pUL36 and pUL37 might occur already in the nucleus [21,22].
Primary envelopment of the newly assembled capsids takes place
in the nucleus [21,22].

In neurons, egressing HSV1 particles are transported over long
distances. For alphaherpesviruses the nature of the particles
undergoing axonal transport from the nucleus to the cell periphery
(termed anterograde transport) is debated, in particular for HSV1
and pseudorabies virus (PrV). Two models of viral assembly have
been suggested. According to the ‘separate model’ (synonym:
subassembly model) [24,26,27], cytosolic capsids lacking an
envelope are transported along microtubules while the viral
tegment proteins and glycoproteins travel separately or in
association with transport vesicles. In this model, assembly of
mature virions occurs at axonal varicosities or at axon terminals
[17,26–32]. Conversely, according to the ‘married model’, viral
particles are transported from the cell body towards the axon
terminal already fully assembled and inside transport vesicles [33–
37]. In both models, the HSV1 particles are transported in the
cytoplasm along microtubules by cellular microtubule motors
(reviewed in [24,38–40]). One particular feature of all cytoskeletal
motors is that they move actively only in one direction: either to
the plus-end-directed microtubule motors, such as kinesin-1 or
kinesin-2 [16,39,43,44]. Several tegument proteins are essential
for intracellular transport of capsids and may contribute to
forming viral motor binding sites. In particular, it has been shown
that the tegument proteins pUL36 and pUL37 are essential for
capsid transport during entry and egress [40–50]. Moreover,
HSV1-GFPVP26 capsids lacking most of the outer tegument
proteins, but still containing inner tegument proteins such as
pUL36 and pUL37, are transported along microtubules in vitro
in the presence of cytosol [18]. Furthermore, Radtke et al. [16]
have shown that pUL36 and pUL37 are accessible on the surface
of capsids that recruit motors in vitro. It has also been suggested
that the capsid protein VP26 is involved in retrograde transport of
capsids [51], although other studies have demonstrated that this
protein is not essential for dynein-mediated transport [52–54].
Furthermore, VP26 is also not required for recruiting dynein and
kinesin onto isolated capsids in vitro [16,18]. The tegument protein
pUS11 was shown to bind to kinesin-1 [55], although it does not
appear necessary to recruit kinesin-1 to capsids in vitro [16].

Little is known about the location and identity of the tegument
proteins bound to capsids in situ during transport. A previous study
of HSV1 virions using cryo electron microscopy and single particle
icosahedral reconstruction has revealed only a small ordered
density of tegument located at the vertices of the capsid [56]. This
density has been suggested to be formed by the inner tegument
protein pUL36. Furthermore, earlier conventional electron
microscopy studies have shown capsids inside cells with substantial
densities bound at the vertices [45]. Together, these results have
suggested that the molecular motor complexes might attach to
the vertices of the capsid but this binding platform has remained
uncharacterized.

Here, we applied cryo electron tomography (cryoET, [57]) to
analyze the three-dimensional structure of HSV1 particles during
anterograde axonal transport. By virtue of this technique, the
rapidly frozen specimen is kept vitrified in near-native conditions
[58,59] and does not suffer from structural re-arrangements
caused by chemical fixation, dehydration or heavy metal staining.
The vast majority of progeny capsids found in axons were
non-enveloped; hence, our data support the separate model of
anterograde axonal transport. Surprisingly, not only cytosolic
capsids containing DNA but also capsids devoid of DNA had been
transported along the axons despite significant differences in the
amount of tegument associated with them. Further, three-
dimensional cryoET snapshots of capsid assembly by secondary
envelopment in axon terminals suggest that secondary envelop-
ment might involve vesicle fusion to form a sufficiently large
enveloping compartment.

Results

Establishing an experimental system of viral intra-axonal
transport accessible to cryoET

Intracelular transport of alphaherpesviruses is often studied in
neurons of dissected nervous ganglia explants, e.g. in sympathetic
neurons of rat superior cervical ganglia (SCG). Unfortunately, this
system is not accessible to analysis by cryoET for two reasons.
First, the size of the explants is typically prohibitive as, upon
freezing, it causes an ice thickness exceeding the penetration limit
of electrons (~1 µm) [57]. Placing the EM grids further away from
the explant to analyze flat regions of the outgrown neurons is
likewise not applicable because the grids then needed to be
removed prior to freezing. In this case, the neurons would be
damaged thus preventing a native in situ analysis. Therefore, we
chose to use primary neurons cultured after dissociation.
In early experiments, we analyzed primary neurons from dissociated rat dorsal root ganglia (DRG) by cryoET. These peripheral sensory neurons present a near-native model for studying HSV1 transport. Unfortunately, also in this system, the prominent thickness of the cell bodies resulted in a specimen thickness that impeded cryoET. The hippocampus is a brain region that is typically infected during herpes simplex encephalitis in humans [60]. Therefore, we next analyzed hippocampal neurons, which are also a close-to native system for the study of HSV1 infection and, in addition, provided extended areas suitable for cryoET of axons.

Fluorescence microscopy imaging of progeny HSV1 capsids in axons of primary neurons

Primary hippocampal neurons cultured on electron microscopy grids (Figure 1A) were infected with HSV1. Infection was followed by live cell imaging using HSV1(KOS)-GFPVP26 [52]. VP26 is a small capsid protein located on top of the hexons [61,62]. At 2 h post infection, the axons contained only occasionally fluorescent HSV1 particles (not shown). Around 16 hours post infection (p.i.), after synthesis of progeny viruses, massive egress of fluorescent viral particles occurred (Figure 1B). Around 60% of the fluorescently labeled particles were motile. The average velocity of the viral particles during anterograde transport was 2.4 μm/s (Figure 2, Table S1), consistent with earlier reports [28,42,48,49]. Despite frequent changes in direction, capsid transport along axons had a preferred orientation towards the cell periphery and anterograde transport on average was faster than retrograde transport (Figure 2, Table S1).

Cytosolic non-enveloped progeny capsids were transported in axons

Samples vitrified at 16 h p.i. were analyzed by cryo electron microscopy. Often, at least two capsids per field of view (1.65×1.65 μm) were recognized by cryo electron microscopy (cryoEM) 2D projection images (Figure 1C). Using cryoET, we identified the transported particles as non-enveloped cytosolic capsids (Figure 1D–F). In the axon, they were consistently found in close proximity to microtubules (Figure 1D–F; arrows).

Axon cytosol contains not only C-capsids but also A- and B-capsids

Besides cytosolic DNA-containing C-capsids, unexpectedly, there were also cytosolic A- and B-capsids present in the axons, although in lower numbers than C-capsids. In 2D projections of thicker cellular specimens, structural features were superimposed upon one another, and thus difficult to interpret. In contrast, in three-dimensional tomographic reconstructions, the capsids were clearly discernible from cytoplasmic vesicles and finer features such as individual capsomers as well as the structural elements in the cytoplasm became recognizable (Figure 1D–F). We could clearly identify cytosolic A-capsids as well as cytosolic B-capsids. While the former were angular and empty, the latter contained densities of the scaffold protein in the capsid lumen. Their morphology was clearly different from that of fully DNA-packaged C-capsids (Figs. 1D–F, S1). We noted that cytosolic A- and B-capsids were not observed after infection with HSV1(KOS) or the HSV1(KOS)-GFPVP26 variant, whereas they comprise the majority in HSV1(F) (Table 1). The microtubules had luminal densities consistent with earlier observations [63] (Figure 1D–F).

Figure 1. Transport of HSV1 capsids in neurons during egress. (A) Bright field image of a hippocampal neuron grown on a holey carbon support film for 7 days. A neurite (white arrow) and cell body (asterisk) are indicated. Bar: 6 μm. (B) Series of time-lapse wide-field fluorescence images of a mid-axon region of a HSV1(KOS)-GFPVP26 infected neuron at 16 hours p.i. Pictures were taken every 2 seconds, as indicated by the number in the lower left corner. The left image shows an overlay of the fluorescence channel with the bright field image. Arrows indicate the positions of individual viral particles. Bar: 5 μm. (C) Cryo electron microscopy (projection image) of an intact axon at 16 h p.i. Cytosolic C-capsids are framed in black and cytosolic A-capsids in black dashed squares. (D–F) Slices through the reconstructed tomographic volume obtained from the area of interest in (C). pm: plasma membrane; mt: mitochondria; black arrows: microtubules. Bars in (C–F): 200 nm.
doi:10.1371/journal.ppat.1002406.g001
While dense material was associated occasionally with cytosolic capsids (data not shown), we could not assign unequivocally such densities to cellular microtubule motors. Overall, the capsids seem to contain very little tegument, and the capsomers were not obscured by tegument or cellular protein complexes but were clearly recognizable.

Icosahedral vertices of axonal capsids are the sites of capsid-tegument interaction

Earlier studies have shown that some tegument proteins are essential during intracellular capsid transport [48–50,64,65]. To analyze the interactions between capsid and tegument during transport, we averaged the densities of cytosolic capsids that were computationally extracted from tomograms of infected neurons. The 14 tomograms acquired of neurons infected either with wild type strains HSV1(F) or HSV1(KOS), or with HSV1(KOS)-GFPVP26 contained a total of 67 cytosolic capsids (Table 1) (Figure 3Ai-iv; Figure 4A, C). Cytosolic A-/B-capsids were compared to the average of 158 nuclear A-capsids, likewise biochemically purified from nuclei of infected cells (Figure 3Bi-iv; Figure 4B, D). The resolution for both groups of nuclear capsid averages was 5.6 nm. Nuclear capsids are known to be virtually devoid of tegument proteins [16–19]. Therefore, a comparison of native cytosolic capsids to biochemically purified nuclear capsids could reveal features on cytosolic capsids that correspond to tegument proteins acquired shortly before nuclear egress or in the cytosol. Indeed, this comparison revealed a prominent extra density, located exclusively at the C-capsid vertices (Figure 4A, C; blue). It was present on top of the pentons and connected further to the positions of the two adjacent triplexes and to one side of the neighboring hexons. Thus, these extra densities were only positioned on hexon-penton interfaces but not on hexon-hexon interfaces.

Cytosolic A-/B-capsids possess less tegument density at the capsid vertices than cytosolic C-capsids

The comparison of cytosolic A-/B-capsids to the nuclear A-capsids showed that cytosolic A-/B-capsids comprised only a small amount of extra density (Figure 4B, D; green). Nevertheless, this extra density was also located exclusively at the vertices, in particular towards one side of the peripentonal hexons. On cytosolic A-/B-capsids, no extra density was present on top of the pentons.

Few enveloped virions in middle regions of axons

Occasionally, there were enveloped virions in regions of the axons that were quite some distance away from both, the soma and the axon terminals (Figure 5), although at much lower frequency than non-enveloped capsids. Of the 73 capsids located in middle regions of axons at 16 h p.i., less than 10% were enveloped while the others were non-enveloped (ratio 6:67). Nevertheless, such enveloped virions were also located in close proximity to microtubules (data not shown).

Secondary envelopment occurs in axon terminals

Secondary envelopment sites were characterized by capsids being in close proximity to groups of vesicles (Figure 6). Three-dimensional analysis revealed that such assembly sites lacked any cytoskeletal elements, but that filamentous actin rather surrounded these assembly sites (Figure 6C, D). In contrast, there were no microtubules in these areas. Notably, the vesicles in assembly sites had different sizes and were characterized by two different morphologies. Some of the vesicles were studded with spike-like

Table 1. Frequency of viral particle types found in middle regions of axons for the HSV1 strains used for infection.

| HSV-1 strain | Cytosolic A-capsids | Cytosolic B-capsids | Cytosolic C-capsids | Enveloped virions | Number of tomograms |
|--------------|---------------------|---------------------|---------------------|------------------|---------------------|
| F            | 6                   | 20                  | 13                  | 3                | 7                   |
| KOS          | 0                   | 0                   | 4                   | 3                | 5                   |
| KOS-GFPVP26  | 0                   | 0                   | 24                  | 0                | 2                   |

Figure 2. Directionality and run lengths of intracellular transported viral particles at 16 h p.i.. Each bar corresponds to an individual single run, 15 in total. Particles that entered or ran out from the field of view during the 10 min observation period were also taken into account. These data are coming from two different observations. For more detail see Table S1. Black bars: anterograde transport, white bars: retrograde transport.

doi:10.1371/journal.ppat.1002406.g002

Table 1. Frequency of viral particle types found in middle regions of axons for the HSV1 strains used for infection.

doi:10.1371/journal.ppat.1002406.t001
densities, protruding from the membrane into the lumen of the vesicle (Figure 6C, black arrowhead, Figure 6D, yellow densities). In contrast, other vesicles showed a smooth luminal surface. Typically, electron-dense material, presumably tegument, was accreted on the cytoplasmic face of vesicles with spike-like densities on their interior side (Figure 6C, black arrows). When those vesicles had a concave cytoplasmic side it was typically facing towards a capsid. By virtue of our three-dimensional reconstructions, we revealed that at least in some cases the volume of individual spike-studded vesicles appeared not to suffice to fully enclose a capsid.

Discussion

In this study, we used cryoET to visualize HSV1 capsid-tegument interactions in 3D during axonal transport in vitrified hippocampal axons. To this end, we first established a close-to-native experimental cell system enabling us to follow intra-axonal herpesvirus transport by both fluorescence microscopy and cryoET. By culturing dissociated hippocampal neurons directly on electron microscopy grids, we were able to circumvent the practical limitations that dissected nerve ganglia explant systems pose for cryoET. Furthermore, this type of primary neurons provides a relevant model for HSV1 since the hippocampus in the brain is infected during herpes simplex encephalitis in humans [60].

Culturing the hippocampal neurons on electron microscopy grids did not impair the course of HSV1-infection. Live-cell imaging using HSV1(KOS)-GFPVP26 revealed a peak in anterogradely transported virus particles at 16h p.i.. This time point is consistent with earlier reports on egressing HSV1 in infected neurons [29,36,66,67]. The average anterograde speed of 2.4 μm/s of the HSV1 particles (Figure 1C, Table S1) agrees well with earlier observations [41]. These transport rates suggest active transport by
a kinesin, e.g., kinesin-1 or kinesin-2 which have been shown to bind to isolated, tegumented HSV1 capsids in vitro [16]. The characteristic pattern of net anterograde transport despite intermittent changes in directionality has likewise been reported before for herpesvirus egress [41,42]. It most likely reflects the engagement of motor complexes with opposite directionality on the same viral particle [16,68]. CryoET is not the adequate tool for a systematic, statistical analysis, since it is limited to axon areas thin enough to be penetrated by the electron beam. Nevertheless, on a population level, the number of egressing HSV1 particles was fully sufficient to characterize particles in transit at higher resolution by cryoET. The majority of the viral particles in mid-axon regions were non-enveloped cytosolic capsids.

We next focused on the organization of tegument proteins on these cytosolic, axonal capsids to identify the interaction platform for the attachment of microtubule motors mediating intracellular transport. For HSV1(F), we detected all three intra-axonal capsid types – 52% contained DNA (cytosolic C-capsids), while the remaining ones lacked DNA (15% cytosolic A- and 33% B-capsids, respectively). We also frequently observed A- and B-type capsids during and after secondary envelopment in axon terminals (data not shown). In contrast to a prevailing hypothesis [13,69], our results indicate that A-/B-capsids leave the nucleus and are actively transported to the cell periphery as it had been suggested previously [3,70]. Cytosolic B-capsids have also been reported for another herpesvirus, simian cytomegalovirus [71]. Whether the fact that the majority of intra-axonal capsids were cytosolic C-capsids reflects their higher efficiency in nuclear egress or just the ratio of nuclear C-capsids to A-/B-capsids remains to be determined. Formally, we cannot exclude that the packaged DNA genome had been lost at a later stage in the cytosol resulting in the appearance of cytosolic A-type capsids. However, it seems very unlikely that cytosolic C-capsids would give rise to cytosolic B-capsids.

Interestingly, we observed cytosolic A-/B-capsids only when infecting with HSV1(F), while neurons infected with HSV1(KOS) (wild-type or -GFPVP26) lacked them (Table 1). Negatsch et al. reported recently for HSV1(KOS) a lack of pUS9 expression [72]. The US9 region of our HSV1(KOS) has the same mutations as reported by Negatsch et al. (2011), while our HSV1(F) lacks any mutations in the pUS9 region (data not shown). pUS9 had earlier been reported to play a role in herpesvirus axonal transport [32,73]. Our results suggest that also nuclear egress of the HSV1(KOS) capsids may be either highly specific for C-capsids or impaired for A-/B-capsids when compared to HSV1(F). Whether this difference due to the changes in the US9 gene remains to be established. Thus, in a situation where nuclear capsid egress is highly specific for C-capsids as observed here for HSV1(KOS), A- and B-capsids might be retarded in the nucleus.

Figure 4. Difference maps between cytosolic capsids and nuclear capsids. (A) Difference map between cytosolic C-capsids and nuclear C-capsids, superimposed onto the nuclear C-capsids average. (B) Difference map between cytosolic A-/B-capsids and nuclear A-capsids, superimposed onto the nuclear A-capsids average. (C, D) Close-up view of a vertex in (A) and (B), respectively. The isosurface thresholds for the difference maps are 1.5σ above the mean density in (A) and 0.5σ in (B). doi:10.1371/journal.ppat.1002406.g004

Figure 5. Enveloped virions in middle regions of axons. (A) Schematic diagram of a neuron indicating the mid-axon region. (B) Cryo electron microscopy (projection image) of a pair of enveloped virions (boxed areas) in a mid-axon region vitrified at 16 h p.i.. Note the bundle of microtubules entering and leaving this area. Bar: 200 nm. (C) CryoET slice through the respective reconstructed tomographic volume for the field shown in (B). Asterisk: enveloped capsids; mt: microtubule; pm: plasma membrane. Bar: 200 nm. doi:10.1371/journal.ppat.1002406.g005
The late time point of vitrification at 16 h p.i. suggests that both cytosolic, axonal progeny C-capsids and A-/B-capsids presented an adequate tegument composition to be transported towards the cell periphery for assembly and exit. The abundance of potential parental, incoming capsids derived from newly produced viruses superinfecting these axons is very low, because there were virtually no capsids in cells that had been vitrified 5 to 20 minutes p.i. even when using an MOI of up to 200 (data not shown).

The combination of cellular cryoET with subvolume-averaging allowed visualizing the tegument density distribution on intra-axonal capsids in unprecedented detail. Subvolume-averaging for macromolecules inside cells has been barely performed so far [74]. The reasons for this are that it is difficult to identify macromolecular complexes within a cellular context, and that the number of complexes of interest within cells is low compared to in vitro particle preparations. Comprehensive knowledge on the protein composition of the intra-axonal cytosolic capsids is lacking since so far they could not be purified for quantitative mass spectrometry analysis. The existing information on these particles is based on data using fluorescently tagged proteins and immunolabelling experiments and therefore incomplete. Here, we obtained novel information by averaging subviral structures in their native surroundings. This will enable future studies on their interactions with other host factors, and will allow to correlate such data derived from in situ / in vivo experiments with the results of biochemical systems reconstituting key intermediate steps in vitro [75].

In our study, subvolume-averaging in situ including icosahedral symmetrisation showed that tegument proteins associated exclusively with the capsid vertices. In accordance with previous studies from isolated virions [61], the capsid protein VP26 did not contact the extra density present on such cytosolic capsids. This supports the notion that VP26, located on top of the capsid hexons [6], but not the vertex pentons, is dispensable for capsid transport [51-54]. Our results furthermore agree with biochemical studies that VP26 is not required for recruiting dynein or kinesin-1 onto capsids [16,18]. In turn, the tegument material exclusively located around the capsid vertices supports the notion that the molecular motors mediating transport might bind to the vertex region as it has been suggested previously [45].

The striking differences in the tegument structure between cytosolic C-capsids and cytosolic A-/B-capsids (Figs. 3 and 4), most notably the presence of extra density on top of the pentons of the cytosolic C-capsids, provided valuable insights into the complex capsid–tegument interaction network. The minor capsid protein pUL25 forming a heterodimer with the protein pUL17 has been attributed to a density termed “elongated C-capsid specific component (CCSC)” in cryoEM reconstructions of nuclear C-capsids [13,76]. The CCSC is barely visible on nuclear A- and B-capsids, consistent with a lower abundance of both proteins that has also been confirmed by proteomic data [13,77]. Both pUL25 and pUL17 have been localized on nuclear A- and B-capsids by cryoEM and TAP pulldown assays [76,78]. In these studies the protein complex has been termed “capsid-vertex specific component (CVSC).” Furthermore, pUL25 can interact with pUL36, the largest herpesvirus tegument protein, in HSV1 [50,79] and in PrV [22] that in turn interacts with the tegument proteins pUL37 and VP16 [65,80-82]. In accordance with these studies, our results show that cytosolic C-capsids were associated with a higher amount of tegument than cytosolic A-/B-capsids. This is consistent with the cytosolic C-capsids comprising higher amounts of pUL17/pUL25, and therefore binding more tegument than cytosolic A-/B-capsids. Nevertheless, this low tegumentation on cytosolic A-/B-capsids appeared to be sufficient for at least some capsid transport from the neuronal soma into the axons. Thus, full coverage of all capsid vertices by tegument seems not to be required for microtubule transport, and tegument recruitment onto even one vertex might be sufficient albeit barely detectable in the icosahedral average reconstruction of cytosolic A-/B-capsids.

Non-enveloped HSV1 cytosolic capsids detected inside hippocampal axons are in agreement with the ‘separate model’ of alphaherpesvirus axonal anterograde transport [17,24,26-31]. Further supporting this model, we identified sites of secondary envelopment at axon terminals (Figure 6). We also observed enveloped virions in mid-axon regions (Figure 5), but at a much lower rate than non-enveloped particles (Table 1). CryoET is not an adequate tool for a statistical analysis, but the ratios between enveloped and non-enveloped capsids nevertheless indicate a trend. Two different scenarios may explain this. First, it is possible that the virions in middle regions of axons underwent secondary envelopment in a varicosity, and that they would eventually exit the cell also from here as reported previously [29]. This would imply that even though enveloped viral particles were sporadically
observed, they might not undergo long distance transport. Although axon terminals appeared as the main envelopment and exit sites for HSV1, some enveloped particles may have been generated in the soma, and only afterwards entered the axons. A recent report comparing PRV and several strains of HSV1 reports that in explanted primary neurons from rat superior cervical ganglia, for HSV, about 75% of the viral particles in the axon and growth cone were enveloped and 25% non-enveloped [67]. Thus, the assembly pathway of HSV1 may be more complex than anticipated by the ‘married’ or ‘separate’ models for HSV1 axonal transport. Further studies comparing a wider range of neurons derived from different structures of the nervous system and other strains of alphaherpesviruses will ultimately reconcile these apparent discrepancies. Furthermore, combinations of different tags on VP26 with additional mutations in US9 or other herpesviral genes may result in complex phenotypes in axonal transport that may not be recognized or remain silent during infection of epithelial cells.

CryoET is the method of choice for visualizing filamentous actin. Our native three-dimensional analysis of the axon terminals revealed that the secondary envelopment sites themselves were devoid of filamentous actin while the actin meshwork surrounding them seemed to form the boundary of an assembly compartment. Given that the dimension of these compartments was around (1 µm)$^3$, these surrounding actin filament structures remained nevertheless unnoticed by fluorescence microscopy so far. Future dedicated studies of such actin cages using correlative fluorescence and electron microscopy are needed to further characterize this feature of assembly sites.

The assembly sites contained numerous vesicles studded on their luminal inside with glycoprotein-like densities, presumably having being transported to these sites independently of cytosolic capsids. Classical electron microscopy techniques cannot visualize these spikes as unequivocally as it has been achieved here. Tegument proteins accumulated on the cytosolic surface of these vesicles and might be the cause of a vesicle indentation to form a concave surface towards the capsids. Further, some of these vesicles did not appear to be large enough to fully envelope one capsid. The close proximity of several of these vesicles suggests that secondary envelopment might involve vesicle fusion to form a sufficiently large enveloping compartment.

In summary, we have characterized a new neuronal infection model that enables investigating axonal transport, assembly and egress of HSV1 in 3D in a close-to-native state. CryoET revealed that the axonal viral particles were predominantly non-enveloped cytosolic capsids. We found that in addition to cytosolic C-capsids, unexpectedly cytosolic A-/B-capsids also underwent axonal transport. The prominent differences in tegumentation between these two capsid types suggest that efficient transport of capsids does not require large amounts of tegument, and occurs in the presence of different amounts of tegument. For both capsid types, the capsid-to-inner-tegument interactions were exclusively limited to the capsid vertices. These interactions are likely crucial for transport by forming a binding platform for microtubule motors. The higher abundance of non-enveloped over enveloped capsids in middle regions of axons, and the secondary envelopment sites at axon terminals favor the separate model for HSV egress for this combination of HSV1 strains and hippocampal neurons. The three-dimensional visualization of secondary envelopment sites revealed insights into a level of detail that allowed us to propose novel aspects of this process like formation of an actin bound compartment and a possible role for fusion of smaller vesicles during envelopment.

Materials and Methods

Viral preparation

HSV1(F), HSV1(KOS) and HSV1(KOS)-GFPVP26 [52] viruses were amplified in BHK-21 cells, and the viral titers were determined by plaque titration on Vero cells as described previously [1,54]. The virus stocks had a titer of $10^9$ PFU/ml.

Fluorescence microscopy and time-lapse analysis

Hippocampal neurons were isolated from 17 days old rat embryos [provided by Boyan Garvalov, MPI Neurobiology, Germany]. IBIDI slides (µ-slide 8 well, Ibidi GmbH) were coated with 1 mg/ml poly-L-lysine (Sigma) in borate buffer (1.24 g boric acid + 1.9 g borax in 400 ml distilled water, pH 8.5) overnight. They were then washed with distilled water three times before adding MEM horse serum medium ( Gibco), which was replaced next day by neurobasal medium (Gibco) supplemented with B27 (Gibco) and glutamine. Neurons were then seeded at a density of 4,500 cells per 1 cm². They were incubated for 7 days at 37°C, 5% CO$_2$ and then infected with HSV1(KOS)-GFPVP26 at an MOI of 50 PFU/cell. Infected neurons were imaged using a 63x oil objective on an Axiowert 200 M light microscope (Zeiss) equipped with an AxioCam HRm camera (Zeiss) and controlled by the Axiovision 4.1 software (Zeiss). For the long time-lapse experiments, viral infection of neurons was monitored by wide field phase contrast and fluorescence imaging every hour over a period of 24 hours. For the short time-lapse experiments, fluorescent pictures were taken every 2 seconds at the same region for 10 min. Fluorescence was detected with a GFP blue band excitation/green band emission filter set (HQ-EGFP; F41-017; AHF Analysentechnik AG). An incubation chamber around the microscope allowed time-lapse observations at 37°C, 5% CO$_2$ and high humidity (EMBL workshop, No. 530010; Cell Biology Trading). In the short time-lapse experiments, the speed and length of several continuous runs were measured in two different observations from two different neurons (Table S1). Some of the particles came in or moved out of the field of view during the observation time. These were also taken in consideration in the measurements.

Infection of neurons on grids

Au grids of 200 mesh with holey carbon support films (Quantifoil GmbH, Jena, Germany) were sterilized on a Petri dish under UV light for 15 min and then coated as described above for the IBIDI slides. Dissociated rat hippocampal neurons were prepared as described [83]. Neurons were plated over the grids at a density of 100,000 cells in a 60 mm diameter Petri dish and incubated 7 days at 37°C and 5% CO$_2$ to enable the growth of axons and dendrites. Neurons were then infected with HSV1 at a MOI of 50. At 16 hours post infection (p.i.), neurons were prepared for cryoET as described below.

Purification of nucleocapsids

BHK-21 cells were infected with 0.01 to 0.02 PFU/cell for 2 to 3 days until detached and collected by sedimentation. They were then washed once in MNT buffer (30 mM MES, 20 mM Tris, pH 7.4, 100 mM NaCl, snap-frozen and stored at -80°C. Nuclear capsids were purified as previously described [16,18,21,84]. Capsids were diluted in three volumes TNE (20 mM Tris, pH 7.5, 500 mM NaCl, 1 mM EDTA) with 10 mM DTT and protease inhibitors, and sedimented by centrifugation (Beckman TL A100.2 rotor, 15 min, 50 kp m, 4°C). The pellets were resuspended in BB80 buffer (80 mM PIPES, 1 mM EGTA, 2 mM MgCl$_2$; pH 6.8 with KOH) with 10 mM DTT, 1 mg/ml soybean trypsin inhibitor, protease inhibitors, 100 µg/ml RNase.
Symmetrized averages were used as templates for the next were averaged. Icosahedral symmetry was applied to the averages. the HSV1 capsid [87] as a template and the oriented subvolumes was determined using a 22 Å resolution structure of sampled by a factor of four (IMOD). Subsequent processing steps 2 was measured along the tilt axis after each tilt and automatically specified for creating up-scaled or down-scaled maps.

Accession numbers
The following HSV-1 capsid maps have been deposited in the Electron Microscopy Data Bank (EMDB) at PDBe [http://www.ebi.ac.uk/pdbe/emdb/]: EMD_1955, cytosolic C-capsids; EMD_1957, cytosolic A-/B-capsids; EMD_1958, nuclear A-capsids; EMD_1959, nuclear C-capsids.

Supporting Information
Figure S1 Types of intracellular progeny capsid found in axons. (A) Cytosolic A-capsid. (B) Cytosolic B-capsid. (C) Cytosolic C-capsid. Bar: 100 nm. (TIF)
Table S1 Measured speeds and directionalities of capsids travelling inside axons at 16 h p.i. (DOC)

Acknowledgments
We thank U. Maurer, M. Beck and C. Hagen (Munich) for insightful discussions and W. Baumeister (Munich) for support and encouragement. We acknowledge K. Radtke and A. Buch (Hannover) for fruitful discussions on capsids and axonal transport; B. Garvalov, I. Ferreira and L. Meyn (Munich) for neuron preparation and P. Desai for HSV1(KOS)-GFPVP26.

Author Contributions
Conceived and designed the experiments: II JTH KG. Performed the experiments: II JTH KG. Analyzed the data: II JTH KG. Contributed reagents/materials/analysis tools: II JTH KD FB BS KG. Wrote the paper: II JTH KD FB BS KG.

References
1. Gruenwald K, Desai P, Winkler DC, Heymann JB, Belnap DM, et al. (2003) Three-dimensional structure of herpes simplex virus from cryo-electron tomography. Science 302: 1396–1398.
2. Maurer UE, Sodeik B, Gruenwald K (2000) Native 3D intermediates of membrane fusion in herpes simplex virus 1 entry. Proc Natl Acad Sci U S A 102: 10559–10564.
3. Schrag JD, Prasad BV, Rixon FJ, Chiu W (1989) Three-dimensional structure of the HSV1 nucleocapsid. Cell 56: 651–660.
4. Booy FP, Newcomb WW, Trus BL, Brown JC, Baker TS, et al. (1991) Liquid-crystalline, phase-locked pattern of encapsidated DNA in herpes simplex virus. Cell 66: 1007–1015.
5. Newcomb WW, Trus BL, Booy FP, Steven AC, Wall JS, et al. (1993) Structure of the herpes simplex virus capsid. Molecular composition of the pentons and the triplices. J Mol Biol 232: 499–511.
6. Zhou ZH, Prasad BV, Jakana J, Rixon FJ, Chiu W (1994) Protein subunit structures in the herpes simplex virus A-capsid determined from 400 kV spot-scan electron cryomicroscopy. J Mol Biol 242: 456–469.
7. Zhou ZH, Dougherty M, Jakana J, He J, Rixon FJ, et al. (2000) Seeing the herpesvirus capsid at 8.5 Å. Science 288: 877–880.
8. Trus BL, Cheng N, Newcomb WW, Homa FL, Brown JC, et al. (2004) Structure and polymorphism of the UL6 portal protein of herpes simplex virus type 1. J Virol 78: 12668–12671.
9. Cardone G, Winkler DC, Trus BL, Cheng N, Heuser JE, et al. (2007) Visualization of the herpes simplex virus portal in situ by cryo-electron tomography. Virology 361: 426–434.
10. Chang JT, Schmid MF, Rixon FJ, Chiu W (2007) Electron cryomicroscopy reveals the portal in the herpesvirus capsid. J Virol 81: 2065–2070.

Lawrence S. Nirenberg Award Paper: III JTH KG. Wrote the paper: II JTH KD FB BS KG.
19. Mettenleiter TC, Klupp BG, Granzow H (2009) Herpesvirus assembly: an update. Virus Res 143: 222–234.
20. Conway JF, Cockrell SK, Copeland AM, Newcomb WW, Brown JC, et al. (2007) Productive herpes simplex virus in brain of elderly normal subjects and Alzheimer’s disease patients. J Med Virol 75: 1105–1110.
21. Bucks MA, O’Regan KJ, Murphy MA, Wills JW, Courtney RJ (2007) Herpes simplex virus utilization of the large secretory vesicle pathway for anterograde transport targets herpesvirus entry and egress in sensory neurons. Proc Natl Acad Sci U S A 104: 163–169.
22. Feierbach B, Bisher M, Goodhouse J, Enquist LW (2007) In vitro analysis of tegument-capsid interactions and DNA in intact herpes simplex virus type 1 virions. J Virol 81: 5494–5498.
23. Mettenleiter TC (2006) Intriguing interplay between viral proteins during herpesvirus assembly: or: the herpesvirus assembly puzzle. Vet Microbiol 113: 163–169.
24. Heymann JB, Cheng N, Newcomb WW, Trus BL, Brown JC, et al. (2003) Herpes simplex virus type 1 tegument forms in the cytoplasm of the cell body. J Virol 77: 9934–9951.
25. Nagel CH, Dohner K, Fathollahy M, Strive T, Borst EM, et al. (2008) Nuclear dynamics of herpes simplex virus capsid maturation visualized by time-lapse cryo-electron tomography: from cells to molecules. Annu Rev Biochem 74: 833–865.
26. Antinone SE, Smith GA (2006) Two modes of herpesvirus trafficking in neurons: separate and married mechanisms. J Virol 85: 5919–5928.
27. Snyder A, Wisner TW, Johnson DC (2006) Herpes simplex virus capsids are transported along microtubules in the cytoplasm of the cell body. J Virol 80: 284–290.
28. Snyder A, Wisner TW, Johnson DC (2006) Herpes simplex virus type 1 motility along microtubules in vitro. Traffic 7: 227–237.
29. Heymann JB, Cheng N, Newcomb WW, Trus BL, Brown JC, et al. (2003) Herpes simplex virus type 1 infection: Efficient dynein-mediated capsid transport without the small capsid protein VP26. J Virol 80: 8221–8224.
30. Reiner J, Smith GA, Gross SP, Enquist LW (2001) Herpesviruses use bidirectional fast-axonal transport to spread in sensory neurons. Proc Natl Acad Sci U S A 98: 3466–3470.
31. Do¨hner K, Ebersold MW, Helenius A (1997) Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. J Cell Biol 136: 1007–1021.
32. Smith GA, Sodeik B (2003) Viral interactions with the host cytoskeleton. J Virol 83: 2058–2066.
33. Antinone SE, Smith GA (2006) Herpes simplex virus type 1 infection: Efficient dynein-mediated capsid transport without the small capsid protein VP26. J Virol 80: 8221–8224.
34. Feierbach B, Bisher M, Goodhouse J, Enquist LW (2007) In vitro analysis of tegument-capsid interactions and DNA in intact herpes simplex virus type 1 virions. J Virol 81: 5494–5498.
35. Do¨hner K, Radtke K, Schmidt S, Sodeik B (2006) Eclipse phase of herpes simplex virus type 1 infection: mechanisms of capsid transport and egress and envelopment of herpes simplex virus capsids analyzed with dual-color fluorescence HSV1(17+). J Virol 82: 3109–3124.
36. Garvalov BK, Zuber B, Bouchet-Marquis C, Kudryashev M, Gruska M, et al. (2010) Structural studies by electron tomography of virion formation and intraaxonal transport of herpes simplex virus type 1. J Virol 84: 5528–5539.
37. Tomishima MJ, Enquist LW (2002) In vivo egress of an alphaherpesvirus from axons. J Virol 76: 8310–8317.
38. Snyder A, Polioceva K, Johnson DC (2008) Herpes simplex virus eGFP and US9 proteins promote transport of both capsids and virion glycoproteins in neuronal axons. J Virol 82: 10613–10624.
39. Do¨hner K, Nagel CH, Sodeik B (2005) Viral stop-go and-go along microtubules: taking a ride with dynein and kinesins. Trends Microbiol 13: 329–327.
68. Gazzola M, Burckhardt CJ, Bayati B, Engelke M, Greber UF, et al. (2009) A stochastic model for microtubule motors describes the in vivo cytoplasmic transport of human adenovirus. PLoS Comput Biol 5: e1000623.

69. Klupp BG, Granzow H, Keil GM, Mettenleiter TC (2006) The capsid-associated UL25 protein of the alphaherpesvirus pseudorabies virus is nonessential for cleavage and encapsidation of genomic DNA but is required for nuclear egress of capsids. J Virol 80: 6235–6246.

70. Baines JD, Cunningham C, Nabanga D, Davison A (1997) The U(L)15 gene of herpes simplex virus type 1 contains within its second exon a novel open reading frame that is translated in frame with the U(L)15 gene product. J Virol 71: 2666–2673.

71. Trus BL, Gibson W, Cheng N, Steven AC (1999) Capsid structure of simian cytomegalovirus from cryoelectron microscopy: evidence for tegument attachment sites. J Virol 73: 2181–2192.

72. Negatsch A, Mettenleiter TC, Fuchs W (2011) Pseudorabies virus UL36 tegument protein physically interacts with the UL37 protein. J Virol 85: 3863–3871.

73. Lyman MG, Curanovic D, Enquist LW (2008) Targeting of pseudorabies virus structural proteins to axons requires association of the viral US9 protein with lipid rafts. PLoS Pathog 4: e1000065.

74. Ben-Harush K, Maimon T, Pala I, Villa E, Medalia O (2010) Visualizing cellular processes at the molecular level by cryo-electron tomography. J Cell Sci 123: 7–12.

75. Forster F, Hegerl R (2007) Structure determination in situ by averaging of tomograms. Methods Cell Biol 79: 741–767.

76. Cheng N, Trus BL, Belnap DM, Newcomb WW, Brown JC, et al. (2002) Handedness of the herpes simplex virus capsid and procapsid. J Virol 85: 7513–7522.

77. Thurlow JK, Murphy M, Stow ND, Preston VG (2006) Herpes simplex virus type 1 DNA-packaging protein UL17 is required for efficient binding of UL25 to capsids. J Virol 80: 2110–2126.

78. Cockrell SK, Huffman JB, Toropova K, Conway JF, Homa FL (2011) Residues of the UL25 protein of herpes simplex virus that are required for its stable interaction with capsids. J Virol 85: 4875–4887.

79. Pasdeloup D, Blondel D, Isidro AL, Rixon FJ (2009) Herpesvirus capsid association with the nuclear pore complex and viral DNA release involve the nucleoporin CAN/Nup214 and the capsid protein pUL25. J Virol 83: 6610–6623.

80. Klupp BG, Fuchs W, Granzow H, Neukirchen D, Mettenleiter TC (2002) Pseudorabies virus UL36 tegument protein physically interacts with the UL37 protein. J Virol 76: 3063–3071.

81. Vittone V, Diefenbach E, Trifitt D, Douglas MW, Cunningham AL, et al. (2005) Determination of interactions between tegument proteins of herpes simplex virus type 1. J Virol 79: 9566–9571.

82. Ko DH, Cunningham AL, Diefenbach RJ (2010) The major determinant for addition of tegument protein pUL48 (VP16) to capsids in herpes simplex virus type 1 is the presence of the major tegument protein pUL36 (VP1/2). J Virol 84: 1397–1405.

83. Witte H, Neukirchen D, Bradke F (2008) Microtubule stabilization specifies initial neuronal polarization. J Cell Biol 180: 619–632.

84. Percud ML, Kemp MC, Randall CC, O’Callaghan DJ (1974) Studies of the molecular anatomy of the L-M cell strain of equine herpes virus type 1: proteins of the nucleocapsid and intact virion. Virology 59: 201–216.

85. Kremer JR, Mastronarde DN, McIntosh JR (1996) Computer visualization of three-dimensional image data using IMOD. J Struct Biol 116: 71–76.

86. Heymann JB, Belnap DM (2007) Bsoft: image processing and molecular modeling for electron microscopy. J Struct Biol 157: 3–18.

87. Cheng N, Trus BL, Belnap DM, Newscomb WW, Brown JC, et al. (2002) Handedness of the herpes simplex virus capsid and procapsid. J Virol 76: 7855–7859.