Tick-Borne Encephalitis Virus Infects Rat Astrocytes but Does Not Affect Their Viability

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Abstract

Tick-borne encephalitis virus (TBEV) causes one of the most dangerous human neuroinfections in Europe and Asia. To infect neurons it must cross the blood-brain-barrier (BBB), and presumably also cells adjacent to the BBB, such as astrocytes, the most abundant glial cell type. However, the knowledge about the viral infection of glial cells is fragmental. Here we studied whether TBEV infects rat astrocytes. Rats belong to an animal group serving as a TBEV amplifying host. We employed high resolution quantitative fluorescence microscopy to investigate cell entry and cytoplasmic mobility of TBEV particles along with the effect on the cell cytoskeleton and cell survival. We report that infection of astrocytes with TBEV increases with time of exposure to TBEV and that with post-infection time TBEV particles gained higher mobility. After several days of infection actin cytoskeleton was affected, but cell survival was unchanged, indicating that rat astrocytes resist TBEV-mediated cell death, as reported for other mammalian cells. Therefore, astrocytes may present an important pool of dormant TBEV infections and a new target for therapeutic intervention.

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Introduction

Tick-borne encephalitis virus (TBEV) is medically important human pathogen that causes one of the most dangerous neuroinfections in humans in Europe and in Asia [1–3]. TBEV is a member of the genus Flavivirus, family Flaviviridae [4]. Mature virus particles are enclosed within lipidic envelope which carry attachment molecules (E protein) for the host cell receptors (heparan sulfate) [1,5–7]. The first and the most important host cells infected by TBEV are likely epidermal Langerhans cells (i.e. dendritic cells; [8]) which transport the virus to the lymph nodes and initiate the spread of infection to lymphoid compartments [3]. In some vertebrate species the virus is neurovirulent and crosses the blood-brain-barrier (BBB), and presumably also cells adjacent to the BBB, such as astrocytes, the most abundant glial cell type. However, the knowledge about the viral infection of glial cells is fragmental. Here we studied whether TBEV infects rat astrocytes. Rats belong to an animal group serving as a TBEV amplifying host. We employed high resolution quantitative fluorescence microscopy to investigate cell entry and cytoplasmic mobility of TBEV particles along with the effect on the cell cytoskeleton and cell survival. We report that infection of astrocytes with TBEV increases with time of exposure to TBEV and that with post-infection time TBEV particles gained higher mobility. After several days of infection actin cytoskeleton was affected, but cell survival was unchanged, indicating that rat astrocytes resist TBEV-mediated cell death, as reported for other mammalian cells. Therefore, astrocytes may present an important pool of dormant TBEV infections and a new target for therapeutic intervention.

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The aim of this study was to test whether astrocytes, the most numerous glial brain cells [17], can be infected by TBEV and to measure cytoplasmic TBEV particle dynamics in the initial phases after the infection, along with the astrocyte viability. The susceptibility to TBEV infection would make astrocytes a potential TBEV reservoir. We used rat astrocytes as a model cell, since rodents of several species are known to be TBEV amplifying hosts and may maintain TBEV through latent persistent infections [3,28].

The results show that TBEV infects rat astrocytes and that the infection of a single cell, which progresses in time-dependent phases, is associated with changes in actin cytoskeleton, but astrocyte viability is unaffected. We propose that astrocytes represent an important reservoir of TBEV brain infection, which makes these cells a new target for therapeutic intervention. Given their tight morphological association with blood vessels, infected astrocytes may possibly affect the BBB and neurons.
Materials and Methods

Ethics Statement

The care for experimental animals and the euthanization of animals was carried out in strict accordance with the following ethical codes and directives: The International Guiding Principles for Biomedical Research Involving Animals developed by the Council for International Organizations of Medical Sciences and the Directive on Conditions for Issue of License for Animal Experiments for Scientific Research Purposes (Official Gazette of the Republic of Slovenia 40/85, 22/87, 43/07). The protocol for the euthanization of the animals used in our study was approved by the Veterinary Administration of the Ministry for Agriculture and the Environment of the Republic of Slovenia (permit No: 34401-29/2009/2), issued on 22.4.2009. We have followed the rule of Three R’s to reduce the impact of research on animals.

Cell Cultures

Astrocyte cultures were prepared from cortices of 3 days old Wistar rats as described [29]. Cells were maintained in high-glucose Dulbecco’s modified Eagle’s medium (Invitrogen, Life Technologies, Carlsbad, NM, USA) containing 10% fetal bovine serum (FBS), 1 mM pyruvate, 2 mM glutamine and 25 μg/ml penicillin/streptomycin at 37°C, 95% air/5% CO2. After reaching confluence, cells were manipulated as described [30]. Prior the experiments cells were sub-cultured onto Lab-Tek™ chambered coverglass (Thermo Scientific) or onto 22 mm-diameter poly-L-lysine-coated coverslips and used within 6 days after plating. Vero E6 cells were used for the preparation of virus stocks and as productively infected control cell type after infection with TBEV. Cells were maintained in Dulbecco’s MEM with high glucose (DMEM GlutaMAX™, Invitrogen) supplemented with 10% FBS. Cultures were incubated at 37°C/5% CO2. Unless stated otherwise all chemicals for maintaining cell cultures and experimental procedures were obtained from Sigma-Aldrich (Dienenhofen, Germany).

TBEV Labelling and Cytotoxicity Test

Viral strains and isolates of TBEV can be classified into three subtypes: the European, the Siberian and the Far Eastern [31]. In this study we used the European TBEV strain Ljubljana 1 [32]. TBEV was grown 7 days on Vero E6 cells. Supernatant was collected and centrifuged twice at 4°C (10 min at 3200 × g and 5 min at 20800 × g in Eppendorf 5804R centrifuge). Pellet was

![Figure 1. Time-dependant internalization of DiD-labelled TBEV into cultured rat astrocytes.](image-url)
Figure 2. The number of endosomes and lysosomes associated with DiD-TBEV particles increased with time p.i. (Aiii). An astrocyte with DiD-labelled TBEV vesicles (TBEV) incubated at 37°C for 4 h and 18 h and with labelled early endosomes (anti-EEA1 1:300). Overlays represent overlapped DiD-TBEV and EEA1 fluorescent signals, indicating the association between DiD-TBEV and endosomes. Bars: 5 μm. (Aii). Prolonged incubation increased the average number of DiD-TBEV labelled vesicles per cell from 14.7±4.3 (4 h) to 27.3±3.2 (18 h) and also the average number of vesicles co-labelled with DiD-TBEV and EEA1 from 3.1±0.8 (4 h) to 7.9±0.9 (18 h). Black bars - DiD-TBEV labelled vesicles, white bars - DiD-TBEV and EEA1 co-labelled vesicles, *P<0.05. (Bi,ii) An astrocyte with DiD-labelled TBEV vesicles (TBEV) incubated at 37°C for 4 h and 18 h and with LAMP1-labellend late endosomes/lysosomes (LAMP1-lysosomal associated membrane protein 1; 1:300). Overlays represent overlapped DiD-TBEV and LAMP1 fluorescent signals, indicating the association between DiD-TBEV and late endosomes/lysosomes. Bars: 5 μm. (Biii). Prolonged incubation increased the average number of DiD-TBEV labelled vesicles per cell from 9.8±1.4 (4 h) to 34.3±4.6 (18 h) and the average number of vesicles co-labelled with DiD-TBEV and LAMP1 from 5.3±1.0 (4 h) to 15.6±2.0 (18 h). Black bars - DiD-TBEV labelled vesicles, white bars - DiD-TBEV and LAMP1 co-labelled vesicles, **P<0.001. (C) Astrocyte co-labelled with anti-LAMP1 (1:300) and anti-EEA1 (1:300). In a single, 1 μm thick optical slice, late endosomes/lysosomes and early endosomes appear to be largely different in size due to different position in z-axis and variable antibody attachments. Arrowheads point to large late endosomes/lysosomes (LAMP1) and early endosomes (EEA1). Bar (bar inset): 5 μm (2.5 μm). n (n) = number of cells (number of vesicles).

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resuspended in astrocyte growth medium and labelled with different concentrations of fluorescent lipophilic Vybrant® DiD labelling solution (DiD, Molecular Probes, Invitrogen) in mM: 20, 50, 100 and 200. Labelling was performed for 2 h at 37°C (Eppendorf Thermomixer Compact, 500 rpm). Afterwards, the unbound dye was removed by buffer exchange into Hepes 145 buffer (50 mM Hepes, 145 mM NaCl, pH 7.4; [33] by using illustra NAP-5 columns with sephadex G-25 DNA grade (GE Healthcare)). Labelled virus (conc. 10^10 copies per ml) was diluted in astrocyte growth medium, aliquoted and stored at 2–8°C. Astrocytes were infected with 10^3–10^7 TBEV.

Cytotoxicity of TBEV in astrocytes and Vero E6 cells was tested at various time intervals: 4 h, 18 h, 48 h, 3 days, 6 days, 10 days and 14 days p.i. with Countess™ Automated Cell Counter (Invitrogen) according to manufacturer’s instructions.

TBEV RNA Concentration

The concentration of TBEV RNA was measured using one-step quantitative real time RT-PCR [34]. Vero E6 cells were infected with TBEV and the virus concentration was measured in the supernatant collected from Vero E6 cells 7 days post infection (p.i.) before and after labelling TBEV with fluorescent lipophilic Vybrant® DiD labelling solution. Further on, in the experiments, we have used the TBEV in concentration of 10^8 copies/ml, labelled with 50 mM DiD.

Imaging

Imaging of fixed and live cells was performed with an inverted confocal microscope (Zeiss LSM 510 META, Carl Zeiss) using oil-immersion objective 63×/NA 1.4. For excitation of DiD dye He/Ne laser was used (633 nm), the emission light was filtered with long pass filter, with the cut off below 650 nm. The conjugate Alexa Fluor 488 was excited by argon laser (488 nm) and the emission light was collected through the band pass filter (505 to 530 nm). The conjugate Alexa Fluor 546 was excited by He/Ne laser and the emission light was filtered with long pass filter 560 nm. To eliminate possible bleed-through, the green and red emission fluorescence was acquired sequentially. In live cells the mobility of vesicles that expressed DiD fluorescence of labelled TBEV was recorded. Time series images were recorded in 2 s intervals for 2 min of total recording time. Experiments were conducted at 37°C (Heatable universal mounting frame, Carl Zeiss).

Immunocytochemistry

The cells were washed with the phosphate buffered saline (PBS), fixed in 2–4% formaldehyde (prepared from paraformaldehyde) in PBS for 3–15 min at room temperature (RT) and permeabilized with Triton X-100 for 10 min at RT. The non-specific back-
ground staining was reduced by incubating cells in blocking buffer, containing 3% bovine serum albumin (BSA) and 10% goat serum in PBS, at 37°C for 1 h. The cells were then stained with primary antibodies, diluted into 3% BSA in PBS and incubated at 37°C for 2 h or at 4°C overnight. When two primary antibodies (raised in different species) were used, the staining was done sequentially. Afterwards, the cells were rinsed in PBS and stained with secondary antibodies at 37°C for 45 min. At the end of the staining protocol the cells were mounted onto glass slides using SlowFade Gold antifade reagent (Molecular Probes, Invitrogen).

Primary antibodies used were: anti-E 1:10 [35], anti-β-Actin 1:200 (Abcam), anti-α-Tubulin 1:100 (Sigma), anti-Clathrin light chain 1:300 (Synaptic Systems), anti-EEA1 1:300 (BD Biosciences) and anti-LAMP1 1:300 (Abcam). Secondary antibodies were Alexa Fluor® 488 goat anti-mouse IgG and Alexa Fluor® 546 goat anti-rabbit IgG (Molecular Probes, Invitrogen).

Figure 4. The percent of directional vesicle periods was less than 20%. Panel A shows vesicle paths in 30 s. Clumped trajectories represent non-directional mobility (NDM) and elongated trajectories represent directional mobility (DM). Circles denote vesicles subjected to mobility analysis. Bar: 2 μm. Graphs B–D represent vesicle directionality (relationship between MD and TL). The proportion of DM periods (MD > 1 μm, dashed line) remained similar during all incubation times, although the number of vesicles significantly increased (see also Figure 1). White circles - NDM periods, grey circles - DM periods, n = number of vesicles. Values MD and TL were calculated at 30 s of recording time.

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Analysis
The mobility of fluorescently labelled vesicles was analysed by ParticleTR software (Celica, Slovenia). To describe vesicle mobility the parameters were calculated as described [36,37]: step length (displacement of a vesicle in the time interval of 2 s), track length (TL, the total length of the analysed vesicle pathway), velocity and maximal displacement (MD; [36]). Vesicle mobility was analysed in cells from three independent astrocyte cultures. The analysis of the vesicle mobility was performed for epochs of 30 s.

Vesicle size was analysed with ImageJ software (available at National Institute of Health, USA, http://rsbweb.nih.gov/ij/). Fluorescent particles above the threshold level 8 pixels² (0.14×0.14 μm²) were determined as vesicles, corresponding to the vesicle size area above 0.1568 μm² to cover a broad span of imaged vesicles with different fluorescence intensities at slightly different z-

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positions within optical slice, similarly as described [38]. The extent of the co-labelled vesicles by fluorescent probes was determined by manually counting the observed fluorescent probes in the green and red channels.

Statistical significance was determined with the Mann-Whitney Rank Sum test. Values presented on graphs are expressed as mean ± s.e.m.

**Results**

**Identifying Initial Steps of TBEV Infection in Astrocytes**

To image intracellular localization of TBEV particles, we pre-labelled them with 50 μM DiD lipophylic dye and afterwards infected cultured astrocytes with conc. of 10^7 RNA copies/ml. Infection with TBEV was confirmed by immunolabelling with antibodies against the viral large envelope protein E [5] (Figure 1A, inset). TBEV labelling of cells increased with the time p.i. as assessed by counting TBEV particles per cell (Figure 1A,B). On one hand, the screening of several time periods following the

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**Figure 5.** MD and TL of DiD-labelled TBEV vesicles exhibiting directional mobility increased with time p.i. Average MD and TL of all vesicle periods are represented in panels A and B. No significant difference was observed neither between MD (in μm) at different times p.i.: 0.63±0.05 (2 h), 0.89±0.07 (4 h), 0.74±0.05 (18 h) (A), nor between TL (in μm): 1.93±0.10 (2 h), 2.23±0.10 (4 h), 2.13±0.03 (18 h) (B). However, a significant increase was observed for MD (in μm) for DM periods (white bars): from 1.69±0.27 (2 h) to 2.68±0.23 (4 h; **P<0.01) and 2.42±0.09 (18 h; *P<0.05) and small, but significant decrease for NDM periods: 0.48±0.02 μm (2 h), 0.43±0.01 μm (4 h) and 0.44±0.001 μm (18 h) (**P<0.01, ***P<0.001 (grey bars) (C). Mean TL (in μm) of DM periods (white bars) slightly, but significantly increased from 3.74±0.35 (2 h) to 5.00±0.29 (4 h; *P<0.05) while of NDM remained similar: 1.68±0.09 (2 h), 1.66±0.06 (4 h) and 1.74±0.02 (18 h) (D). n = number of vesicles.

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Figure 6. TBEV affected cytoskeleton morphology, but not cell shape nor cell viability. A–F, Immunocytochemical labellings of TUBULIN (anti-α-Tubulin 1:100) and ACTIN cytoskeleton (anti-β-Actin 1:200). Squared area is 2× enlarged in adjacent columns. Substantial rearrangement of actin cytoskeleton was observed after 3 and 6 days p.i. (E,F). Cell shape remained preserved at all times p.i. Bars: 10 μm (whole cell), 5 μm (enlarged panels).
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exposure of cells to TBEV revealed that the number of TBEV particles per cell increased: $2.9 \pm 1.2 \ (0.5 \ h \ p.i.), \ 4.9 \pm 1.4 \ (2 \ h \ p.i., \ P \leq 0.05), \ 7.4 \pm 1.3 \ (4 \ h \ p.i., \ P \leq 0.01) \ and \ 31.5 \pm 2.8 \ (18 \ h \ p.i., \ P \leq 0.001)$ (Figure 1A). We analyzed up to 31 cells from three different animals. On the other hand, we also determined the fraction of cells in which at least 3 DiD-TBEV particles were observed in a coverslip with cultured astrocytes. Clearly, the percent of infected cells increased as a function of post-infection time: $68 \pm 9\% \ (4 \ h), \ 92 \pm 2\% \ (18 \ h; \ P \leq 0.05; \ Figure \ 1B)$. Next, to confirm that the localization of DiD-TBEV particles was intracellular, not merely at the cell surface, we immuno-labelled cells with antibodies against several proteins of the endosomal pathway. DiD-TBEV particles co-localized with clathrin light chain antibodies, $57\% \ (n = 11) \ at \ 4 \ h \ p.i. \ and \ 48\% \ (n = 16) \ at \ 18 \ h \ p.i. \ (P > 0.1; \ data \ not \ shown), \ confirming \ clathrin-dependent \ endocytosis \ mediates \ TBEV \ entry$. Additionally, DiD-TBEV particles were found co-localized with early endosomes (labelled with early endosomal antigen 1 (EEA1); [39]): $22\% \ (at \ 4 \ h \ p.i.) \ and \ 30\% \ (at \ 18 \ h \ p.i.; \ calculated \ from \ Figure \ 2Aiii) \ and \ with \ late \ endosomes/lysosomes (labelled \ with \ late \ endosomal/lysosomal \ associated \ membrane \ protein \ 1 \ (LAMP1; \ [40])); \ 54\% \ (at \ 4 \ h \ p.i.) \ and \ 43\% \ (at \ 18 \ h \ p.i.; \ calculated \ from \ Figure \ 2Biii). \ A \ few \ DiD-TBEV \ loaded \ vesicles \ appeared \ enlarged; \ they \ were \ comparable \ in \ size \ to \ late \ endosomes/lysosomes (LAMP1) and early endosomes (EEA1) in non-infected cells (Figure 2C). Taking into account that EEA1 and LAMP1 markers overlap in approx. $10\%$ [41], we estimated that approximately $60–70\%$ of DiD-TBEV particles were co-localized with either early endosomes or late endosomes/lysosomes and the rest were observed before entry into endosomal/lysosomal pathway, which is consistent with a multistep endocytic virus entry into animal cells [42].

**DiD-TBEV is Predominantly Loaded in Small Subcellular Structures**

We observed that internalized DiD-TBEV vesicles were predominantly small, as evident from vesicle area distributions (Figure 3A–C) and that their number increased with time p.i. (41%...
Viability Remains Unchanged

With Increasing p.i. time DiD-TBEV Particles Acquire Increased Directionality

Large vesicles with fluorescence area above 1.078 $\mu^2$ were immobile. On the other hand, small vesicles were mobile and were subjected to mobility analysis. Speed, track length (TL) and maximal displacement (MD) were calculated as described previously [36]. In 30 s of recording time vesicle pathways appeared predominantly clumped, meaning that vesicles exhibited non-directional mobility (NDM). However, some vesicles displayed directional mobility (DM), as evident from elongated trajectories (Figure 4A). The percent of DM periods (Figure 4, MD>1 $\mu$m, (dashed line)) remained below 20% during all incubation times, although the percent of small vesicles increased from 41% (2 h) to 72% (18 h, Figure 3). MD and TL of DM periods and NDM periods were distinct (Figure 5). Mean MD of DM periods significantly increased: from 1.69±0.28 $\mu$m (2 h) to 2.63±0.23 $\mu$m (4 h; P<0.05) and 2.42±0.09 $\mu$m (18 h; P<0.01) (Figure 5C). And mean TL of DM periods significantly increased from 3.74±0.35 $\mu$m (2 h) to 5.00±0.29 $\mu$m (4 h; P<0.05, Figure 5D) and to 4.36±0.11 $\mu$m (18 h). On the other hand, the mean MD and TL of NDM periods slightly decreased; MD: 0.40±0.02 $\mu$m (2 h) to 0.43±0.01 $\mu$m (4 h; P<0.01) and 0.44±0.001 $\mu$m (18 h; P<0.001) (Figure 5A), TL: 1.68±0.09 $\mu$m (2 h) to 1.66±0.06 $\mu$m (4 h; P<0.01) and 1.74±0.02 $\mu$m (Figure 5B). The average speed of DM periods were 0.12±0.01 $\mu$m/s (2 h) and 0.17±0.01 $\mu$m/s (4 h) $\mu$m/s and 0.15±0.003 $\mu$m/s (18 h) and of NDM periods 0.06±0.003 $\mu$m/s (2 h), 0.06±0.001 $\mu$m/s (4 h) and 0.06±0.01 $\mu$m/s (18 h).

TBEV Induces Disintegration of Actin Filaments While Cell Viability Remains Unchanged

Cytoskeleton rearrangements of host cells are reported for several viral infections (Herpesvirus, [43]; TBEV, [27]). To examine whether TBEV infection triggered changes of the rat astrocyte cytoskeleton, molecular motor-associated microtubules and actin filaments were labelled in astrocytes at several p.i. times (4 h, 18 h, 48 h, 3 days and 6 days). No obvious alterations of cytoskeleton were noticed in rat astrocytes until day 2 p.i. Then, after day 3 p.i. significant reorganization of actin filaments was observed, while microtubules appeared unaffected (Figure 6).

Finally, to assess whether TBEV triggers the cytopathic effect (CPE) in primary rat astrocytes, we recorded fields of view of TBEV infected primary rat astrocytes and control Vero E6 cells and measured their viability for 14 days (Figure 7). The CPE (morphologically altered cell shape, detached cells) was observed in Vero E6 cells at day 3 p.i., whereas in astrocytes no CPE was recorded (Figure 7A). Moreover, the results of the trypan blue exclusion viability test show that the viability of primary rat astrocytes was not affected by TBEV infection (Figure 7 B). On the other hand, in the control Vero E6 cells, which were used to multiply TBEV, the viability was significantly affected already after 10 h (66±2%, P<0.001) and was further reduced to 16±2% (P<0.001) at day 14 p.i., compared to non-infected cells (95±2%) (Figure 7B). Successful replication of TBEV in both cell types was confirmed by measuring the virus load (TBEV copies/cell) at different times p.i. (Figure 8). Moreover, the supernatant collected from astrocytes at the end of the experiment (14 days) still reduced the viability of Vero E6 cells (39%) at day 7 p.i. and triggered CPE (Figure 8), confirming the presence of infective TBEV. From these data we conclude that TBEV infection does not significantly affect the viability of rat astrocytes, implying that these cells could act as a potential TBEV reservoir.

Discussion

Despite the high clinical importance of TBEV neuroinfection (long-term neurological disabilities, over 1% mortality rate, growing incidence in Europe), nothing is known about the TBEV infection of the most abundant neuronal supportive glial cells, astrocytes, and their possible implication in active or dormant TBEV infections. Recent data indicate that TBEV can be detected in rodent organs for longer periods of time and that the brain tissue stands out by high virus load [34]. In this report experimental evidences show that astrocytes, the most abundant mammalian glial cells become infected with TBEV, which makes them a potential mediator of brain infection and a reservoir of brain TBEV in rodents.

TBEV internalization into astrocytes increased with longer post-infection time. Although the mechanism of internalization remains to be elucidated, a number of different proteins have been identified as potential flavivirus receptors on the cell surface, however there is no direct evidence for a role of any of these during TBEV entry into host cells, and some of the results are controversial [44]. Glycosaminoglicans, such as heparan sulphate (HS), are expressed on different tissues [45]. Frequently, HS appears to serve as a primary attachment molecule that concentrates viral particles on the cell surface and may facilitate the subsequent binding to more specific receptor molecules [7,46]. An involvement of HS during attachment and entry of TBEV has been demonstrated, although it appears that more than one single type of host-cell molecule is involved [3,5,7,47]. In astrocytes mimicking injury response, upregulation of HS and higher sulfation of heparan sulphate proteoglycans occurs [48]. Therefore, one possible explanation of increased time-dependent TBEV entry into astrocytes could be attributed to an increased expression of HS on astrocyte membrane of infected astrocytes. However, this was not monitored in our experiments. The internalization of TBEV particles into astrocytes was consistent with confirmed clathrin-dependent entry of several members of Flaviviridae family: West Nile virus (WNV) [2], Dengue virus [49,50], Hepatitis C virus [51] and Bovine Viral Diarrhoea virus (BVDV) [52]. And their localization in late endosomes/lysosomes was consistent with other flaviviruses: BVDV, [52]; WNV, [53]; DIL-labelled dengue virus, [50].

TBEV-loaded vesicles observed following 18 h post-infection time were predominantly smaller or comparable in size to early endosomes (300 nm, [2]; 300–400 nm, [54]); and much smaller from late endosomes which have on average 700 nm in diameter.
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Author Contributions

Conceived and designed the experiments: MP MK JJ TAZ RZ. Performed the experiments: MP MK JJ. Analyzed the data: MP MK JJ. Contributed reagents/materials/analysis tools: TAZ RZ. Wrote the paper: MP MK JJ TAZ RZ.
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