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REVIEW ARTICLE

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Tick-borne encephalitis virus: molecular determinants of neuropathogenesis of an emerging pathogen

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ABSTRACT

Tick-borne encephalitis virus (TBEV) is a zoonotic agent causing severe encephalitis. The transmission cycle involves the virus, the Ixodes tick vector, and a vertebrate reservoir, such as small mammals (rodents, or shrews). Humans are accidentally involved in this transmission cycle. Tick-borne encephalitis (TBE) has been a growing public health problem in Europe and Asia over the past 30 years. The mechanisms involved in the development of TBE are very complex and likely multifactorial, involving both host and viral factors. The purpose of this review is to provide an overview of the current literature on TBE neuropathogenesis in the human host and to demonstrate the emergence of common themes in the molecular pathogenesis of TBE in humans. We discuss and review data on experimental study models and on both viral (molecular genetics of TBEV) and host (immune response, and genetic background) factors involved in TBE neuropathogenesis in the context of human infection.

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Tick-borne encephalitis virus; neuropathogenesis; neuroinvasiveness; neurovirulence

1. Introduction

The spread of tick-borne encephalitis (TBE) has become a major concern in Europe and Asia over the past 30 years, with both the expansion of risk areas into new regions and the emergence of new endemic areas (Amato-Gauci and Zeller 2012; de Graaf et al. 2016). Indeed, TBE virus (TBEV) infection, encompassing a wide spectrum of diseases ranging from asymptomatic to full-blown encephalitis and even death, is currently a common cause of viral central nervous system (CNS) infection in many countries. Moreover, TBEV infection may lead to long-term or permanent neurological sequelae (Karelis et al. 2012; Zambito Marsala et al. 2014; Šmit and Postma 2015; Lenhard et al. 2016). TBEV belongs to the genus *Flavivirus* in the family *Flaviviridae*. Three closely genetically related TBEV subtypes with different vectors and endemic areas are described: the European subtype (TBEV-Eu), transmitted by *Ixodes ricinus,* and the Siberian (TBEV-Sib) and Far Eastern (TBEV-FE) subtypes, transmitted by *Ixodes persulcatus*. In addition to the three main subtypes, two other subtypes have been recently described; the Baikalian (TBEV-Bkl) subtype comprising 13 strains found in East Siberia and Northern Mongolia (Kozlova et al. 2018), and the Himalayan subtype (TBEV-Him), found in wild rodents in the Qinghai-Tibet Plateau in China (Dai et al. 2018). All three main subtypes are associated with varying courses of the disease and various degrees of severity (Lindquist and Vapalahti 2008). The mechanisms involved in the development of TBE are very complex and likely multifactorial. In fact, the TBEV-Eu, TBEV-FE and TBEV-Sib strains show different degrees of neuroinvasiveness and neurovirulence, which may explain the different clinical courses observed. However, despite these classical and observed pictures, it is important to note that each subtype contains low- and high- pathogenicity strains (i.e. the TBE-Eu strain Hypr shows pathogenicity similar or even higher than that of the TBEV-FE strain Sofjin). Environmental factors in some areas or genetic dispositions of the indigenous human populations may also contribute to the different disease spectra. Recently, data on blood–brain barrier (BBB) crossing by the virus, new putative viral factors, and new advances in the reverse genetic approach have produced novel insight into and perspectives on the understanding of TBE pathogenesis. This review summarizes data on the molecular structure of TBEV,

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and experimental *in vivo* and *in vitro* models of TBEV infection, as well as on the clinical presentation and pathogenesis of TBE in humans, with the particular purpose of emphasizing suspected viral determinants of TBE neuropathogenesis. We focused on the genomic specifics of TBEV strains associated with variable disease severity.

2. Molecular structure and replication cycle of TBEV

The viral cycle in cells infected by flaviviruses leads to the production of three types of particles: immature noninfectious, partially mature and mature virions (Füzik et al. 2018). Mature virions are approximately 50 nm in diameter and have a relatively simple molecular organization, with a nucleocapsid surrounded by a lipid bilayer containing the two envelope glycoproteins: E (envelope) and M (membrane). The nucleocapsid is composed of a single positive-stranded RNA and the capsid protein C (Lindenbach and Rice 2001; Gritsun et al. 2003). The genome length is approximately 11 kb (ranging from 10,405 to 11,103 nucleotides). The differences are due to the variable length of the 3′ untranslated region (UTR), which ranges from 31 to 728 nucleotides in different strains. The genomic 5′-UTR is conserved, with a length of131 nucleotides. In these regions, the RNA forms secondary stem-loop structures that probably serve as cis-acting elements for genome amplification, translation, or packaging (Lindenbach and Rice 2001; Gritsun et al. 2003). The genome contains one open reading frame (ORF) encoding a polyprotein of 3414 amino acids (aa) that is processed co- and posttranscriptionally into three structural proteins (C, M, and E) and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) by cellular and viral proteases (Lindquist and Vapalahti 2008) (Figure 1).

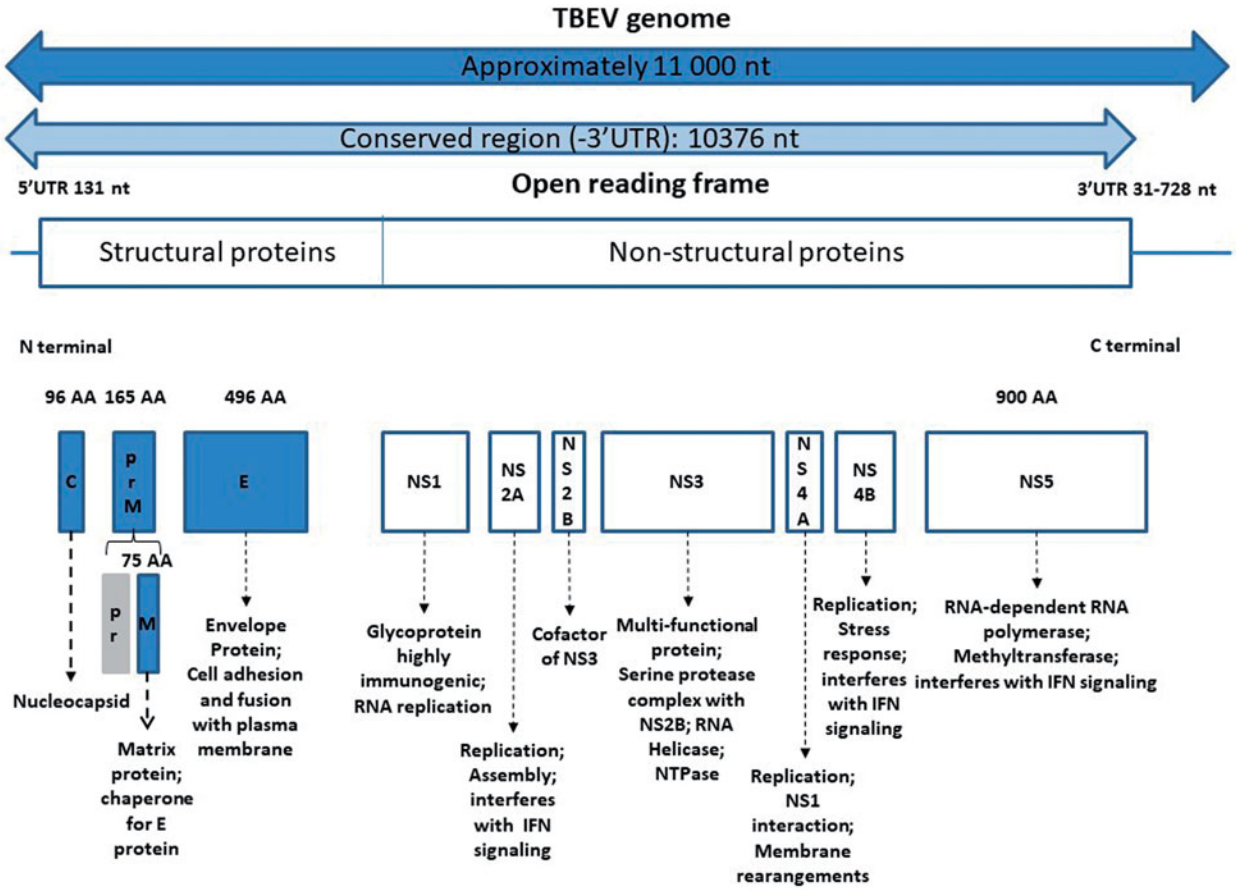

Figure 1. Genome organization of TBE viruses. Schematic view of the genome organization of TBE viruses and the produced polyprotein and its cleavage products. The genome length in nucleotides (Nt) is indicated in brackets. Structural proteins (C, E, and prM/M) are shown in blue boxes and nonstructural proteins in white boxes, with their respective lengths in amino acids (AA). The precursor M (prM) protein corresponds to the precursor form of the membrane (M) protein, present at the surface of immature and noninfectious virions. The dotted black arrows indicate the suspected or known function of each protein.

2.1. Structural proteins

The E protein (496 aa, 50 kDa) is the major surface protein of the viral particle, and the most important antigen (Figure 1). This protein interacts with yet unknown cell receptors and mediates virus–cell membrane fusion. Two major receptor candidates are suggested for TBEV entry in mammalian cells, laminin-binding protein (LBP) and the αVβ3 integrin (Malygin et al. 2009). Heparin sulfate proteoglycans have been shown to mediate, at least in part, viral particle adherence to permissive cells (Kroschewski et al. 2003). In mammalian hosts, the E protein also induces the production of virus-neutralizing antibodies involved in the protective immune response, and its externally accessible region is composed of three distinct domains (DI, DII, and DIII) that are the major targets of neutralizing antibodies. In the mature virion, E proteins are dimerized in a head-to-tail orientation and float on the viral surface. Flat dimers extend in a direction parallel to the viral membrane, with residues important for antibody binding exposed on the outer surface of the protein (Rey et al. 1995; Füzik et al. 2018). In this conformation, the fusion peptide located in the tip of the distal domain is hidden under the proximal part of the dimer partner. Upon cell entry via receptor recognition and endocytosis, acidification of the endosomes triggers an irreversible conformational change (Figure 2). Subsequently, E proteins form homotrimeric spikes, and the fusion peptide is exposed at the tip towards the endosomal membrane, resulting in fusion and the release of the infectious viral genome into the cytoplasm (Füzik et al. 2018).

Viral replication occurs in pockets formed by endoplasmic reticulum (ER) membranes, into which the virus buds and subsequently follows the secretory pathway to exit the cell (Figure 2). Virus assembly occurs in the ER, where protein C (96 aa, 11 kDa), a highly basic protein, packages the RNA genome into nucleocapsids on the cytoplasmic side of the ER membrane (Figure 2). The molar ratio of E protein to C protein in a mature virion has been estimated to be approximately to 1:3 (Schwaiger et al. 2014).

Assembly of the viral envelope containing the precursor M (prM) and E proteins occurs through budding from the nucleocapsid into the ER lumen (Chambers, Hahn, Galler 1990). The prM and E proteins have double-membrane anchors. Each monomer of the E protein contains two transmembrane segments at its carboxy-terminal end, and the second transmembrane region is known to be important for virion formation (Orlinger et al. 2006). This assembly leads to the formation of the immature, noninfectious form of the virion, in which the glycoproteins E and prM are exposed in a heterodimeric form on the viral surface (Lorenz et al. 2002; Elshuber et al. 2003). Immature virions are transported through the host secretory pathway. The prM protein (26 kDa) acts as a chaperone for the E protein to facilitate its correct folding and protects it from ongoing premature irreversible conformational changes during transport of the immature virion through the secretory pathway (Figure 2).

In the acidic vesicles of the late trans-Golgi network, the prM protein on the surface of immature virions is cleaved by the host cell protease furin during exocytosis to generate infectious, M-containing mature virions (Stadler et al. 1997). This final activation cleavage leads to the reorganization of the E protein into fusion-competent homodimers. Infectious mature virions are released from the cell through fusion of the transport vesicles with the host cell plasma membrane (Wengler and wengler 1989) (Figure 2). M proteins (75 aa, 8 kDa) contain one lateral membrane helix, two transmembrane helices and an N-terminal loop region that interacts with E proteins (Füzik et al. 2018).

The E protein also has the ability to form, with prM, smaller enveloped capsid-less virus-like 30-nm icosahedral particles, termed recombinant subviral particles (RSPs) (Allison et al. 1995a). RSPs assemble in the ER and are transported through the secretory pathway in the same way as whole virus particles (Lorenz et al. 2003). Since these particles do not have the structural C protein and viral genomic RNA, they are not infectious.

2.2. Nonstructural proteins

Nonstructural proteins have several enzymatic functions (RNA-dependent RNA polymerase machinery, or serine protease activity needed for the polyprotein cleavage) and seem to have a role in modifying innate immune responses (Lindenbach and Rice 2001).

The NS1 protein (46 kDa) is a highly conserved glycoprotein with 12 invariant cysteine residues. This protein was previously called “soluble antigen” and induces protective immune responses in immunized animals (Jacobs et al. 1992) and infected individuals (Albinsson et al. 2018) (Figure 1). This protein performs a distinct function according to its conformation as a dimer or hexamer. In its dimeric form, the NS1 protein is involved in the replication process (Bugrysheva et al. 2001). However, when the NS1 protein is associated with a membrane, it forms a hexameric, ring-like structure of approximately 10 nm in diameter, which exerts immunomodulatory activities (Winkler et al. 1988).

The NS3 protein (70 kDa) is multifunctional, with nucleoside triphosphatase (NTPase) and helicase

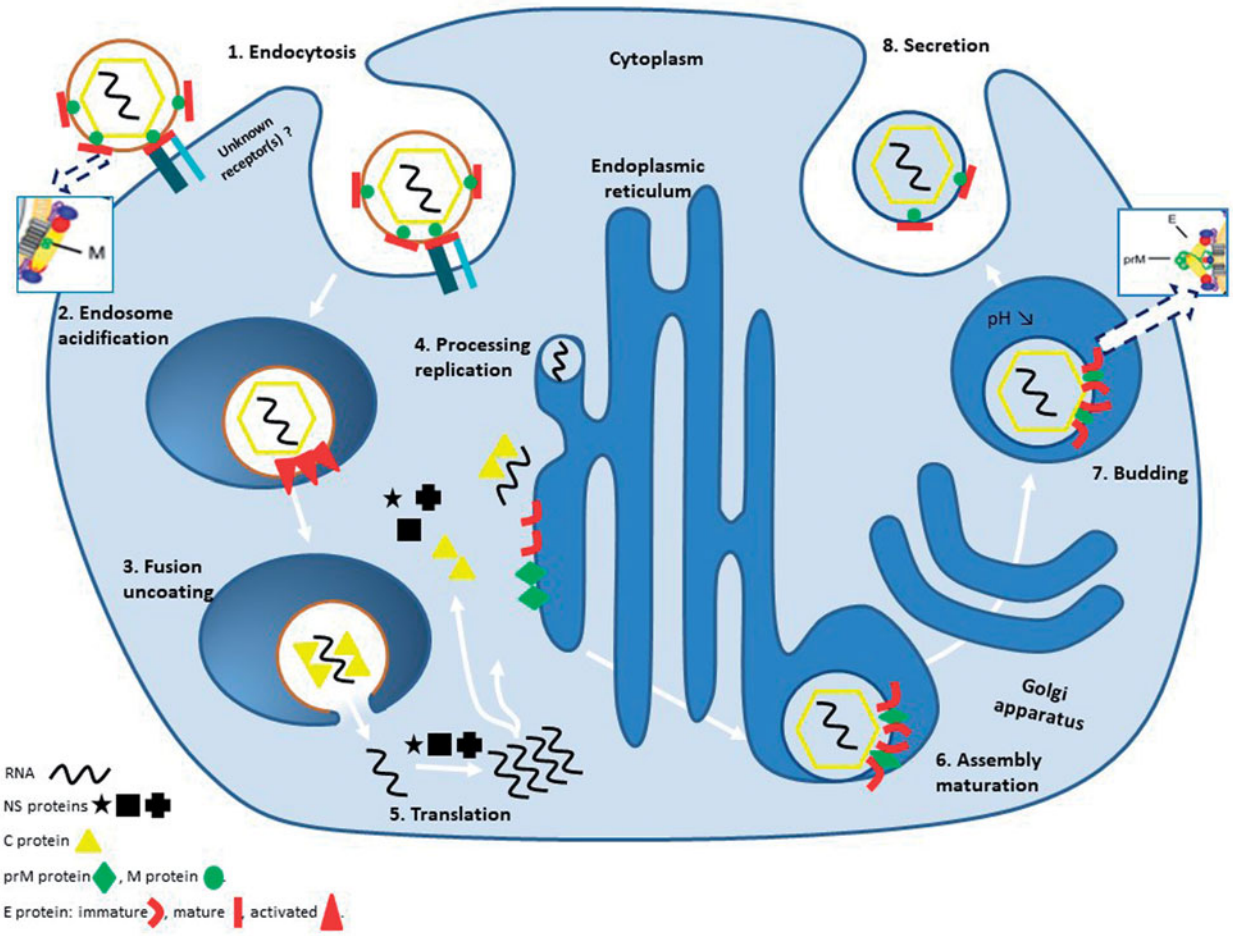

Figure 2. TBE virus life cycle. The E protein interacts with cell receptors and mediates virus–cell membrane fusion. In the mature virion, E proteins are dimerized and float on the virus surface with the fusion peptide hidden under the proximal part of the dimer partner. Upon cell entry by receptor recognition and endocytosis (1 – Endocytosis), endosomal acidification triggers an irreversible conformational change (2 – Endosome acidification). Subsequently, the fusion peptide is exposed at the tip towards the endosomal membrane, resulting in fusion and the release of the infectious viral genome into the cytoplasm (3 – Fusion uncoating). Viral replication takes place in pockets formed by endoplasmic reticulum (ER) membranes (4 – Processing replication), into which the virus buds and subsequently follows the secretory pathway. Virus assembly occurs in the ER, where the C protein packages the RNA genome into nucleocapsids (6 – Assembly maturation). Assembly of the viral envelope containing the precursor M (prM) and E proteins occurs through budding from the nucleocapsid into the ER lumen, leading to the formation of immature, noninfectious virions (7 – Budding). Immature virions are transported through the host secretory pathway. In the acidic vesicles of the late trans-Golgi network, the prM protein on the surface of immature virions, is cleaved by the host cell protease furin during exocytosis to generate infectious, M-containing mature virions. Infectious mature virions are released from the cell through fusion of the transport vesicles with the host cell plasma membrane (8 – Secretion).

domains in the C terminal region (Wengler and Wengler 1991) and a serine protease domain in the N terminal region (Chambers, Weir, Grakoui 1990) (Figure 1).

The NS5 protein is the largest (900 aa, 103 kDa) and the most conserved among the flaviviruses, and contains two domains, a C-terminal RNA-dependent RNA-polymerase (RdRp) domain, and the N-terminal methyltransferase core, which enables NS5 to exhibit the (nucleoside-2′-O-)-methyltransferase activity required for methylation of the cap structure at the 5′ end of the RNA genome (Egloff et al. 2002) (Figure 1). Furthermore, the NS5 protein has been identified as an interferon antagonist (Best et al. 2005).

During the infectious cycle, the NS3 and NS5 (RdRp) proteins form polymerase complexes that are probably associated with membranes through the NS1 and NS2A (22 kDa) proteins (Lindenbach and Rice 2001). The NS3 protein in association with the NS2B (protease component, 14 kDa) protein provides virus-specific serine protease activity for the cleavage of newly synthesized virus polyprotein (Stadler et al. 1997).

The NS4 region of the viral genome encodes two hydrophobic proteins, NS4A and NS4B (Figure 1). NS4B

(252 aa, 27 kDa) is the larger of the two proteins and contains five hydrophobic regions with four transmembrane domains. NS4A (16 kDa) and NS4B probably facilitate appropriate orientation of the polyprotein within intracellular membranes, and thereby ensuring correct cleavage and the function of polymerase complexes. (Lindenbach and Rice 2001).

3. Tools to study TBE neuropathogenesis

The neuropathogenesis of TBE is determined by both the capacity of the virus to enter the CNS after peripheral inoculation, called neuroinvasiveness, and its ability to replicate and cause damage within the CNS, termed neurovirulence (Maximova and Pletnev 2018). Several experimental approaches (animal models and *in vitro* models) are available to assess neuropathogenicity (Table 1).

3.1. Animal models

Many experimental animal models have been used to produce experimental infection and to investigate the neuropathogenicity of TBEV, including large mammals (e.g. dogs (Gresíková et al. 1972), cows (Gresíková 1958), sheep, roe deer, and monkeys (Votiakov et al. 1975, 1982) and small mammals (e.g. Syrian golden hamsters (Zlontnik et al. 1976), hedgehogs (Kozuch et al. 1967), lizards (Gresikova-Kohutova and Albrecht 1959), moles (Kozuch et al. 1966), and mice (Kozuch

Table 1. Major benefits and drawbacks of TBEV *in vitro* and *in vivo* study models.

Model	Benefits	Drawbacks	Main discoveries	Ref.
Animal models				
Large mammals				
Sheep	Susceptible to TBEV infection	Rarely develop clinical symptoms, except if intracerebrally inoculated	Association of pathogenesis with each TBEV subtype	Votiakov et al. (1975, 1982) and Mansfield et al. (2016)
Monkeys		Many limitations (ethical considerations, high cost) High variability in experimentally induced clinical presentation according to *Macaca* species		Gritsun et al. (2003), Votiakov et al. (1975, 1978), Slonim and Závadová (1977), Zlontnik et al. (1976), and Süss et al. (2007, 2008)
Small mammals (rodents)				
Syrian hamsters	Susceptible to TBEV infection	Very artificial experimental model of TBE		Frolova et al. (1987) and Zlontnik et al. (1976)
Laboratory mice	Most practical, available and frequently used animal models	Susceptibility to TBEV closely related to the age of the mice No clear correlation between the virulence of strains in mice and strain pathogenicity in humans	Association of pathogenesis with each TBEV subtype Site-directed mutations/ deletions studies	Andzhaparidze et al. (1978), Brinton and Perelygin (2003), Rumyantsev, Murphy, Pletnev et al. (2006), Engel et al. (2010), and Palus et al. (2013)
Isolated wild-type viral strains	Isolated from brains of fatal cases in humans or mice	Culture of TBEV in laboratory conditions affects both the genotypic and the phenotypic characteristics of the viral strain	Association of pathogenesis with each TBEV subtype	Andzhaparidze et al. (1978), Malenko et al. (1982), Romanova et al. (2007), Růzek et al. (2009b), and Leonova et al. (2017)
Reverse genetic systems				
PCR-based methods	Recovery of infectious viruses directly from nucleic acids encoding the viral genomes	Heterogeneous cDNA template population	Site-directed mutations/ deletions studies	Gritsun et al. (1995)
Infectious cDNA clone		Difficult and laborious protocols Not suitable for routine use in diagnostic laboratories		Pletnev et al. (1992, 1993), Mandl et al. (1997, 2000), Gritsun et al. (2001), Maximova et al. (2008), Ashgar et al. (2016), and Khasnatinov et al. (2016)
Infectious-subgenomic-amplicon (ISA) method	Rescue infectious virus from clinical and/or animal samples	Reliability and efficacy of this method have to be confirmed	Isolation of infectious single-stranded positive-sense RNA viruses from clinical or animal samples	Aubry et al. (2014) Aubry, Nougairede, de Fabritus et al. (2015), Aubry, Nougairede, Gould et al. (2015) and De Fabritus et al. (2015, 2016)

et al. 1981; Monath and Heinz 1996). Large mammals are accidental hosts in the TBEV transmission cycle, and are not able to further spread the virus. Experimental infections had been produced in dogs, cows, or roe deer. In the past decade, these species have been used as sentinel species in endemic areas (Pfeffer and Dobler 2011; Imhoff et al. 2015).

Experiments in which sheep were inoculated intracerebrally with either TBEV-Sib or TBEV-FE have demonstrated high neurovirulence of TBEV-FE, with a tropism for glial cells, and persistent infections with TBEV-Sib, with a tropism for neuronal cells (Votiakov et al. 1975, 1982). Subcutaneous inoculation of European strains into sheep induces viremia and viral excretion in milk. The virus is detected mostly in lymphatic system organs, where it persists for only a few days post infection (Gritsun et al. 2003; Mansfield et al. 2016).

Several species of monkeys have been used to study TBE neuropathogenesis (e.g. *Macaca mulatta*, *Macaca cynomolgus*, and *Macaca sylvanus*). *Macaca mulatta* monkeys are less sensitive to TBEV infection than *Macaca cynomolgus* monkeys (Frolova and Pogodina 1984). Intracerebral or intraspinal inoculation of TBEV leads to the development of clinical and histopathological signs of encephalitis. The severity of the disease correlates with the virulence of the TBEV strain used. Animal studies conducted in monkeys, that produced different forms of experimental TBE, support the hypothesis of high TBEV-FE neurovirulence and persistent infection with TBEV-Sib (Votiakov et al. 1975, 1982; Fokina et al. 1982; Frolova and Pogodina 1984; Gritsun et al. 2003). Subcutaneous inoculation of European strains leads to an asymptomatic infection with viremia and seroconversion (Slonim and Závadová 1977). Monkeys infected intranasally or intracerebrally with European TBEV develop chronic encephalitis with degenerative spongiform lesions and astrocytic proliferation (Zlontnik et al. 1976). Süss et al. (2007, 2008) described the first case of TBE in a monkey (*Macaca sylvanus*) after natural exposure (tick bite) in a TBE risk area. The TBEV-Eu strain (closely related to the Neudoerfl strain) was present in the brain. Clinical illness similar to that observed in a typical severe human TBE case was observed (Süss et al. 2007, 2008). More recently, other monkey species have been used experimentally (e.g. *Cercopithecus aethiops* and *Macaca fascicularis*) to study the efficacy of novel vaccines and treatment preparations against TBE using TBEV-Eu strains (Pripuzova et al. 2013).

Syrian golden hamsters are sensitive to TBEV infection, but adult hamsters show reduced susceptibility. Siberian strains produce chronic disease in this animal model (Frolova et al. 1987). Asymptomatic infection with European strains can produce seroconversion and development of histopathological changes in the brains of infected hamsters (Zlontnik et al. 1976).

Mice are suitable animal models for infection with TBEV because the symptoms and physiopathological markers are similar to those observed in severe cases of the disease in humans. However, asymptomatic forms of TBE in humans are much more common than clinical forms, rendering mice unsuitable for modelling sublethal and chronic forms of TBE. Laboratory mice (e.g. ICR, Swiss Albino or BALB/c mice) are the most practical and most commonly used animal models for TBE, since most laboratory mouse strains are more susceptible to TBEV infection than wild mice, (Mandl 2005). This characteristic is due to the high susceptibility of most laboratory mouse strains to flavivirus infection, which is genetically mapped to a stop codon mutation in the coding region of the 2′–5′-oligoadenylate synthetase gene Oas 1 b (Brinton and Perelygin 2003; Palus et al. 2013). Intracranial or peripheral inoculation of wild-type strains of TBEV into mice usually results in a lethal infection that resembles severe cases of TBE in humans (Mandl 2005). However, the severity of the disease is closely related to the age of mouse (Andzhaparidze et al. 1978). Intracranial inoculation of TBEV into suckling or juvenile mice allows the study of neurovirulence (Mandl 2005; Rumyantsev, Murphy, Pletnev et al. 2006; Engel et al. 2010), while neuroinvasiveness is investigated by peripheral (subcutaneous or intraperitoneal) inoculation of juvenile or adult mice (Mandl 2005; Pletnev et al. 2006; Engel et al. 2010).

3.2. Viral strains

Isolation of viral pathogens from clinical and/or animal samples has traditionally relied on either cell cultures or laboratory animal model systems. Wild-type viral strains are isolated from the brains of human patients with fatal cases and stored at −20 °C as lyophilized samples (Leonova et al. 2017). Conversely, several wild-type strains have been used to produce TBEV infection in animal models and/or on cell cultures systems, to study particular aspects of neuropathogenesis. Among the many viral strains used, here are some of the most commonly used TBEV strains for *in vivo* or *in vitro* infection test. The TBEV-Sib strain Vs was isolated from a patient with a chronic form of TBE. This strain was largely used to model chronic TBE in monkeys and Syrian golden hamsters (Malenko et al. 1982). The TBEV-FE Soph-K strain was inoculated into Syrian golden hamsters

(intracranially or subcutaneously), producing clinical disease in a few animals (14%), although pathological lesions characterized as meningoencephalitis were found in the CNS of the majority of the animals (Andzhaparidze et al. 1978). Furthermore, monkeys inoculated intracerebrally with this strain show an asymptomatic infection with subacute disseminated meningoencephalitis, with a progradient chronic course lasting 3 months, providing a model of progressive degenerative CNS disease (Andzhaparidze et al. 1978). The TBEV-FE Sofjin strain is often inoculated in mouse models (Leonova et al. 2017). Regarding the TBEV-Eu subtype, the Neudoerfl and Hypr strains are thoroughly characterized and have been extensively used to produce infection in mouse models; the Hypr strain has even been used in cell lines of neural origin (Růzek et al. 2009a, b). However, culturing TBEV under laboratory conditions is known to affect both the genotypic and phenotypic characteristics of the virus (Romanova et al. 2007). Thus, both the Hypr and Neudoerfl strains are usually used as low-passage viruses in such experiments.

3.3. In vitro models

3.3.1. Reverse genetic systems

The viral genomic RNA is infectious and produces progeny virus when transfected into susceptible cells (Mandl et al. 1997). Several approaches are available to generate infectious viruses *in vitro*. The first important step in these different processes is the generation of complementary DNA (cDNA). For TBEV, the two classical approaches are PCR-based methods for constructing recombinant virus and plasmid-based methods to produce infectious clones.

The first method, the PCR-based method, was developed by Gritsun et al. (1995) and consists of the production of infectious RNA transcripts of TBEV, in which uncloned, full-length cDNA is generated by RT-PCR amplification and subsequently used as an *in vitro* transcription template (Gritsun et al. 1995). The major drawback of this approach lies in the heterogeneity of the cDNA template population, making the method unsuitable for rigorous examination of the impact of single mutations on the phenotypic characteristics of derived virus populations. In the plasmid-based infectious clone approach, cDNA is cloned into a plasmid under the control of a specific promoter, which enables *in vitro* transcription of viral RNA (Mandl et al. 1997, 2000; Gritsun et al. 2001; Ashgar et al. 2016). Infectious cDNA clones represent a useful experimental system for the specific mutagenesis of TBEV, exhibiting measurable biological properties (replication and infection abilities) allowing comparison to parental wild-type strains (Takano et al. 2011). Site-directed mutagenesis of infectious cDNA clones is a useful technique for the characterization of virulence determinants. Some of these cDNA clones consist of intratypic chimeras (composed of two different strains of TBEV) (Khasnatinov et al. 2016) or heterotypic chimeras of TBEV and dengue-4 virus (TBEV/DEN4) (Pletnev et al. 1992, 1993). Using a chimeric cDNA clone of dengue 4 virus containing the substituted TBE M and E genes from a virulent Far Eastern strain (Sofjin strain), Pletnev et al. (1992, 1993) showed that viral chimeras could infect and replicate in mosquito cells. They also showed that the viral chimeras acquired the capacity to invade the CNS of mice, demonstrating that the E protein determines the cellular tropism of flaviviruses (Pletnev et al. 1992, 1993). The chimeric TBEV/DEN4Δ30 cDNA clone contains the prM and E structural protein genes of the FE-TBEV strain Sofjin and a 30-nucleotide deletion in the 3′-UTR of the DEN4 segment of the chimeric genome. This deletion is genetically stable and was shown to attenuate DEN1, DEN4, and West Nile/DEN4 viruses in mice, monkeys and humans (Rumyantsev et al. 2006). Maximova et al. (2008) showed that the TBEV/DEN4Δ30 clone demonstrates high viral replication and virus-associated histopathology in the CNS, with lesions in the cerebral hemispheres but progressively decreasing towards the spinal cord (Maximova et al. 2008).

The major advantages of the cDNA clone approach are that the genome is defined, and that it can be manipulated. In contrast, natural viral isolates are present as quasispecies making site-directed mutagenesis studies difficult and nonspecific. However, the generation of infectious cDNA clones are laborious and difficult due to the instability and toxicity of some viral sequences in bacteria (Aubry, Nougairede, de Fabritus et al. 2015).

The infectious-subgenomic-amplicon (ISA) method is the most recent technique for generating TBEV clones. Three PCR amplicons that have a cytomegalovirus (CMV) promoter at the 5′-UTR and 70- to 100-bp overlapping regions are produced. The amplicons are mixed and introduced into cells, where they recombine and produce infectious viruses (Aubry et al. 2014; Aubry, Nougairede, Gould 2015). The ISA method is an effective approach to rescue infectious viruses from clinical and/or animal samples that may have deteriorated during the collection and storage period. Attenuated TBEVs have successfully been produced using the ISA method combined with random codon re-encoding (De Fabritus et al. 2015, De Fabritus et al. 2016).

3.3.2. Molecular constructs

Constructs obtained from recombinant plasmids that express prM and the full-length E proteins of TBEV (i.e. TBEV strain Oshima 5–10) have been shown to produce virus-like particles (VLPs), which are smaller than virions (30 nm versus 50 nm in diameter) and have surface properties and fusion activities similar to those of infectious viruses. These constructs are currently used to assess antiviral candidates, as they mimic early events of viral entry. VLPs are also, excellent immunogens and excellent candidates for a recombinant vaccine against infections with TBEV (Allison et al. 1995a; Schalich et al. 1996; Yoshii et al. 2003).

3.3.3. Cell line culture

TBE viruses used for *in vitro* experiments are usually grown in primary chicken embryo cells (CE) (Heinz and Kunz 1981), in porcine embryonic kidney (PEK) or pig kidney epithelial (PS) cells (Ershova et al. 2016), or in baby hamster kidney-21 (BHK-21) cells (Mandl et al. 2000). These cell lines were used to investigate the infectivity of virus stocks (Mandl et al. 1997; Holzmann et al. 1997). Simian Vero B4 cells and human lung carcinoma A549 cells have also been used for infectivity measurements (Ashgar et al. 2016; Maximova et al. 2008). Palus et al. (2017) used primary human brain microvascular endothelial cells (HBMECs) to study BBB crossing. These cells support TBEV entry into the brain via a transcellular pathway, but without altering the expression of key tight junction proteins and cell adhesion molecules or compromising BBB integrity (Palus et al. 2017). Růzek et al. (2009b) inoculated several neural cell lines (neuroblastoma (UKF-NB-4), medulloblastoma (DAOY) and glioblastoma cells) with TBEV strain Hypr (Růzek et al. 2009b). All the neural cell lines tested are susceptible to TBEV infection and produce approximately 100- to 10,000-fold higher virus titers than the cell lines of extraneural origin, indicating the high susceptibility of neural cells to TBEV infection. Viral strains can also be cultured in tick cell lines to study viral replication and maturation processes (Senigl et al. 2004), or host antiviral responses by transcriptomic and proteomic analysis (Weisheit et al. 2015).

4. Pathogenesis and pathology of TBE in humans

4.1. Clinical signs

TBEV is usually transmitted by tick bites or, rarely, by the consumption of unpasteurized milk products from infected cattle. The average incubation period is 8 days after a tick bite (range, 4–28 days) (Zambito Marsala et al. 2014). The incubation period can be shorter (2–4 days) in cases of food-borne infection (Hudopisk et al. 2013).

All three TBEV subtypes are traditionally associated with varying disease courses (i.e. a monophasic, or biphasic course, or a chronic form) and various degrees of severity, although clinical pictures could be various among a same subtype according to the viral strain in cause.

4.1.1. TBEV-Eu subtype

TBEV-Eu strains characteristically cause a milder biphasic infection. Few infected people develop clinical symptoms (only 20–30%). The first viremic phase, starting approximately 8 days after the tick bite, is characterized by a flu-like syndrome and lasts 5 days (range, 2–10 days). This presentation is followed by an asymptomatic period of 7 days (range, 1–21 days). The second stage occurs in 20–30% of patients, with neurological disorders of differing severity (Lenhard et al. 2016; Taba et al. 2017). Meningoencephalitis is the most frequent clinical presentation (20–60% of patients). Meningoencephalomyelitis and meningoencephaloradiculitis are the most severe forms of the disease and affect approximately 10% of patients (especially older individuals). In serious infections, death may occur, usually due to diffuse brain edema and brain engagement or medulla oblongata involvement. Magnetic resonance imaging (MRI) findings may be abnormal with nonspecific T2 hyperintense signals, particularly in the thalamus, brainstem, cerebellum and peripheral nervous system (Zambito Marsala et al. 2014; Lenhard et al. 2016). The electroencephalogram (EEG) shows abnormalities in 50–70% of patients, with encephalitis symptoms (Zambito Marsala et al. 2014; Lenhard et al. 2016). Disease in children is less severe than in adults, with reported case fatality rates of less than 2%. Old age, male sex, diabetes, disease severity in the acute stage, meningoencephaloradiculitis, meningoencephalomyelitis (Lenhard et al. 2016), abnormal MRI findings, and pleocytosis (CSF cell count >300/L), BBB impairment and low anti-TBE neutralizing antibody titers are associated with poor prognosis (Karelis et al. 2012; Zambito Marsala et al. 2014; Taba et al. 2017). Although sequelae, also termed "postencephalitic syndrome", are less frequent with TBEV-Eu strain infection than infection with the other two subtypes, some signs can persist for several months or years. Mild sequelae comprise emotional lability, asthenia, and intermittent headache. Moderate sequelae include ataxia, paresis of the extremities, and mild cognitive disorders. Severe

sequelae are persistent neurological deficits (especially hemiparesis), intensive postencephalitic complaints, and severe cognitive disorders impacting quality of life (Karelis et al. 2012). In a prospective cohort study, Lenhard et al. (2016) found that long-term outcomes for patients with meningoencephaloradiculitis were considerably worse (median modified Rankin Scale (mRS) = 4) than those for patients with meningoencephalitis (mRS = 1, $p < 0.0001$) or meningitis (mRS = 0, 57.7% of the cohort) (Lenhard et al. 2016).

4.1.2. TBEV-FE subtype

TBEV-FE strains cause the most severe form of CNS disorder with recorded case fatality rates of 5–20% (Süss 2008b). The onset of disease is acute (sudden high fever associated with a meningeal syndrome and neurological impairments and/or paralysis) with no biphasic course of disease presentation. However, recent serological evidence suggests that asymptomatic or unspecific manifestations may exist in cases of infection with TBEV-FE strains (Yoshii et al. 2017). The clinical picture is severe, with coma, focal meningoencephalitis, encephalitis, and myelitis, involving the brainstem and spinal cord structures. Patients usually develop prolonged sensations of fatigue during recovery. Significant and diffuse lesions in the central and peripheral nervous systems have been reported. In the most severe forms, there is extensive involvement of the CNS, with major damage to neurons in different parts of the brain and spinal cord (Votiakov et al. 1975, 1982).

4.1.3. TBEV-Sib subtype

TBEV-Sib strains usually induce a less severe clinical presentation than Far-Eastern viruses, and are associated with a high prevalence of the nonparalytic febrile form of encephalitis. Moreover, there is a tendency for patients to develop a chronic progressive form of TBE in 1–1.7% of the cases. Chronic TBE forms are usually defined by clinical presentation including Kozhevnikov's epilepsy (more commonly known as epilepsia partialis continua in Western countries), lateral sclerosis, progressive neuritis, progressive muscle atrophy, and a Parkinson-like disease; these forms affect mainly working-age people and children (Poponnikova 2006). The recorded fatality rates range from 6 to 8%. Asymptomatic infections produced by these viruses have also been identified (Jääskeläinen et al. 2016). Experiments in animals (i.e. monkeys, Syrian golden hamsters) provide evidence of the possible association of chronic forms of TBE with TBEV-Sib strains. A prolonged incubation period is observed (15–24 days post-infection) followed by a progressive development of neurological symptoms (Pogodina et al. 1981a, 1981b; Fokina et al. 1982; Frolova and Pogodina 1984). Both mutations (T277V and E279G) in the *TBEV NS1* gene (Gritsun et al. 2003) and an inappropriate T-cell immune response (Naslednikova et al. 2005) have been suspected to be associated with chronic progressive disease (Lindquist and Vapalahti 2008).

4.2. TBE neuropathogenesis

A schematic representation of TBE pathogenesis is shown in Figure 3. The virus is transmitted by saliva from the tick during the first minutes of its blood meal (Hermance and Thangamani 2018). The tick bite triggers the activation of the innate immune response via the expression of highly conserved for pathogen recognition molecules (Toll-like receptors, e.g. TLR3) and signal transduction pathways (NF-kappa B, Janus kinase (JAK)-signal transducer and activator of transcription (STAT) proteins, retinoic acid-inducible gene-I-like receptor (RIG-1), and protein kinase R (PKR)) (Figure 3) (Robertson et al. 2009). After inoculation, the virus replicates mainly in immature dendritic cells (DCs) of the skin (Figure 3). However, viral antigens have also been observed in neutrophils, and monocytes of skin explants from tick-feeding sites (Labuda et al. 1996).

It has been suggested that tick saliva facilitates the transmission of TBEV to vertebrates (Labuda et al. 1993). Several studies have previously shown that tick saliva modulates TBEV infection in DCs. Fialová et al. (2010) observed an increase in the proportion of virus-infected cells and a decrease in the virus–induced expression of TNFα and interleukin-6 (IL-6) when TBEV-infected DCs were treated with tick saliva (Fialová et al. 2010, Shevtsova et al. 2017). Lieskovská et al. (2018) found that viral replication was significantly increased by saliva of the *Ixodes ricinus* tick, inducing enhancement of Akt pathway activation in TBEV-infected DCs. The Akt pathway is a signal transduction pathway that promotes survival and growth in response to extracellular signals. This pathway plays a critical regulatory role in various cellular processes, such as apoptosis, autophagy, RNA processing, endocytosis, and translation, and is therefore targeted by many viruses. The authors suggested that tick saliva provides pro-survival and anti-apoptotic signals to infected DCs via the upregulation of Akt, which may have positive consequences for TBEV replication and transmission (Lieskovská et al. 2018).

Infected immature DCs transport TBEV to nearby draining lymph nodes and lymphoid organs where it replicates in B cells, T cells, and macrophages (Figure 3).

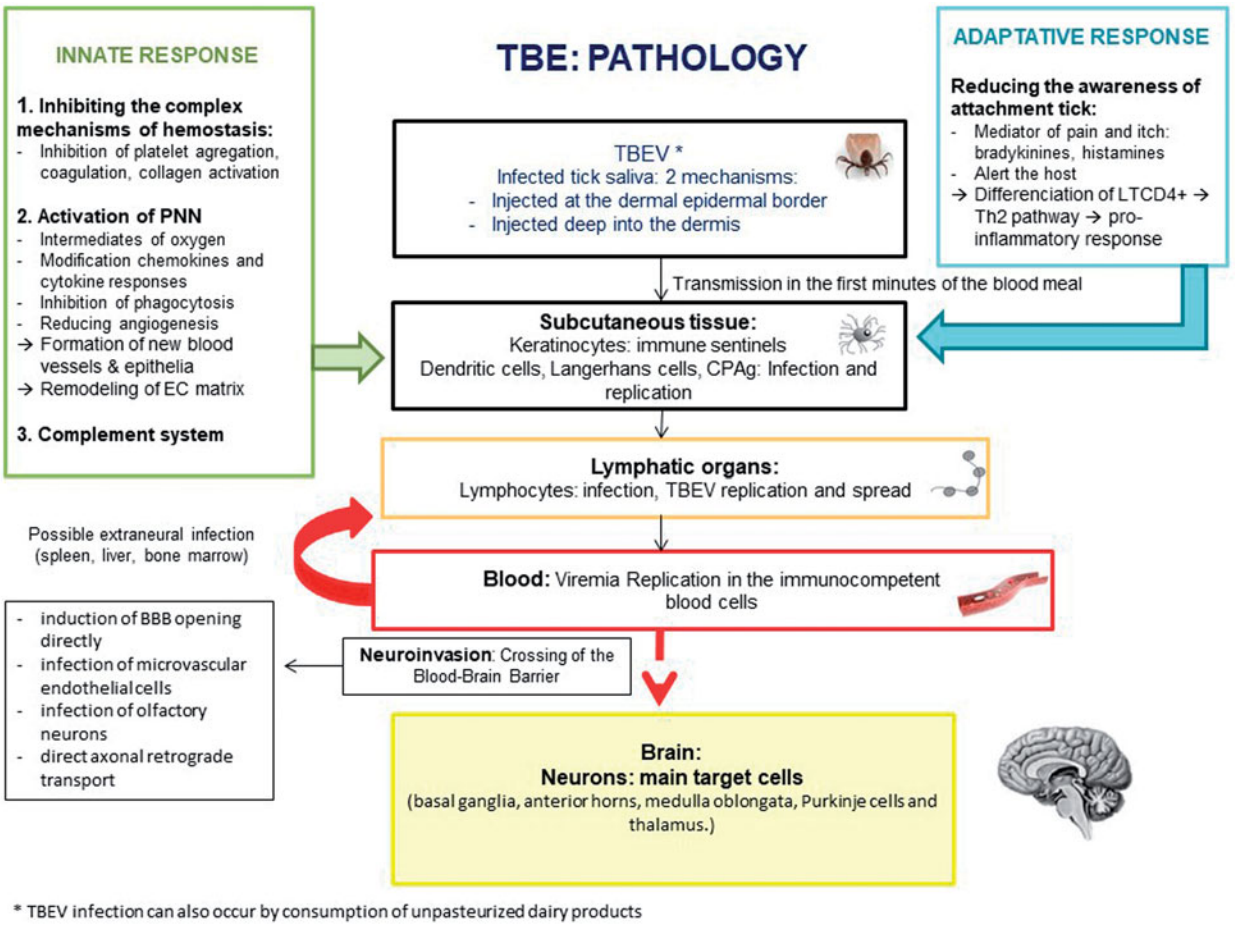

Figure 3. TBE: Pathology. Schematic representation of TBE pathology from initial infection via a tick bite to the occurrence of brain infection, through viremia and blood–brain barrier crossing. Major events due to the innate immune response are summarized in the green box; and those due to the adaptive response, in the blue box.

DCs undergo a maturation process that grants them the ability to activate naive T cells to T helper type 1 (Th1), Th2, and cytotoxic T-lymphocyte (CTL) effector cells (Robertson et al. 2009; Růzek et al. 2010). *In vitro*, infection of DCs with TBEV inhibits DC maturation and selective inhibition of IL-12 secretion, reducing T cell proliferation (Robertson et al. 2014).

In the lymph nodes, an initial inflammatory reaction occurs with activation of monocytes, macrophages, and polymorphonuclear neutrophils and induction of the complement system. Then, C3 convertase formation amplifies the complement-mediated response. Viremia occurs after viral replication and spread through efferent lymphatic vessels and the thoracic duct. TBEV has been isolated from human blood leukocytes during the first days after the tick bite, indicating that the virus is replicating in blood cells (Růzek et al. 2010). Many extraneural tissues are infected in humans (especially the spleen, liver, and bone marrow); the release of the virus from these tissues enables viremia to persist for several days (Růzek et al. 2010; Zambito Marsala et al. 2014).

TBEV enters the CNS by the hematogenic pathway (Figure 3). Several suspected mechanisms may lead to brain invasion: (i) infection of olfactory neurons and peripheral nerves, (ii) viral entry into vascular endothelial cells of brain capillaries, or (iii) crossing of the BBB (Robertson et al. 2009; Růzek et al. 2010). The most likely hypothesis is that the virus enters the cerebral parenchyma through the blood vessels after attachment to endothelial cells by interaction with adhesive proteins (e.g. VCAM-1) and infects the systemic and cerebral capillary endothelium (Robertson et al. 2009; Růzek et al. 2010; Zambito Marsala et al. 2014; Palus et al. 2017). There is also another suspected mechanism, termed the "Trojan horse" mechanism in which the virus is transported by infected immune cells that traffic to the CNS (Palus et al. 2017).

4.2.1. TBE virus target cells

TBEV preferentially infects large neurons in the grey matter of the basal ganglia, anterior horns, medulla oblongata, spinal cord, brainstem, Purkinje cells, pons,

cerebellum, and thalamus (Dietmann et al. 2016). Oligodendrocytes are rarely infected, while infected astrocytes seem to be a potent source of pro-inflammatory mediators, with possible pathologic consequences to the nervous tissue (Palus et al. 2014). Viperin (virus-inhibitory protein, ER-associated, IFN-inducible) is an interferon-stimulated gene (ISG) with broad-spectrum antiviral activity against multiple flaviviruses *in vitro*. Its activity *in vivo* restricts neurotropic infections to specific regions of the CNS. Lindqvist et al. (2018) examined both the regional and cell-type specificity of viperin in the defense against infection by several model neurotropic flaviviruses, including TBEV in primary neural cultures. *In vitro*, viperin restricts TBEV replication in primary cortical neurons but not in cerebellar granule cell neurons. Interferon-induced viperin is also very important for the control of TBEV replication in primary cortical neurons. These recent findings show that viperin restricts the replication of neurotropic flaviviruses in the CNS in a regional and cell-type-specific manner (Lindqvist et al. 2018).

Recently, *in vitro* infection of astrocytes with TBEV has been confirmed in several studies, in primary rat, human and mouse astrocytes (Růzek et al. 2009b; Potokar et al. 2014; Palus et al. 2014; Lindqvist et al. 2016). Astrocytes are an abundant and heterogeneous neuroglial cell type and a key element fostering CNS homeostasis, and are one of the first CNS cell types to become infected during viral infections of the CNS. Moreover, these cells are morphologically closely aligned with neuronal synapses, blood vessels, and ventricle cavities, and thereby are able to functionally interact with neurons and endothelial cells. These findings indicate that astrocytes epitomize a potential mediator of brain infection and a reservoir of brain TBEV in rodents. In humans, brain astrocytes may have a similar role (Potokar et al. 2019; Zorec et al. 2019).

4.2.2. Direct cytopathogenic effect *of TBE virus*

TBEV infection generates several types of brain damage (cytotoxic T cell and neutrophil infiltration, microglial cell proliferation, hyperemia, edema, neurophagia, dysfunction of the infected cells, neuronal degeneration, necrosis, and apoptosis). These are probably the consequence of viral infection and the resulting inflammatory response (Růzek et al. 2010). In fact, TBEV RNA is rarely detected in CSF (Saksida et al. 2005, 2018; Nagy et al. 2018; Veje et al. 2018) or in the brain in fatal cases (Kuivanen et al. 2018; Tomazic et al. 1997; Tomazic and Ihan 1997; Lipowski et al. 2017), which renders it difficult to attribute a direct cytopathogenic effect to TBEV. Moreover, during the early phase, glucose hypermetabolism has been observed, reflecting the inflammatory reaction. During the last phase, glucose hypometabolism signals neuronal dysfunction (Dietmann et al. 2016). The balance between the direct viral cytopathogenic effect and the resulting effect of the inflammatory response may differ depending on the TBEV subtype. In fact, during infection with TBEV-FE subtypes, rapid neuronal degeneration occurs due to direct replication of the virus in these cells. When inoculated into experimental animals, TBEV-FE strains directly infected and damaged neurons in the brain, resulting in severe encephalitis (Votiakov et al. 1975, 1982). TBEV-Eu subtypes generally do not produce severe infection in neurons (Bílý et al. 2015). Damage to neurons is only observed in some animals as a secondary inflammatory effect arising from infection of glial cells. In fact, TBEV-Eu strains initially do not replicate in or damage neurons even after intracerebral inoculation. The primary target of TBEV-Eu is lymphoid tissue, and the virus subsequently appears in the brain 6–9 days after inoculation (predominantly in the cerebellum) in animals that develop encephalitis (Votiakov et al. 1975, 1982). Interestingly, TBEV-Eu and TBEV-FE subtypes have different sensitivities to dextran sulfate, implying that these two viruses could have different receptor specificities that could contribute to their different pathogenicity.

4.2.3. The role of the immune response on neuropathogenesis

The immune system has a dual function, which is both protective (via interferons, B-lymphocytes, and antibodies) and damaging (via cytotoxic T cells, and microglial cells) (Zambito Marsala et al. 2014). In particular the cellular immune response may contribute both to host resistance against infection and to pathological reactions affecting the CNS and causing neural damage with severe consequences for brain function, possibly leading to fatal outcomes.

In contrast to the humoral immune response, cellular responses elicited by natural infection have been poorly studied until recently, and there are only a few studies of T cell responses to TBEV infection in humans (Blom et al. 2015, 2018).

In particular, CD8+ T cells play a key role in neuropathogenesis, as data collected in animal models and patients have shown a prevalent intrathecal cellular response, including Th1 CD4+ lymphocytes and cytotoxic CD8+ lymphocytes, which are likely involved in disease immunopathogenesis and neuronal damage (Růzek et al. 2010). Only a few studies have shown an inverse topographic correlation between inflammatory

changes in postmortem human brains (consisting primarily of CD8+ T cells and macrophages/microglia infiltration in brain tissue in fatal cases) and the distribution of viral antigen. These findings suggest that immunopathological effects caused by T cells may influence the disease outcome (Gelpi et al. 2005, 2006). In agreement with the idea of a key role played by the CD8+ T cells in the neuropathogenesis of TBEV infection, Růzek, Vancová, Tesarov et al. (2009) demonstrated prolonged survival of CD8-deficient and SCID mice compared to that of immunocompetent mice, following experimental viral infection in a murine model. Moreover, adoptive transfer of CD8+ T cells into TBEV-infected SCID mice was found to decrease the mean survival time. However, in contrast to these results, when mice were infected with a low-pathogenic strain, CD8+ T cells instead appeared to contribute to increased survival (Růzek, Vancová, Tesarov et al. 2009).

Blom et al. (2015) studied the primary T cell-mediated immune response in patients diagnosed with TBE, with a particular emphasis on the CD8+ T cell response during the second stage of disease. During the neurological phase, peripheral blood CD8+ T cells were strongly activated within 1 week of hospitalization, and a large portion of these CD8+ T cells expressed high levels of perforin and granzyme B, and low levels of the anti-apoptotic protein Bcl-2. TBEV-specific CD8+ T cells were mainly monofunctional in the acute stage of disease and tended to become more polyfunctional in the convalescent phase. While TBEV-specific T cells have a high content of both perforin and granzyme B, the implication of the perforin-dependent mechanism in immunopathogenesis during acute TBEV infection remains to be investigated (Blom et al. 2015).

During the neurological phase of the disease, peripheral blood CD4 T cells show only low or at most moderate levels of activation (Blom et al. 2015, 2018). TBEV-specific CD4+ T cells from naturally infected patients show a higher level of polyfunctionality (producing IL-2, TNF α, and IFN γ) in the convalescent phase of disease, than TBE vaccine-specific T cells (Aberle et al. 2015).

Both humoral and cell-mediated immunity are usually required to clear TBEV infection from a vertebrate host. B lymphocytes secrete protective antibodies (Grygorczuk et al. 2018). IgM antibodies appear between the 8th and 14th days after the beginning of infection and can be detected in serum up to 10 months after acute infection. IgG antibodies usually appear 6 weeks after infection and persist throughout the lifetime, conferring protective immunity against reinfection (Holzmann 2003; Taba et al. 2017). However, TBEV has been suggested to cause both pathogenic and protective cross-reactivity (Günther et al. 1997; Phillpotts et al. 1985; Ferenczi et al. 2008).

Little is known about natural killer cells in TBE. NK cells may also play a protective role in human TBEV infection. However, responses mediated by these cells may also be associated with the development of symptoms during the course of TBEV infection (Blom et al. 2016, 2018). The presence of cytokines, interferons, interleukins, and chemokines in serum or cerebrospinal fluid (CSF) indicates the activation of inflammatory mediators and may be involved in brain damage (Taba et al. 2017). These mediators are secreted by monocytes, dendritic cells, microglial cells, and sometimes astrocytes (Palus et al. 2014). Type I interferons (IFN-α and IFN-β) play a vital role in antiviral immunity. These molecules activate intracellular signaling pathways (e.g. JAK-STAT) that result in the activation of transcription factors (IRF-9, and ISGF-3). These factors bind to DNA promoter regions and activate the synthesis of interferons, cytokines, chemokines, and growth factors (Robertson et al. 2009).

However, the ability of TBEV to modulate the host immune response, in a manner dependent on the genotypic and phenotypic variability of the TBEV population can impact the course and outcome of the disease (Robertson et al. 2009; Överby et al. 2010; Leonova et al. 2014; Krylova et al. 2015).

4.3. Host genetic factors affecting TBE neuropathogenesis. The progression and severity of infectious diseases often depend on multiple factors including the causative agent (e.g. viral strain and/or subtype), the infectious viral dose, the environment and the host's genetic susceptibility. Human genetic susceptibility to TBEV-induced disease is currently being studied. It has been previously reported that a nonfunctional chemokine receptor 5 (CCR5), produced by a 32-bp deletion in the coding region of the CCR5 gene, and a functional Toll-like receptor 3 (TLR3) predispose adults to clinical TBE. Independent of age, nonfunctional CCR5D32 mutation is a significant risk factor for the development but not the disease severity of clinical TBE. The polymorphism of the TLR3 gene predisposes only adults to clinical TBE and may be associated with disease severity (Kindberg et al. 2008, 2011; Barkhash et al. 2013; Mickienė et al. 2014). Other polymorphisms in genes encoding 2′-5′-oligoadenylate synthetase 2 (OAS2), 2′-5′-oligoadenylate synthetase 3 (OAS3), interleukin-28 (IL-28), interleukin-10 (IL-10), matrix metalloproteinase-9 (MMP-9), and CD209 (DC-SIGN) have been studied (Barkhash et al. 2012, 2016, 2018).

5. Molecular determinants affecting TBE neuropathogenesis

The reasons that TBEV strains are associated with varying disease evolution and severity remain poorly understood. However, analysis of viral genomic sequences suggests that mutations occurring in viral proteins may have an impact on the neuropathogenesis of TBE (Kellman et al. 2018).

5.1. Envelope protein (E protein)

The E protein mediates the tropism and attachment of TBEV to the host cell and can therefore be a crucial determinant of neurovirulence and/or neuroinvasiveness (Pletnev et al. 1992; Mandl et al. 2000).

As previously explained, each monomer consists of three structurally distinct domains and a transmembrane anchor region (positions 450–496). The three N-terminal domains form an ectodomain that covers the virion surface. Domain I is the central domain, and domain II corresponds to the dimerization domain. Domain I includes the only glycosylation site of TBEV at Asn154 (Bressanelli et al. 2004; Füzik et al. 2018). Glycosylation of the TBEV E protein is important for the secretion of the virus from infected cells (Goto et al. 2005). Domain II contains a fusion loop (residues 100–109) positioned at the tip of the ectodomain, that is involved in the process of fusion with the endosomal membrane and in the delivery of the viral genome into the cytoplasm of the host cell (Füzik et al. 2018). TBEV E protein domain III (positions 300–395) exhibits an immunoglobulin constant domain-fold structure and can fold independently from the rest of the protein (Bhardwaj et al. 2001). E proteins of TBE viruses share 12 conserved cysteine residues, which form six disulfide bridges at defined locations. The anchor domain, also named domain IV, comprises a stem region formed by three peripheral membrane helices and a transmembrane region (two helices) (Füzik et al. 2018).

5.1.1. E protein DIII *domain*

TBE complex viruses share a high degree of amino acid similarity in the E protein (77–98%), especially in domain III (80–95%), probably reflecting a low rate of evolutionary change (Bhardwaj et al. 2001). Regarding the potential impact of E protein domain III genomic variability on neuropathogenesis (neurovirulence and neuroinvasiveness), several levels of investigation could be considered: (i) genomic variability between the three subtypes, (ii) key point mutations among strains of a viral subtype, and (iii) viral fitness at the quasispecies level.

Despite the slow rate of viral evolution, point mutations in conserved sites might result in crucial changes in viral properties. Amino acid alignment of TBEV-Eu, TBEV-FE, and TBEV-Sib strains identified "serological amino acids" distributed throughout the E protein as clusters of variable amino acids and differentiated the viruses in accordance with their antigenic, geographic, and pathogenetic characteristics. The amino acid residues at positions 313, 317, 331, and 349 differ in the three main TBEV subtypes (Ecker et al. 1999; Gritsun, Lashkevich, Gould et al. 2003). However, by analysing 398 available amino acid sequences of TBEV E protein domain III, Ershova et al. (2016) showed that the amino acid residues at position 349 are not specific for any of the TBEV subtypes, but the amino acid residues at positions 313, 317, and 331 exhibit the main differences among t the three TBEV subtypes. The three residues at positions 313, 317, and 331 are located on the protein surface (Ershova et al. 2016).

For each viral subtype, a number of mutations located on the upper lateral surface of domain III were identified that modulate neuropathogenesis in an immunodeficient mice model (Figure 4). Indeed, some residues in the TBEV-Eu strain E protein were shown to be crucial for neuroinvasiveness and/or neurovirulence: (i) using the Neudoerfl strain, Mandl et al. (2000) showed that virulence was dependent on specific residues (E308, E310, E311, and E315) located in an area that is considered to be the potential receptor-binding determinant (Mandl et al. 2000; Engel et al. 2010); (ii) the amino acid substitution G368R in the Neudoerfl strain has been shown to be associated with the loss of neuroinvasiveness (Holzmann et al. 1997); (iii) the amino acid substitutions Y384H and G386R may result in localized changes in the charge because flanking amino acids may be important in disulfide bond formation (Holzmann et al. 1990); and (iiii) Wallner et al. (1996) compared the genomic sequences of two European strains: strain Hypr strain (a high-passage strain isolated from humans) and strain 263 (a low-passage strain isolated from ticks) to the prototype strain. In contrast to strain 263, strain Hypr showed significantly higher neuroinvasiveness in mice than the prototype strain. They identified a small number of specific amino acids, especially at position 390, that may impact neuropathogenesis (Wallner et al. 1996).

Finally, one should remember that TBEV circulates as a quasispecies between vertebrate and invertebrate hosts, which impose different selective pressures on the virus population. Minor variants may also have a great

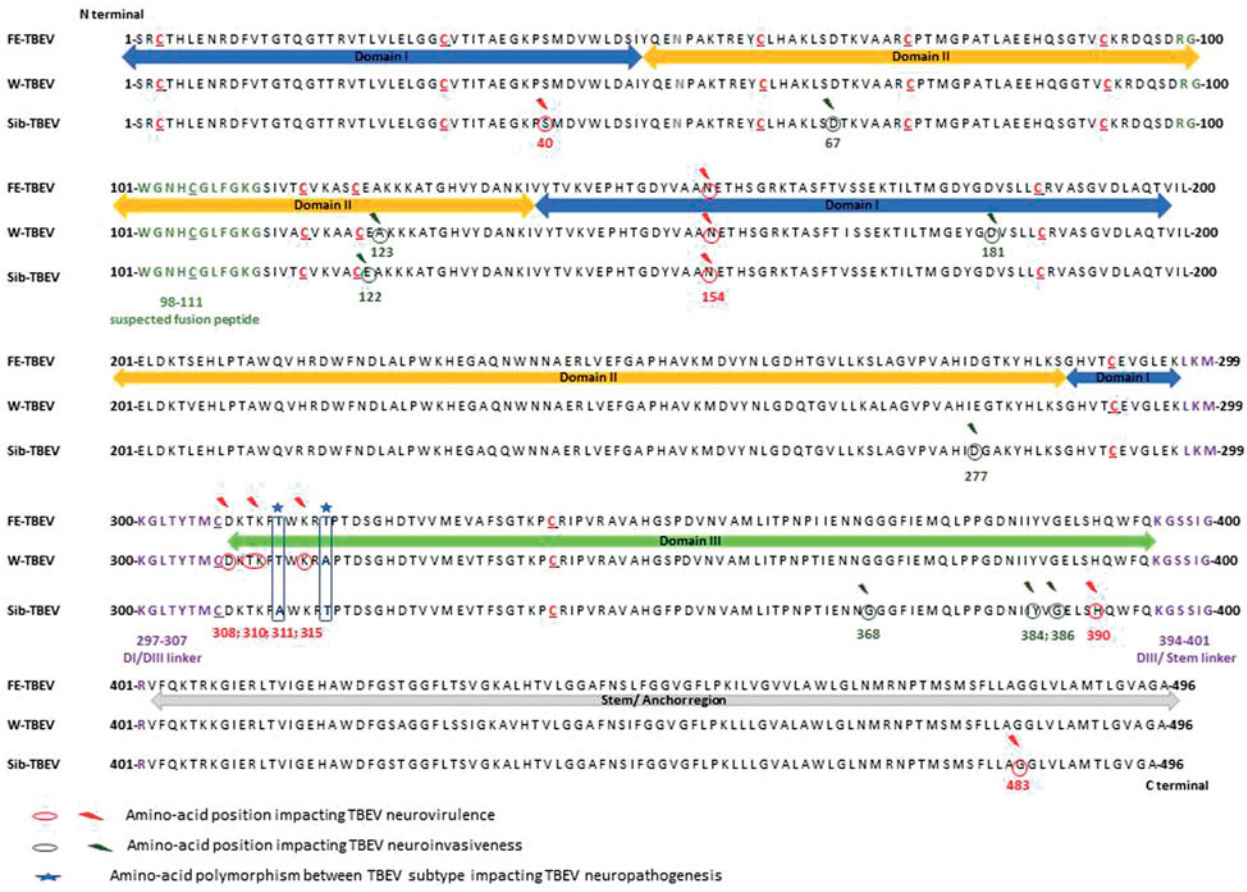

Figure 4. Structural alignment of the prototype strains of the three TBEV subtypes. Protein E amino acid (AA) sequence alignment of the prototype strain of the three TBE virus subtypes. The Far Eastern TBEV subtype (FE-TBEV) is represented by the Sofjin strain, the Western TBEV subtype (W-TBEV) is represented by the Neudoerfl strain, and the Siberian TBEV subtype (Sib-TBEV) is represented by the Vasilchenko strain. The colour-coded bar below the sequence indicates theDI, DII, and DIII domains and the stem anchor region in blue, yellow, green, and grey, respectively, according to Bressanelli et al. 2004. The segments between the domains (linkers) are shown in purple (the DI/DIII and DIII/stem linkers). The amino acid positions suspected of impacting TBEV neurovirulence are indicated by red circles and red arrows, and those likely involved in neuroinvasiveness are indicated by green circles and green arrows. Reported amino-acid polymorphisms between TBEV subtypes known to impact TBEV neuropathogenesis are represented by blue stars.

impact on viral properties, such as neuroinvasiveness. Litov et al. (2018) showed that viable TBEV variants, which are different from the major population phenotype and can become a major part of the viral population under favorable environmental conditions, can exist long term at abundances of less than 1% (Litov et al. 2018).

5.1.2. E protein DI, DII, and stem/anchor domains

The mutations D67G, E122G, and D277A (domain DII) introduced in haemagglutination-deficient strains (Siberian subtype) induced increased hydrophobicity and positive charge on the virion surface (Figure 4). Interestingly, all three engineered virus mutants exhibited lower neuroinvasiveness in laboratory mice than the wild-type virus. However, the D67G mutation, identified in a strain isolated from a fatal case, also appears to enhance the ability of TBEV to cross the human BBB, a likely explanation for fatal encephalitis (Khasnatinov et al. 2009) (Figure 4). Holzmann et al. (1997) showed that the domain DI Y181D mutant and domain DII A123K mutant of the TBEV-W Neudoerfl strain, exhibited significantly reduced neuroinvasiveness after subcutaneous inoculation into GP Swiss albino mice (Holzmann et al. 1997) (Figure 4).

The N154L mutation, introduced by site-directed mutagenesis within the E protein glycosylation site in domain DI, significantly decreased the neurovirulence of TBEV-DEN-4 chimeras in mice and restricted viral replication in cell culture (Pletnev et al. 1993) (Figure 4). In the Oshima 5–10 strain (TBEV-FE subtype) the amino acid substitution S40R/P at position 40 in the DI domain was responsible for a reduction in plaque sizes

infectious virus yields in cell culture and for reduced neurovirulence in mice (Hayasaka et al. 2004) (Figure 4). Goto et al. (2003) observed a loss of neuroinvasiveness of the A483G mutant compared to that of the Oshima 5–10 parental strain (TBEV-FE subtype) in mice. This substitution is located in the anchor domain of the E protein (Goto et al. 2003) (Figure 4).

5.2. NS5 protein

Leonova et al. (2013) observed some key substitutions S634T, R677K, I692V, and A724S in the NS5 protein of FE-subtype strains, more precisely in the RNA-dependent RNA polymerase domain (RdRp) of NS5, which may affect the pathogenicity of the strain (Leonova et al. 2013; Belikov et al. 2014). Using a cDNA clone of the Oshima strain (FE-subtype), Hayasaka et al. (2004) showed that changes in residues 378 (V378R/A) and 674 (R674K) affected neurovirulence (after intracerebral inoculation) and neuroinvasive properties (after subcutaneous injection) in laboratory BALB/c mice (Hayasaka et al. 2004).

Engel et al. (2010) assessed a chimeric attenuated virus vaccine candidate containing the structural protein genes of TBEV (Sofjin strain, FE-subtype) on a dengue virus genetic background (TBEV/DEN4). This molecular construction showed a high level of neurovirulence in both mice and monkeys (Gritsun, Lashkevich, Gould 2003; Engel et al. 2010). Introduction of, on the one hand, the E(315) mutation or the NS5(654, 655) mutations, and on the other hand the both the E(315)+NS5(654, 655) mutations resulted in stepwise attenuation of the virus both *in vitro* and *in vivo*, with low, moderate, and high levels of attenuation, respectively (Engel et al. 2010).

5.3. 3′ Untranslated region

The UTRs contain several conserved structural stem loops that are important for the replication, translation initiation, and packaging processes (Mandl 2005). The 3′-UTR of TBEV can be divided into two domains: a 5′-terminal variable region heterogeneous in both in length and sequence and a 3′-terminal highly conserved core element containing a sequence that is essential for viral genome replication (Wallner et al. 1996; Kofler et al. 2006). The variable region of the 3′-UTR is considered essential for the natural transmission cycle of TBEV, but does not seem to be involved in viral replication and virulence in mammals (Mandl et al. 1998). The sequence and length of the variable region vary among TBEV strains, with some strains containing a poly (A) tract in the variable region. Deletions of sequences in the variable region were identified in many TBEV strains of all three subtypes. These strains were either isolated from vertebrates and ticks and passaged in mammalian cell culture or isolated from clinical samples (Mandl et al. 1998; Leonova et al. 2013; Belikov et al. 2014; Formanová et al. 2015). However, the position and size of these deletions are not conserved and vary depending on the strain.

Belikov et al. (2014) described viral variants with extensive deletions covering almost the entire 3′-UTR in strains of the FE-subtype isolated from patients, that produced either subclinical infection or severe disease. The longest deletion in the 3′-UTR was found in strains belonging to the cluster of FE-subtype strains inducing subclinical disease. This pattern suggests that extensive deletion may dramatically reduce pathogenicity. The authors hypothesized that extended deletion, if it did not affect the conservative end portion of the 3′-UTR (325 nucleotides), had a moderate impact on pathogenicity (Belikov et al. 2014).

However, regarding 3′-UTR deletions in TBEV-Eu subtypes, conflicting results have been observed. Some deletions introduced into the 3′-UTR of the Neudoerfl strain produced strong attenuation of virulence in a mouse model (Mandl et al. 1998), while the presence of extended deletions in the 3′-UTR was associated with high virulence in the Hypr strain, which has a shorter 3′-UTR than the Neudoerfl strain (Wallner et al. 1996). The mechanism of the occurrence of these deletions, and the role and importance of these deletions in the evolution of the viral population remain uncertain and require additional studies. Moreover, a few Eu-subtype strains contain a poly(A) tract located in the variable region of the 3′-UTR, which seems to have an impact on virulence in laboratory mice (Wallner et al. 1996; Mandl et al. 1998). More recent studies also showed that both the length of the variable region and the presence of the poly(A) tract could modulate the virulence of FE-subtype strains (Sakai et al. 2014, 2015). Ashgar et al. (2016) observed various lengths of the poly(A) tract in a blood-feeding tick. This group showed that viruses with a long poly(A) tract were attenuated in cell culture but more virulent when inoculated in laboratory mice (Ashgar et al. 2016).

5.4. Impact of the other viral proteins

Except for the E protein and the NS5 protein, the roles of NS proteins in virus–host interactions are poorly understood, and only a few data, mainly obtained on FE-subtype strains, are available on the impact of point

mutations and/or deletions observed in these viral regions on neuropathogenesis (Leonova et al. 2013; Belikov et al. 2014; Formanová et al. 2015). Leonova et al. (2013) observed a deletion of amino acid 111 associated with three key amino acid substitutions in some strains that potentially impacted viral pathogenesis (Leonova et al. 2013). In fact, the introduction of certain deletions and amino acid substitutions into the C protein disrupts the assembly of infectious particles (Schalich et al. 1996; Mandl 2005). Some point mutations located at positions 45 and 46 of the NS3 protease may affect the budding process of viral particles and resulted in low pathogenicity of the strains, especially regarding neuroinvasiveness in laboratory mice (Leonova et al. 2013; Belikov et al. 2014). Moreover, mutations mapped in the close vicinity of the NS2B-NS3 serine protease active site may determine the neuropathogenicity of TBEV (Růzek et al. 2008). Mutations in the NS1 protein have been associated with chronic progressive disease in cases of TBEV-Sib subtype infection (Gritsun, Lashkevich, Gould 2003).

The major suspected molecular determinants of TBEV neuropathogenesis are identified in the E protein, especially in domain III and the 3′-UTR (Kellman et al. 2018). However, data collected from clinical samples, observed in tick or mammalian cells, or obtained in laboratory mice, are not always concordant. These discrepancies reflect the complex interplay of TBEV during the transmission cycle of this zoonosis. It could be of interest to study other viral proteins such as the prM/M protein, which is an important actor in the viral entry process in greater detail.

6. Conclusion and perspectives

TBE is a viral CNS infection that may result in long-term neurological sequelae. As an emerging zoonosis, TBE is exhibiting an increasing incidence in Europe, with a subsequent increase in its health burden. There is currently a growing interest in developing a better understanding of factors of TBE pathogenesis. In fact, the severity of TBE may depend on various factors including the inoculation dose, exposure time, characteristics of the human host (age, sex, immune status, and susceptibility based on the host's genetic background), and characteristics of the TBEV genome. In the CNS, if the primary targets of TBE viruses are neurons, key aspects of neuronal damage are still poorly understood. Moreover, effective *in vitro* astrocytes infection open new insights on the way TBEV could diffuse in the CNS.

New promising approaches, such as the ISA method, could help address this issue. In fact, this procedure allows the isolation of infectious single stranded positive-sense RNA viruses, such as TBEV from clinical or animal samples and permits the isolation of infectious viruses from clinical and/or animal samples that may have deteriorated during the collection and storage period. Virus isolation remains essential to provide a reference infectious virus that can be used for phenotypic and genotypic characterization as well as for a variety of studies on the fundamental basis of viral pathogenesis. This is particularly difficult during TBEV infection, as TBEV RNA is rarely detected in CSF, or in the brain in fatal cases.

Some viral determinants of viral neuropathogenesis are currently highlighted, such as DIII domain of the E protein domain DIII or the 3′-UTR. Extensive data on both viral neurovirulence and neuroinvasiveness are essential for understanding the disease process and for developing live attenuated vaccines or new therapeutic strategies.

Disclosure statement

All authors have contributed directly at different levels in the preparation of this paper. None of the authors has a conflict of interest.

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