Tick-borne encephalitis: Development of *in vitro* and *in vivo* models and evaluation of siRNA for antiviral therapy

PhD Thesis submitted by

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Bern, Dean of the Faculty of Medicine

Bern, Dean of the Faculty of Science

Bern, Dean of the Vetsuisse Faculty Bern
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### Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AAV</td>
<td>Adeno-associated viruses</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSL</td>
<td>Biosafety level</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double stranded RNA</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>LGTV</td>
<td>Langat virus</td>
</tr>
<tr>
<td>miRNA</td>
<td>micro RNA</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotide</td>
</tr>
<tr>
<td>LV</td>
<td>Lentivirus</td>
</tr>
<tr>
<td>OHCs</td>
<td>Organotypic hippocampal slice cultures</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>ssRNA</td>
<td>single stranded RNA</td>
</tr>
<tr>
<td>TBE</td>
<td>Tick-borne encephalitis</td>
</tr>
<tr>
<td>TBEV</td>
<td>Tick-borne encephalitis virus</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
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</tbody>
</table>
Summary

Tick-borne encephalitis virus (TBEV) and Langat virus (LGTV) are members of the tick-borne encephalitis virus serocomplex, a group of closely related flaviviruses that cause serious neurological illness and hemorrhagic fevers in humans [1]. TBEV is transmitted by the bite of infected ticks and is the causative agent of tick-borne encephalitis (TBE), a potentially fatal neurological infection that affects patients in Europe and Asia. Despite the availability of a vaccine, approximately 5000-10000 cases of TBEV infection occur every year in Europe and Russia for which no effective antiviral therapy is available. LGTV is a naturally attenuated tick-borne flavivirus with a low pathogenicity for humans and previously used in clinical trials as a live vaccine against TBEV. Even though natural infections of LGTV in humans have never been detected, 0.005% of patients who received the LGTV-based vaccine developed encephalitis with severe neurological sequelae. LGTV does not pose a significant epidemiological threat in comparison with TBEV and can therefore be handled under biosafety level 2 conditions.

In this project the high genetic and antigenic similarity of LGTV with TBEV was exploited for the development of in vitro and in vivo models of TBEV infection.

In the first part the antiviral effect of RNA mediated interference (RNAi) by small interfering RNA (siRNA) against LGTV was evaluated in cell cultures and organotypic hippocampal cultures (OHCs). Nineteen siRNA sequences were analyzed for their antiviral potential on HeLa cells. The most efficient siRNA molecule, targeting a highly conserved sequence within the 5’ untranslated region (UTR), was 100% conserved between different members of the TBEV complex. This sequence (D3) was further investigated for its potential in the inhibition of LGTV replication on OHCs. A 1000-fold reduction in the number of infectious particles was achieved on brain slices.

In the second part an infant rat model of TBE using LGTV was developed. Infant Wistar rats were inoculated intracisternally with LGTV and assessed for clinical symptoms of disease, inflammatory response in the brain and histological findings at days 2, 4, 7 and 9 post-
infection. Infection with LGTV led to a reduced weight gain and development of motor impairments. The cyto/chemokines RANTES, IFN-γ, IL-6, IFN-β and MCP-1 were significantly increased in the CSF of infected animals and their brains exhibited characteristic histopathological features of TBE. The comparison of the inflammatory response profiles between CSF samples of human patients infected with TBEV with those from LGTV-infected animals showed similarities. The newly established rat model using LGTV shows important clinical and histological features mimicking those observed in human TBE. Thus the model provides a novel tool to investigate mechanisms of disease and to evaluate new therapeutic strategies against encephalitogenic flaviviruses.

In the third part of the project we produced preliminary results on the in vivo application of siRNA in the developed rat model.
1. Introduction

1.1 Tick-borne encephalitis virus

1.1.1 Biological-molecular characteristics

Tick-borne encephalitis virus (TBEV) is a member of the genus *Flavivirus* within the family *Flaviviridae*. flaviviruses can be divided into mosquito-borne viruses, tick-borne viruses and viruses with no known vectors. Important human pathogens within the mosquito-borne virus group are Japanese encephalitis virus, Yellow fever virus, Dengue virus and West Nile virus. In the tick-borne flaviviruses group, TBEV, but also the Powassan virus, Louping ill virus, Omsk hemorrhagic fever virus and Kyasanur Forest disease virus, are important pathogens causative agents of potentially fatal neurological infections in human across Europe and Asia (Table 1-1). Based on serological- and sequence analyses TBEV is geographically and genetically divided into three subtypes, the European (TBEV-Eu), the Siberian (TBEV-Sib) and the Far Eastern (TBEV-FE) subtypes. Nucleotide and amino-acid sequence analyses have confirmed the divergent nature of these three subtypes despite their close biological similarities [2, 3].

All flaviviruses, which include more than 70 enveloped RNA virus species, have a single-stranded (+) 11-kilobase genome encoding for a single open reading frame (ORF) [4, 5]. This ORF encodes for a single polyprotein which is co- and post-translationally cleaved by cellular and viral proteases. It consists of three structural proteins (C; prM; E) and seven non-structural proteins (NS1; NS2A; NS2B; NS3; NS4A; NS4B; NS5). The coding region is flanked by two untranslated regions (5’UTR; 3’UTR) (Figure 1-1, A). The 5’ end of the genome has a cap but the 3’ end lacks a poly-A tail. Non-structural proteins have several functions, e.g NS5 provides the RNA-dependent RNA polymerase machinery and NS3 and NS2B provides a serine protease for the cleavage of the polyprotein.

Mature virions are composed of a capsid structure, which encloses and protects the viral genome, surrounded by a host-derived lipid bilayer containing the two envelope
glycoproteins, E (envelope) and M (membrane) (Figure 1-1, B). The E protein works both as a ligand to the cell surface receptor and as a fusion protein [6].

Table 1-1. Pathogenic flaviviruses [7-11].

<table>
<thead>
<tr>
<th>Virus species</th>
<th>Principal vector species</th>
<th>Geographic distribution</th>
<th>Principal host species</th>
<th>Human disease</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tick-borne</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kyasanur Forest disease</td>
<td><em>Haemaphysalis</em> spp</td>
<td>India</td>
<td>Monkeys</td>
<td>Haemorrhagic fever</td>
</tr>
<tr>
<td>Langat</td>
<td><em>Ixodes granulatus</em></td>
<td>Malaysia, Thailand, Siberia</td>
<td>Unknown</td>
<td>Encephalitis</td>
</tr>
<tr>
<td>Louping ill</td>
<td><em>Ixodes</em> spp</td>
<td>UK, Ireland, Norway</td>
<td>Sheep, gouse, hares</td>
<td>Encephalitis</td>
</tr>
<tr>
<td>Omsk hemorrhagic fever</td>
<td><em>Dermacentor</em> ssp</td>
<td>Western Siberia</td>
<td>Muskrats</td>
<td>Haemorrhagic fever</td>
</tr>
<tr>
<td>Powassan</td>
<td><em>Ixodes</em> spp</td>
<td>Russia, USA, Canada</td>
<td>Small mammals</td>
<td>Encephalitis</td>
</tr>
<tr>
<td>Tick-borne encephalitis</td>
<td><em>Ixodes</em> spp</td>
<td>Europe, Asia</td>
<td>Rodents</td>
<td>Encephalitis</td>
</tr>
<tr>
<td><strong>Mosquito-borne</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dengue</td>
<td><em>Aedes aegypti</em></td>
<td>Tropics, subtropics</td>
<td>Human beings</td>
<td>Fever, rash, vasculopathy</td>
</tr>
<tr>
<td>Japanese encephalitis</td>
<td><em>Culex tritaeniorhynchus</em></td>
<td>Asia</td>
<td>Birds</td>
<td>Encephalitis</td>
</tr>
<tr>
<td>West Nile</td>
<td>Mosquitoes, ticks</td>
<td>Worldwide</td>
<td>Birds</td>
<td>Encephalitis</td>
</tr>
<tr>
<td>Yellow fever</td>
<td><em>Aedes</em> spp / <em>Haemagogus</em> spp</td>
<td>Africa, South America</td>
<td>Monkeys</td>
<td>Pantropic</td>
</tr>
</tbody>
</table>
Figure 1-1: Schematic representation of the genome structure of TBEV (A) and its structural organization (B) [5]. Langat genome represents a single open reading frame depicted with the structural and non-structural protein coding regions, flanked by the 5’ and 3’ untranslated regions (UTRs). The TBEV virion is a small (50 nm), round, enveloped particle. The viral nucleocapsid (blue) encloses the viral genome and is surrounded by a lipid envelope (yellow) in which two glycoproteins M (or prM in the immature virion) (green) and E (red) are anchored.

1.1.2 Replication cycle

The first step in the TBEV life cycle is the binding of the mature virus particle to the still unknown host surface receptor. Virions are internalized by receptor-mediated endocytosis. Low pH in the endosomal compartment triggers the fusion of viral membranes with endosomal membranes which leads to the release of the nucleocapsid into the cytoplasm. After uncoating, the positive single-stranded RNA genome is translated into a single polyprotein which is co- and post-translationally processed by viral and cellular proteases into viral structural and non-structural proteins. RNA replication occurs via the production of minus-strand RNA which serves as template for the synthesis of new positive-strand RNAs [5]. Genome replication occurs on intracellular membranes and new virus particles are assembled on the surface of the endoplasmatic reticulum (ER) into immature virions particles. Maturation occurs in the trans-Golgi network when prM is cleaved to M by furin allowing conformational rearrangements of the E protein. Mature virions are released by
exocytosis [4]. A schematic representation of TBEV replication cycle is depicted in the Figure 1-2.

![Schematic diagram of TBEV life cycle](image)

**Figure 1-2**: Schematic diagram of TBEV life cycle [5]. After binding to a specific receptor, TBEV enters into the cell by endocytosis. The low pH of the endosome induces fusion of the virion envelope with cellular membranes. Following uncoating, RNA genome is released into the cytoplasm where it functions as mRNA. Viral RNA replication occurs on intracellular membranes where immature virions are assembled. After cleavage of the protein prM, infectious virions are released from the cell by exocytosis.

### 1.1.3 Epidemiology

The natural hosts of all three TBEV subtypes are small rodents which act as both amplifying as well as reservoir host [8]. Humans are dead-end infection hosts for TBEV and do not play a role in the maintenance of the virus in nature. In the natural environment, ticks act as vector and reservoir for the virus. *Ixodes ricinus* is the dominant tick species across Europe and the most epidemiologically important vector for the European TBEV subtype. The vector for the Siberian and Far-Eastern subtypes is *Ixodes persulcatus* [12] (Figure 1-3). Transmission of the virus from infected to non-infected ticks may occur by co-feeding on vertebrates without the need for the host to develop a significant viremia [13, 14]. However,
the most frequent transmission route of TBEV occurs by feeding on viremic animals. Generally, throughout Europe *I. ricinus* becomes active and starts feeding on a range of hosts in spring and early summer [8]. Ticks have an extended life cycle which can last up to 5 years and once the infection occurs they remain infected for the whole duration of their life [5, 15]. The prevalence of ticks infected with TBEV in endemic areas of Europe usually varies from 0.5% to 5% [16, 17]. In Switzerland a mean virus prevalence of 0.46% of infected ticks was observed in endemic areas in 2010 [18]. In Russia prevalence rates of 40% have been recorded in certain endemic regions [19].

The most common transmission route of TBEV to humans is by the bite of an infected tick (Figure 1-4). Transmission occurs through the infected saliva, which is secreted by the tick during feeding. Interestingly, the amount of TBEV in tick saliva increases up to 100-fold during feeding [6, 15]. In rare cases TBEV can be transmitted to humans after the consumption of unpasteurized milk products from viremic livestock [20]. Laboratory-acquired cases of TBE infection have also been reported associated with accidental needle-stick injuries or aerosol infections [21]. It is estimated that around 3000 TBEV infection cases occur annually in Europe and 11000 cases in Russia with the highest incidence of infections occurring between May-June and September-October, the seasonal feeding peak for *Ixodes* species [19]. Only 50-60% of the TBE patients have recognized the tick bite [22]. Interestingly, 70-95% of human infections are either sub-clinical or totally asymptomatic [19, 23].

In the last 30 years an increase of 400% in TBE cases was registered in Europe and the virus is now found in previously unaffected areas [18, 24]. This important increase of TBE incidence can be linked to many factors including changes in climate, vector distribution as well as social, economic, demographic, ecological and political factors.


Figure 1-3: Tick species which act as vectors for TBEV. *Ixodes ricinus* (A) is the dominant tick species across Europe and the most important transmission vector for the European TBEV subtype (TBEV-Eu). *Ixodes persulcatus* (B) inhabits regions of the Urals, Siberia and far-eastern Russia and is the main vector for the Siberian (TBEV-Sib) and Far Eastern TBEV (TBEV-FE) subtypes. Source: www.eurospiders.com and http://de.academic.ru/.

![Tick species](image)

Figure 1-4: Transmission cycle of TBEV [25]. In a dash blue arrows circle the four developmental stages of the tick, from egg to larva, nymph and adult. Solid red lines in the transmission cycle show the presence of TBEV in ticks, which after being infected carry the virus throughout the entire lifespan. Thickness of red arrows shows the prevalence of the tick. In general humans are dead-end hosts and do not play a role in maintaining the virus cycle [6].
1.1.4 Pathogenesis

The initial step after the tick bite is the replication of TBEV in epidermal Langerhans cells, dendritic cells of the skin resulted to be the first and most important host cells to be infected. The virus progresses from the site of the bite to draining lymphatic nodules where it replicates and amplifies [5]. Replication of the virus in the lymphoid compartments and other tissues leads to viremia and systemic infection, a prerequisite for the viral invasion of the central nervous system (CNS) (Figure 1-5). The mechanism by which TBEV crosses the blood-brain barrier (BBB), enters the brain and invades the CNS still remains unclear.

Several hypotheses have been postulated for TBEV CNS invasion. One possibility is a virus penetration due to a cytokine-mediated permeabilization of the BBB [26]. Other hypotheses involve infection of brain endothelial cells or entry through the olfactory bulb [27]. In the CNS, neurons are the primary targets for TBEV although other cell types may also be infected [28].
1.1.5 Tick-borne encephalitis

Tick-borne encephalitis develops once TBEV invades the CNS. TBE has become a growing public health problem in Europe, Russia and Asia in the past decades [8, 30]. Within Europe, Czech Republic has one of the highest incidence rates with 400-1000 TBE cases reported yearly [30]. Russia has the highest incidence of TBE cases in the world (3000-11000 cases annually) [30].

Clinical course and symptoms severity can be influenced by a variety of factors, e.g virus strain, infectious dose, age and immune status of the patient. Usually TBE is more severe in adult than children. Human infections with different subtypes of TBEV may vary a lot in the
clinical course and in the severity and outcome of the disease. The incubation period of all TBEV strains lasts on average between 7 and 14 days after the tick bite. Infections caused by the European TBEV strain usually display a biphasic course [22, 31, 32]. The first viremic phase appears with flu-like symptoms such as fever, fatigue, muscle pain, general malaise, headache and body pain lasting up to 1 week. After recovery and a symptom-free interval of around one week, 20-30% of infected patients develop the neurological phase of the disease with a clinical spectrum ranging from mild meningitis to severe meningoencephalitis, meningoencephalomyelitis or meningoencephaloradiculitis [31]. In the remaining patients the disease resolves without a second phase. The fatality rate in adult patients is less than 2% and residual long-term sequelae were reported in 10-50% of the patients with different levels of severity [33, 34]. The most obvious feature of TBE in patients is ataxia, followed by paresis or paralysis of one or more limbs, meningeal signs, febrile headache and cognitive disorders [23, 34]. TBE viral antigens were immunohistochemically detected in large neurons of human brains after fatal TBE [28]. Lesions are predominantly observed in thalamus, cerebellum, brainstem and nucleus caudatus, but the exact mechanisms of neuronal death and tissue destruction remain unclear [35, 36]. Observations in post-mortem human brains as well as in mouse models show a TBEV-mediated rapid microglia/macrophage activation and a generally strong inflammatory response [37]. These findings suggest immune-mediated neuronal damages rather than direct TBEV-induced apoptosis. The inflammatory response in TBE consists of mononuclear inflammatory infiltrates, astrocytosis, perivascular cuffing and microglial nodules [37]. Pro-inflammatory cyto- and chemokines such as MCP-1 (CCL-2), RANTES (CCL-5), IP-10 (CXCL10), IP-9 (CXCL11) and interferon gamma (IFN-γ) were up-regulated in CSF of TBE patients [38]. Interestingly, a clinical study demonstrated an association between the mutation affecting the chemokine receptor CCR5 (CCR5Δ32) and TBE [39]. CCR5 is a chemokine receptor that promotes trafficking of leukocytes and thus plays a central role in the host immune response. It was shown that the loss of CCR5 function in human is associated with a higher disease severity in TBE patients. Similar
findings were found for infections with WNV, where CCR5 deficiency resulted in 100% susceptibility to severe symptomatic disease [40].

The Far Eastern TBE virus subtype in most of the cases does not develop a biphasic disease course but causes severe clinical CNS infections with important neurological sequelae and a case fatality rate of up to 35%. The Siberian subtype develops clinical illness similar to the European subtype with a biphasic disease form and a case fatality rate of about 2% [9, 34]. An interesting characteristic of TBEV-Sib infection is its ability to cause chronic diseases [34, 41]. These chronic forms have not so far been observed in Western Europe. Children less than 4 years of age are less frequently affected by severe TBE neuronal disease when infected with the European TBEV subtype. However, infection with the Far Eastern subtype can progress into severe CNS disease in children as well [15, 42, 43].

1.1.6 Diagnosis

After the incubation period from 7 to 14 days, patients infected with TBEV usually develop the first viremic phase consisting in the sudden onset of an uncharacteristic influenza-like illness with fever, headache and joint pain. During this first phase the virus can be isolated from the blood or detected into the brain by reverse-transcriptase-polymerase chain reaction (RT-PCR). However, patients within the initial viremic phase do not usually consult a physician and hospitalization normally takes place only in the second phase of the disease, when first neurological symptoms develop [22]. At this time point, the virus has already been cleared from the blood and from the CSF and the production of specific antibodies has started (Figure 1-6). Development of a humoral immune response in the second phase enables diagnosis of TBE by enzyme-linked immuno-sorbent assays (ELISAs) used for the specific detection of TBEV-immunoglobulin M (IgM) and TBEV-IgG in serum and CSF. IgM-antibodies are detectable in serum until 6-7 weeks after infection, whereas IgG-antibodies persist for the whole life and mediate a life-long immunity that prevents reinfection [6, 22]. Low levels of neutralizing serum antibodies usually correlates with a higher disease severity
The primary target for neutralizing antibodies is the E protein, although antibodies specific for prM and nonstructural proteins have also been observed [44]. Because of the high similarity of the antigenic structure within the E protein between several flaviviruses and the similar clinical features, diagnostic difficulties may occur. Cross-reactive antibodies induced by other flavivirus infections/vaccinations are able to interfere in the diagnostic immunoassay analysis without, however, mediate protection against the specific virus. This issue is especially relevant in regions where different flaviviruses co-circulate and is an increasing problem in travel medicine due to the popularity of new destinations where other flaviviruses such as Dengue virus, Japanese encephalitis or Yellow fever virus are endemic. In such cases a neutralization assay is necessary to assess immunity. Neutralization studies are performed with infectious virus particles requiring biosafety level 3 laboratories, making this test technically difficult, time consuming and expensive.

**Figure 1-6:** Biphasic course of TBEV infections. Development of the humoral immune response with the production of specific antibodies (IgM and IgG) in serum and CSF [22].
1.1.7 Therapy and prevention

There is still no specific drug available with demonstrated efficacy against TBEV and in general against any flavivirus infection. Treatment of viral encephalitis is based exclusively on the management of complications caused by the disease [45]. Early diagnosis of the infection and treatment of clinical symptoms with an accurate medical care are essential to improve the chance of survival in patients with severe disease [46]. Active vaccination with formaldehyde-inactivated virus is the most effective method to protect against TBE.

In Western Europe two vaccines are currently available: FSME-IMMUN (Baxter Vaccine AG, Vienna, Austria), based on the Neudörfl strain of TBEV-Eu, and Encepur (Chiron-Behring, Marburg, Germany), based on the K23 strain of TBEV-Eu. Both vaccines consist of whole purified TBEV-Eu strain, propagated in chick embryo fibroblasts, inactivated with formaldehyde and used with aluminium hydroxide as adjuvant [23, 47, 48]. Both FSME-IMMUN and Encepur require 3 doses for complete immunization. FSME-IMMUN is the vaccine most widely used in Europe and a recent study showed the ability of the vaccine to induce equivalent levels of neutralizing antibodies against TBEV strains of the European, Far Eastern, and Siberian subtypes [47].

In Russia two vaccines are available: TBE vaccine Moskow (TBE-Moskow) (Institute of Poliomyelitis and Viral encephalitis, Moskow, Russia), prepared from the Sofjin strain of the Far Eastern TBEV subtype, and ENCEVIR (Virion, Tomsk, Russia) based on the strain 205 of TBEV-FE. Both vaccines consist of formaldehyde-inactivated TBE virions and use aluminium hydroxide as adjuvant. The TBE-Moskow vaccine was approved for use in 1982 and since then more than 25 million people in Russia and neighboring countries have received this vaccine. ENCEVIR was licensed in 2001 and shows similar immunization ability compared to TBE-Moskow. For both vaccines the primary course of vaccination consists of three doses (http://www.who.int/biologicals/vaccines/tick_borne_encephalitis/en/index.html).
1.2 Langat virus as a model for tick-borne encephalitis virus

Langat virus (LGTV) was first isolated in Malaysia in 1956 from *Ixodes granulatus* [49]. LGTV is classified as a member of the tick-borne encephalitis virus serocomplex (or TBE serogroup), a group of closely related flaviviruses including Louping-ill virus, Omsk hemorrhagic fever virus, Kyasanur Forest virus, Powassan virus and Tick-borne encephalitis virus [1]. The natural transmission cycle involves various species of rats but detailed information on the natural transmission of the virus is missing [9]. LGTV has an attenuated virulence for human hosts and was used as a vaccine candidate against TBE for several years [50]. The vaccination campaign was abandoned due to 35 patients in a group of 650’000 which developed a severe meningoencephalitis resulting in permanent neurological sequelae (Occurrence 1:18000, 0.005%) [19, 51]. LGTV shares more than 74% nucleotide identity with TBEV. Due to its low pathogenicity, it can be handled under biosafety level 2 (BSL-2) conditions. In the natural environment, LGTV does not cause disease in rodents, but it was shown to develop into encephalitis when inoculated intracerebrally in young laboratory mice. The virus is completely avirulent for adult mice following subcutaneous or intraperitoneal inoculation [52, 53].

1.3 RNA interference (RNAi)

RNA interference (RNAi) is a conserved biological response to double-stranded RNA (dsRNA) resulting in the degradation or translation inhibition of homologous messenger RNA. This sequence-specific, post-transcriptional gene silencing process constitutes a key component of the innate immune response to viral infection in both plants and invertebrates and regulates the expression of protein-coding genes in all eukaryotes. Whether RNAi has an antiviral activity in mammals is still an open debate. At present there are no studies showing that endogenous RNAi is involved as antiviral mechanism in mammals [54].

RNAi was discovered in 1998 by Andrew Fire and Craig C. Mello in the nematode worm *C. elegans* in response to long double-stranded RNA, which resulted in the complementary
sequence-specific gene silencing. For this discovery they were awarded the Nobel Prize in Physiology or Medicine in 2006 [55]. In 2001 Elbashir and coworkers showed that synthetic 21-nucleotide siRNAs could trigger RNAi in mammalian cells as well [56].

1.3.1 Mechanism

The RNAi pathway is an evolutionary conserved cellular mechanism for regulating gene expression based on RNA molecules [57]. Based on their origins and biogenesis, there are two main categories of small RNAs molecules involved in RNAi-mediated gene silencing: micro RNAs (miRNAs) and small interfering RNAs (siRNAs) [58].

MiRNA are generated by endogenous transcripts in the nucleus. They are processed by an enzyme complex consisting of RNAse III Drosha into pre-miRNAs and then specifically exported into the cytosol by exportin-5. In the cytoplasm pre-miRNA is processed by the RNAase III enzyme Dicer into miRNA. MiRNAs function as key components in the regulation of endogenous genes, impacting nearly all types of biological pathways. It is believed that almost 1000 miRNAs regulate gene expression of almost one third of vertebrate genomes [59, 60].

In contrast, siRNAs are exogenously administered or derived from long double stranded RNA, and can potentially inhibit the expression of any target gene and defend the genome integrity in response to the presence of foreign nucleic acids such as viruses or transposons [61].

In the cytoplasm, both miRNAs and siRNAs initiate the same pathway, bind to mRNA and induce mRNA cleavage or translational repression. The RNAi pathway using siRNA molecules requires a perfect base pair (bp) matching between the siRNA antisense strand and the target mRNA sequence, which results in the sequence specific degradation of the mRNA molecule. Conversely, the miRNA pathway results either in translational repression when the homology between the miRNA and its miRNA target is imperfect or, in case of a perfect homology, in the mRNA cleavage [62]. Discrimination between endogenous miRNA
and non-self siRNA by the cellular machinery is modulated by chemical structures at the ends of the RNA duplexes [63].

The main components and essential steps of the RNAi mechanism using siRNA as effector molecule are schematically represented in Figure 1-7. The initial step of the RNAi pathway is the processing of long dsRNA into 21-23 nucleotides (nt) siRNA fragments by the cellular RNase-III enzyme Dicer. The siRNA molecules are subsequently loaded into a multiprotein complex known as RNA-induced silencing complex (RISC). The double stranded siRNA molecules are then unwinded into single strands in an ATP-dependent process generating the active RISC complex guided only by the antisense strand. The active components of the RISC are endonucleases called argonaute proteins which, together with the antisense strand, recognize and cleave the complementary mRNA target sequence.

In plants, insect and nematodes, siRNA molecules are produced from long viral dsRNA intermediates synthesized during the viral replication. These small interfering RNA molecules are amplified through the activity of a cellular RNA-dependent polymerase resulting in the production of virus-specific siRNAs targeting the complementary viral RNA [64, 65]. This step amplifies the RNAi response in a catalytic cycle that persists until no target viral RNA is left [66]. In contrast to C.elegans, where RNAi is stable and long lasting, gene silencing by transfected siRNA duplexes in mammalian cells is transient. This is because mammalian cells lack the RNA-dependent RNA polymerase able to amplify the siRNA molecules. As a result, gene silencing through introduction into the cytoplasm of synthesized siRNAs is dependent on the number of siRNA molecules transfected into the cells. By each cell division these become progressively diluted [58].
1.3.2 Application

Since the discovery of RNAi in 1998 by Mello and Fire, this technology has rapidly become a powerful tool in biology and potentially a new therapeutic strategy in biomedicine [55, 67]. Due to its ability to efficiently silence gene expression, RNAi is today one of the most widely used nucleic acid-based sequence specific gene silencing method in biomedical research. Applications range from functional genomics to promising novel therapeutic tools for treating human diseases, from genetic disorders to viral infections.

Figure 1-7: RNAi mechanism. Long dsRNA is introduced into the cytoplasm and cleaved into 21-23 siRNA fragments by the enzyme Dicer. SiRNA is then incorporated into the RISC complex. The active RISC together with the antisense siRNA strand targets and degrades the complementary mRNA (Adapted from http://integratedhealthcare.eu/1/en/rna_interference/1498/).
1.3.2.1 Functional gene analysis

RNAi is a powerful reverse genetic approach using the silencing of genes in a sequence specific manner for the investigation of gene function. RNA molecules used to silence gene expression are either chemically synthesized or expressed within the cell after the introduction of plasmids or viral vector systems [68]. RNAi-based screenings allow large-scale functional analysis either in mammalian cell cultures or in vivo [69, 70]. High-throughput whole genome in vivo screens based on the RNAi-technique have been extensively carried out in C. elegans and D. Melanogaster [71, 72]. RNAi in worms is systemic and heritable and can be achieved using long (<500 bp) dsRNA introduced by different strategies such as microinjection into the body cavity, feeding with bacteria expressing dsRNA, or by soaking the worms in a solution of dsRNA. In fruit flies RNAi-based gene function studies are obtained with the introduction of short hairpin dsRNA that provides heritable genetic knockdown or by dsRNA injection into the abdomen. Genome-wide RNAi screens have elucidated a large variety of biological issues in developmental biology, embryogenesis, cell signaling, aging, metabolic regulation, neurodegenerative diseases and immunity, among others [73-80].

1.3.2.2 Gene therapy

SiRNAs as therapeutic molecules developed very rapidly and the first clinical trial of a siRNA-based drug started in 2004, just three years after the discovery of synthetic siRNA-induced RNAi in mammalian cells [56]. Theoretically, designed siRNA molecules can be used to silence almost any gene in the genome. The numbers of RNAi-based preclinical and clinical trials have grown over the past years and include studies in ocular and retinal disorders [81], cancer therapy [82], respiratory disorders (Table 1-2) and viral infections (Table 1-3) [83, 84].

The first clinical trial conducted using siRNA started in 2004 as a therapy against the age related macular degeneration (AMD), a genetic disease affecting the retina and causing
blindness. In cancer the evolving understanding of the molecular pathways responsible for carcinogenesis has created opportunities for new antitumoral therapies employing RNAi technology. The liver was one of the first organs targeted in the development of RNAi-based therapies for cancer but today several other tumor cells have been successfully targeted by siRNA in preclinical studies [85-87].

Table 1-2: Current clinical trials for genetic disorders using the siRNA-based technology (Adapted from [58, 84] and clinicaltrial.gov).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Disease</th>
<th>Target</th>
<th>Company</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bevasiranib</td>
<td>Age-related macular degeneration (AMD)</td>
<td>VEGF</td>
<td>Acuity Pharmaceuticals (Philadelphia, PA)</td>
<td>Terminated Phase III</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Allergan Inc. (Irvine, CA)</td>
<td>Completed Phase II</td>
</tr>
<tr>
<td>Sirna-027</td>
<td>Age-related macular degeneration (AMD)</td>
<td>VEGFR1</td>
<td>Merck-Sirna Therapeutics (Whitehouse Station, NJ)</td>
<td>Completed Phase I</td>
</tr>
<tr>
<td>REDD14NP</td>
<td>Age-related macular degeneration (AMD)</td>
<td>RTP801</td>
<td>Quark Pharmaceuticals (Fremont, CA)</td>
<td>Completed Phase I</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Silence Therapeutics (London, UK)</td>
<td></td>
</tr>
<tr>
<td>ALN-VSP02</td>
<td>Liver cancer</td>
<td>VEGF, KSP</td>
<td>Alnylam (Cambridge, MA)</td>
<td>Completed Phase I</td>
</tr>
<tr>
<td>I5NP</td>
<td>Acute renal failure</td>
<td>P53</td>
<td>Quark Pharmaceuticals (Fremont, CA)</td>
<td>Completed Phase I</td>
</tr>
<tr>
<td>CALAA-01</td>
<td>Solid tumor</td>
<td>RRM2</td>
<td>Calando Pharmaceuticals (Pasadena, CA)</td>
<td>Active Phase I</td>
</tr>
<tr>
<td>Proteasome siRNAs</td>
<td>Metastatic melanoma</td>
<td>LMP2, LMP7, MECL1</td>
<td>Duke University</td>
<td>Active Phase I</td>
</tr>
</tbody>
</table>

1.3.2.3 Antiviral gene silencing

The antiviral RNAi-based mechanism has the ability to target all types of viral genomes (ssDNA, dsDNA, ss(+)-RNA, ss(-)-RNA and dsRNA). For a potent and stable RNAi-based inhibition of virus replication, it is ideal to target viral genes essential for virus replication. Since its discovery about 20 years ago, several plant and animal viruses have been inhibited in vitro and/or in vivo by the RNAi mechanism. A list of all viruses targeted with nucleotide-based technologies were recently reviewed by Shah and Schaffer [88] and includes important human pathogens such as Hepatitis B virus (HBV), Herpes simplex virus and
Human immunodeficiency virus (HIV), between others. Some RNAi-based antiviral compounds have been tested in clinical trials with the most promising being the ALN-RSV01, a siRNA molecule directed against the respiratory syncytial virus (RSV) (Table 1-3). ALN-RSV01 targets RSV mRNA encoding the nucleocapsid protein and is the first siRNA-based drug targeting a microbial pathogen to be tested in human. ALN-RSV01 is administered to the human respiratory tract as a nasal spray [89].

Unfortunately, targeting of viral factors may lead to escape from RNAi and development of resistance to the antiviral activity of siRNA molecules. The most convenient strategies to reduce the chance of escape are the targeting of highly conserved viral genes or silencing host genes essential for viral replication [90]. A well-known example of non-essential host factor is the chemokine receptor CCR5, co-receptor for HIV essential for virus entry. Targeting CCR-5 receptor with siRNA was shown to increase resistance against HIV infection in both in vitro and in vivo systems [91, 92].

Tab. 1-3: Clinical trials for antiviral RNAi therapy (Adapted from [83] and clinicaltrial.gov)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Indication</th>
<th>Target</th>
<th>Company</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPC3649</td>
<td>Hepatitis C virus</td>
<td>miR-122</td>
<td>Santaris Pharma (Horshilm, Denmark)</td>
<td>Completed, Phase II</td>
</tr>
<tr>
<td>ALN-RSV01</td>
<td>RSV in volunteers</td>
<td>RSV nucleocapsid</td>
<td>Alnylam (Cambridge, MA)</td>
<td>Completed, Phase II</td>
</tr>
<tr>
<td>ALN-RSV01</td>
<td>RSV in lung transplant patients</td>
<td>RSV nucleocapsid</td>
<td>Alnylam (Cambridge, MA)</td>
<td>Completed, Phase II</td>
</tr>
<tr>
<td>ALN-RSV01</td>
<td>RSV in lung transplant patients</td>
<td>RSV nucleocapsid</td>
<td>Alnylam (Cambridge, MA)</td>
<td>Active, Phase IIb</td>
</tr>
</tbody>
</table>

1.3.3 SiRNA delivery

In 2001 it was shown for the first time by Elbashir and coworkers that transfection of synthetic siRNAs can induce RNAi-mediated gene silencing in mammalian cells [56]. Since then the development of stable siRNA molecules for the targeting of desired cells, tissues and organs remains the biggest issue in the development of RNAi-based drugs. Indeed unmodified and unprotected siRNAs are highly unstable molecules in vivo, easily degraded.
by nucleases in the blood. Moreover, due to their large molecular weight (~13 kDa) and polyanionic nature, naked siRNAs do not easily penetrate hydrophobic plasma membranes. Besides stabilizing chemical modifications used to increase siRNA half-time, RNAi can be achieved essentially by two nucleic acid based strategies: i) transient specific gene silencing effect through the introduction into the cytoplasm of small double stranded RNA molecules (siRNAs); ii) long-term and stable knock-down through the delivery of gene expression cassettes expressing short hairpin RNA (shRNA) [58].

1.3.3.1 Chemical modifications

Unmodified siRNA molecules are very rapidly degraded in the bloodstream (half-life of less than 15 min in serum) and can induce an interferon response from the innate immune system [93, 94] (see chapter 1.3.4). Moreover, unmodified siRNAs can also induce specific off-target effects, as they interfere with natural miRNA pathways [95]. Chemical modifications of siRNA molecules can increase their stability, decrease their toxicity, and improve the pharmacokinetic and pharmacodynamic properties of the molecule without affecting the silencing activity [96]. There are two main classes of chemical modifications used for the in vivo delivery of siRNAs: the backbone and the sugar modifications (Figure 1-8). The backbone modifications involve alterations of the phosphate ester linkages (PO₄) in the nucleic acid. The most common backbone modification consists in the replacement of the phosphodiester linkages with phosphothioate (PS) which provides an increased nuclease resistance and more favorable pharmacokinetic properties [97]. The most widely used sugar modifications of siRNAs are the 2’ modifications of the sugar ring, namely O’methyl (2’-OMe), fluoro (2’-F) and methoxyethyl (2’-MOE). These modifications often improve the silencing potency, the stability and overall pharmacokinetic and pharmacodynamic properties [98].
1.3.3.2 Non-viral siRNA delivery

Administration of chemically modified 21-25 nt siRNA duplexes is one of the most popular clinical application for RNAi-based therapies. To penetrate the hydrophobic cellular membrane, negatively charged siRNA molecules need assisting carriers. Cationic lipids are one of the best studied and most promising carriers for RNAi delivery. Positively charged lipids possess the ability to bind and condense siRNA through electrostatic interactions and transport the enclosed nucleic acids across the cellular membrane into the cytoplasm of target cells (Figure 1-9). Oligofectamine, Lipofectamine, Lipofectin, RNAiFect are some of the commercially available lipid-based transfection reagents for siRNA delivery in vitro. DOTAP
(N-[1-(2,3-dioleyloxy)]-N,N,N trimethylammonium propane) and Oligofectamine have been used for the in vivo delivery of siRNA [99]. However, systemic administration of cationic liposomes in vivo can activate the immune system and induce cytokines-related cell toxicity. This immune activation is supposed to be triggered by TLR4 [100, 101]. Toxicity varies with the precise composition of the lipids employed.

Nanoparticles cationic polypeptides have also been extensively used to obtain enhanced intracellular delivery of siRNAs. Polyethylenimine (PEI) is the most promising and efficient polycationic polymer used for development of efficient gene delivery vectors [102]. PEI polymers can complex with siRNA and are taken up by the cells by endocytosis. High molecular weight PEIs show the highest transfection efficiency. However, cytotoxicity limits their applications.

Figure 1-9. Widely used non-viral delivery strategies include siRNAs encapsulated within liposomes (A) or nanoparticles (B) [103].

1.3.3.2 Viral shRNA delivery

Viral vector systems, mostly based on Adeno-associated viruses (AAV) and Lentiviruses (LV), have been shown to be an efficient strategy for obtaining gene silencing for an extended period of time in a wide range of mammalian cells and animal models. Especially for chronic diseases, cancer or AIDS, long term gene silencing is highly desirable and viral delivery of silencing RNA represents an ideal delivery vehicle. In viral vectors, siRNA, called
shRNA, is expressed intracellularly from plasmid DNA. ShRNAs are generally transcribed from pol III promoters such as U6 and H1 by RNA polymerase III which drives high levels of constitutive shRNA expression. ShRNAs contain perfect stems of 21-25 bp with a tight terminal hairpin loop.

Lentiviruses constitute a subclass of retroviruses with two copies of an ssRNA genome in an enveloped capsid. LVs can transduce both dividing and non-dividing cells which makes them good vector candidates for the delivery of silencing RNA within the central nervous system (e.g. neurons) [58]. When the virus enters the cell, viral RNA genome is reverse-transcribed into DNA, which is then inserted into the host genome at a random position by the viral integrase enzyme. The vector, now called provirus, remains in the genome and is passed to daughter cells during division (Figure 1-10). The integration of small RNA within the host genome is highly unpredictable with the risk for insertional mutagenesis. This makes long-term effects of lentiviral vectors difficult to predict [104]. Lentivirus vectors can accommodate up to 7.5 kb amounts of DNA and are lowly immunogenic [58].

Adeno-associated viruses are non-pathogenic small ssDNA viruses. They infect humans and some other primate species and can transduce both dividing and non-dividing cells with a low reactivity with cellular immune response. Contrary to LVs, AAVs largely persist extrachromosomally with a low genome integration rate and a minimal risk of insertional mutagenesis, making them a very attractive delivery system for gene therapy. However, this may also represent a negative aspect since the genetic information is less stable and may be lost during cell division [105]. AAVs vectors have a limited packaging capacity of 5 kb which is sufficient for the insertion of at least eight individual shRNA cassettes [106].
1.3.4 Off-target effects

When designing therapeutic strategies involving siRNA molecules it is important to know all the properties of the RNA strand that is used for the target of the desired gene. The introduction of exogenous siRNA molecules can indeed induce off-target effects which can lead to false interpretation of the data obtained. Off-target effects can be classified into specific and non-specific. The former consist in the silencing of undesired genes as a consequence of an imperfect pairing between the siRNA guide strand with the non-target transcript, usually in the 3'UTR [60]. As explained in the chapter 1.3.1, siRNA and miRNA pathways have a number of similarities and they partially share the same silencing machinery. As a consequence, siRNA can enter the natural miRNA pathway and function as miRNA, silencing targets with partial sequence complementarity. Those specific off-target effects can be minimized with the introduction of chemical modifications on the sense and/or antisense siRNA strand, with a reduction in the applied siRNA concentration and with an accurate siRNA design. A multitude of software and algorithms have been developed to optimize siRNA design and reduce undesired specific off-target effects.
Nonspecific off-target effects consist in the activation of the immune system in response to exogenous siRNA molecules or vehicles, such as cationic lipids, required to deliver siRNA. RNA molecules trigger the innate immune response in mammals by activating a type-I interferon (IFN) response. Innate immune activation by siRNA is a significant undesirable side effect in vivo due to the toxicity and possibly strong inflammation associated with massive cytokine production [108]. Initially it was believed that only dsRNA bigger than 30 base pairs were able to stimulate an immune response through the activation of the kinase PKR with the consequent shut down of global gene expression. However, subsequent studies have demonstrated that even short dsRNA molecules were associated with nonspecific immunostimulatory effects [109-112]. Activation of the innate immunity through siRNA can be either Toll-like receptor (TLR)-mediated or non-TLR-mediated. RNA-sensing TLRs are expressed in endosomes and include TLR-3 and TLR-7/8, sensing dsRNA and ssRNA, respectively. TLRs located on the cell membrane, such as TLR-2 and TLR-4, recognize lipopeptides and lipopolysaccharides, respectively. TLR-4 was shown to induce an immune response after binding to cationic lipids [113]. Non-TLR-mediated innate immune response is triggered by siRNA binding to proteins such as the dsRNA-binding protein kinase (PKR) or the retinoic acid inducible gene 1 (RIG-I) [114, 115]. Non-specific target effects can be reduced with the introduction of chemical modifications in the siRNA molecule, by local application of low siRNA doses and by optimizing the siRNA design avoiding pro-inflammatory sequences.
3. Aims of the project

This study evaluated the potential of RNA interference (RNAi) by small interfering RNA (siRNA) as a specific therapy for tick-borne encephalitis (TBE). The flavivirus Langat virus was used as a model for Tick-borne encephalitis virus (TBEV) infection. Three aims were defined for this study.

3.1 Aim 1

To identify siRNA formulations able to efficiently inhibit Langat virus replication in an in vitro model based on
a) Immortalized cell cultures and
b) Organotypic hippocampal cultures (OHCs).

To this end siRNA sequences targeting different regions of the Langat genome were designed and screened for their ability to interfere with viral replication on HeLa cells. SiRNA-mediated virus inhibition was evaluated with real-time RT-PCR, immunoperoxidase focus assay and immunostaining. The most efficient and most conserved siRNA sequence was selected and tested for its antiviral activity on organotypic hippocampal brain cultures (OHCs).

3.2 Aim 2

To develop an infant rat model of viral encephalitis using Langat virus as a representative member of the Tick-borne encephalitis virus complex (TBE serogroup). To this end, infant rats were intracisternally infected with Langat virus and clinical symptoms, laboratory parameters (cyto- and chemokines secretion in the CSF), behavioral experiments and histopathological findings were evaluated for up to 9 days post-infection. The newly established model was compared to clinical and laboratory parameters of human patients affected by TBE and to other TBEV-infected mouse models.
3.3 Aim 3

To evaluate the efficacy of siRNA formulations as antiviral agents \textit{in vivo}. The selected siRNA sequence from Aim 1, efficiently inhibiting Langat virus replication \textit{in vitro}, was used for the \textit{in vivo} application. For the evaluation of RNAi \textit{in vivo} we used the infant rat model developed in Aim 2. Animals were treated at different time points with siRNA before and after infection with Langat virus. The antiviral effect of siRNA was evaluated by RT-PCR from CSF and brain tissues.

The project was designed as a collaborative study between the Institute for infectious diseases in Bern and the Spiez Laboratory in Spiez. Experiments were performed in both Institutes based on the infrastructural needs.
4. Results

4.1 SiRNA inhibits replication of Langat virus, a member of the tick-borne encephalitis virus complex in organotypic rat brain slices

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Abstract

Tick-borne encephalitis virus is the causative agent of tick-borne encephalitis, a potentially fatal neurological infection. Tick-borne encephalitis virus belongs to the family of flaviviruses and is transmitted by infected ticks. Despite the availability of vaccines, approximately 2000-3000 cases of tick-borne encephalitis occur annually in Europe for which no curative therapy is available.

The antiviral effects of RNA mediated interference by small interfering RNA (siRNA) was evaluated in cell culture and organotypic hippocampal cultures. Langat virus, a flavivirus highly related to Tick-borne encephalitis virus exhibits low pathogenicity for humans but retains neurovirulence for rodents. Langat virus was used for the establishment of an in vitro model of tick-borne encephalitis. We analyzed the efficacy of 19 siRNA sequences targeting different regions of the Langat genome to inhibit virus replication in the two in vitro systems. The most efficient suppression of virus replication was achieved by siRNA sequences targeting structural genes and the 3′ untranslated region. When siRNA was administered to HeLa cells before the infection with Langat virus, a 96.5% reduction of viral RNA and more than 98% reduction of infectious virus particles was observed on day 6 post infection, while treatment after infection decreased the viral replication by more than 98%. In organotypic hippocampal cultures the replication of Langat virus was reduced by 99.7% by siRNA sequence D3.

Organotypic hippocampal cultures represent a suitable in vitro model to investigate neuronal infection mechanisms and treatment strategies in a preserved three-dimensional tissue architecture. Our results demonstrate that siRNA is an efficient approach to limit Langat virus replication in vitro.
Introduction

Tick-borne encephalitis virus complex represents a group of closely related viruses endemic in Europe and Asia causing serious neuroinfections and hemorrhagic fevers [23]. Tick-borne encephalitis virus (TBEV) belong to the *Flaviviridae* family and can be divided in three main subtypes, the European, the Siberian and Far Eastern subtypes [8]. TBEV are transmitted to humans by the bite of infected ticks and, in rare cases, through the consumption of infected unpasteurized milk [20, 116]. Following an incubation period of 3-8 days after a tick bite, the virus replicates locally in epidermal dendritic cells and spreads via lymph vessels to the blood stream where a short but significant viremia occurs and extraneural tissues are infected. It is during this viremic phase that the virus crosses the blood-brain barrier and invades the central nervous system (CNS) by a still unknown mechanism. Once the virus has reached the CNS, active infection causes inflammation, lysis of cells and cellular dysfunction [34, 117]. Clinical manifestations of TBEV infections typically follow a biphasic course. The first viremic phase is characterized by flu-like symptoms such as fever, headache and muscle pain. After 3-7 days of an asymptomatic phase, 20-30% of the patients develop the second meningoencephalitic phase with neurological disorders of varying severity [5, 33, 117, 118]. The most common clinical feature of TBE patients is ataxia followed by paresis or paralysis of one or more extremities [31].

Two efficient formalin-inactivated whole virus vaccines are available in Europe and provide a high degree of protection against the disease. Nevertheless more than 2500 TBE cases have been registered yearly in Europe in the past ten years and a continuous increase in TBE morbidity was observed in the last years [24, 30]. Up to today no specific therapeutic options are available for flaviviral infections and an effective therapy for TBEV infection would be highly desirable [19]. Results from *in vitro* and *in vivo* studies indicate that therapeutics based on RNA interference (RNAi) could be effective against viral infections, and small interfering RNA (siRNA) molecules are promising candidates for future clinical applications [104, 119-121]. Indeed, several RNAi-based antiviral drugs are currently being tested in clinical trials [83, 84, 89, 90].
RNA interference is a conserved post-transcriptional gene silencing process which leads to the specific degradation of RNA within the cytoplasm of eukaryotic cells [55, 57, 66]. Long double-stranded RNA molecules are initially processed by the enzyme Dicer into 21-25 nucleotides long small interfering RNA. In the cytoplasm siRNAs are incorporated into the multiprotein RNA-induced silencing complex (RISC) which results in sequence specific association and degradation of the complementary mRNA [55, 122]. SiRNA mediated intervention strategies take advantage of this conserved mechanism by artificially introducing siRNA molecules into the cytoplasm which ultimately leads to the degradation of target RNA molecules. It was shown that the application of siRNA molecules with sequences complementary to viral genomic RNA or RNA replication intermediates allows to significantly reduce the number of virus progeny in infected cells. Using this strategy the replication of human pathogenic flaviviruses such as West Nile virus (WNV), Dengue virus (DEN) and Japanese encephalitis virus (JEV) was successfully inhibited both in vitro and in vivo [104, 123-127] and recently inhibition of TBEV by RNAi was demonstrated in human embryonic kidney cells (HEK293T) [128]. Sensitive target sequences were identified within the structural (Capsid (C), membrane protein (M) and envelope (E)), non-structural (mostly NS5 and NS3) and the 3’ untranslated region of the flavivirus 11 kb open reading frame (ORF).

We used organotypic hippocampal brain slice cultures (OHCs) infected with Langat virus (LGTV) as a model to analyze the effectiveness of RNAi in the inhibition of the virus replication. OHCs is a well established model based on in vitro cultured brain slices which was adapted for this virus encephalitic model from previous work [129]. Organotypic cultures offer unique advantages over other in vitro models, in that they reproduce important aspects of the in vivo situation. Organotypic cultures retain the three-dimensional tissue architecture of the brain with a preserved cellular composition including neurons, microglial cells and astrocytes [129, 130]. The use of OHCs allowed the culture of Langat virus in an in vitro system reproducing some aspects of TBEV infection of the brain. LGTV is a member of the tick-borne encephalitis virus complex sharing high nucleotide homology with TBEV. It has an attenuated virulence for the human host and was used as a vaccine candidate against TBEV.
for several years, but was abandoned due to the sporadic occurrence of encephalitis cases (1:18000) [50, 51]. Because of its relative avirulence for humans it can be handled under BSL-2 conditions. LGTV retains neurovirulence for rodents and is therefore considered a suitable virus-model for the pathogenic TBEV in mice and rats [52, 53, 131, 132]. Using Langat virus in the organotypic brain culture model we could demonstrate the effectiveness of siRNA in inhibiting TBE virus replication in a three-dimensional neuronal tissue architecture.
Results

Evaluation of siRNA sequences targeting Langat virus genome. A total of 19 siRNA sequences (Q1-Q6 and D1-D13) targeting genes within the structural (S), the non-structural (NS) and the 3’ and 5’ untranslated regions (UTRs) of Langat genome (Table 1 and Figure 1A) were tested. All siRNA sequences were evaluated for their capacity to inhibit replication of Langat virus in HeLa cells when transfected 4h before the infection occurred. Fourteen out of the 19 specific siRNA sequences were able to induce a significant reduction of viral genome copy numbers in comparison to the nonsense siRNA used as a negative control (Figure 1B). Inhibition of virus replication was most efficient with siRNA molecules targeting sequences located within the structural region (Q5, Q6, D5, D12) and the 5’ UTR (D3 and D8), reducing viral genome copy numbers by up to 85%. Only Q1, D1 and D10, all targeting the 3’ UTR, had no effect on virus replication. A comparative analysis of all siRNA target sequences with the genomes of five members of the Tick-borne encephalitis virus complex revealed that siRNA sequences D1, D3, D8, D10 and D13 located within the 3’UTR, 5’UTR and the core region were strongly conserved with 1 to 2 nucleotide divergence between different viral subtypes. SiRNA sequence D3 was the only one with 100% sequence identity in all three TBEV subtypes and in the Omsk hemorrhagic fever virus (Table S1). SiRNA sequence D3 targeting 5’ UTR was selected for further experiments on HeLa cells and organotypic cultures.

Inhibition of virus replication by siRNA D3 in HeLa cells. To determine the inhibitory effect of siRNA sequence D3 over time the replication profile of Langat virus was assessed daily during the 6 days incubation period in cells treated with specific siRNA D3 and in cells treated with nonsense siRNA as controls. While virus replication was continuous in HeLa cells treated with nonsense siRNA, the number of viral genome copies was reduced by 96.5% (1.4 logs) (Figure 2A) and the number of infectious particles by 98.8% (2 logs) (Figure 2B), from day 2 to day 6 post infection in cells transfected with siRNA D3. To exclude unspecific inhibition of virus replication trough an effect of the transfection reagent on cell viability we performed a LDH-based cytotoxicity assay on cells under the different treatment
regimes. No significant difference in cytotoxicity was observed between transfected and infected cells and untransfected and uninfected cells demonstrating that cell viability was comparable between the four groups (Figure S1).

We analyzed the effectiveness of siRNA on viral replication when administered after the initiation of the infection. To this end HeLa cells were first infected with Langat virus at MOI of 10, 1, 0.1 or 0.01 and 1 h later transfected with siRNA sequence D3. Treatment of infected HeLa cells with siRNA sequence D3 resulted in a reduction of viral genome copies of 1.6 logs (MOI of 10), 2.4 logs (MOI of 1), 2.4 logs (MOI=0.1) and 5 logs (MOI=0.01) reduction compared to cells transfected with the nonsense siRNA sequence which corresponds to an inhibition of more than 98% for all the infectious doses. At the lowest infectious dose of 0.01 MOI the virus titer was reduced under the detection limit.

**Langat virus replication in the organotypic hippocampal cell culture model.** OHCs are an excellent model for the investigation of neuronal infection mechanisms and new treatment strategies. The ability of Langat virus to infect and replicate in OHCs was demonstrated by real-time RT-PCR and immunofluorescence (Figure 4). Using a red fluorescent antibody, cells expressing Langat virus proteins were specifically stained. Figure 4 A shows a strong expression pattern of viral proteins in OHC infected with Langat virus in contrast to uninfected OHC depicted in Figure 4 B. Co-staining of Langat virus proteins (red) and Fox 3 (green), a marker for neuronal cells, shows strong co-localisation, especially in the cornu ammonis (CA) and dentate gyrus (DG) (Figure 4 C, D, E, F, G, H).

**Antiviral activity of siRNA in infected organotypic hippocampal cell cultures.** To assess the capacity of siRNA to inhibit virus replication in a complex three-dimensional neuronal network, inhibition experiments were performed on OHCs. Transfection of OHCs with the specific siRNA sequence D3 before and after the infection led to an efficient inhibition of virus replication reducing the number of viral genome copies in OHC homogenates from $5.4 \times 10^5$ to $1.7 \times 10^3$ copies/µl (2.7 logs) and the number of infectious particles from $2.7 \times 10^2$ to an average of 0.5 ffu/µl (4.3 logs) (Figure 5 A and B) corresponding to a reduction of more than 99.6% as compared to nonsense siRNA treated OHCs. This marked reduction of viral
progeny in organotypic cultures treated with siRNA was confirmed by immunostaining methods. While in OHCs transfected with the non-targeting siRNA the expression of viral proteins led to strong red fluorescent signal in a high percentage of the cells, in OHCs transfected with the specific siRNA sequence D3 no red fluorescence was detectable, confirming an efficient reduction of viral protein levels in all cells (Fig. 5 C and D).

**Off-target effects.** Several studies suggest that transfection of cells with siRNAs may result in the activation of the interferon pathway and affect virus replication via interferon mediated antiviral activity [115, 133, 134]. To investigate whether the treatment of OHCs with siRNA leads to the activation of the IFN system, expression levels of IFN-β mRNA were quantified by real time RT-PCR in non-infected OHCs treated with different siRNA sequences and compared to untreated or poly I:C treated cultures. While treatment of HeLa cells with poly I:C, used as a positive control, leads to a strong upregulation of IFN-β mRNA, none of the siRNA sequences lead to an increase in the IFN-β mRNA expression 24 h after the second transfection,(Figure S3).
Discussion.

Therapeutic options for the treatment of TBE are lacking and specific therapies are urgently needed since the incidence of TBE is rising. Several studies have shown that siRNA molecules have the potential to be used as a specific therapeutic strategy against viral infections. Due to difficulties in delivery, toxicity and the stimulation of unspecific immune response few approaches were continued in vivo. For TBE virus infections detailed investigations determining optimal target sequence and inhibitory capacity for synthetic siRNA are lacking. The majority (11/19) of the 19 siRNA sequences screened in this study were capable of reducing Langat virus titer by more than 80% in comparison to nonsense siRNA when transfected into the cells before the infection (Figure 1 B). The most effective reduction in viral replication was achieved with siRNA sequences targeting the 3’ UTR and the structural genes. SiRNA sequence D3 (5’UTR) and Q6 (Envelope) showed the highest capacity to inhibit viral replication, reducing the number of viral RNA genome copies from 1x10E6/\(\mu\)l (nonsense siRNA) to 5.5x10E4/\(\mu\)l (siRNA D3) and 6.6x10E4/\(\mu\)l (siRNA Q6) respectively, corresponding to a reduction of more than 93% (Figure 1 B). In addition to its strong antiviral activity sequence D3 is highly conserved between different members of the tick-borne encephalitis virus complex and was therefore selected for further experiments on HeLa cells and organotypic brain cultures. Using the siRNA sequence D3 we could demonstrate that the application of siRNA after the infection was still effective. In fact the inhibitory effect was comparable to the results obtained when siRNA was applied before infection. These results indicate that it is feasible, at least in the paradigm tested herein, to inhibit replication of TBEV in vitro once the infection has been initiated.

For further in vitro analysis we developed an encephalitis model based on infection of rat organotypic hippocampal brain cultures with Langat virus. The use of an attenuated virus strain has several advantages compared to the work with human pathogenic TBE strains. Despite its relative avirulence for the human host, LGTV shares a high nucleotide homology with pathogenic TBEV, nevertheless LGTV can be worked under BSL-2 conditions. In rodents Langat virus is able to cause viremia, infect the central nervous system and thereby
causing encephalitis and neuronal lesions replicating clinical and histopathological features observed in human cases of TBE [52, 135]. Our immunohistochemical results about infection of neuronal cells by LGTV is in agreement with previous findings of fatal TBE human cases where neurons represent the main target for TBEV infection [28]. By measuring virus replication in organotypic hippocampal cultures and staining viral proteins within the neuronal cells we could demonstrate that Langat virus productively infects neurons of rat brain slices (Figure 4C, D, E, F, G, H). In many aspects OHCs fill the gap between dissociated cell cultures and in vivo animal experiments. OHCs are ex vivo brain slices and constitute an intact neuronal network with a well-preserved representation of the most important brain cells, including neurons, astrocytes and microglia [136, 137]. This in vitro model of neuronal network allows the investigation of the mechanisms underlying neuropathogenicity of TBE and, as we have shown in this study, the efficiency of new antiviral drugs under conditions resembling those encountered in vivo [138, 139]. Our results obtained in OHCs transfected with the siRNA sequence D3 support findings from several in vivo studies showing that neuronal cells could be transfected with siRNA and are competent to develop a strong siRNA mediated antiviral activity [104, 127, 140, 141]. Applying siRNA sequence D3 on organotypic cell cultures an inhibition of Langat virus replication by more than 99.6% was achieved, both on the level of viral RNA and on the number of infectious particles. After entering the human body, tick-borne flaviviruses develop a high viremia which is considered to be a prerogative for the subsequent invasion of the CNS [15]. The antiviral potential of our siRNA sequences may help to attenuate the viremia and thus prevent the development of encephalitis or, if encephalitis is already established, to limit the number of infected neurons to a minimum thereby minimizing the damage in the brain caused by either the virus itself or the immune response to the infection.

The siRNA sequence D3 targets a well conserved stretch of the 5’UTR of Langat virus sharing 100% homology with all 3 subtypes of TBEV, the European, the Siberian and the Far Eastern subtype as well as with the Omsk hemorrhagic fever virus and 95% (18 nucleotides out of 19) with Louping ill virus (see Table S1). We therefore consider D3 a promising
candidate for future in vivo studies on TBEV infections. Other siRNA sequences such as D5, D8, D12, D4, Q5 and Q6 were also highly effective in our in vitro analysis and may be used in the combination with D3. As it has been suggested in other studies the combination of 2 or more specific siRNA simultaneously targeting multiple viral genome regions may enhance the inhibitory effect and reduce an eventual viral escape [124, 142].

An unspecific stimulation of the interferon response by the siRNA sequence D3 is unlikely since no upregulation of the IFN-β gene was detected. Cationic lipid reagents, such as Lipofectamine RNAiMAX and Dharmafect 2 used in this study, are promising carriers for the siRNA delivery in vivo [143, 144]. However they are known to cause side effects such as cytotoxicity and non-specific activation of intracellular signalling pathways [100, 101]. To determine toxicity of the transfection regimen the LDH release was assessed in several experiments but no increase in LDH level was observed in HeLa cells or in organotypic cell cultures treated with the transfection reagents and siRNA.

Despite these encouraging results, development of reliable transfection methods remains the biggest hurdle in the progress of in vivo antiviral RNAi-based drug therapies. In case of encephalitogenic viruses such as TBEV, carriers for therapeutic siRNA molecules must be able to cross the blood brain barrier [145]. For these reasons the development of new strategies which allow highly efficient transfection of siRNA molecules is a crucial step on the way towards the clinical use of RNAi-based therapies. Organotypic cultures represent a valuable tool to evaluate the efficiency of different transfection strategies with siRNA and to determine their potential to induce the interferon system in a conserved neuronal network.

In summary, our study provides further support for the use of RNAi technology in the development of antiviral drugs against encephalitogenic tick-borne flaviviruses. Organotypic brain cultures were used for the first time as a successful in vitro approach in an RNAi-based antiviral therapy.
Material and Methods

Cell culture and virus amplification. Vero and HeLa cells were cultured in Eagle’s minimum essential medium (MEM) supplemented with 10% foetal bovine serum, 1.25% L-Glutamin, 1% Non-essential amino acids, 1% Penicillin-Streptomycin and 0.5% Neomycin-Bacitracin (Biochrom AG, Berlin, Germany). Langat virus strain TP21 was kindly provided by Daniel Růžek (University of South Bohemia, České Budějovice, Czech Republic). Virus was cultured on 80% confluent Vero cells in 75-cm² culture flasks (Sigma-Aldrich, Buchs, Switzerland) and grown for 7 days at 37°C in cell culture medium containing 2% FBS. Virus containing cell culture supernatant was titrated and used for infection and transfection assays.

Immunoperoxidase focus assay (IPFA). Virus quantification was performed by IPFA. Serial dilutions of virus cultures (100 µl/well) were inoculated on 24 well plates with 80% confluent Vero cells. Viral adsorption was allowed for 1 h at 37°C on a rocking platform and wells overlaid with 200 ul of pre-warmed cell culture medium (2% FBS) and 500 ul MEM containing 1% methylcellulose (Sigma-Aldrich), 5% FBS, 1.25% L-Glutamin, 1% Non-essential amino acids, 1% Penicillin-Streptomycin and 0.5% Neomycin-Bacitracin. Plates were incubated for 6 days at 37°C. Cells were washed with PBS pH 7.4, fixed with 4% formaldehyde in PBS for 1 h and cell membrane permeabilized by adding 1% Triton in PBS for 5 min. Following two washing steps with PBST (PBS + 0.05% Tween), 500 µl monoclonal anti-flavivirus antibody (Anti-Flavivirus Group A clone D1-4G2-4-15, Millipore AG, Switzerland) diluted 1:1000 in blocking buffer (PBST containing 10 % FBS and 4% Skimmed Milk powder (Hochdorf Nutritec AG, Sulgen, Switzerland)) was added for 1h at room temperature (RT). After two washing steps with PBST cells were incubated for 1h with the secondary goat anti-mouse-HRP antibody (Anti-mouse IgG (H+L); Kirkegaard & Perry Laboratories, Gaithersburg, USA) diluted 1:1000 in blocking buffer. Viral spots were visualized after thorough washing by adding the HRP substrate AEC (3-amino-9-ethylcarbozole) (Fluka, Switzerland) in N,N-Dimethylformamide (Fluka, Switzerland) diluted
in acetic acid 0.05 M (Fluka, Switzerland). Viral titers were determined as focus forming units (FFUs) per millilitre.

Organotypic cultures. Organotypic hippocampal cultures were prepared from 5 days-old Wistar rats as previously described by us [130]. Briefly, rat pups were sacrificed by a lethal dose of Pentobarbital i.p (G. Streuli & Cie. SA, Uznach, Switzerland). The brain was removed and submerged in ice-cold dissection medium consisting of Hank’s balanced salt solution (HBSS; Gibco Life Technologies, Basel, Switzerland) with 6 mg/ml glucose and 10 µg/ml Penicillin-Streptomycin. Hippocampus was isolated and cut perpendicular to the axis into 400 µm thick-sections by a McIlwain tissue chopper (Mickle Laboratory, Guilford, UK). Slices with intact hippocampal morphology were selected and individually transferred on a semiporous (0.4 µm) membrane of the Transwell inserts (Corning Inc., Corning, NY). Inserts were placed in a 24-well plate in contact with 200 µl serum-free Neurobasal™ medium (Gibco) supplemented with B27 Supplement (20µl/ml, Gibco). Before transfection slices were incubated at 37°C with 5% CO₂ for 4 days. Medium containing B27 Supplement was changed every day for the first two days.

siRNAs. Six siRNA sequences (Q1-Q6) were designed and synthesized by Qiagen (Hilden, Germany) using the BioPredsi algorithm [146]. Thirteen sequences (D1-D13) (siSTABLE) were designed using the Dharmacon online tool siDESIGN® Center (http://www.dharmacon.com/designcenter) and synthesized by the same company (Thermo Fisher Scientific, Lafayette, USA). SiRNA sequences were chosen according to the algorithm score.

Comparative analysis of all siRNA sequences with genomes of tick-borne flaviviruses was performed using GeniusPro Version 5.5.7 (Table S1).

Transfection and infection assays. Transfection and infection experiments were performed on HeLa cells and organotypic cell cultures. HeLa cells were seeded to a confluency of 60-70% on 24-well plates the day before transfection. When infection of HeLa cells was performed after transfection, cells were first transfected with 200 nM siRNA for 4. SiRNAs were complexed with the transfection reagent Lipofectamine RNAiMAX (Invitrogen, Basel,
Switzerland) according to the manufacturer’s instructions and lipid-siRNA complexes added to the cells with 1 ml of serum- and antibiotic-free MEM. After 4h incubation, cells were washed and infected with 200 µl Langat virus in culture medium at MOI of 10. Virus inoculum was removed after 1 h and cells were incubated with 1 ml antibiotic-free MEM containing 2% FBS for 6 days. Virus titer was assessed by determining the number of genome copies in 100 µl cell supernatant by real-time RT-PCR and number of infectious particles assessed by immunoperoxidase focus assay (IPFA).

When infection was performed before transfection, HeLa cells were infected with Langat virus at a MOI of 10, 1, 0.1 or 0.01. One hour after the infection the inoculum was removed and cells were transfected with 200 nM siRNA/Lipofectamine RNAiMAX complexes as described above. The siRNA/Lipofectamine complexes were removed after 4 hours and cells incubated for 6 days at 37°C. Virus titer was assessed by determining the number of genome copies in 100 µl cell supernatant by real-time RT-PCR 6 days after infection.

Organotypic hippocampal cultures were prepared as described above. After slicing OHCs were cultured for 4 days to allow recovery. OHCs were transfected with 800 nM siRNA complexed with the lipid-based transfection reagent Dharmafect 2 (Thermo Fisher Scientific, Lafayette, USA) for 24 h according to the manufacturer’s instruction. Lipid-siRNA complexes were prepared in 200 µl antibiotic-free Neurobasal Medium (NBM) whereof 100 µl were given to the medium underneath the membrane and 100 µl were added drop wise onto the slice. One day after incubation the OHC were infected with 2x10E6 FFU Langat virus in 200 µl NBM. After 1 h inoculum was removed and OHCs transfected for the second time. Viral genome copy number and number of infectious particles were assessed 2 days after the second transfection step.

**LDH cytotoxicity test.** HeLa cells transfected with 200 nM siRNA (D3 or nonsense) and untransfected cells were infected with Langat virus 4 h after transfection and incubated for 6 days at 37°C. During 6 days samples of 100 µl supernatant were taken daily and LDH activity was quantified according to the manufacturer’s instructions applying the Cytotoxicity
Detection KitPLUS (LDH) (Roche Diagnostics, Rotkreuz, Switzerland) by measuring optical density (OD) at 490 nm.

**Viral RNA extraction.** Nucleic acid extraction from cell supernatant or OHC homogenate was performed using the BioRobot EZ1 Workstation (Qiagen). 100 µl cell culture supernatant were inactivated in 400 µl AVL viral lysis buffer (Qiagen) and viral RNA isolated applying the EZ1 Virus mini kit v2.0 (Qiagen). OHCs were homogenized in 800 µl Qiazol (Qiagen) by the TissueLyser System (Qiagen) for 2 min at 25 Hz. Extraction was performed with the Universal RNA Tissue Kit (Qiagen) following the manufacturer’s instructions. RNA was eluted in a final volume of 50 µl.

**Primers.** The specific primers and probes for the detection of Langat virus strain TP21 were designed using Primer Express software v3.0 (Applied Biosystems, Foster City, CA) and purchased from Microsynth (Balgach, Switzerland). LGTV forward primer 5’-TGTGTGGAGCGGCGATT-3’; LGTV reverse primer 5’-TAAGGGCGCGTTCCATCTC-3’; probe 5’-AGCCACGCTTCCAGAGGAGCACC-3’. Detection of interferon β, OAS-2 and STAT-1 genes was performed using the corresponding pre-designed QuantiTect Primer assays (Qiagen).

**One step real-time reverse transcriptase PCR.** Viral RNA was quantified by one-step real-time RT-PCR using the QuantiFast probe RT-PCR kit (Qiagen) according to the manufacturer’s protocol with the following cycling conditions: reverse transcription at 50°C for 10 min, an initial PCR activation step at 95°C for 5 min, 40 cycles of two-step cycling for 10 s at 95°C and 30 s at 60°C.

**Fluorescence microscopy.** Organotypic hippocampal slices were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline for 90 min at 4°C and cryopreserved in 18% sucrose solution in PBS for 3-4 days. Slices were cut into 12 µm thick sections with a Jung CM 1800 cryostat (Leica Microsystems, Glattbrug, Switzerland) according to a procedure described elsewhere [130]. The sections were collected in PBS, transferred to a chrome-alum-gelatine-coated glass slide, air dried and kept in PBS. Organotypic sections were incubated with primary antibodies directed against Langat virus (LGT-I-13A10; 1:1000
Fort Detrick, USAMRIID, USA) and FOX3 (rabbit polyclonal; 1:1000, Millipore). Primary and secondary antibodies were diluted in TBS (pH 7.6) containing 0.5% bovine serum albumin. Sections were washed three times with PBS and then exposed to the secondary antibody for 45 min at RT in the dark. Secondary antibodies used were: donkey anti-mouse Cy3 (1:1000; Jackson, West Grove, PA) and anti-rabbit Alexa Fluor 488 (1:1000; Invitrogen). After washing slides were counterstained with Dapi for 1 min, washed and mounted with Mowiol® (Merck, Darmstadt, Germany) containing 2.5 % Dabco® (Sigma-Aldrich). Stained OHCs were photographed with a Zeiss fluorescent microscope (Axiophot, Zeiss, West Germany).
Acknowledgement

We would like to thank Johanna Signer (Spiez Laboratory) for the excellent technical support.

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Langat virus was kindly provided by D. Růžek (Institute of Parasitology, and University of South Bohemia, Czech Republic)

Langat virus antibody was kindly provided by C. Schmaljohn (Fort Detrick, USAMRIID USA).
References

Table. 1. Antisense sequences and positions of siRNAs used for inhibition.

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LGTV-specific siRNA sequences inhibit production of viral RNA in HeLa cells. 19 siRNA sequences (Q1-Q6 and D1-D13) targeting genes within the whole open reading frame and the two UTRs of LGTV genome were analyzed for their antiviral potential on HeLa cells (A). After transfection with siRNA, cells were infected with Langat virus (MOI=10) and six days later virus replication was assessed by quantitative real-time RT-PCR (B). Results are shown as a percentage of virus inhibition compared to the control cells transfected with the non-coding siRNA. Data are presented as mean ± SD of three independent experiments. Fourteen (Q2, Q3, Q4, Q5, Q6, D3, D4, D5, D6, D8, D9 D11 D12 and D13) out of 19 were significantly reduced (p<0.05) compared to a theoretical mean of 100 expressed by cells treated with nonsense siRNA; measured by One sample t test.
Inhibitory effects of siRNA D3 on viral replication on HeLa cells over time. The replication profile for Langat virus was determined for the time period of 6 days. Indicated are the numbers of RNA copies (A) and the number of infectious particles (B) for cell cultures treated with nonsense siRNA or with specific siRNA D3.
Antiviral effect of siRNA D3 in relation to the infectious dose of Langat. HeLa cells were infected with Langat virus at different infectious dose (MOI of 10, 1, 0.1 or 0.01) and one hour after infection cells were treated with 200 nM siRNA sequence D3 or with nonsense siRNA. Virus titer was assessed by quantitative real-time RT-PCR 6 days after infection in the cell culture supernatant. The reduction of viral RNA in siRNA D3 treated cells is statistically significant compared to nonsense siRNA treated cells for all four MOI (p<0.05). The data are presented as mean ± SD of three independent experiments and significance calculated using an unpaired T-test.
Expression of Langat virus proteins in infected organotypic hippocampal cultures.

OHCs were infected with 2x10E6 FFU Langat virus for 7 days and immunostained with an anti-Langat virus antibody (LGTV; red) (A). Uninfected slices are shown as a control (B). Double staining of viral proteins (LGTV; red) (D 10x; G 40x) and neurons (FOX3; green) (E 10x; H 40x) on infected OHCs showed colocalisation (C 10x; F 40x). Cell nuclei were counterstained with Dapi (blue).
Antiviral effect of RNAi on rat organotypic hippocampal cultures. OHC were transfected with 800 nM specific siRNA D3 or with nonsense siRNA 24h before and 1 h after the infection with LGTV. OHC were incubated for 6 days and virus titer assessed. The number of genome copies (A) and the number of infectious particles (B) measured in OHCs pre- and posttreated with siRNA D3 or nonsense siRNA are indicated. Expression of viral proteins was analyzed by immune staining of Langat virus proteins (red) in microtome slices of OHCs treated with specific siRNA D3 (D) or with nonsense siRNA (C). Cell nuclei were stained with Dapi (blue). Figures C and D are representative images of six independent experiments.
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## Supplementary data

### Table S1. Comparative analysis of siRNA sequences with members of the Tick-borne encephalitis virus complex

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Results of the comparative analysis are shown as “number of identical nucleotides (nt)/total number of siRNA nt”.
Results Project 1

**Figure S1**

LDH release of HeLa cells as an index of cytotoxicity over time. Transfected HeLa cells (siRNA D3 or nonsense siRNA) infected with Langat virus compared to untreated cells infected with Langat virus and to untreated and uninfected cells used as negative control. Cytotoxicity results are expressed as % LDH release of total LDH determined from homogenized HeLa cells used as positive control.
**Figure S2**

**LDH cytotoxicity assay on transfected organotypic hippocampal cultures.** OHCs transfected twice with siRNA (D3 or nonsense), untransfected OHCs infected with Langat virus or untreated OHCs were tested for cytotoxicity by measuring LDH release in 100 µl of the medium surrounding the slices 48 h after the second transfection. No difference in cytotoxicity was measured between transfected and control groups. Cytotoxicity results are expressed as % LDH release of total LDH determined from homogenized OHCs used as positive control.
Expression level of IFN-β in transfected OHCs. Expression levels of IFN-β mRNA was assessed in OHCs treated with siRNA D3 or nonsense siRNA 48h after the second transfection and compared to IFN-β mRNA levels found in untreated OHCs and in OHCs activated with poly I:C. Data showed that transfection of OHCs did not result in upregulation of IFN-β mRNA expression over the level of untreated slices. The data are presented as the mean ± SD from three independent experiments.
4.2 A tick-borne encephalitis model in infant rats infected with Langat virus

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To be submitted.
Abstract

Tick-borne encephalitis virus (TBEV) and Langat virus (LGTV) are closely related viruses within the tick-borne encephalitis virus complex, a group of flaviviruses that cause neurological diseases in humans. TBEV is the causative agent of tick-borne encephalitis (TBE), a severe brain infection that can cause long lasting neurological sequelae. LGTV has an attenuated virulence for human hosts and was previously used in clinical trials as a live vaccine against TBEV. Even though natural infections of LGTV in humans have never been detected, it was shown to develop encephalitis in 1 of every 18'500 people who received the vaccine. The pathogenesis of TBEV and the mechanisms of brain injury underlying the neurofunctional sequelae of TBE are poorly understood and currently no effective therapy is available.

With the aim to study the pathogenicity of TBEV, we developed an infant rat model of tick-borne encephalitis using Langat virus.

Infant Wistar rats were inoculated intracisternally with Langat virus and assessed for clinical symptoms of disease, inflammatory response in the brain and histological findings.

Infection with LGTV led to meningoencephalitis including gait disturbances, hypokinesia and reduced weight gain or weight loss. In the cerebrospinal fluid (CSF) RANTES, IFN-γ, IL-6, IFN-β and MCP-1 were significantly increased in infected animals. Infected brains exhibited characteristic histopathological features of TBE including subarachnoid pleocytosis, perivascular cuffs and glial nodules. Immunohistochemistry documented the presence of LGTV in the thalamus, hippocampus, midbrain, frontal pole and Purkinje cells of the cerebellum.

This newly established rat model of tick-borne flavivirus-meningoencephalitis mimics important clinical and histopathological features of human TBE. The model provides a novel tool to investigate mechanisms of disease and to evaluate new therapeutic strategies against encephalitogenic flaviviruses.
Introduction

Tick-borne encephalitis virus (TBEV) is a neurotropic virus causing tick-borne encephalitis (TBE), a potentially fatal viral infection of the brain [6, 34]. TBEV is transmitted to humans through the skin after the bite of an infected tick. The virus progresses from the dendritic cells of the skin to draining lymph nodes where it replicates and is amplified. When viremia is established the virus gains access to the central nervous system by a still unknown mechanism [5, 6]. TBE usually follows a biphasic clinical course. The viremic febrile first stage, lasting up to one week and characterized by unspecific flu-like symptoms, is followed by a second neurological phase. Clinical features developed during TBE can be of different severity, ranging from mild meningitis to severe meningoencephalitis [31, 34]. Despite the availability of effective vaccines against TBEV, between 5'000 and 10'000 TBE clinical cases are reported annually across Europe and Russia with a fatality rate of 2% in Europe and up to 35% in East Europe [6, 30, 33]. No specific therapeutic interventions against TBE are available once the infection occurs.

TBEV is not the only flavivirus able to induce severe disease in humans. The genus flavivirus comprises ~ 70 viruses which are transmitted to humans by either mosquitoes or ticks [7, 12]. Tick-borne flaviviruses are primarily found in Europe and Asia and include Powassan virus, Kyasanur Forest disease virus, Omsk hemorrhagic fever virus, Louping ill virus, Langat virus (LGTV) and TBEV [8, 19, 23]. These viruses are genetically and antigenically closely related and are all members of the mammalian tick-borne flavivirus serogroup [1, 12]. They are transmitted by *Ixodes* and *Dermacentor* tick species and can cause mild febrile illness, biphasic fever, encephalitis or haemorrhagic fever in humans [7]. LGTV is the only non-pathogenic Tick-borne flavivirus for humans under natural conditions. For this reason and for its close antigenic affinity with TBEV, LGTV was tested in human trials as a potential vaccine for prevention of TBEV. Unfortunately, LGTV vaccination resulted in unexpectedly high incidence (~1:18'500) of neurologic disease causing permanent neurological sequelae among vaccines [19, 50, 51].
In the present study the close affinity between LGTV and TBEV was exploited for the development of an *in vivo* model of TBE in infant rats. In the natural environment, LGTV does not cause disease in rodents but it was shown to develop encephalitis when inoculated subcutaneously or intracerebrally in young laboratory mice [147, 148]. In adult mice (> 4 week-old) LGTV is generally apathogenic when inoculated subcutaneously or intraperitoneally [10, 52, 149].

The aim of this study was to evaluate the use of a low pathogenic tick-borne flavivirus as a model for the human pathogen TBEV and to compare the clinical parameters of the disease developed by LGTV with the encephalitis caused by TBEV in animal models and humans patients. For the first time a rat model, particularly adequate for the assessment of behavioral experiments and the evaluation of neuronal damages after central nervous system (CNS) viral infection, was used as host model organism for TBE [150]. After intracisternal injection of infant rats with LGTV we characterize the disease by describing clinical symptoms, inflammatory response, viral replication into the brain and histopathological finding. Regulation of inflammatory cyto- and chemokines response in infected animals was compared to CSF samples of TBEV human patients.
Material and Methods

Animals and virus. All animal studies were approved by the Animal Care and Experimentation Committee of the Canton Bern, Switzerland, and Swiss national regulation for animal protection. The animals used in the present study were suckling pups of Wistar strain (11 day old rats kept with the mother) supplied by Charles River Laboratories. Langat virus strain TP21 was kindly provided by Daniel Růžek (University of South Bohemia, České Budějovice, Czech Republic) and cultured in PC12 cells. The high concentrated virus stock was produced after seven days of infection by ultrafiltration with an Amicon ultra-15 centrifugal filter unit (Millipore, Zug, Switzerland) with a 100’000 molecular weight cutoff. Virus quantification was performed by immunoperoxidase focus assay.

Langat virus inoculation. Animals were infected by intracisternal injection of 25 µl virus suspension containing 10E6 focus forming units of Langat virus using a 32-gauge needle. PC12 cell medium supernatant centrifuged for 10 min at 1200 rpm was inoculated in control rats. The body weight was measured daily for 9 days. At day 2, 4, 7 and 9 p.i animals were sacrificed by an overdose of intraperitoneally administered pentobarbital (150 mg/ kg) and perfused with 30 ml of ice-cold phosphate buffer saline (PBS) via the left cardiac ventricle.

Viral RNA extraction. At d2, d4, d7 and d9 p.i viral load was measured by quantitative real-time RT-PCR from 2 µl cerebrospinal fluid (CSF) obtained by puncture of the cisterna magna, and from the sagittal right hemisphere of the perfused brain tissue. Nucleic acid (NA) extraction from previously centrifuged CSF was performed with the QIAsymphony SP/AS instrument (Qiagen), using the QIAsymphony Virus/Bacteria Midi kit (Qiagen) in accordance to the instruction provided by the manufacturer. 2 µl CSF were diluted in 48 µl AVE Buffer and supplemented with 3 µg RNA diluted in 150 AVL buffer. RNA was eluted in a final volume of 50 µl.

Viral RNA extraction from brain homogenates was performed using the BioRobot EZ1 Workstation (Qiagen). The sagittal half brain hemisphere, divided into cerebellum, middle brain and frontal pole, were homogenized and virus inactivated in 800 µl Qiazol (Qiagen) by
the TissueLyser System (Qiagen) for 5 min at 25 Hz. Extraction was performed with the Universal RNA Tissue Kit (Qiagen) following the manufacturer’s instructions. RNA was eluted in a final volume of 60 µl.

**Primers.** The specific primers and probes for the detection of Langat virus strain TP21 were designed using Primer Express software v3.0 (Applied Biosystems, Foster City, CA) and purchased from Microsynth (Balgach, Switzerland). Langat virus primer forward 5’-TGTGTGGAGCGGCGATT-3’; reverse 5’-TAAGGGCGCGTCCATCTC-3’; probe 5’-AGCCACGCTTCCAGGAGGACCC-3’. Detection of interferon β gene expression was performed using the corresponding pre-designed QuantiTect Primer assays (Qiagen).

**One step real-time reverse transcriptase PCR.** Viral RNA was quantified by one-step real-time RT-PCRs using the QuantiFast probe RT-PCR kit (Qiagen) according to manufacturers protocol with the following cycling conditions: reverse transcription at 50°C for 10 min, an initial PCR activation step at 95°C for 5 min, and 40 cycles of two-step cycling for 10 s at 95°C and 30 s at 60°C.

**Neurofunctional behavioral tests.** Balance, coordination and physical conditions of infected vs control animals were analyzed with a rotarod performance test at day 2, 4, 7 and 9 postinfection. Rats were placed on a rotating drum (Ugo Basile srl, Comerio, Italy), accelerating from 4 to 40 revolutions/min over the time course of 5 min. The latency to fall was measured in seconds. Each trial was repeated three times each day. Spontaneous locomotor activity of infected and control rats was measured with an open field test at day 2, 4, 7 and 9 p.i. Rats were individually placed in the middle of an arena (90 x 90 x 40 cm) (Hugo Sachs Elektronik - Harvard Apparatus, March-Hugstetten, Germany) for 2 minutes and the distance (cm) moved recorded with the video tracking system Ethovision® (Noldus Information Technology, Wageningen, Netherlands). Each trial was repeated three times.

**Measurement of cytokines/chemokines concentration.** Concentration of cyto- and chemokines in the CSF was measured by Bio-Plex 200 suspension array system (Bio-Rad, Hercules, CA, USA) using microsphere-based multiplex assays. The protein concentration of two cytokines (IL-6 and IFN-γ) and two chemokines (RANTES and MCP-1) was assessed in
25 µl of centrifuged CSF using the rat cytokine/chemokine immunoassay (RCYTO-80k, Millipore AG, Zug, Switzerland). Concentration of IFN-β into the CSF was measured by RT-PCR.

CSF samples of nineteen human patients with Tick-borne encephalitis were provided by the Bern university hospital and analyzed for the same cyto- and chemokines expression by Bio-Plex 200 using a Human cytokine/chemokine immunoassay kit (MPXHCYTO-60k, Millipore AG, Zug, Switzerland). The manufacturer’s protocol of each kit was followed.

**Histopathology.** After perfusion with ice cold PBS pH 7.4 brains were removed and the left hemisphere post-fixed for 7 days in methanol:acetic acid (95:5) solution at 4°C and processed for paraffin embedding. 10 µm sagittal sections were produced using a Leica microtome. After deparaffinization and re-hydration, slices were incubated with primary antibodies directed against Langat virus (LGT-I-13A10; 1:1000), Iba-1 (rabbit polyclonal; 1:400, Wako), PCP-2 (H60) (rabbit polyclonal; 1:50, Santa Cruz Biotechnology) and FOX3 (rabbit polyclonal; 1:1000, Millipore). Primary and secondary antibodies were diluted in TBS containing 0.5% bovine serum albumin. Sections were washed three times with PBS and incubated with their secondary antibody for 45 min at room temperature in the dark. Secondary antibodies used were: donkey anti-mouse Cy3 1:1000 (Jackson, West Grove, PA) and anti-rabbit Alexa Fluor 488 1:1000 (Invitrogen). After washing, slides were counterstained with DAPI for 1 min, washed and mounted with Mowiol® (Merck, Darmstadt, Germany) containing 2.5% Dabco® (Sigma-Aldrich). Digital pictures of stained sections were obtained with a Zeiss fluorescent microscope (Axiophot, Zeiss, West Germany).

**Statistical analysis.** Student T test two-tailed or nonparametric Mann Whitney Test were used for statistical analysis to assess the significant differences of weight changes, behavioral experiments and cyto-/chemokines expression in the CSF of infected versus control groups. All the results are presented as mean ± standard deviation and p-values <0.05 were considered statistically significant. All statistical analyses were performed using the statistical program GraphPad Prism (version 5.02).
Results

**Clinical observations.** 11-day-old suckling rats were infected intracisternally (i.c) with 10E6 focus forming units (FFU) of LGTV. Starting a day 4 p.i, animals infected with LGTV started to gain less, or lose weight compared to the controls (Figure 1). The significant decreasing in body weight corresponded with the first appearance of clinical symptoms such as gait disturbances, hypokinesia, ataxia and paresis. No mortality was observed. At day 7 p.i animals partially recovered and symptoms became less severe.

**Behavioral tests.** Neurological illness and physical impairments started 4 d.p.i. Neurobehavioral assessment was investigated using two different behavioral tests at day 2, 4, 7 and 9 p.i. Balance, coordination and physical conditions were measured with a rotarod performance test. Infected animals showed significantly reduced rotarod performance ability at days 4, 7 and 9 p.i compared to the controls (Figure 2). The general locomotor activity and the willingness to explore new environment was measured with an open field test. Even though the difference was not statistically significant, infected animals showed constant lower motorial activity compared to mock infected animals at each time point (data not shown).

**Virus titer in the brain and CSF.** We observed viral replication from d2 to d4 p.i in the tissue of the three investigated brain regions (cerebellum-middle brain-frontal pole). The highest viral RNA titer was observed at day 4 p.i in all the three regions with the highest peak of 10E10 viral RNA/g tissue in the cerebellum (Figure 3 A). At day 7 and 9 p.i we did not observe further viral replication in the brain tissue but a persisting high virus titer in all three regions was observed until 9 d.p.i. The cerebellum contained the highest amount of viral RNA during the nine days. In the CSF viral RNA constantly declined and was almost completely cleared at day 9 p.i (Figure 3 B).

**Cyto- and chemokines analyses.** To identify inflammatory mediators relevant during TBE we tested four different immunoregulatory factors for differential expression in the cerebrospinal fluid of mock- and LGTV-infected rats on days 2, 4, 7 and 9 after infection. The mean CSF level of the chemokines RANTES (also known as CCL-5) and the monocyte...
chemotactic protein-1 (MCP-1, also known as CCL-2) were significantly increased in the infected rats at day 2, 4, 7 and 9 p.i and at days 4 and 7, respectively. The expression of cytokines IFN-\(\gamma\), IL-6 and IFN-\(\beta\) were significantly upregulated on day 4 p.i. IFN-\(\gamma\) remains upregulated until d9 p.i, whereas the level of IL-6 and IFN-\(\beta\) expression is reduced at day 7 and 9 p.i (Figure 4). A group of 18 human patients with TBE were compared with a control group consisting of 9 patients, in whom infection with TBEV was excluded. The concentration of RANTES, IFN-\(\gamma\) and IL-6 was significantly increased in the CSF of TBE patients in comparison with controls. There was no significant difference in the MCP-1 concentration between the two groups (Figure 5).

**Virus localization and cellular tropism within the CNS.** Immunofluorescence staining was performed to localize viral infection and investigate cellular tropism of LGTV in brains of infected rats. The presence of LGTV antigens was confirmed in different regions of the brain such as cerebellum, midbrain, hippocampus, thalamus and frontal pole. Little reactivity was observed in the cerebral cortex (Figure 6 A). Interestingly, virus cell-type tropism was detected in the cerebellum. Cerebellar Purkinje cells, the largest neurons in the mammalian nervous system, appeared to be one of the primary targets for LGTV. Immunostaining analyses of the cerebellum showed a perfect colocalisation of LGTV-infected cells with PCP-2-expressing cerebellar Purkinje cells (Figure 6 C and D). In contrast, FOX-3-positive granule neurons within the cerebellum were largely unaffected by LGTV infection (Figure 6 B).

**Brain histopathology.** Microglia proliferation with formation of microglial nodules was observed in the cerebral cortex of infected rats by Nissl staining (Figure 7). We visualized a diffuse presence of rod-like reactive microglial cells within the entire sagittal half brain sections by immunostaining with Iba1 antibody specific for activated microglia. Prominent perivascular cuffs and leukocytes infiltration were present in infected brains but no important neuronal damages were observed.
Discussion.

The development of an animal model for the study of pathogenesis and infection mechanisms of TBEV is a prerequisite for the design of new therapeutic drugs specific for the treatment of TBE. Tick-borne encephalitis virus and Langat virus are genetically distinct but closely related flaviviruses members of the mammalian TBE serocomplex [25]. TBEV is an important emerging human pathogen and the most common cause of arboviral encephalitis in Europe for which no curative therapies are available to date [151]. LGTV has an attenuated virulence for human hosts although sharing more than 74% nucleotide identity with TBEV. In the present study we used Langat virus as an experimental in vivo model for TBEV infection.

During the neurological phase of TBE patients develop clinical symptoms such as ataxia, confusion and altered consciousness. In the brain TBEV infection causes rapid microglia/macrophage activation and a diffused strong inflammatory response with astrogliosis, formation of microglial nodules, perivascular lymphocytic cuffing and varying degrees of neuronal loss [28, 37, 151]. Pathological findings are predominantly observed in thalamus, cerebellum, brainstem, basal ganglia and nucleus caudatus [35-37].

Clinical features, histopathological findings and inflammatory responses observed in our rat model infected with LGTV are similar to those observed in humans with TBE. Starting at day 4 p.i animals developed ataxia, loss of balance, paralysis of one or more limbs and motoric difficulties. This was reflected by a severe inability to walk on a rotating beam. Histopathological markers for viral encephalitis such as perivascular lymphocytic infiltrates, reactive astrocytes and microglial nodules were diffusely observed in infected brains. We visualized a predominant infection of the cerebellum with a cellular virus tropism for cerebellar Purkinje cells. These cells play an important role in cerebellar function and are key elements in motor learning and motor coordination [152]. In human cases of TBE, labelling of TBEV antigens within the cerebellum was also consistently found in Purkinje cells confirming similarities of our Langat model with human TBE [28].
The comparison of the inflammatory response in the CSF of animals infected with Langat virus with human patients infected with TBEV showed similar expression patterns. In both cases we observed an up-regulation of the chemokine RANTES (CCL-5) and of the cytokines IL-6 and IFN-γ. In LGTV infected animals upregulation of RANTES, a chemokine that attracts lymphocytes, starts at day 2 p.i, increases strongly at day 4 p.i and remains upregulated at day 7 and 9 p.i. High expression of RANTES in response to flaviviviral infections has been observed in a wide range of cell cultures [153-155], in animal models [118, 156, 157] and in the CSF of human patients infected with TBEV or other encephalitogenic flaviviruses [158, 159]. This confirms the critical role of the chemokine RANTES in the pathogenesis of flaviviral infection in the CNS as a trigger for the accumulation of inflammatory cells at the site of the infection. IFN-γ, an important antiviral mediator playing a critical role during the acute stage of TBE, was highly expressed at day 4 p.i followed by a rapid decline during the next days [160, 161]. Similarly, the proinflammatory cytokine IL-6 was exclusively secreted at day 4 p.i. For the chemokine MCP-1 we also observed an up-regulation at day 4 and 7 p.i in infected animals and even though human CSF samples analyzed in this study do not show a similar expression patter, previous studies have confirmed the fundamental role of MCP-1 in the pathomechanism of inflammation in the CNS during TBE [157, 162]. To summarize, the profile of cyto- and chemokine expression within the CSF of LGTV-infected animals correlates with previous findings in TBEV-infected patients validating our animal model.

In the experimental model developed in this study we consistently observed the appearance of the first neurological disorders as well as the loss of weight and secretion of inflammatory mediators at day 4 p.i. At this time point the highest Langat virus titer was measured in the brain tissue. These results provide evidence of an acute phase of the disease starting at day 4 p.i decreasing rapidly in the following days.

In contrast to other animal models of TBE using the highly pathogenic Tick-borne encephalitis virus [118, 148, 163, 164], suckling rats inoculated intracisternally with 10E6 FFU of Langat virus do not succumb to the infection. Animals develop clinical symptoms
such as self limiting ataxia and loss of balance which are however less severe compared to those developed with TBEV. Nevertheless, after the acute phase animals remain persistently infected and behavioral impairments measured with the rotarod test persist until day 9 p.i. Compared to TBEV, Langat virus has the important advantage to be handled under biosafety level 2 (BSL-2) conditions, which is economically and technically more advantageous than BSL-3 facilities necessary for TBEV.

In conclusion, our model recapitulates important features of human TBE including clinical symptoms, inflammatory parameters and histopathological findings. Langat virus model offers the possibility to investigate selected aspects of the pathogenicity of tick-borne flaviviruses and is an interesting alternative for in vitro and in vivo assays for the study of TBE infection.

Acknowledgement

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Langat virus was kindly provided by D. Růžek (Institute of Parasitology, and University of South Bohemia, Czech Republic)

Langat virus antibody was kindly provided by C. Schmaljohn (Fort Detrick, USAMRIID USA).
References


Weight curves of infected versus control animals. At day 4 p.i infected animals start to show a reduced weight gain compared to mock-infected controls. Values are mean ± SD (d0 n=36; d2 n=36; d4 n=30; d7 n=18; d9 n=6 in each group). *P<0.05; ***P<0.005; ****P<0.0001 based on two-tailed unpaired T-test.
Rotarod performance test. At day 4 (n=24 in each group), day 7 (n=18 in each group) and 9 (n=6 in each group) p.i animals infected with LGTV showed a significantly reduced rotarod performance ability compared to the controls. No difference was observed at day 2 p.i (n=30 in each group). Values are mean ± SD. ****P<0.0001 based on two-tailed unpaired T-test.
Virus titer in the CSF and brain tissue. In the brain tissue (A), divided into the three regions cerebellum, middle brain and frontal pole, we observed a viral replication during the first 4 days p.i with a maximal peak at day 4 p.i. At day 7 and 9 p.i viral load in the brain is only partially reduced. The amount of viral RNA into the CSF declines over time (B).
Inflammatory mediators in rats following infection with 10E6 FFU of Langat virus. The levels of RANTES (A), MCP-1 (B), IL-6 (C) and 1 IFN-γ (E) in the CSF were measured by Luminex method, whereas expression of IFN-β (D) by quantitative real-time RT-PCR in infected and control animals at day 2 (n=23 in each group), 4 (n=16 in each group), 7 (n=12 in each group) and 9 (n=6 in each group) post infection. Asterisks show pairs that exhibit
significant differences using the two-tailed nonparametric Mann Whitney test or parametric unpaired t test (* P<0.05; ** P<0.01; ***P<0.001; **** P<0.0001).

Figure 5

Inflammatory mediators in CSF of human patients infected with TBEV. RANTES (A), IL-6 (C) and IFN-γ (D) were significantly upregulated in infected patients, whereas we did not observe a difference for the chemokine MCP-1 (B) between the two groups. Results are represented as mean ± SD of 18 patients infected with TBEV and 9 controls. ** P<0.01, ***P<0.001 based on two-tailed nonparametric Mann Whitney test.
Localization of LGTV antigens within the brain. (A) Sagittal half brain sections of infected rats at day 4 p.i stained with LGTV antibody and Dapi. Viral antigens (LGTV; red) are present in different brain regions including cerebellum (C), midbrain (Mb), hippocampus (Hc), thalamus (Th) and frontal pole (FP). (B) In the cerebellum Langat virus antibody (LGTV; red) were detected in the purkinje cell layer and no granular neurons (green; FOX3) were infected by the virus. (C-D) Cerebellar protein specific for purkinje cells (green; PCP-2) colocalize with Langat virus antigens (red, LGTV) within the cerebellum.
Microglial nodules in LGTV infected brains. Microglial nodules (shown with arrows) are histological hallmark for viral encephalitis and were detected within the cerebral cortex on infant rats infected with LGTV.
4.3 RNAi-based therapy against tick-borne encephalitis in vivo – Preliminary results

Introduction

Tick-borne encephalitis (TBE) is an emerging flaviviral disease of great importance for public health for which no specific treatments are available. RNA interference (RNAi) represents a promising approach for the development of effective specific antiviral drugs. The protective effect of RNAi against other important flaviviral infections such as West Nile virus, Japanese encephalitis virus and Yellow fever virus was previously demonstrated in vitro and in vivo [101, 122-126]. In a recent study, replication of Tick-borne encephalitis virus (TBEV), causative agent of TBE, was successfully inhibited in human embryonic kidney cells (HEK293T) by siRNA. However, there are still no publications having shown an RNAi-induced protective effect against TBEV in vivo [128].

After the selection in vitro of an effective siRNA sequence inhibiting Langat virus (LGTV) replication, used herein as a virus model for TBEV, and the development of a TBE model in rats, we began to evaluate RNAi in vivo. In this preliminary study two delivery strategies were chosen, injection of naked siRNA and lipid formulations. The molecules were applied intracisternally before and after infection with LGTV. The antiviral effect of siRNA was evaluated according to viral titer in the brain and cerebrospinal fluid (CSF) and to secretion of inflammatory mediators in the CSF.

So far, three experiments were performed and no inhibitory effects were observed. New delivery strategies need to be evaluated.
Material and methods

Animals and virus. See Material and Methods in chapter 4.2

Virus inoculation and siRNA application. Animals were infected by intracisternal injection of 25 µl virus suspension containing 10E6 focus forming units of Langat virus using a 32-gauge needle. 25 µl siRNA (20µM) sequence D3 were applied 1 h before infection, 1 day, 2 days and 3 days after infection by intracisternal injection. As a control an unspecific siRNA was applied (siGENOME non-targeting siRNA #1, Dharmacon). Application of siRNA was performed using different strategies: naked siRNA without formulation; complexed with the lipid-based transfection reagent Dharmfect 2 (Thermo Fisher Scientific, Lafayette, USA); complexed with the mixture of cationic lipids JetSI/DOPE (Polyplus transfection, New York, USA). For lipid formulations the manufacturer’s instructions were followed. Cisternal CSF samples were taken by puncture of the cisternal membrane at day 2 and 4 p.i.

At day 4 p.i animals were sacrificed by an overdose of intraperitoneally administered pentobarbital (150 mg/ kg) and perfused with 30 ml of ice-cold phosphate buffer saline (PBS) via the left cardiac ventricle.

Viral replication was documented by quantitative real-time RT-PCR from 2 µl cerebrospinal fluid (CSF) at days 2 and 4 p.i and from the perfused right hemisphere of the brain at day 4 p.i.

Viral RNA extraction. See Material and Methods in chapter 4.2

Primers. See Material and Methods in chapter 4.2

One step real-time reverse transcriptase PCR. See Material and Methods in chapter 4.2

Measurement of cytokines/chemokines concentration. CSF cyto- and chemokines concentration was measured by Bio-Plex 200 suspension array system (Bio-Rad, Hercules, CA, USA) using microsphere-based multiplex assays. Concentration of two cytokines IL-6 and IFN-γ and chemokine RANTES was assessed in 25 µl centrifuged CSF using the rat cytokine/chemokine immunoassay (RCYTO-80k, Millipore AG, Zug, Switzerland).
Results

**In vivo application of naked siRNA.** Quantitative analysis by RT-PCR of viral RNA in the CSF at day 2 and 4 post infection did not show a significant difference between animals injected with siRNA sequence D3 and the unspecific sequence (Figure 1).

Secretion of the chemokine RANTES was significantly down-regulated at day 4 post infection in siRNA D3-treated animals. For cytokines IFN-γ and IL-6 no expression differences were observed between treated and control groups at both time points 2 and 4 dpi (Figure 2).

**In vivo application of siRNA complexed with lipid-based transfection reagents.** When siRNA was formulated with Dharmafect 2 or with JetSI/DOPE no significant reduction in the viral load in both CSF and brain tissue was measured in siRNA D3-treated animals. No difference in the inflammatory response mediated by RANTES, IFN-γ and IL-6 in the CSF was measured between the two groups.
Figure legends

Figure 1

Langat virus RNA in the CSF and brain tissue of animals treated with naked siRNA. Viral RNA measured by RT-PCR in the CSF (A) and brain tissue (B) of rats injected with unformulated siRNA sequence D3 versus nonsense. Results are shown as mean ± SD of 8 animals. Statistic was performed using an unpaired T-test.
Cyto- and chemokines expression in the CSF of animals treated with naked siRNA. RANTES, IFN-γ and IL-6 secretion in the CSF of animals treated with siRNA D3 and controls was measured by Luminex. A significant reduction in the expression of RANTES in animals treated with siRNA sequence D3 was observed at day 4 p.i. (*P<0.05). No differences in the expression level of IL-6 and IFN-γ were detected at day 2 and 4 post-infection. Statistic was performed with a Mann Withney U test (n=8).
Discussion

One major limitation in the development of RNAi-based therapies is the poor uptake of siRNA in most mammalian cells in vivo. Moreover, naked siRNAs are rapidly degraded by endo- and exonucleases in vivo, which restricts their expected effects to a short period. Nevertheless, delivery of non-complexed, naked siRNA by direct injection into the CNS is one possible strategy for siRNA delivery to brain cells. Even though some reports have described a poor uptake of naked siRNA with inefficient gene silencing in the brain or neuronal cells [165, 166], other studies were successful in the application of non-complexed siRNA into the CNS. Dorn and colleagues showed an siRNA-mediated silencing of the pain-related cation channel P2X3 resulting in a reduced pain response after intrathecal injection of naked siRNA [167]. Another group was able to reduce the expression of the metabolism-related protein Foxo1 by 50% after direct injection of siRNA into the hypothalamus [168]. In contrast to these successful applications of naked siRNA for the knockdown of endogenous disease-related genes, inhibition of neurotropic viruses after delivery of naked siRNA to the CNS have not been demonstrated so far.

The data obtained in this preliminary in vivo study using naked siRNA showed no difference in the viral titer in the CNS between siRNA treated and control groups. Nevertheless, we observed a significantly lower expression of the chemokine RANTES at day 4 post infection in animals treated with siRNA D3. This suggests that even though siRNA D3 did not significantly reduce the viral titer in the brain, it somehow still influenced the inflammatory response caused by the virus. The exact mechanism responsible for this effect should be further investigated.

To improve the inhibition of viral replication by siRNA, we tested two lipid-based transfection reagents for a more efficient delivery to brain cells. Dharmafect 2 was successfully applied for the in vitro transfection of OHC. JetSi, a mixture of cationic lipids with the fusogenic lipid dioleoylphosphatidylethanolamine (DOPE), is one of the few commercially available lipid-based transfection reagents previously shown to successfully deliver siRNA into the brain.
Unfortunately, in our experimental setting, siRNA D3 combined to the two reagents showed neither an inhibitory effect on LGTV replication nor a reduced inflammation in the CSF.

Infections of the CNS by Yellow fever virus, Japanese encephalitis virus and West Nile virus were successfully inhibited by RNAi in vivo in previous studies [104, 124, 127, 171]. To our knowledge, these are the only publications showing a protective effect of siRNA in vivo against encephalitogenic viruses. Delivery strategies chosen by these groups were injection of plasmids or lentiviral vectors for the endogenous expression of shRNA, lipid-mediated transfections (JetSI/DOPE) or peptide/siRNA complexes. These encouraging data provide important clues proving that RNAi-based strategies for treating acute and deadly viral infections may be feasible. However, the little number of published studies showing a siRNA-mediated antiviral effect in the brain also emphasizes the numerous challenges still linked to this research field.

Although we were not able to significantly reduce viral replication in vivo so far, the potent viral inhibitory effects of siRNA sequence D3 obtained on hippocampal cultures (more than a thousand fold reduction, see Results Project 2) remain encouraging. New delivery and application strategies such as shRNA and other lipid- and protein-based transfection reagents will have to be considered.
5. Discussion and Outlook

Tick-borne encephalitis is an important and severe neurological illness affecting humans in large areas of Europe and Asia [34]. Despite the availability of a vaccine, the number of human cases of TBE in Europe has increased by almost 400% in the last 30 years. No specific antiviral therapy for the treatment of infections with TBEV and in general with flaviviruses is available [24]. RNAi technology using small interfering RNA molecules was considered as antiviral approach against TBEV.

In this project we have investigated the potential of siRNA as specific antiviral therapy against TBEV in vitro and in vivo.

In the first part of this work we identified several siRNA sequences able to induce a strong antiviral effect against Langat virus, a flavivirus strain closely related to Tick-borne encephalitis virus and to other human pathogens such as Omsk hemorrhagic fever virus, Louping ill virus and Kyasanur forest disease virus. The inhibitory effect was demonstrated in immortalized human HeLa cell line and organotypic hippocampal brain cultures, an interesting model system here successfully applied for the first time in an RNAi-based antiviral study. Using the most potent siRNA on HeLa cells infection (identified as D3), we were able to induce a siRNA-based virus inhibition of 90% compared to slices treated with a non-specific siRNA sequence in organotypic brain cultures. This is to our knowledge the most efficient and strong antiviral effect of siRNA on Flavivirus replication published so far. Although, in the present study, the antiviral effect was demonstrated only against the attenuated Langat virus strain, siRNA sequence D3 is also likely to be effective in treating other tick-borne flaviviruses. Indeed, the selected target sequence D3, located within the 5’ UTR, is highly conserved not only between all three TBEV subtypes but also between different members of the Tick-borne serogroup. The ability of siRNA sequence D3 to attenuate encephalitis caused by several related viruses within the TBE serogroup will be the subject of further investigations.
In the second part of this project we were able to establish an *in vivo* model of Tick-borne encephalitis using the low-pathogenic Langat virus. After intracisternal inoculation of infant rats, animals developed clinical symptoms, laboratory parameters and histopathological features similar to those observed in human patients infected with TBEV. This newly established *in vivo* model represents a tool for the study of neuropathogenesis of neurotropic flaviviruses and for the development of new therapeutic interventions against TBEV or related viruses. Langat virus resulted to be an adequate virus model for the highly pathogenic TBEV with the important advantage to be handled under biosafety level 2 conditions. However, LGTV probably will not display the full spectrum of features observable for TBEV infection, being less virulent.

In the third part of the project preliminary experiments were performed for the *in vivo* application of siRNA using knowledge gathered from the two first parts, i.e. the established TBE rat model and the antiviral siRNA sequence D3, the most potent candidate identified in the *in vitro* study. Injection of naked siRNA or siRNA complexed with lipid-based transfection reagents were the two strategies chosen for these first experiments. Both strategies were previously used for *in vivo* delivery of siRNA molecules to the brain [168]. Unfortunately, using these experimental settings, our preliminary data did not show a reduction of viral replication after siRNA application to the brain. Possible explanations for these first negative results can be an inefficient delivery to the target cells due to a poor uptake of the siRNA by the neuronal cells or a rapid degradation of the RNA molecules after injection in the CNS. Novel delivery strategies with improved cellular uptake need to be further analyzed. Self-produced liposomes composed of different chemical compositions combined with selected surface peptides will be evaluated for their ability to deliver siRNA to target cells. Fluorescent labeled siRNA can be used to trace the fate of the delivered siRNA in the cells increasing the knowledge about the delivery efficacy. The developed *in vitro* model based on organotypic hippocampal cultures will be a helpful tool for the preliminary evaluation of delivery efficacy of the new formulations.
Antiviral applications of RNAi have been extensively exploited in several studies and a wide range of siRNA molecules have been discovered to be efficient against many different important human pathogens, such as HIV [172], Hepatitis C [173], Herpes simplex virus [174], West Nile virus [123], Japanese encephalitis [127], Chikungunya virus [175], Dengue virus [125], between others. The intense interest in the development of this powerful approach is driven by the urgent needs of new antiviral therapeutics. Indeed, important human diseases are still not covered by effective vaccines and efficient therapeutic antiviral treatments are limited. The majority of the published data about antiviral effects of siRNA refer to *in vitro* experiments performed with a wide range of different mammalian cell types. While *in vitro* studies provide a broad range of very promising siRNA molecules, successful application of RNAi-based antiviral drugs in animal models are severely limited. The large discrepancy between the number of *in vitro* and *in vivo* data lets assume that the specific delivery of siRNA molecules to the intracellular site of action is still a largely unsolved issue which needs intensive research.

In conclusion, our study provides further support on the use of siRNA for the development of antiviral therapy against encephalitogenic viruses. We successfully developed an *in vitro* model based on organotypic brain cultures for the use of siRNA as antiviral molecule against a tick-borne flavivirus. A highly conserved siRNA molecule with a potent antiviral activity was identified in OHCs. Moreover, we developed a novel *in vivo* model for tick-borne encephalitis using for the first time an attenuated strain member of the Tick-borne flavivirus complex.
6. References


References


References


7. Acknowledgements

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8. Appendix

8.1 Declaration of Originality

Last name, first name: Maffioli Carola

Matriculation number: 03-719-606

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.

All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.

I am aware that in case of non-compliance, the Senate is entitled to divest me of the doctorate degree awarded to me on the basis of the present thesis, in accordance with the “Statut der Universität Bern (Universitätsstatut; UniSt)”, Art. 20, of 17 December 1997.

Place, date Signature

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