Infection and injury of human astrocytes by tick-borne encephalitis virus

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Tick-borne encephalitis (TBE), a disease caused by tick-borne encephalitis virus (TBEV), represents the most important flaviviral neural infection in Europe and north-eastern Asia. In the central nervous system (CNS), neurons are the primary target for TBEV infection; however, infection of non-neuronal CNS cells, such as astrocytes, is not well understood. In this study, we investigated the interaction between TBEV and primary human astrocytes. We report for the first time, to the best of our knowledge, that primary human astrocytes are sensitive to TBEV infection, although the infection did not affect their viability. The infection induced a marked increase in the expression of glial fibrillary acidic protein, a marker of astrocyte activation. In addition, expression of matrix metalloproteinase 9 and several key pro-inflammatory cytokines/chemokines (e.g. tumour necrosis factor α, interferon α, interleukin-1β, IL-6, IL-8, interferon γ-induced protein 10, macrophage inflammatory protein, but not monocyte chemotactic protein 1) was upregulated. Moreover, we present a detailed description of morphological changes in TBEV-infected cells, as investigated using three-dimensional electron tomography. Several novel ultrastructural changes were observed, including the formation of unique tubule-like structures of 17.9 ± 0.15 nm diameter with associated viral particles and/or virus-induced vesicles and located in the rough endoplasmic reticulum of the TBEV-infected cells. This is the first demonstration that TBEV infection activates primary human astrocytes. The infected astrocytes might be a potential source of pro-inflammatory cytokines in the TBEV-infected brain, and might contribute to the TBEV-induced neurotoxicity and blood–brain barrier breakdown that occurs during TBE. The neuropathological significance of our observations is also discussed.

INTRODUCTION

Tick-borne encephalitis (TBE) is a serious viral infection of the central nervous system (CNS) caused by tick-borne encephalitis virus (TBEV). TBEV is a single-stranded positive-sense RNA virus belonging to the genus Flavivirus, family Flaviviridae (Mansfield et al., 2009). More than 13 000 clinical cases of TBE, including numerous deaths, are reported annually in Europe and north-eastern Asia (Mansfield et al., 2009). Despite the medical importance of this disease, some crucial steps in the development of encephalitis remain poorly understood. In humans, TBEV may produce a variety of clinical symptoms. The clinical spectrum of acute TBE ranges from symptoms of undifferentiated febrile illness or mild meningitis to severe meningoencephalitis with or without myelitis (Haglund & Günther, 2003; Růžek et al., 2010). Chronic TBE occurs less frequently and has been reported only in some regions of Russia, mainly in Siberia and the Far East, where this form comprises 1–3 % of all TBE cases (Gritsun et al., 2003). Major hallmarks of TBEV neuropathogenesis are neuroinflammation followed by neuronal death and disruption of the blood–brain barrier (BBB) (Růžek et al., 2009a, 2011; Palus et al., 2013, 2014). The response of TBEV infection in the brain is characterized by massive inflammatory events, including the production of cytokines (e.g. IFN-γ, TNF-α, and IL-1β, IL-6 and IL-10) and chemokines [e.g. monocyte chemotactic protein (MCP)-1/CCL2, IFN-γ-induced protein 10 (IP-10)/CXCL10, macrophage inflammatory...
Fig. 1. TBEV can infect human primary astrocytes. (a) HBCAs grown and fixed on slides at days 3 and 10 post-infection (p.i.) were stained with anti-flavivirus envelope antibody (green) and counterstained with DAPI (blue). TBEV-infected HBCAs immunostained with flavivirus-specific antibody demonstrated virus replication in the cytoplasm, with antigen aggregates forming at day 3 p.i. At later time points (10 days p.i.), only the brightly stained aggregates of viral antigen were observed. Mock-infected HBCAs stained with primary and secondary antibodies were used as a negative control, and did not exhibit any TBEV antigen staining. (b) TBEV titres in culture supernatant from HBCAs collected at 0, 1, 2, 3, 7, 9 and 15 days p.i. were determined by plaque assay using porcine kidney stable cells. Viral titres are expressed as p.f.u. ml⁻¹. Data represent means ± SEM. (c) Total RNA extracted from HBCA cell lysates at 0, 2, 7, and 15 days p.i. was used to determine the number of
protein (MIP-1α and RANTES) (Palus et al., 2013). Microglia and astrocytes are classically believed to serve as the predominant source of these cytokines and chemokines in the CNS, and therefore may act as important processors of neuroinflammation and neurodegeneration (Ramesh et al., 2013). The pro-inflammatory chemokines attract immunocompetent cells to the CNS (Réaux-Le Goazigo et al., 2013), including CD8+ T-cells, which may mediate immunopathology during TBE (Růžek et al., 2009a). Moreover, the pro-inflammatory molecules can further activate downstream apoptotic signalling pathways in neurons, resulting in neuronal death (Kumar et al., 2010) or inducing breakdown of the BBB (Růžek et al., 2011; Erickson et al., 2012; Palus et al., 2014). We used a rodent model to demonstrate that TBEV infection is associated with the dramatic BBB breakdown that occurs during the later stages of infection. The BBB breakdown most likely represents a bystander effect of virus-induced cytokine/chemokine overproduction in the brain (Růžek et al., 2011). However, the specific cell types that express these cytokines and chemokines have not been characterized.

Although neurons are primary targets after TBEV enters the CNS (Hirano et al., 2014), other brain cells may also be infected (Potokar et al., 2014). Infection of non-neuronal CNS cells including astrocytes has, albeit infrequently, been reported in cases of flavivirus encephalitis (Desai et al., 1995; Nogueira et al., 2002; German et al., 2006; Balsitis et al., 2009; de Araújo et al., 2009; Sips et al., 2012). It was shown recently that TBEV infects cultured primary rat astrocytes without affecting their viability. Therefore, it was suggested that astrocytes might represent an important reservoir of TBEV in brain during the infection (Potokar et al., 2014). Astrocytes are the most abundant glial cell population in the human brain (Nedergaard et al., 2003) and have various leading roles in the brain, including integrating neuronal functions, neuronal support and regulation of the BBB. Thus, astrocytes serve as a structural and functional bridge between endothelial cells of the BBB and neurons; together, they form the ‘neurovascular unit’ (Stanimirovic & Friedman, 2012), which regulates blood flow, the integrity of the BBB and neuronal activity in response to physiological and pathophysiologically changes (Hussmann et al., 2013).

Astrocytes are key players in the inflammatory response during neural infections caused by flaviviruses, namely Japanese encephalitis (Bhowmick et al., 2007; Yang et al., 2012) and West Nile encephalitis (Diniz et al., 2006; Verma et al., 2011; Hussmann et al., 2013); however, their role in the development of TBE remains largely unstudied. Here, we aimed to investigate the sensitivity of primary human astrocytes to TBEV infection, virus growth, virus-induced astrocyte activation, and cytokine and chemokine production.

We demonstrate here for the first time, to the best of our knowledge, that TBEV is capable of productive, persistent infection in primary human astrocytes, and that this infection is associated with astrocyte activation and the production of various pro-inflammatory cytokines and chemokines.

On the ultrastructural level, the infection causes massive morphological changes that include the proliferation and rearrangement of the rough endoplasmic reticulum (RER) and lead to the formation of new compartments with an optimal microenvironment that provides functional sites for protein synthesis, processing and RNA replication, whilst providing protection against the host immune system (Welsch et al., 2009; Gillespie et al., 2010; Offerdahl et al., 2012; Miorin et al., 2013). These newly transformed compartments are represented by vesicles or vesicle packets that contain a pore opening to the cytosol (Offerdahl et al., 2012; Miorin et al., 2013) and convoluted membranes with a putative polyprotein processing function (Welsch et al., 2009). A number of other functions have been ascribed to the proliferation of this membrane network, including the concentration of virus replication machinery, the provision of a solid-state platform for viral protein synthesis and replication, and the sequestration of viral dsRNA (the replicative form) from innate immune sensors (Overby et al., 2010; Offerdahl et al., 2012).

We also used electron tomography to provide important insights into the three-dimensional (3D) morphology of the infected cells, and, to the best of our knowledge, this is the first description of the 3D architecture of the tubule-like structures found in the RER of TBEV-infected human astrocytes. Taken together, our findings suggest that astrocytes can significantly contribute to the development of inflammation in the CNS during TBE. This information may facilitate novel strategies for treating this important neural infection.

RESULTS

TBEV can infect and replicate in human astrocytes

We employed a plaque assay and immunofluorescence staining for viral antigen to determine TBEV infection and replication kinetics in primary human brain cortex astrocytes (HBCAs) (Fig. 1). Viral antigen was not detected in mock-infected HBCAs (Fig. 1a) or in cells stained with secondary antibody alone. Based on a total of 23 000 cells counted in at least seven independent fields, approximately 11 % of HBCAs were infected with TBEV at day 3, 14 % at day 7, 10 % at day 10 and 18 % at day 15 post-infection (p.i.) (Fig. 1d).

TBEV replication was quantified using a plaque assay in TBEV-infected cell supernatants collected daily from days 0
to 3 and then at 7, 9, 10 and 15 days p.i. Productive TBEV replication in the form of release of virions was first detected at day 2 after infection, and day 2 also represented the limit of virus production (Fig. 1b). Intracellular TBEV replication assessed by quantitative real-time reverse transcription (RT)-PCR also confirmed virus replication for the first 15 days p.i., and the number of TBEV RNA copies increased in a time-dependent manner (Fig. 1c). We also used phase-contrast microscopy to examine TBEV-infected astrocytes for cytopathogenic effect (CPE) and cell death; neither was observed at any time point (data not shown).

Immunofluorescence staining revealed that the TBEV antigen was distributed mostly diffusely throughout the entire body of the astrocyte at early time points after infection (Fig. 1a). However, at later time points (as early as day 3 after infection), we observed brightly staining aggregates of viral antigen. A co-localization study with protein disulfide isomerase family A, member 3 (PDIA3) antigen (also known as Erp57, Er-60 and GRP58) suggested that the antigen was localized primarily in extremely hypertrophied and rearranged endoplasmic reticulum of the cells as early as day 3 p.i. (Fig. 2).

**TBEV induces the expression of multiple pro-inflammatory cytokines/chemokines in human astrocytes**

Pro-inflammatory cytokines, such as IL-1β and TNF-α, play an important role in mediating neuronal death and neuroinflammation in various diseases. Therefore, we investigated the effect of TBEV infection on the mRNA expression of key pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8, IFN-α and TNF-α. We also measured the expression of MCP-1/CCL2, MIP-1β/CCL4 and IP-10/CXCL10 mRNAs in infected and control astrocytes (Figs 3 and 4). On day 1 p.i., we observed no significant increase in the mRNA expression of any cytokine/chemokine.

The expression of CCL2/MCP-1 mRNA did not change significantly at any time point (Fig. 3f). Robust upregulation of TNF-α mRNA was detected at 3 and 4 days p.i.; however, the expression was decreased at 15 days p.i. (Fig. 3a). IFN-α mRNA expression was slightly upregulated only at day 4 p.i. (Fig. 3b). Although the expression of IL-1β, IL-6 and IL-8 mRNAs increased from day 3 to 4, no upregulation was observed at day 15 p.i. (Fig. 3c–e, respectively). We observed strong increases of CCL4/MIP-1β and CXCL10/IP-10 mRNA expression from 3 to 4 days p.i.; however, at day 15, the expression level for these molecules was similar in infected cells and control cells (Fig. 4a, c).

We used ELISA to investigate the release of secreted MIP-1β/CCL4 and IP-10/CXCL10 cytokines/chemokines in the culture medium of TBEV-infected and control cells. The amount of soluble MIP-1β/CCL4 did not increase until day 4 p.i. At day 5 p.i., the amount of soluble MIP-1β/CCL4 was significantly increased (Fig. 4d). Basal levels of IP-10/CXCL10 in culture media were very low. Starting on day 2

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**Fig. 2.** TBEV antigen is co-localized with PDIA3 antigen in infected HBCAs at later times p.i. HBCAs grown and fixed at day 3 p.i. were stained with anti-flavivirus envelope antibody (green) and anti-PDIA3 antibody (red), and counterstained with DAPI (blue). Co-localization of TBEV and PDIA3 antigens was observed at all investigated time points p.i. Three representative examples are shown. Mock-infected HBCAs stained with primary anti-flavivirus and secondary antibodies (or cells stained with secondary antibodies only) were used as negative controls and did not exhibit any TBEV or PDIA3 antigen staining (not shown).
p.i., the amount of soluble IP-10/CXCL10 increased substantially ($P<0.001$; Fig. 4b).

**TBEV infection induces the production of matrix metalloproteinase 9 by astrocytes**

The expression of matrix metalloproteinases (MMPs), especially MMP-9, correlates with BBB disruption during many neuroinflammatory diseases. Therefore, we investigated the effect of TBEV infection on the production of MMP-9 by astrocytes. The release of soluble MMP-9 into the culture medium of mock- and TBEV-infected astrocytes was detected using ELISA. Starting at day 2 after infection, the amounts of soluble MMP-9 increased continuously until the end of the experiment, with a dramatic increase at day 7 (Fig. 5).

**TBEV infection is associated with the activation of infected astrocytes, as demonstrated by increased glial fibrillary acidic protein (GFAP) expression**

To provide additional evidence that TBEV infection per se causes astrocyte activation, we measured the production of...
GFAP, a marker of astrocyte activation, in mock-infected and TBEV-infected astrocytes, as well as in cells treated with lipopolysaccharide (LPS) at various times p.i. Flow cytometry indicated that the intensity of GFAP production increased significantly in TBEV-infected HBCAs at 3, 7 and 15 days p.i. compared with mock-infected and LPS-treated cells, clearly demonstrating astrocyte activation (Fig. 6a, b). The intensity of GFAP production in the TBEV- and mock-infected cells was also visualized with fluorescence microscopy using a specific anti-GFAP antibody (Fig. 6c).

**TBEV causes dramatic ultrastructural morphological changes in infected astrocytes**

We used transmission electron microscopy and electron tomography to investigate ultrastructural changes in mock- and TBEV-infected HBCAs at 3 and 9 days p.i. At 3 days p.i., we observed rearranged cisterns of the RER with typical virus-induced vesicles and viral particles, as described elsewhere (Fig. 7a) (Růžek et al., 2009b). Next, we observed that many enveloped TBEVs were crowded into the Golgi complex (Figs 7b and 8b). In contrast, at 9 days p.i., we observed a lower number of viral particles in the cisterns of the RER (Fig. 7c) and in vacuoles close to the Golgi complex (Fig. 7d). At 9 days p.i., we observed intra-mitochondrial electron-dense granules that formed annular structures in the mitochondrial matrix (Fig. 7c). Moreover, these cells contained both swollen mitochondria with mitochondrial cristae located in the periphery, and mitochondria that apparently lacked any alteration in morphology (Fig. 7e). Furthermore, some infected cells displayed other...
morphological features of cells undergoing necrotic cell death, such as chromatin aggregates in the nuclear periphery, irregularly shaped cells and surface blebs, and enlarged cisternal space in the endoplasmic reticulum (Fig. 7c–e). In contrast, neither virus replication nor ultrastructural alterations were evident in several other cells from the same

**Fig. 6.** TBEV infection activates HBCAs, as demonstrated by increased GFAP production. (a) Flow cytometry analysis of GFAP production in mock-infected and TBEV-infected HBCAs at 3, 7 and 15 days p.i. is shown as overlapping histograms of relative fluorescence intensity of the analysed cells. (b) The mean fluorescence intensity (MFI) of GFAP-positive cells in culture after mock infection or TBEV infection and LPS treatment at 3, 7 and 15 days p.i. was determined by flow cytometry; it was significantly increased in TBEV-infected HBCAs at all time points investigated. (c) HBCAs grown and fixed at days 3 and 7 p.i. were stained with anti-GFAP antibody (green) and counterstained with DAPI (blue). HBCAs stained with secondary antibody alone were used as a negative control and did not exhibit any GFAP antigen staining (not shown). *P<0.05; **P<0.01; ***P<0.001.
culture and the same time interval (Fig. 7f), which is in accordance with immunofluorescence staining for viral antigen. The ultrastructure of these cells was similar to that of the mock-infected HBCAs (Fig. 8a). Morphological changes induced by TBEV infection involved almost all cell compartments (RER, Golgi complex, mitochondria and phagosomes) as visible on the 3D model of the infected cells (Fig. 8b).

**TBEV induces the formation of tubule-like structures in the endoplasmic reticulum of some infected astrocytes**

We observed tubule-like structures, which were located inside the RER cisternal space of only a very few of the infected astrocytes (Fig. 9 and Movie S1, available in the online Supplementary Material). The tubule-like structures were laid out in many parallel groupings of bundle-like fascicles. The electron density of these tubule-like structures was consistent throughout their shape (Fig. 10a–c), and they were 17.9 nm (± 0.2 nm; n=101) in diameter. In contrast, when viewed using the electron microscope, cellular microtubules outside the ER appeared to be less electron-dense on the inside and were bordered on the outside by two dense lines; the diameter of these microtubules was 20.3 nm (microtubules in Fig. 10d–f). All enveloped viral particles were observed in the lumen of the RER, and most were directly connected to the tubule-like structures (Fig. 11a–d). The diameter of the enveloped
viral particles was 42.5 nm (±0.7 nm; n=12), and the diameter of the nucleocapsid was 26.1 nm (±0.7 nm; n=12). Several TBEV-induced vesicles that were nearly spherical and ranged from 60 to 90 nm in diameter were observed in close proximity to viral enveloped particles. Some TBEV-induced vesicles were connected in a manner identical to that described regarding the tubule-like structures (Fig. 11e–h). We also noted the presence of a subviral particle enclosed in a vesicle in close proximity to structures (Fig. 11e–h). We also noted the presence of a subviral particle enclosed in a vesicle in close proximity to viral enveloped particles. Some TBEV-induced vesicles were connected in a manner identical to that described regarding the tubule-like structures (Fig. 11e–h). We also noted the presence of a subviral particle enclosed in a vesicle in close proximity to the tubule-like structures. The presence of subviral particles (Fig. 11i, j) indicated defective virus assembly.

DISCUSSION

Although TBEV is a significant cause of encephalitis in humans, relatively little attention has been given to TBEV infection of cells in the human CNS. Neurons are primary targets for TBEV (Hirano et al., 2014) in the CNS, and other brain cells may also be infected (Potokar et al., 2014). Although TBEV antigen was not detected in astrocytes in a study investigating brains from fatal human TBE cases (Gelpi et al., 2005), data from other studies indicate that non-neuronal CNS cells including astrocytes are also, albeit infrequently, infected in cases of flavivirus encephalitis (Desai et al., 1995; Nogueira et al., 2002; German et al., 2006; Balsitis et al., 2009; de Araújo et al., 2009; Sips et al., 2012). Infection of non-neuronal cells might play some role in the entry of the virus into the CNS, development of neuroinflammation and viral persistence in the CNS during chronic infection. A recent report demonstrated that primary rat astrocytes are sensitive to TBEV infection, although the infection did not affect cell viability (Potokar et al., 2014). Therefore, it was suggested that astrocytes might represent an important reservoir for dormant TBEV during chronic brain infection (Potokar et al., 2014), for example in cases of chronic TBEV infections reported in humans in Siberia and the Far East (Gritsun et al., 2003). Moreover, an increasing number of studies have demonstrated the important role of astrocytes during encephalitis caused by other flaviviruses, such as West Nile virus and Japanese encephalitis virus (Chen et al., 2000, 2004; Diniz et al., 2006; Kumar et al., 2010; Verma et al., 2011; Yang et al., 2012; Hussmann et al., 2013; Hussmann & Fredericksen, 2014). However, cultured astrocytes were not sensitive to infection with dengue virus (Imbert et al., 1994). In this study, we showed that primary human astrocytes could be infected with TBEV and produce relatively high virus titres (Fig. 1). The viability of the infected cells was not altered during the monitored time interval after infection (15 days), which is in accordance with the findings of a study on primary rat astrocytes (Potokar et al., 2014). In agreement with other authors (Potokar et al., 2014), we can conclude that primary human astrocytes are much more resilient to TBEV infection than other cell types, such as human neuroblastoma, glioblastoma and medulloblastoma cells (Rúžek et al., 2009b). However, the number of infected cells in the culture did not exceed 20 % during the entire investigated period, suggesting that only a fraction of the cells is sensitive to infection, whilst the rest remain resistant. It remains unknown exactly what renders some cells sensitive to the infection and others resistant.

TBEV infection in the brain is associated with the induction of several cytokines and chemokines. Accumulation of cytokines and chemokines in the CNS may accentuate the progression of encephalitis instead of restricting virus replication (Ramesh et al., 2013). Although viral infection is not generally as robust in human glial cells as in neurons, they secrete much higher levels of immune mediators, such as cytokines and chemokines (Verma et al., 2011). Therefore, during the inflammatory response, astrocytes and other glial cells may influence the balance between host protection and neurotoxicity. We reported previously in a mouse study...
that high expression of various cytokines/chemokines during TBE is able to mediate immunopathology, and might be associated with a more severe course of infection and increased fatality (Palus et al., 2013). In the present study, we observed that TBEV infection of astrocytes is associated with the dramatically increased production of various pro-inflammatory cytokines and chemokines. In particular, quantitative real-time RT-PCR and ELISA indicated that the expression/production of IL-1\(\beta\), IL-6, IL-8, IFN-\(\gamma\), TNF-\(\alpha\), IP-10/CXCL10 and MIP-1\(\beta\)/CCL4 was significantly elevated in TBEV-infected astrocytes (Figs 3 and 4), which is consistent with other studies describing cytokine/chemokine production by flavivirus-infected astrocytes (Verma et al., 2011; Yang et al., 2012; Hussmann & Fredericksen, 2014). The greatest increase in cytokine/chemokine production was observed between days 2 and 3 p.i. (Figs 3 and 4). This finding was consistent with the time of peak virus production in HBCAs (Fig. 1b).

Cytokines such as TNF-\(\alpha\) and IL-1\(\beta\) have been reported as potent inducers of neuronal injury (Brabers & Nottet, 2006; Ghoshal et al., 2007; McColl et al., 2008; Kumar et al., 2010; Verma et al., 2011). IL-1\(\beta\), IL-6 and IL-8 are endogenous pyrogens that exert multiple downstream inflammatory signalling pathways (Verma et al., 2011). These cytokines are elevated during various CNS infections, including TBE (Palus et al., 2013). Increased concentrations of pro-inflammatory cytokines, including TNF-\(\alpha\) and IL-6, were detected in sera from TBE patients, and their elevated levels corresponded with the acute phase

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**Fig. 9.** Tubule-like structures observed in TBEV-infected HBCAs at 3 days p.i. This is a 3D reconstruction of a dual axis tomogram (a) and a slice of the tomogram (b). Tilt series images were collected in the range ±65° in 0.65° increments. The final reconstructed section thickness was approximately 60 nm, which was divided into 75 slices. Pixel resolution: 0.81 nm. Bars, 200 nm. This tomogram is shown in Movie S1.
of the disease (Atrasheuskaya et al., 2003). The chemokine IP-10 has the ability to attract activated T-cells in the CNS (Klein et al., 2005). Excessively high levels of IP-10 in the CNS can be very harmful to the host (Sasseville et al., 1996; Westmoreland et al., 1998; Sui et al., 2006), possibly by activating a calcium-dependent apoptotic pathway (Sui et al., 2004). In human TBE patients, higher levels of IP-10 can be detected in serum, as well as in cerebrospinal fluid (Lepej et al., 2007; Zajkowska et al., 2011). The attraction of CD8+ T-cells to the CNS by IP-10 can have important consequences for viral clearance, as well as for immunopathological reactions observed during TBE (Růžek et al., 2009a). Similar to our study, astrocytes have been described as a predominant source of IP-10 in Japanese encephalitis (Bhowmick et al., 2007). The expression of MCP-1/CCL2, a compound that is able to disrupt the integrity of the BBB and modulate the progression of neuroinflammation (Yao & Tsrka, 2014), is highly upregulated in TBEV-infected brain tissue (Palus et al., 2011). However, its expression was not increased in astrocytes after TBEV infection in the present study, suggesting that astrocytes are not responsible for the production of this cytokine in the CNS during TBE. Together, TBEV-infected astrocytes produce a variety of cytokines that can mediate a diverse range of neurodegenerative functions, including disruption of the BBB, chemotaxation of peripheral immune cells into the CNS and neuronal damage.

We reported previously that TBE is associated with the disruption of BBB integrity, which is most likely caused by cytokine/chemokine overproduction in the brain (Růžek et al., 2011). In human TBE patients, higher levels of MMP-9 (a compound with multiple functions, including disruption of the BBB) have been observed in serum (Palus et al., 2014) and cerebrospinal fluid (Kang et al., 2013). However, it was not clear which cells were involved in MMP-9 production and BBB disruption. In the present study, we observed that TBEV-infected astrocytes produced large quantities of MMP-9 (Fig. 5), and therefore might represent the main cell population responsible for the increase of BBB permeability during TBE. The entry of TBEV into the CNS precedes the breakdown of the BBB (Růžek et al., 2011). The invasion of TBEV into the CNS brings the virus into close proximity with the second component of the BBB, astrocytes (Hussmann et al., 2013). TBEV-activated astrocytes then produce MMP-9, which might cause the BBB breakdown. Moreover, MMP-9 is capable of causing neuronal apoptosis (del Zoppo, 2010). Additional evidence of astrocyte activation by TBEV was demonstrated by the increased production of GFAP (Fig. 6). GFAP is involved in many important CNS processes, including cell communication and BBB function. Increased GFAP expression/production as a marker of astrocyte activation has been documented in many studies (Brodie et al., 1997; Zhou et al., 2004; Pozner et al., 2008; Watanabe et al., 2008; Kumar et al., 2010; Fan et al., 2011; Ojeda et al., 2014), represents one of the earliest responses to CNS injury and is a distinguishing feature of reactive astrogliosis (Montgomery, 1994). The activation of glial cells including astrocytes represents one of the major histopathological features of TBE (Környey, 1978; Gelpi et al., 2006). The astrocyte activation leads to a downstream cascade of inflammatory cytokine production that results in the death of neurons (Kumar et al., 2010; Pekny et al., 2014). In our
study, GFAP production was higher in TBEV-infected astrocytes than in mock-infected or LPS-stimulated astrocytes. Treatment with LPS had no effect on GFAP production at 3 and 7 days after treatment, which is in accordance with a previous study that demonstrated that LPS downregulates the expression of GFAP mRNA (Letournel-Boulland et al., 1994). However, on day 15 post-treatment, LPS treatment increased GFAP production in astrocytes compared with controls, although at a lower level than in TBEV-infected cells (Fig. 6a, b).

We demonstrated previously that TBEV infection of human neural cells (neuroblastoma, medulloblastoma and glioblastoma cells) is associated with a number of morphological changes. The infection of medulloblastoma and glioblastoma cells led to proliferation of the RER and extensive rearrangement of cytoskeletal structures (Růžek et al., 2009b). With the exception of typical remodelling of the RER (Figs 7 and 8), here we have described the morphology and 3D organization of TBEV-induced structures, called tubule-like structures, located in the RER of infected HBCAs (Figs 9–11). Tubular structures (also called elongated vesicles or rod-like particles) of sizes ranging from 50 to 100 nm in diameter and 100 nm to 3.5 μm in length have been reported previously inside the RER of other cells infected with either tick-borne flaviviruses (Lorenz et al., 2003; Offerdahl et al., 2012) or mosquito-borne flaviviruses (Welsch et al., 2009). Tubules with closed ends and without pores or connections to other tubules or other structures have been observed in the RER of ISE-6 tick cells persistently infected with Langat virus (Offerdahl et al., 2012). Tubular structures measuring 50 nm in diameter have been found in mammalian COS-1 cells expressing proteins prM and E of TBEV (Lorenz et al., 2003). We supposed that enveloped virus particles were also connected to tubule-like structures in TBEV-infected neuroblastoma cells (Růžek et al., 2009b; Fig. 5). However, the tubular structures that we observed in TBEV-infected astrocytes differed from those reported previously with

![Fig. 11. 3D architecture of tubule-like structures observed in TBEV-infected HBCAs. The connection (arrow) between tubule-like structures and enveloped virions in the model (a, c, e, g) and in a slice of a dual axis tomogram (b, d, f, h). Enlargement of Fig. 9(a), areas c (a, b), d (c, d), e (e, f) and f (g, h) is shown. Supposed subviral particle enclosed in the vesicle outside the RER, detail from Fig. 9(a), area g, is shown in the tomogram top view (i) and side view (j). Tilt series images were collected in the range ±65° in 0.65° increments. The final reconstructed section thickness was approximately 60 nm, which was divided into 75 slices. Pixel resolution, 0.81 nm. Bars, 50 nm.](image-url)
respect to diameter and the appearance of the inner part of the structure. The presence of these tubule-like structures solely in the RER and their small diameter dispute their microtubular origin. Although the function and origin of the tubule-like structures remain unexplained, these structures appear to be a feature of persistent infection (Lorenz et al., 2003; Offerdahl et al., 2012). We propose that viral activity leads to the production of tubule-like structures, either directly as a result of defective virus assembly, or due to disruption of the host’s cellular metabolism.

In TBEV-infected HBCAs at 9 days p.i., we observed the presence of swollen mitochondria next to mitochondria without any ultrastructural alterations. This finding might indicate irreversible injury of some astrocytic mitochondria that is connected with the permeability of mitochondrial membranes and the uptake of water from the cytosol. The remaining mitochondria inside astrocytes may help maintain the energy balance, supporting cell survival and thus contributing (as the glial cells) to neuronal protection. The absence of TBEV particles and lack of ultrastructural alteration in other HBCAs in the culture is in accordance with immunofluorescence staining and the observation of Potokar et al. (2014) regarding the resistance of astrocytes to TBEV-mediated cell death. In contrast to previous observations of TBEV infections of neural cells, we did not observe any signs of apoptosis (Růžek et al., 2009b) or the formation of structures described recently as laminal membrane structures in neurons (Hirano et al., 2014).

In summary, these results demonstrate for the first time, to the best of our knowledge, that cultured human primary astrocytes are sensitive to TBEV infection and are a potential source of pro-inflammatory cytokines in TBEV-infected brain cells, which might contribute to TBEV-induced neurotoxicity and/or BBB breakdown during TBE.

METHODS

**Virus and cells.** The TBEV strain Neudoerfl, a prototype strain of the European subtype, kindly provided by Professor F. X. Heinz from the Medical University of Vienna, was used in all experiments. The virus has been characterized extensively, including its genome sequence (GenBank accession no. U17495) and the 3D structure of its envelope protein, E (Rey et al., 1995). The virus was passaged four times by infecting suckling mice intracranially before its use in the present study.

HBCAs were purchased from ScienCell at passage 1 and propagated in C3C Complete Medium with 10% serum (ACBR) at 37 °C and 5% CO₂. In all experiments, low-passage-number cells were used. Porcine kidney stable (PS) cells (Kozuch & Mayer, 1975) were grown at 37 °C in L-15 medium supplemented with 3% newborn calf serum and a 1% antibiotic mixture of penicillin and streptomycin (Sigma).

**Viral growth in HBCAs.** Monolayer HBCA cultures grown in 96-well plates were inoculated with virus diluted in the culture medium to an m.o.i. of 5. Virus-mediated CPE was investigated using light microscopy. At 0, 1, 2, 3, 5, 7, 9 and 15 days p.i., supernatant medium from appropriate wells was collected and frozen at −70 °C. Titres were determined by plaque assay.

**Plaque assay.** Virus titres were assayed on PS cell monolayers, as described previously (De Madrid & Porterfield, 1969). Briefly, 10-fold dilutions of the virus sample were placed in 24-well tissue culture plates and PS cells were added in suspension (0.6 × 10⁻⁵–1.5 × 10² cells per well). After incubation for 4 h, the suspension was overlaid with carboxymethylcellulose (1.5% in L-15 medium). After incubation for 5 days at 37 °C, the plates were washed with PBS, and the cell monolayers were stained with naphtholene black. Infectivity was expressed as p.f.u. ml⁻¹.

**Immunofluorescence staining.** Infected and non-infected cells on slides were subjected to cold acetone:methanol (1:1) fixation for 10 min, rinsed in PBS and blocked with 10% FBS. Cells were labelled with flavivirus-specific mAb (1:250; Sigma-Aldrich) or with anti-GFAP antibody conjugated with Alexa Fluor 488 (1:200, eBioscience) for 1 h at 37 °C. Flavivirus-specific, mouse mAb and anti-PDIA3 rabbit antibody (1:250; Sigma-Aldrich) were used for double labelling. After washing with Tween 20 (0.05%, v/v) in PBS, the cells were labelled with anti-mouse, goat secondary antibody conjugated with FITC (1:500; Sigma-Aldrich) or anti-rabbit, goat secondary antibody conjugated with Atto 550 NHS (1:500, Sigma-Aldrich) for 1 h at 37 °C. The cells were counterstained with DAPI (1 µg ml⁻¹; Sigma) for 30 min at 37 °C, mounted in 2.5% 1,4-diazabicyclo(2.2.2)octane (Sigma) and examined with an Olympus BX-51 fluorescence microscope equipped with an Olympus DP-70 CCD camera.

**Flow cytometry.** HBCAs were cultured in a 96-well plate at a concentration of 5 × 10⁴ cells ml⁻¹ and infected with the TBEV strain Neudoerfl (m.o.i. of 5) 1 day after seeding. Mock-infected or LPS-treated (at a final concentration of 100 ng ml⁻¹) cells were used as controls. Cells were harvested at 3, 7 and 15 days p.i. Astrocytes were fixed with a Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Briefly, the cultured cells were washed twice with Dulbecco’s PBS (Sigma-Aldrich), trypsinized, inactivated with FCS and centrifuged at 160 g for 5 min at 4 °C. Harvested cells were fixed and permeabilized for 45 min according to the manufacturer’s protocol. An anti-flavivirus group antigen antibody (1:500; Merck-Millipore) and a secondary FITC-conjugated anti-mouse polyclonal antibody (1:500; Sigma-Aldrich) were used to stain the TBEV-positive cells. An anti-GFAP antibody (1:100, Santa Cruz) conjugated to Alexa Fluor 488, was used to stain GFAP-positive cells. The cells were washed with 1× permeabilization buffer after each staining and centrifuged at 300 g for 5 min at room temperature. Finally, the cells were resuspended in 1% FCS and used for flow cytometry analysis on a BD FACS Canto II with BD FACS Diva software. Obtained data were analysed using Flowing Software 2.0, version 2.5.1 (Perttu Terho, University of Turku, Finland).

**Quantitative real-time RT-PCR.** Total RNA was extracted from TBEV-infected HBCAs and control cells and cDNA was synthesized by reverse transcription using an Ambion Cells-to-CT kit (Applied Biosystems) according to the manufacturer’s instructions. The synthesized cDNAs were used as templates for quantitative real-time PCR. The PCR was performed using pre-developed TaqMan Assay Reagents [Assay IDs: IL-6 (Hs00985639_m1), IL-1β (Hs01555410_m1), IL-8 (Hs00174103_m1), IFN-α (Hs00819693_sH), TNF-α (Hs00174128_m1), MIP-1β/CCL4 (Hs09999148_m1), MCP-1/CCL2 (Hs00234140_m1) and IP-10 (Hs01124251_g1)] and TaqMan Gene Expression Master Mix (Applied Biosystems) on aRotor Gene-3000 (Corbett Research). Human β-actin (Hs00900670_m1) and glyceraldehyde 3-phosphate dehydrogenase (Hs03929097_g1) were used as housekeeping genes. The amplification conditions were as follows: 2 min at 50 °C (to allow UNG to destroy any contaminating templates); 10 min at 95 °C (to denature UNG and activate the
enzymes); 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min.

To calculate the fold change in gene expression, the cycle threshold ($C_t$) of the housekeeping genes was subtracted from the $C_t$ of the target gene to yield $\Delta C$. Change in expression of the normalized target gene was expressed as $2^{-\Delta \Delta C}$, where $\Delta \Delta C = \Delta C_{\text{sample}} - \Delta C_{\text{control}}$, as described previously (Livak & Schmittgen, 2001).

Viral RNA was quantified in cells grown in 96-well plates at 0, 2, 7 and 15 days p.i. with TBEV strain Neudoerfl (m.o.i. of 5). The cells were lysed using an Ambion Cells-to-CT kit and subsequently subjected to RNA purification with a QIAamp Viral RNA Mini kit (Qiagen) according to the manufacturer's instructions. Viral RNA was quantified using a TBEV Real-time RT-PCR kit (Liferiver) on a Rotor Gene-3000 (Corbett Research) following the manufacturer's instructions.

ELISA. We used human ELISA kits (Invitrogen) to measure the effect of TBEV exposure on cytokine/chemokine and MMP-9 expression in HBCAs. Mock-infected and TBEV-infected HBCAs were plated in 96-well plates at a density of $2 \times 10^4$ cells per well and infected 24 h later. The cells were infected with TBEV (m.o.i. of 5) and mock infected with the same dilution of brain suspension without virus. At 0, 1, 2, 3, 4, 5, 9 and 15 days p.i., supernatant medium from appropriate wells was collected and frozen at $-70$ °C. Cell supernatants were then assayed for CXCL10/IP10 (Human ELISA kit, KAC2361; Invitrogen), MMP-9 (Human ELISA kit, KHC3061; Invitrogen) according to the manufacturer's instructions.

Transmission electron microscopy and electron tomography. TBEV-infected and control HBCAs that had been grown on sapphire discs were high-pressure frozen at either 3 or 9 days p.i. in the presence of 20% BSA diluted in growing medium using a Leica EM PACT2 high-pressure freezer. Freeze substitution (Leica EM AFS2) was carried out in 2% osmium tetroxide diluted in 100% acetone at $-90$ °C for 16 h, and then warmed up at a rate of 5 °C h$^{-1}$ to remain at $-20$ °C for 14 h, and finally warmed up again at the same rate to a final temperature of 4 °C. Samples were rinsed three times in anhydrous acetone at room temperature and infiltrated stepwise in acetone/xylene mixtures with SPI-pon resin (SPI) (acetone : SPI ratios of 2 : 1, 1 : 1 and 1 : 2, for 1 h at each step). The samples, now in pure resin, were polymerized at 60 °C for 48 h.

Sections were prepared using a Leica Ultracut UCT microtome (Leica Microsystems) and collected on 300 mesh copper grids. Staining was performed using alcoholic uranous acetate for 30 min and in lead citrate for 20 min. Images were obtained using a JEOL 2100F or JEOL 1010 transmission electron microscope. For electron tomography, protein A-conjugated 10 nm gold nanoparticles (Auron) were added to both sides of each section as fiducial markers.

Tilt series images were collected in the range of $\pm 65 \degree$ with 0.65° increments using a 200 kV JEOL 2100F transmission electron microscope equipped with a high-tilt stage and Gatan camera (Orius SC 1000) and controlled by SerialEM automated acquisition software (Mastronarde, 2005). Images were aligned using the fiducial markers. Electron tomograms were reconstructed using the IMOD software package. Manual masking of the area of interest was employed to generate a 3D surface model (Kremer et al., 1996).

Statistical analysis. Data were analysed using version 5.04 of the GraphPad Prism5 software program (GraphPad Software). ELISA measurements of increased chemokine and cytokine production were analysed using one-way ANOVA (Tukey’s multiple comparison test). All other data were analysed using one-way ANOVA (Newman–Keuls multiple comparison test). Differences of $P<0.05$ were considered statistically significant.

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