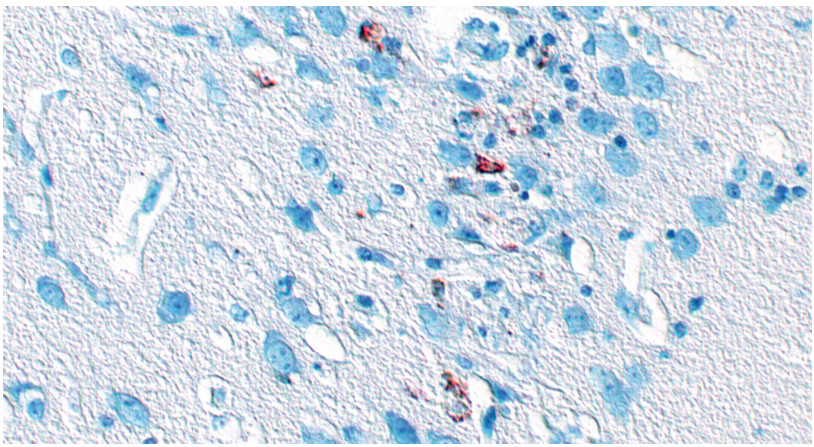
JULIA SEHL

Characterization of a Pseudorabies virus mutant lacking the pUS3 kinase and the tegument protein pUL21 *in vitro* and *in vivo* and the evaluation of an improved animal model for human Herpes Simplex Encephalitis



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Dr. med. vet.

beim Fachbereich Veterinärmedizin

der Justus-Liebig-Universität Gießen

eingereicht von

Julia Sehl

Tierärztin aus Kyritz

Gießen 2020

Mit Genehmigung des Fachbereichs Veterinärmedizin

der Justus-Liebig-Universität Gießen

Dekan: Prof. Dr. Dr. h.c. Martin Kramer

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2. Gutachter: Prof. Dr. Dr. h.c. Thomas C. Mettenleiter

Tag der Disputation: 25. August 2020

To my family and Stefan

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Abbreviations

aa	Amino acid
ATP	Adenosine triphosphate
Bad	Bcl-2 agonist of cell death
Bcl-2	B-cell lymphoma 2
BoHV-1	Bovid alpha Herpes Virus 1
Bid	BH3 interacting-domain death agonist
Bim	Bcl-2-like protein 11
CCL	CC-chemokine ligand
CD	Cluster of differentiation
CNS	Central nervous system
CXCL	C-X-C chemokine motif ligand
DRG	Dorsal root ganglion
FeHV-1	Felid alpha Herpes Virus 1
HHV	Human Herpes Virus
HSE	Herpes simplex encephalitis
HSV-1	Herpes Simplex Virus 1
IFN	Interferon
IL	Interleukin
INM	Inner nuclear membrane
IRF	Interferon regulatory factor
LAT	Latency associated transcript
MDBK	Madin-Darby bovine kidney
Me5	Mesencephalic trigeminal nucleus
MHC	Major histocompatibility complex
MTD	Mean time to death
NEC	Nuclear egress complex
NS	Nervous system
ONM	Outer nuclear membrane
ORF	Open reading frame
PK	Porcine kidney
PNS	Peripheral nervous system
PNSp	Perinuclear space

Pr5	Principal sensory nucleus
PrV	Pseudorabies Virus
RK	Rabbit kidney
SCG	Superior cervical ganglion
Sp5	Spinal trigeminal nucleus
SPEV	Embryonic porcine kidney cell
SuHV-1	Suid alpha Herpes Virus 1
TBK_1	TANK-binding kinase 1
$TCID_{50}$	Tissue culture infectious dose 50
TG	Trigeminal ganglion
TLR	Toll like receptor
TNF	Tumor necrosis factor
$TRAF_3$	Tumor necrosis factor receptor-associated factor 3
TRIF	Toll/IL-R domain-containing adaptor inducing IFN-β
U_L	Unique long
$UNC_{93}B1$	Unc-93 homolog B1
U_S	Unique short
VPM	Ventral posteromedial nucleus
VZV	Varicella Zoster Virus

1 Initial remarks and objectives

Alphaherpesviruses such as Herpes Simplex Virus 1 (HSV-1), Varicella Zoster Virus (VZV) or Pseudorabies virus (PrV) are neurotropic infectious agents able to cause severe diseases in humans or animals and establish life-long latency in their host (Mettenleiter *et al.* 2019). PrV is a threat to many mammalian species, except for humans, non-human primates, horses and birds. While swine as the natural host can survive an infection, the disease is invariably fatal in dogs, cats, cattle, sheep and rodents. There, PrV infection is typically characterized by intense pruritus, which eventually leads to auto-mutilation and severe general illness. PrV exhibits a strong neuroinvasive phenotype, but is also able to infect a multitude of different cells *in vitro* and *in vivo* (Smith 2012). After initial replication in epithelial cells, PrV enters sensory as well as vegetative neurons, which transport the virus to peripheral ganglia and eventually to the CNS (Babic *et al.* 1994, Klopfleisch *et al.* 2006). In young pigs and non-porcine animals a fulminant meningoencephalitis develops (McFerran and Dow 1965, Crandell *et al.* 1982, Henderson *et al.* 1995, Brittle *et al.* 2004, Zhang *et al.* 2015). Due to its pronounced neurotropism PrV has been extensively and successfully used as neuroanatomical tracer, particularly in rodents (Card and Enquist 2014). However, the precise functions of the approx. 70 different PrV proteins during infection and especially for neuroinvasion are not fully understood.

The functional role of many different herpesviral proteins has been investigated *in vitro* and *in vivo*, which enabled deeper insights into herpesvirus biology and increased the knowledge towards pathogenicity. In the past, the function of different PrV proteins in neuroinvasion has been clarified using a murine intranasal infection model (Klopfleisch *et al.* 2004, Klopfleisch *et al.* 2006, Maresch 2011). The role of viral proteins was characterized by determining the mean time to death (MTD) of animals after infection with the corresponding gene deletion mutants. In these experiments one PrV mutant stood out of others, which was deleted in two viral genes, UL21 coding for a capsid-associated tegument protein and US3, which encodes the alphaherpesvirus specific protein kinase. Infection with this mutant resulted in significantly prolonged survival times in mice despite productive infection (Maresch 2011). However, the molecular and pathobiological basis for this remained unclear.

Different approaches have been pursued to elucidate the precise roles of pUL21 and the pUS3 kinase in the viral replication cycle. While not much is known on the function

of pUL21, pUS3 has been reported to be involved in many pathways with numerous viral and cellular substrates (Deruelle and Favoreel 2011, Kato and Kawaguchi 2018). However, to date the functional role of the two pUS3 isoforms expressed by PrV and HSV, and their impact on viral replication, particularly in nuclear egress, is not known.

HSV-1 is a human pathogen and up to 90% of the global population is infected whereas natural infection of animals occurs only rarely (Smith and Robinson 2002, Muller *et al.* 2009, Sekulin *et al.* 2010). HSV-1 is mostly associated with mild to moderate mucocutaneous lesions such as *Herpes labialis* or *Herpes keratitis*, but occasionally an infection can lead to severe meningoencephalitis, also referred to as Herpes Simplex Encephalitis (HSE) (Steiner and Benninger 2018). Necrotizing lesions of HSE are primarily present in the frontal and mesiotemporal lobe, but neither the way of viral invasion nor the reason for this relatively restricted neurotropism are known. Despite treatment, the mortality rate of this life-threatening infection can be high or patients suffer from severe long-term sequelae urging the need for in-depth research. Several animal models for HSE have been established. Particularly in mice, HSV-1 establishes productive infection in the brain, but the animals fail to mirror the human disease properly or they die acutely (Kollias *et al.* 2015, Mancini and Vidal 2018).

VZV or human alphaherpesvirus 3 (HHV-3) is the pathogenic agent of Varicella or Chickenpox in young children (Grahn and Studahl 2015). In adults, VZV causes Herpes Zoster, which occurs after reactivation of latent virus from trigeminal or dorsal root ganglia (DRG) and subsequent infection of peripheral nerves innervating associated dermatomes (Richter *et al.* 2009). Intense itching and pain are mostly reported from patients. In rare cases, VZV is also able to induce severe neurological disorders and is considered as one of the most important pathogens causing encephalitis, meningitis, radiculitis or myelitis (Nagel and Bubak 2018).

In the present thesis, the overall objective was to clarify the molecular, the immunological and pathobiological basis for the survival of mice infected with PrV mutants simultaneously lacking the pUS3 kinase and pUL21. A previously generated mutant, PrV-ΔUL21/US3, devoid of both proteins, productively replicated in the mouse, but infected animals showed an extremely prolonged MTD or were even able to survive (Maresch, 2011). To analyze this phenomenon in more detail, new virus mutants were generated, which were only defective in the pUS3 kinase function (PrV-US3Δkin) in

absence or presence of the UL21 deletion (PrV-ΔUL21/US3Δkin). The mutants were analyzed in cell culture and in mice.

The function of the pUS3 kinase activity and the role of the different pUS3 isoforms in the PrV replication cycle was initially characterized in different cells in culture (**paper I**). Further, PrV-US3Δkin and the corresponding double mutant PrV-ΔUL21/US3Δkin were tested in the standardized mouse model. The MTD was found to be comparable to mutants lacking the US3 open reading frame completely indicating that the kinase function is the major determinant. To unravel the basis for the exceptional ability of mice to survive an infection, the development and kinetics of clinical signs, viral spread and inflammatory response to infection with the different PrV mutants were monitored in close intervals (**paper II**).

In summary, the data generated for this thesis uncovered that mice infected with PrV-ΔUL21/US3Δkin present an improved animal model for HSE and furthermore mimic certain aspects of VZV infection. This animal model for human disease may help to better understand and to control the severe sequelae of alphaherpesviral brain infection in man.

2 Introduction

2.1 The herpesvirus family

The order *Herpesvirales* consists of three families: *Herpesviridae, Alloherpesviridae* and *Malacoherpesviridae* (Davison *et al.* 2009). Eight different species of herpesviruses are known to infect humans whereas more than 170 other species have been associated with disease in mammalians, reptiles, amphibians and fish (Pellett and Roizman 2007). The *Herpesviridae* include mammalian, bird and reptile viruses whereas the *Alloherpesviridae* contain fish and amphibian viruses. Two viruses infecting mollusks are included in the *Malacoherpesviridae* (Davison *et al.* 2009, https://talk.ictvonline.org/taxonomy/). Herpesviruses are complex viruses consisting of a linear double-stranded DNA genome of between 125 to 290 kilo base pairs (kbp) enclosed in a *T=16* icosahedral capsid which is surrounded by a protein-containing tegument. The tegument is divided into an inner, capsid-associated layer and an outer layer proximal to the envelope (Mettenleiter *et al.* 2009). The host-membrane derived lipid bilayer, called envelope, carries virus-encoded mostly glycosylated proteins (Fig. 1) (Pilling *et al.* 1999, Mocarski 2007, Davison *et al.* 2009).

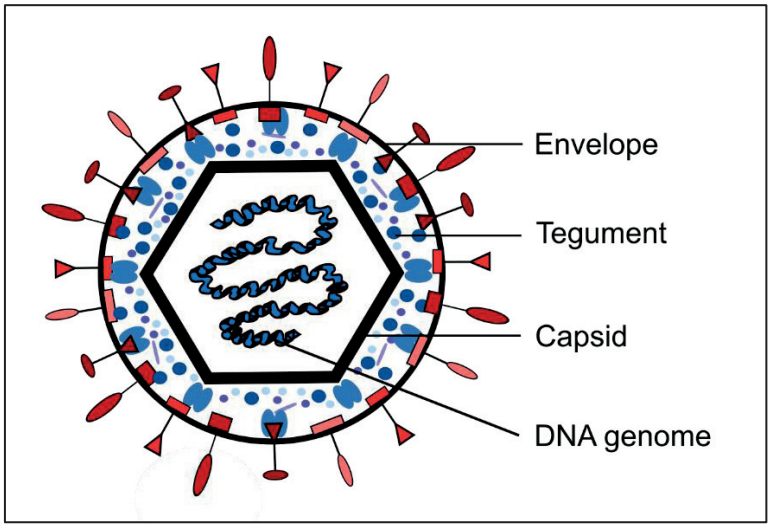

Fig. 1 Structure of a herpes virion. Herpesvirus particles comprise a double-stranded DNA genome, a capsid, a proteinaceous tegument and a viral glycoprotein-coated envelope. Adapted from Pomeranz *et al.* 2005.

Based on the genome, length of the replication cycle and cell type associated with latency, mammalian herpesviruses are subdivided into three subfamilies *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* (Pellett and

Roizman 2007). Alphaherpesviruses establish life-long latency in sensory neurons of their infected hosts. Restricted viral gene expression, often reduced to a single transcript, called latency associated transcript (LAT), and several microRNAs are detectable during latency (Mettenleiter *et al.* 2019).

Alphaherpesviruses infect a broad host range and include different human and animal pathogenic viruses which are subclassified into five genera: *Simplexvirus, Varicellovirus, Iltovirus, Mardivirus* and *Scutavirus* (Mettenleiter *et al.* 2019). Human alphaherpesviruses belonging to the genera *Simplexvirus* or *Varicellovirus* are the HSV-1 (Human Herpes Virus 1, HHV-1), HSV-2 (HHV-2) and VZV (HHV-3), respectively (Fig. 2). Animal viruses assigned to the genus *Varicellovirus* encompass the Equid alphaherpesvirus 1 (EHV-1), Bovine alphaherpesvirus 1 (BoHV-1), Felid alphaherpesvirus 1 (FeHV-1) and Suid alphaherpesvirus 1 (SuHV-1), the latter usually referred to as Pseudorabies virus (PrV) or Aujeszky's disease virus (Mettenleiter *et al.* 2019) (Fig. 2).

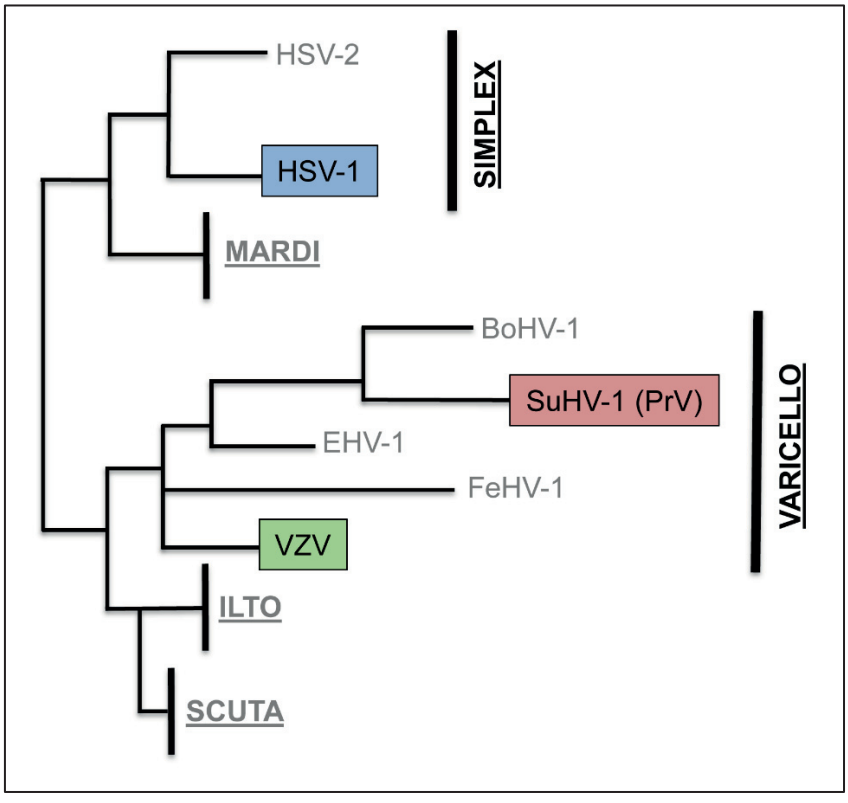

Fig. 2 Simplified phylogenetic tree of alphaherpesviruses. The tree was constructed by comparing the amino acid sequences of the glycoprotein K. VZV (green) and SuHV-1 (red) belong to the genus *Varicellovirus* whereas HSV-1 (blue) is part of the genus *Simplexvirus*. Adapted from Rider *et al.* 2019.

The Gallid alphaherpesvirus 1 as the pathogenic agent of infectious laryngotracheitis in chickens belongs to the genus *Iltovirus*. Several other avian herpesviruses such as the Anatid alphaherpesviurs 1 or Columbid alphaherpesvirus 1 belong to the genus *Mardivirus*. The genus *Scutavirus* includes the Chelonid alphaherpesvirus 5 and Testudinid alphaherpesvirus 3 of turtles (https://talk.ictvonline.org/taxonomy/).

Herpesviral replication cycle

Herpesviral infection is initiated after viral glycoprotein-mediated attachment to cell surface receptors such as nectin-1, which is followed by membrane fusion and entry of the capsid and tegument into the host cell (Struyf *et al.* 2002, Krummenacher *et al.* 2004) (Fig. 3). After release of most tegument proteins intracytoplasmic capsids with the inner layer of the tegument are transported to the nucleus along microtubules (Sodeik *et al.* 1997). At nuclear pores, the viral genome is released followed by transcription of immediate-early, early and late viral genes and replication of viral DNA (Knipe 1989, Schang *et al.* 1999, Shahin *et al.* 2006). Subsequently, nuclear egress takes place by which newly assembled nucleocapsids are released from the nucleus by budding at the inner nuclear membrane (INM). The primary envelope of virions located in the perinuclear space (PNSp) then fuses with the outer nuclear membrane and capsids are released into the cytoplasm (Mettenleiter 2002, Roller and Baines 2017). After partial tegumentation in the cytosol and by budding into trans-Golgi derived vesicles the capsids receive their final envelope and are transported within vesicles to the plasma membrane. From here, they are released into the extracellular space after fusion of the vesicle membrane with the plasma membrane (Turcotte *et al.* 2005, Mettenleiter *et al.* 2009, Hogue *et al.* 2014, Wild *et al.* 2017). Then, herpesviruses can infect additional cells, as e.g. neuronal cells of the peripheral nervous system (PNS) (Miranda-Saksena *et al.* 2018).

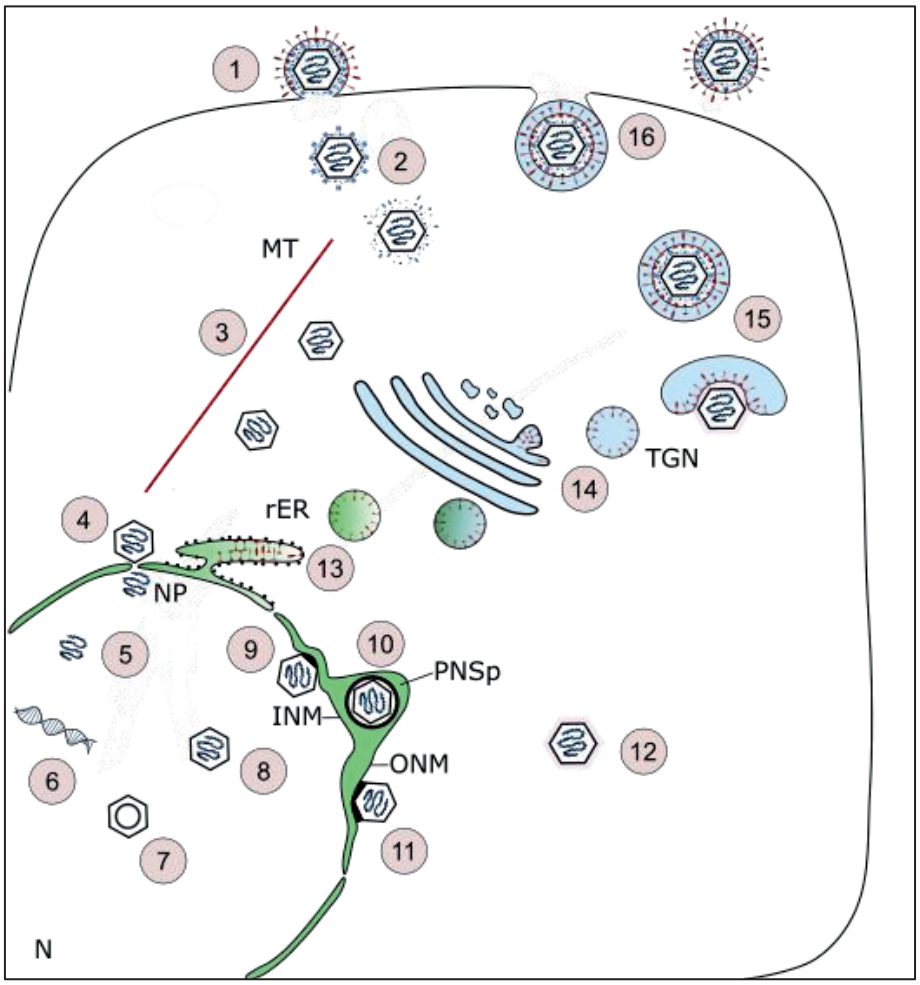

Fig. 3 Herpesviral replication cycle. (1) Attachment, membrane fusion and penetration of the herpesvirus and (2) capsid release into the host cell cytoplasm. (3) Nucleocapsids are transported towards the nucleus (N) via microtubules (MT). After capsid docking at the nuclear pores (NP) (4) the viral genome is released into the N (5). Following viral DNA transcription and replication (6), the viral genome is incorporated into newly assembled capsids (7). Mature capsids (8) then leave the nucleus by budding through the inner nuclear membrane (INM) (9). Primary enveloped virions, which are located in the perinuclear space (PNSp) (10) subsequently leave the PNSp after fusion of the primary envelope with the outer nuclear membrane (ONM) (11) and nucleocapsids are released into the cytoplasm (12). Glycoproteins are synthesized at the rough endoplasmic reticulum (rER) (13) and mature during transit into the trans-Golgi network (TGN). Cytoplasmic nucleocapsids acquire tegument proteins and their final envelope by budding into vesicles of the TGN containing the processed glycoproteins (14). Mature virions in these vesicles (15) are transported to the plasma membrane and released from the cell (16). Adapted from Kramer and Enquist, 2013.

Tissue tropism of alphaherpesviruses

Alphaherpesviruses are pantropic which means they are able to infect various tissues and cell types. At the same time, they are highly neuroinvasive although the

neurotropic properties differ significantly (Mettenleiter 2003, Steiner *et al.* 2007, Smith 2012). In cattle, BoHV-1 causes infectious bovine rhinotracheitis, abortion, and genital infections referred to as infectious pustular vulvovaginitis and balanoposthitis since mucous membranes of the respiratory and genital tract are primarily infected (Muylkens *et al.* 2007). EHV-1 infection in horses is primarily associated with abortion and rhinopneumonitis, but occasionally leads to severe myeloencephalopathy (Pusterla and Hussey 2014). In contrast, after initial replication in epithelial cells PrV directly infects the nervous system, primarily in piglets and non-porcine species while in adult pigs mainly respiratory symptoms occur (Mettenleiter *et al.* 2019). A similar variation is evident for the human alphaherpesviruses HSV-1, HSV-2 and VZV. Dermal, orolabial and genital lesions occur frequently, but infection can also lead to severe CNS disease such as meningoencephalitis (Gupta *et al.* 2007, Steiner and Benninger 2018). In general, neurotropic viruses are capable to access the central nervous system (CNS) and establish productive infection in the brain and spinal cord leading to neurological disorders (Koyuncu *et al.* 2013, Luethy *et al.* 2016).

The ability of a virus to enter cells of the nervous system (NS) is specified as neuroinvasiveness (Card 2001), while neurovirulence describes the capacity to cause disease within the NS (Card and Enquist 1995). A prerequisite of neuroinvasiveness is that virus particles enter and spread within neuronal cells (Salinas *et al.* 2010, Taylor and Enquist 2015). Viruses need modifying or adaptor proteins, which facilitate their directed transport in neurons, and they have to be capable to leave and to spread to neighboring neuronal cells (Taylor and Enquist 2015). This directed neuronal spread of PrV and HSV-1 has been widely used for neuroanatomical retrograde and anterograde tracing, respectively, to map neural circuits (Kristensson *et al.* 1974, Ugolini and Kuypers 1986, Strack and Loewy 1990, Mettenleiter 2003, Ekstrand *et al.* 2008, Lanciego and Wouterlood 2020).

2.2 The herpesviral genome

The PrV and HSV-1 genomes consist of a unique long (U_L) and a unique short region (U_S) (Fig. 4). Whereas in PrV only the U_S region is flanked by inverted repeat sequence, both segments are bounded by inverted repeats in HSV-1 (Ben-Porat *et al.* 1983). This results in four different isomers of the HSV-1 genome, but only in two in PrV. Historically, the PrV genome has been characterized using BamHI restriction fragment

length polymorphism (Kaplan 1973), but nowadays complete genome sequences are available (Klupp *et al.* 2004, Szpara *et al.* 2014). The PrV genome with a length of approx. 143 kbp encompasses 72 open reading frames (ORF) encoding 70 different proteins displaying significant homologies to HSV-1 (Klupp *et al.* 2004, Pomeranz *et al.* 2017). The PrV genome encodes 11 different glycoproteins (Klupp *et al.* 2004). So far, more than 15 different tegument proteins and 6 capsid proteins have been identified (Mettenleiter 2002, Klupp *et al.* 2004). Others are components of the envelope, secreted or non-structural proteins (Klupp *et al.* 2004).

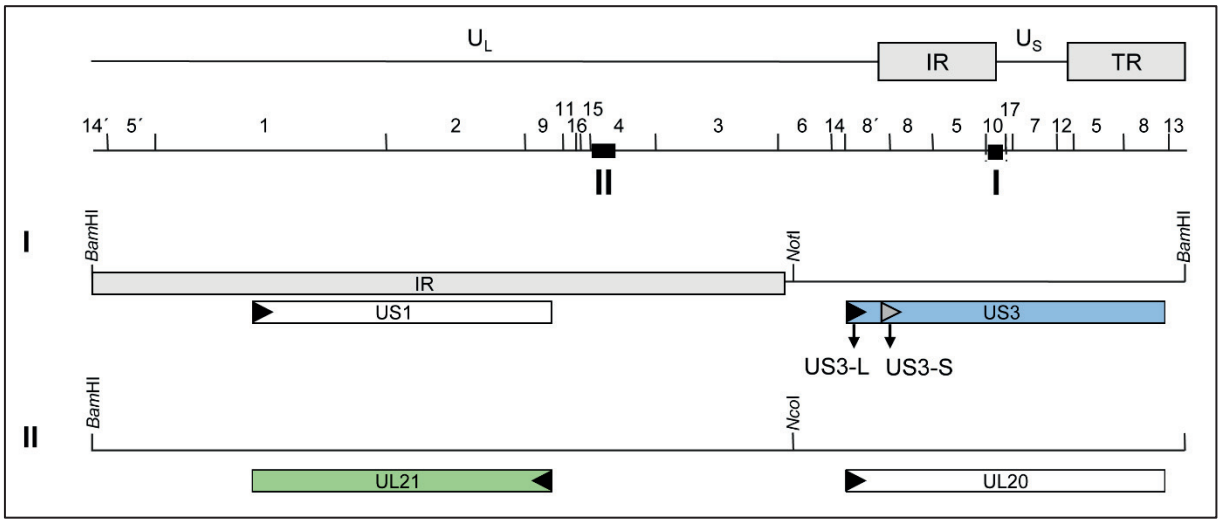

Fig. 4 Schematic map of the PrV genome. The PrV genome consists of the unique long (U_L) and unique short (U_S) regions as well as internal (IR) and terminal (TR) inverted repeat sequences. The positions of BamHI restriction recognition sequences are indicated and fragments are numbered according to their size. The black bars I and II indicate coding regions of the US3 (I) and the UL21 gene (II). Arrowheads highlight transcriptional start sites and orientation of the genes. Restriction sites given in I and II were used for cloning. (I) Enlarged map of BamHI fragment 10, which contains the coding region of the US1 and the adjacent US3 genes. US3 is transcribed into two different mRNAs, encoding the long (US3-L) and the short (US3-S) isoform. (II) Enlargement of the BamHI fragment 4 encoding the UL20-UL21 gene region. The UL21 gene is transcribed in antiparallel direction to the UL20 gene. Adapted from Klupp *et al.* 2005, Sehl *et al.* 2020a.

2.3 The alphaherpesviral serine/threonine kinase pUS3

pUS3 encoded by the US3 gene in the U_S region is conserved among the *Alphaherpesvirinae* (Frame *et al.* 1987, Purves *et al.* 1991, Wagenaar *et al.* 1995, Klupp *et al.* 2001) (Fig. 3). pUS3 is a serine/threonine kinase, present in primary and mature virions, and involved in a multitude of processes during viral replication *in vitro* and *in vivo* (Granzow *et al.* 2004, Kato and Kawaguchi 2018). The multifunctionality of

pUS3 encompasses different processes such as immune escape (Cartier and Masucci 2004, Rao *et al.* 2011, Imai *et al.* 2013, Xiong *et al.* 2015, Rao *et al.* 2018), apoptosis (Leopardi *et al.* 1997, Benetti *et al.* 2003, Geenen *et al.* 2005, Benetti and Roizman 2007, Wang *et al.* 2011), cytoskeletal rearrangement (Favoreel *et al.* 2005, Van Den Broeke *et al.* 2009, Van Den Broeke *et al.* 2009, Jacob *et al.* 2013, Jacob *et al.* 2015), intercellular viral spread (Jansens *et al.* 2017) and nuclear egress of herpesviral particles (Mettenleiter *et al.* 2013). Despite this, the different alphaherpesviral pUS3 homologs are dispensable for viral replication *in vitro* but viral titers are slightly reduced dependent on the virus species and cell culture used (Olsen *et al.* 2006, Deruelle and Favoreel 2011). In PrV, a US3-deleted mutant (ΔUS3) replicates to titers which are up to 5 to 10-fold lower than wildtype PrV titers in cells of porcine or rabbit origin (Wagenaar *et al.* 1995, Klupp *et al.* 2001), as is true for HSV-1 lacking pUS3 in Vero cells (Reynolds *et al.* 2002, Ryckman and Roller 2004).

In vivo, pUS3 has been reported to play a more pivotal role. At least in pigs, PrV-ΔUS3 was attenuated and induced only very mild disease (Kimman *et al.* 1994). In contrast, in mice and rats, PrV-ΔUS3 infection was only slightly different from wildtype infection (Klopfleisch *et al.* 2006, Olsen *et al.* 2006). Rats intraocularly infected with PrV-ΔUS3 displayed a delayed onset of clinical signs, but severity was comparable to wildtype infected animals (Olsen *et al.* 2006). In intranasally PrV-ΔUS3 infected mice the MTD of animals was only slightly prolonged, but resulted in severe disease comparable to wildtype infected mice (Klopfleisch *et al.* 2006). However, in a murine herpes stromal keratitis (Koyanagi *et al.* 2014) or intracerebral infection model (Morimoto *et al.* 2009) no viral antigen was detectable in the brain and virulence was highly decreased after infection with HSV-1-ΔUS3, respectively, pointing to a more critical role of pUS3 for HSV-1 virulence.

In PrV and HSV-1, the US3 gene encodes two isoforms (Rixon and Mcgeoch 1985, Van Zijl *et al.* 1990). In PrV, the major, but shorter transcript is translated into a 334-amino-acids (aa) protein (pUS3-S, 41kDa) whereas the minor, but longer transcript is translated into a 388 aa polypeptide (pUS3-L, 53kDa). In HSV-1, the large pUS3 (481aa, 70kDa) is the major form while the minor form is designated as US3.5 (405aa, 50kDa) (Poon *et al.* 2006). In transient transfection experiments, PrV pUS3-S is predominantly found in the nucleus whereas pUS3-L seems associated with mitochondria, the plasma membrane and the cytoplasm (Calton *et al.* 2004).

Therefore, the different subcellular localizations of the two isoforms may indicate different roles of pUS3 (Deruelle and Favoreel 2011). All pUS3 homologs consist of a kinase domain encompassing a highly conserved adenosine triphosphate (ATP)-binding domain and a catalytic active site. Certain aa residues, particularly the central lysine in the ATP-binding domain or the aspartate in the catalytic center are often mutated to inactivate kinase activity (Deruelle and Favoreel 2011).

The role of pUS3 in nuclear egress

Nuclear egress is a regulated process characterized by sequential envelopment-deenvelopment (Skepper *et al.* 2001). During nuclear egress intranuclear nucleocapsids bud through the INM into the PNSp thereby acquiring a primary, transient envelope. This primary envelope then fuses with the ONM releasing the capsids into the cytoplasm (Mettenleiter *et al.* 2013). The nuclear egress of herpesviruses is mediated by the conserved heterodimeric complex consisting of viral pUL31, a nuclear matrix-associated phosphoprotein, and pUL34, a type II membrane protein which form the nuclear egress complex (NEC) at the INM (Klupp *et al.* 2000). This pUL31-pUL34-complex, which is conserved among the *Herpesviridae* is required for primary envelope-mediated nuclear egress. If absent, nuclear egress is impaired and capsids accumulate in the nucleus. It has been shown that pUL31 and pUL34 are phosphorylated by the pUS3 kinase (Purves *et al.* 1991, Klupp *et al.* 2001, Ryckman and Roller 2004, Mou *et al.* 2009). In the absence of pUS3, primary virions accumulate in large invaginations of the INM, which indicates a role of pUS3 for efficient deenvelopment of virus capsids at the ONM (Wagenaar *et al.* 1995, Klupp *et al.* 2001, Reynolds *et al.* 2001, Reynolds *et al.* 2002). However, whether this defect is the reason for the observed titer reduction of ΔUS3 mutants is not known.

Functional characterization of the two isoforms of PrV pUS3 in the viral life cycle, and how kinase activity affects nuclear egress was of central importance for **paper I**. The pUS3 kinase deficient mutant (PrV-US3Δkin) was further analyzed in **paper II** in a murine intranasal infection model to assess neurovirulence and neuroinvasion compared to a PrV mutant defective in both pUL21 and pUS3.

2.4 The tegument protein pUL21

PrV pUL21, encoded in the U_L region is a capsid-associated inner tegument protein of approx. 60kDa, which is conserved among the alphaherpesviruses (De Wind *et al.* 1992, Radtke *et al.* 2010, Metrick *et al.* 2015) (Fig. 3). pUL21 is known to be dispensable for viral replication in PrV, HSV-1 and BoHV-1 (Baines *et al.* 1994, Klupp *et al.* 2005, Muto *et al.* 2012, Shahin *et al.* 2017). A deletion of UL21 (ΔUL21) in PrV or HSV-1 resulted in titers which are up to 10-fold reduced compared to wildtype virus (De Wind *et al.* 1992, Baines *et al.* 1994, Klupp *et al.* 2005, Mbong *et al.* 2012). In contrast, in HSV-2, the protein is essential for the production of viral progeny (Le Sage *et al.* 2013).

Although different processes in the viral replication cycle have been attributed to pUL21, the specific role of this protein and detailed mechanistic functions are still unclear. Previous studies proposed that pUL21 is involved in packaging of viral DNA during capsid maturation in the nucleus (De Wind *et al.* 1992, Wagenaar *et al.* 2001). Later experiments showed that pUL21 rather has cytosolic functions since it was found mainly in the cytoplasm and DNA cleavage was found to be unimpaired (Baines *et al.* 1994, Klupp *et al.* 2005). However, recently it has been reported that HSV-1 pUL21 promotes egress of capsids from the nucleus (Le Sage *et al.* 2013, Sarfo *et al.* 2017), which supports the observations by de Wind et al. (1992) and Wagenaar et al. (2001).

To date, different other key functions have been attributed to pUL21. For instance, it has been reported that HSV-1 pUL21 promotes the formation of long cellular processes in non-neuronal cells indicating a role of pUL21 in intracellular transport processes via the interaction with microtubules (Takakuwa *et al.* 2001). A delay of protein synthesis in the early stages of the replication cycle has been observed after deletion of UL21 in HSV-1 (Mbong *et al.* 2012). Furthermore, a functional role in virion assembly was identified since the incorporation of tegument proteins pUL46, pUL49 and pUS3 depends on pUL21 (Michael *et al.* 2007). In this context, pUL21 has been proven to build a complex with pUL16 in PrV (Klupp *et al.* 2005) as well as with pUL16 and pUL11 in HSV-1 resulting in a tripartite complex, which might function as a nucleocapsid-envelope-anchor (Loomis *et al.* 2003, Mettenleiter *et al.* 2006, Harper *et al.* 2010, Han *et al.* 2012). In PrV, BoHV-1 and HSV-1, pUL21 is required for efficient cell-to-cell spread (Klupp *et al.* 1995, Muto *et al.* 2012, Shahin *et al.* 2017, Finnen and Banfield 2018).

In vivo, point mutations in the PrV-Bartha UL21 gene contribute to its avirulence in pigs since it was shown that pigs infected with PrV lacking pUL21 survived unlike wildtype infected animals (Klupp *et al.* 1995). In mice, either infected intraocularly or intranasally with PrV-ΔUL21 survival times are prolonged compared to wildtype infection (Klopfleisch *et al.* 2006, Yan *et al.* 2019). Mice inoculated intranasally with $1x10^6$ PFU of PrV-Kaplan died approximately 49 h p.i. whereas animals infected with PrV-ΔUL21 succumbed to death at around 71 h p.i. (Klopfleisch *et al.* 2006). After intraocular infection with $1x10^5$ PFU mice survived slightly longer (Yan *et al.* 2019). Wildtype and PrV-ΔUL21 infection led to a survival time of 87 h and 103 h, respectively. Intranasal instillation produced survival times of 108 h after wildtype infection and 117 h after PrV-ΔUL21 infection. These results confirm that pUL21 is an important determinant for PrV virulence and neuroinvasion, in both pigs and mice (Klupp *et al.* 1995, Klopfleisch *et al.* 2006, Yan *et al.* 2019).

pUL21 interacts with microtubules not only in non-neuronal cells (Takakuwa *et al.* 2001) but also in neuronal cell cultures giving an initial molecular insight how pUL21 could affect neuroinvasion (Yan *et al.* 2019). In detail, it was demonstrated that pUL21 plays an important role in retrograde neuronal transport processes by interacting with a dynein protein, called Roadblock-1, thus facilitating efficient viral spread. This is in line with the previous results (Curanovic *et al.* 2009) showing that mutations or the complete absence of pUL21 in the attenuated live-vaccine strain PrV-Bartha enhanced retrograde transport in neurons.

In summary, although different functions were identified for pUL21 the molecular details as well as viral and cellular interaction partners for pUL21 are unknown. In **paper II** a PrV mutant deleted in the UL21 gene with dysfunctional pUS3 kinase function (PrV-ΔUL21/US3Δkin) was characterized *in vitro* and *in vivo* in comparison to wildtype PrV (PrV-Kaplan) infection and the corresponding single mutants (PrV-ΔUL21 and PrV-US3Δkin).

Interaction between pUS3 and pUL21

So far, neither structural nor functional interactions between pUS3 and pUL21 have been described. In the absence of pUL21 in PrV, less pUS3 and pUL49 are incorporated into mature virus particles (Michael *et al.* 2007). In PrV-Bartha virions also

less pUS3 and pUL49 is incorporated (Lyman *et al.* 2003) and it could be shown that this effect is caused by mutations in the UL21 gene (Michael *et al.* 2007).

2.5 Pseudorabies Virus and Aujeszky's disease

PrV infects a broad range of animal species including pigs as the natural host, but also carnivores, ruminants as well as lagomorphs and rodents such as mice and rats. Birds as well as horses have been shown to be highly resistant against an infection with PrV (Mettenleiter 2000). Recently, few human infections have been reported in China, which presented as encephalitis, endophthalmitis or retinal necrosis (Wang *et al.* 2019, Yang *et al.* 2019). PrV causes Aujeszky's disease (AD) which is also known as Pseudorabies due to the rabies-like clinical appearance of infected animals. The disease was first described in 1849 in cattle in Switzerland and referred to as the „mad itch" syndrome due to severe and excessive pruritus which eventually leads to automutilation in affected animals (Mettenleiter *et al.* 2019). In 1902, the veterinary pathologist and microbiologist Aladár Aujeszky identified the pathogenic agent of the disease.

For productive infection, high virus-containing doses >$1x10^{4-5}$ tissue culture infectious dose 50 ($TCID_{50}$) are necessary for both, porcine and non-porcine animals (Wittmann 1991). In pigs, PrV is transmitted through direct or indirect contact, and particularly in feral pigs and wild boar the virus primarily spreads via the veneral route (Romero *et al.* 2001). In non-porcine animals, which constitute dead-end hosts, transmission does rather not occur (Crandell *et al.* 1982, Thawley and Wright 1982, Mocsari *et al.* 1987). Thus, PrV poses little to no danger to lab workers (Pomeranz *et al.* 2005) and animal experiments are possible in the pig as well as in mice or rats, which makes PrV a suitable model to study molecular details and pathomechanisms of herpesviral disease.

Aujeszky's disease in pigs

In pigs, two distinct clinical pictures evolve after PrV infection, which are dependent on the age and immunological status of the animal as well as on the transmission route and virulence of the virus strain (Nauwynck 1997, Schmidt *et al.* 2001). Among unspecific clinical signs including fever or anorexia, especially young piglets show

neurological impairment such as incoordination, ataxia, trembling and vomiting. Suckling pigs usually die within 24 hours without any clinical signs. Fever and respiratory signs such as rhinitis and coughing occur in adult pigs due to predominant infection of the respiratory tract (Iglesias *et al.* 1992, Gillespie *et al.* 1996). Likewise, abortion or stillbirth, mummification or fetal resorption results from PrV infection in pregnant sows (Kluge and Mare 1974). Pruritus may also develop, but is not as severe as in non-porcine species (Ezura *et al.* 1995).

After oronasal infection of pigs, PrV replicates in the epithelium of the upper respiratory tract, particularly in the oral and nasal mucosa (Kritas *et al.* 1994). PrV is then transported to tonsils and lymph nodes of the head via lymphatic vessels (Mettenleiter *et al.* 2019). Via different cranial nerves including the olfactory (I), trigeminal (V) and glossopharyngeal nerve (IX) PrV invades the CNS resulting in lytic or latent infection of neurons, and can be further transported to higher brain areas causing non-suppurative meningoencephalitis (Pomeranz *et al.* 2005). Further, PrV infects lymphocytes and macrophages and causes viremia, which leads to infection of a multitude of other organs (Page *et al.* 1992, Mettenleiter 2000).

At necropsy, gross lesions are present mostly in young pigs and include epithelial necrosis of tonsils, trachea or oesophagus (Corner 1965, Narita *et al.* 1990). Rhinitis is frequently reported. Hemorrhagic necrosis is described to be present in different organs such as liver, spleen, lung, small and large intestine, adrenal glands and placenta (Olander *et al.* 1966, Narita *et al.* 1990, Ezura *et al.* 1995). Histologically, the epitheliotropism and neurotropism of PrV is obvious. Epitheliotropic lesions are mainly present in young, aborted or stillbirth piglets. Lesions include foci of coagulative necrosis in the liver, tonsils, lung, spleen, placenta and adrenal glands, accompanied with intranuclear Cowdry type A inclusion bodies (Olander *et al.* 1966, Sabo *et al.* 1968, Ezura *et al.* 1995). Pulmonary changes are variable, and range from edema and mild cellular infiltration to hemorrhagic, necrotizing pneumonia. Also necrotizing vasculitis and panencephalitis comprising the cerebral cortex, brainstem and spinal ganglia were reported in piglets (Corner 1965, Sabo *et al.* 1968, Narita *et al.* 1990, Verpoest *et al.* 2017).

Aujeszky's disease in mice

In other animals than pigs such as dogs, cats, cattle or small ruminants as well as rodents, PrV infection is always fatal and mostly restricted to the NS, primarily associated with intense itching and skin lacerations (Hagemoser *et al.* 1980, Crandell *et al.* 1982, Thawley and Wright 1982, Quiroga *et al.* 1995, Quiroga *et al.* 1998). Laboratory animals such as mice are highly susceptible to PrV infection and have been widely used in research. Mice are infectable via different inoculation methods such as the subcutaneous, intravenous, intraocular, intracerebral, intraperitoneal and intramuscular route (Fraser and Ramachandran 1969, Babic *et al.* 1994, Brittle *et al.* 2004, Klopfleisch *et al.* 2004, Klopfleisch *et al.* 2006). However, experimental intranasal infection was highly reproducible (Babic *et al.* 1994, Klopfleisch *et al.* 2004, Klopfleisch *et al.* 2006). Infection of mice with the laboratory-adapted strain PrV-Kaplan typically resulted in the development of severe clinical signs such as hunching, depression, intense pruritus and automutilation within a short time after infection. In all experiments, the animals did not survive longer than three days post infection (p.i.). Typical CNS disorders such as ataxia, shaking or seizures were rather uncommon. In these experiments, it was demonstrated that wildtype PrV mainly infected the trigeminal nerve, trigeminal sensory nuclei in the brainstem as well as parts of the autonomic nervous system of the head (Babic *et al.* 1994, Klopfleisch *et al.* 2004, Klopfleisch *et al.* 2006). In another study, subcutaneous inoculation of PrV into the flank also led to automutilation and rapid death of mice without neurological signs (Brittle *et al.* 2004), but in contrast, animals infected with the attenuated PrV-Bartha showed a different clinical picture. Due to more widespread neuroinvasion towards the cerebral cortex, CNS disorders were present in these animals, but itching did not occur. However, all PrV-Bartha infected animals died of the infection, even though slightly later. Thus, two modes of infection have been shown for PrV in mice: i) the mad-itch syndrome, which is rapidly fatal and ii) neurological disease without itching resulting in a slightly prolonged survival time.

Since intranasal infection in mice reveals high reproducibility this inoculation route has been greatly contributed to unravel the functional role of PrV proteins including glycoproteins (Mettenleiter 2003) or tegument proteins (Klopfleisch *et al.* 2004, Klopfleisch *et al.* 2006). PrV mutants lacking different genes, singly or in combinations,

have been tested after intranasal infection of mice and classified based on their MTD (Klopfleisch *et al.* 2004, Klopfleisch *et al.* 2006).

Intranasal infection of mice with PrV lacking US3 and UL21

Preliminary experiments in mice showed that infection with PrV-ΔUL21/US3, a mutant deleted in both pUL21 and pUS3, resulted in extremely prolonged survival times or even in survival compared to infection with PrV-Kaplan or the single deletion mutants PrV-ΔUS3 and PrV-ΔUL21 (Maresch 2011). In contrast, mice infected with the wildtype strain PrV-Kaplan showed a MTD of approximately 50 h p.i. An infection with PrV-ΔUS3 resulted in a MTD of about 56 h p.i. whereas survival in mice was slightly prolonged after PrV-ΔUL21 infection with 71 h p.i. (Klopfleisch *et al.* 2006). Clinical signs were severe and comparable between all viruses.

Three independent experiments were conducted (Maresch 2011). In the first experiment, only one out of 20 intranasally infected mice showed apathy, lateral recumbency and dyspnea 408 h p.i. In a second experiment, 16 mice were inoculated. Almost all animals developed clinical signs including nasal brigde edema, mild pruritus and dermal erosions. Seven male mice died after 288 h p.i. whereas the others survived. In a third trial, fourteen female and thirteen males were infected. Five males died after 288 h p.i. and one female was euthanized 408 h p.i. Thirteen animals remained asymptomatic whereas the remaining mice recovered from the infection.

Based on these preliminary data in the mouse, a functional relationship between pUS3 and pUL21 could be assumed, and formed the basis for the investigations presented in this thesis. To investigate the phenotype of PrV-ΔUL21/US3 in more detail additional mutants were generated which were only defective in the pUS3 kinase function (PrV-US3Δkin, solely tested in **paper I**) or, in addition, comprised the UL21 deletion (PrV-ΔUL21/US3Δkin, tested in **paper II**). Detailed characterization of these mutants was performed in cell culture as well as in the mouse.

2.6 Herpes Simplex Virus 1 and Herpes Simplex Encephalitis

Seropositivity in humans for HSV-1 is reported with 80 to 90% worldwide. An infection is typically associated with dermal lesions, mainly in the face, but also in the genital region (Smith and Robinson 2002). Primary infection of mucocutaneous surfaces or

damaged skin in the oral region leads to epithelial lesions, known as cold sores or *Herpes labialis.* Those lesions are a prerequisite for viral particles to enter the PNS via sensory nerve endings, which innervate the mucosa or the skin. Following retrograde transport life-long latency is established in the trigeminal ganglion (TG) or the DRG (Lafferty *et al.* 1987, Richter *et al.* 2009). Upon reactivation the virus travels within neurons in an anterograde direction to the axon termini in the periphery to cause recurrent infection located within the same cutaneous region innervated by TG or DRG and does rarely spread to other anatomical locations in immunocompetent persons (Gilden *et al.* 2007). HSV-1 is also able to infect the corneal epithelium causing *Herpes keratitis*, which eventually may lead to blindness (Chentoufi *et al.* 2012). HSV-1 is mainly transmitted via orolabial contact (Smith and Robinson 2002).

Herpes Simplex Encephalitis (HSE)

Sporadically, HSV-1 induces severe encephalitis (Steiner and Benninger 2018) and is considered as the most common cause of brain inflammation (Whitley *et al.* 1989). The incidence of HSE is between 2 and 4 cases/1,000,000 (Hjalmarsson *et al.* 2007, Scheld *et al.* 2014) with reported mortality rates of up to 70%, when untreated (Whitley and Lakeman 1995, Tyler 2009, Steiner and Benninger 2013, Whitley and Baines 2018). Up to 30% of patients die despite treatment and only 9% of treated patients fully recover (Mielcarska *et al.* 2018). Surviving patients suffer from severe long-term sequelae including neurological alterations such as permanent cognitive and memory problems or epilepsy (Mcgrath *et al.* 1997, Raschilas *et al.* 2002, Whitley and Baines 2018). In some cases even relapse of inflammation is possible (Skoldenberg *et al.* 2006). Patients, men and women equally affected, are either between three months and 20 years old or in the major part of cases belong to the elderly generation of above 50 years (Steiner and Benninger 2013).

HSE typically locates asymmetrically to the frontal and mesiotemporal lobes of the forebrain (Esiri 1982, Booss and Kim 1984, Kennedy *et al.* 1988, Nicoll *et al.* 1991), but manifests rarely in the brainstem (Livorsi *et al.* 2010). Patients present with unspecific clinical signs including fever, headache or neck pain. Further, typical neurological signs such as aphasia, disorientation and mental status changes occur, which affect their behavior and personality due to involvement of the limbic system.

Even convulsions or seizures are frequently reported (Raschilas *et al.* 2002, Steiner and Benninger 2018).

To date, it remains unknown whether HSE results from primary or recurrent infection, and why and how the infection is mainly established in restricted areas of the brain. The primary infection is supported by the fact that the frontal and mesiotemporal lobe are synaptically connected to the trigeminal or olfactory pathways which are considered to be infected by HSV-1. In support of this, in more than 50% of patients, the viral strains responsible for HSE are different from the one that caused cold sores (Whitley *et al.* 1982, Steiner 2011). Since HSV-1 can spread from the latently infected TG to the forebrain, reactivation from latency is also feasible (Davis and Johnson 1979, Lafferty *et al.* 1987). Further, viral DNA has been identified in brain tissue indicating the establishment of latency not only in ganglia of the PNS but also in the CNS. This points to reactivation and subsequent recurrent infection in the CNS itself (Fraser *et al.* 1981, Mori *et al.* 2004).

Histopathologically, HSE is characterized by meningeal, perivascular and parenchymal inflammatory mononuclear infiltrates mainly consisting of lymphocytes and histiocytes. Typically, neuronal necrosis as well as reactive gliosis, neuronophagy and satellitosis are detectable following infection (Booss and Kim 1984, Kennedy *et al.* 1988, Nicoll *et al.* 1991, Wnek *et al.* 2016). Patients suffering from HSE are usually not immunocompromised. However, congenital deficiencies in the innate immune response were shown to increase the susceptibility to HSE. In this respect, mutations in the intracellular protein Unc-93 homolog B1 ($UNC_{93}B1$) as well as in TANK-binding kinase 1 (TBK_1), tumor necrosis factor receptor-associated factor 3 ($TRAF_3$), Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF), interferon regulatory factor (IRF) were found leading to impaired Toll-like receptor (TLR) 3 dependent interferon (IFN) I response (Casrouge *et al.* 2006, Perez De Diego *et al.* 2010, Sancho-Shimizu *et al.* 2011, Herman *et al.* 2012, Mork *et al.* 2015). IFNs I are crucial for primary antiviral response against HSV-1 infection, and induce the production of further pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), interleukin 6 and 8 (IL-6 and 8) or different chemokines such as CC-chemokine ligand 5 (CCL5) or C-X-C motif chemokine ligand 10 (CXCL10) which limit the spread of the virus by stimulating the infiltration of immune cells towards the brain (Wuest and Carr 2008, Paludan *et al.* 2011).

To date the pathogenesis of HSE is still incompletely understood. Particularly, the mechanisms leading to HSE are unknown. The exact pathway of HSV-1 spread into the brain as well as the reason for the restricted tropism to the frontal and mesiotemporal lobe are unresolved yet. Further, it remains to be elucidated which host and viral factors either may contribute to control the infection or may lead to these life-threatening neuropathological processes. There is also the question of why HSE does not occur in the vast majority of HSV-1 infections.

Animal models for HSE

Since studying HSV infections in humans is difficult, animal models have been developed to better understand biological and pathobiological aspects of the disease (Kollias *et al.* 2015, Mancini and Vidal 2018). Mice, rats, guinea pigs and rabbits are often used for this purpose. Mice have the same cell surface receptors as humans, which facilitate HSV-1 infection (Petermann *et al.* 2015). Further, mice are easy to modify genetically (Jones 2011). Since their genome has been well-characterized and various gene knockout strains are available, mice are suitable models for studying immunopathological processes. In addition, latency has been successfully established in mouse models, but since mice lack the ability of spontaneous virus reactivation, rabbits and guinea pigs were used for this purpose (Wagner and Bloom 1997, Ramakrishna *et al.* 2015, Matundan *et al.* 2016). In general, mice are highly susceptible to HSV-1 infection and exhibit diffuse inflammation throughout the brain or only in the brainstem, but, however, do not develop the typical lesion in the frontal and mesiotemporal lobe as seen in humans (Shivkumar *et al.* 2013, Kollias *et al.* 2015, Menasria *et al.* 2017).

In mice, the development of disease and severity of illness is dependent on different parameters such as the mouse strain, the animal's age and genetic background, the virus strain and the inoculation route used (Kollias *et al.* 2015). Supporting this, C57BL/6 mice are the most resistant to HSV-1 infection whereas C3H or BALB/c mice are highly susceptible (Kastrukoff *et al.* 1986, Kastrukoff *et al.* 2012). Further, young and neonate mice are more vulnerable to infection due to the immature immune system compared to older animals (Wilcox *et al.* 2015).

To induce HSV-1 encephalitis in mice the intranasal, intraocular or intracerebral route of infection were often utilized (Mancini and Vidal 2018). After intranasal inoculation, it

was shown that the virus entered the trigeminal and the olfactory nerves which also occurs after infection in humans (Shivkumar *et al.* 2013, Menasria *et al.* 2017). Viral transport to the trigeminal ganglion and to the CNS has also been confirmed in an ocular scarification infection model (Reinert *et al.* 2016). Nonetheless, mice rather developed lesions in the brainstem and cerebellum, but lacked focal neuroinflammatory changes in the forebrain as reported for HSE patients (Mancini and Vidal 2018). Moreover, intranasal infection with HSV-1 can lead to undesired side effects such as lung involvement and is considered to be less reproducible than other instillation methods (Kollias *et al.* 2015). Even though intracerebral inoculation is not a natural route of primary HSV-1 infection, this method is favorable to evade the PNS and simplifies investigation of the inflammatory response of brain resident cells (Kopp *et al.* 2009, Vilela *et al.* 2011). However, this inoculation route also resulted in diffuse inflammation of the brain (Wang *et al.* 2012). Other routes of infection such as cutaneous flank and food pad inoculation (Engel *et al.* 1997, Van Lint *et al.* 2004) or intraperitoneal and intravascular inoculation (Fujioka *et al.* 1999, Chen *et al.* 2016) are used to analyze the peripheral or systemic immune response, respectively.

Depending on the virulence of the virus strain animals developed mild or severe illness but usually succumb to death shortly after infection (Hudson *et al.* 1991, Sergerie *et al.* 2007, Kopp *et al.* 2009, Wang *et al.* 2013). In general, a suitable animal model fulfills various aspects that resemble human disease (Kollias *et al.* 2015). For HSE, none of the various animal models combines the different critical factors to understand the pathogenesis of HSE. Therefore, an improved animal model by using PrV-ΔUL21/US3Δkin in mice has been proposed in **paper II** to study HSE.

2.7 Varicella Zoster Virus related diseases

VZV typically causes vesicular skin rash, referred to as Varicella or Chickenpox primarily in young children, and Herpes Zoster (shingles) in adults (Grahn and Studahl 2015). Worldwide, more than 90% of people are infected (Laing *et al.* 2018). VZV can be easily transmitted via aerosols (Tellier *et al.* 2019). After replication in respiratory epithelial cells in the pharynx, the virus is transferred to T lymphocytes within the tonsils (Arvin *et al.* 2010). Following T cell trafficking VZV is primarily transported to the skin where, after initial control by the innate immune system, massive replication eventually leads to vesicular lesions (Moffat *et al.* 2007). Although complications are rather

uncommon in children, VZV can cause severe CNS disease or pneumonia as well as hepatitis (Kusne *et al.* 1995, Mirouse *et al.* 2017, Skripuletz *et al.* 2018). As other alphaherpesviruses VZV also leads to latent infection of different ganglia including cranial nerve, DRG or autonomic ganglia (Richter *et al.* 2009, Grahn and Studahl 2015). In contrast to HSV-1 or PrV, the mechanisms how VZV reaches the ganglia from infected lymphocytes and how latency is established are unclear.

Reactivation of VZV presents as Herpes Zoster, which usually proceeds in adulthood. This is most likely due to decreased VZV-specific cell mediated immunity or immunosuppression, and can be associated with severe CNS complications such as meningoencephalitis, meningitis, meningoradiculitis, myelitis and vasculopathy (Nagel and Gilden 2014). Herpes Zoster is caused by VZV traveling via retrograde spread to the associated dermatomes of the ganglion from where it reactivated (Nagel and Gilden 2014). Although VZV can induce rash in the facial, cervical or lumbosacral regions, lesions are predominantly found in the thoracic location. Patients suffer from severe itching and pain which is referred to preherpetic neuralgia, which can persist and become chronic, and is then termed postherpetic neuralgia (Steiner and Benninger 2018). Chronic radicular pain, known as zoster sine herpete, usually occurs without skin lesions (Gilden *et al.* 2010).

Meningoencephalitis is reported in immunocompetent as well as in immunosuppressed patients, and can either occur along with Varicella or Herpes Zoster (Grahn and Studahl 2015, Skripuletz *et al.* 2018). Affected patients mainly show mental status alterations whereas seizures are rare (Granerod *et al.* 2010, Ellis *et al.* 2014, Nabi *et al.* 2014). Compared to HSE, which involves primarily the frontal and mesiotemporal lobe, meningoencephalitis caused by VZV is not assigned to typical locations, and can either present as acute disseminated encephalomyelitis or distinct cerebellitis (Bozzola *et al.* 2014, Chai and Ho 2014, Nagel *et al.* 2020). The more diffuse inflammation pattern has been attributed to vasculopathy, which is nowadays accepted as the key pathologic feature of VZV associated meningoencephalitis (Gilden 2002, Gilden *et al.* 2009, Nagel and Gilden 2016).

Myelitis caused by VZV is mainly associated with Herpes Zoster and paradoxically asymptomatic. The pathogenesis is still unclear, but it is assumed that the virus is able to spread from infected DRG to the spinal cord (Steiner and Benninger 2018).

Ganglionitis is another frequently reported manifestation of VZV reactivation which mainly includes the TG and DRG of the cervical, thoracic, lumbar and sacral region (Skripuletz *et al.* 2018), and can turn into chronic (Gilden *et al.* 2003, Birlea *et al.* 2014). In support of that, latent VZV DNA was detected in different ganglia of the head and neck including the TG (Richter *et al.* 2009). In **paper II**, analogies to VZV infection including itching, associated skin lesions as well as ganglionitis and myelitis are described based on results after infection of mice with a PrV mutant deleted in the genes encoding pUL21 and lacking pUS3 kinase activity.

2.8 Neuroanatomy and alphaherpesviral neuroinvasion

The NS is devided into the CNS and the PNS. The CNS consists of the brain and the spinal cord surrounded by cerebrospinal fluid and covered by three layers of meninges (Schnell and Maher De Leon 1998). Generally, the brain is composed of three parts, the forebrain, midbrain and hindbrain, which are further subdivided into telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon (Fig. 5A) (Wurst and Bally-Cuif 2001). The telencephalon includes the cerebral cortex as well as subcortical structures such as the caudate nucleus, amygdala or hippocampus (Clowry *et al.* 2018). Depending on the location, the cerebral cortex is termed corresponding to associated cranial bones (Bruner *et al.* 2015). These include the frontal, temporal, parietal and occipital lobe (Fig. 5B). Like the telencephalon the diencephalon belongs to the forebrain which comprises the thalamus, hypothalamus, and epithalamus (Puelles 2019). In the mesencephalon (midbrain) the periaqueductal grey, ventral tegmental area, parts of the reticular formation, the substantia nigra and the cerebral peduncles are located (Moreno-Bravo *et al.* 2012). The hindbrain includes the metencephalon consisting of the pons, parts of the reticular formation and the cerebellum as well as the myelencephalon where different sensory, motor as well as cranial nerve nuclei are located (Johns 2014). The mes-, met- and myelencephalon and parts of the hypothalamus together constitute the brainstem (Angeles Fernandez-Gil *et al.* 2010). The myelencephalon merges into the caudal joining spinal cord (Johns 2014).

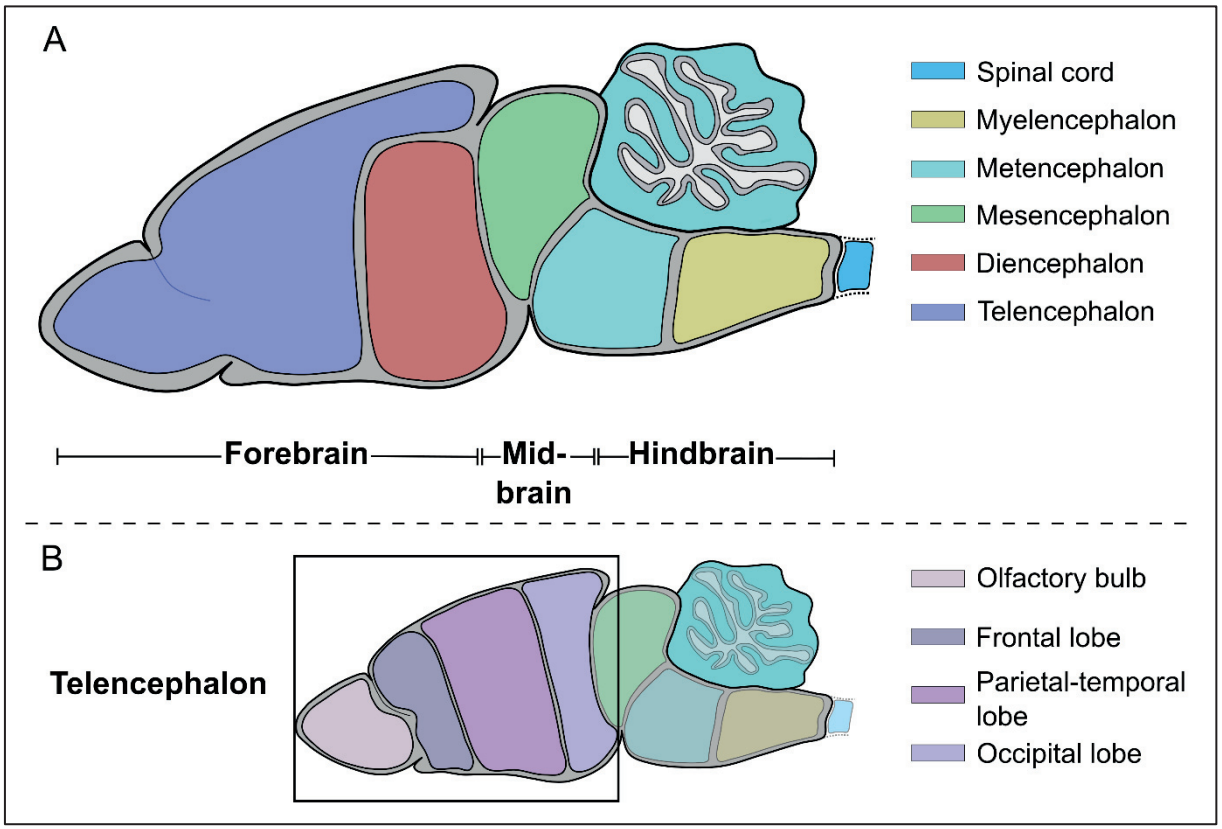

Fig. 5 Schematic illustration showing the different parts of the mouse brain. A) The brain is subdivided into three parts: the forebrain, midbrain and hindbrain, which comprise the tel-, di-, mes-, met- and myelencephalon. B) Lobes of the telencephalon are designated according to their adjacent cranial bones and include the frontal, parietal-temporal and occipital lobe. The olfactory bulb is the most cranial part of the forebrain. For simplification, the diencephalon is not included in this image. Adapted from Sehl *et al*. 2020b.

Neuroinvasion of alphaherpesviruses

Prior to entering the NS especially alphaherpesviruses like HSV-1 or PrV require replication and spread in non-neuronal epithelial cells of the oronasal region. Subsequently sensory nerve-endings are invaded by binding to specific receptors located at the synapse such as nectin-1 and 2 or cluster of differentiation (CD) 155 (Spear *et al.* 2000, Mettenleiter 2003). The sensory neuron is a highly polarized cell consisting of an axon as well as distal and proximal dendrites (Rasband 2010) (Fig. 6). Following fusion with the plasma membrane (axolemma), adaptor and motor proteins are recruited in the cytoplasm to facilitate virus capsid transport along microtubules to the cell body (soma). These motor proteins facilitate cargo transport in an ATP-dependent directional process. Intraaxonal retrograde transport from synapses to the cell body is mediated by the motor protein dynein whereas anterograde transport is

provided by kinesins. Following retrograde spread within neurons viruses reach the cell body of the pre-synaptic cell, whereas a post-synaptic cell is infected upon anterograde spread (Taylor and Enquist 2015) (Fig. 6). Although retrograde transport is most important and predominant during CNS invasion of herpesviruses, anterograde spread is also possible. Once in the soma, especially in the sensory TG or DRG alphaherpesviruses establish latency in the nucleus, and can be periodically activated which results in anterograde spread to the site of primary infection, or they undergo transsynaptic spread to invade higher areas of the NS (Smith 2012).

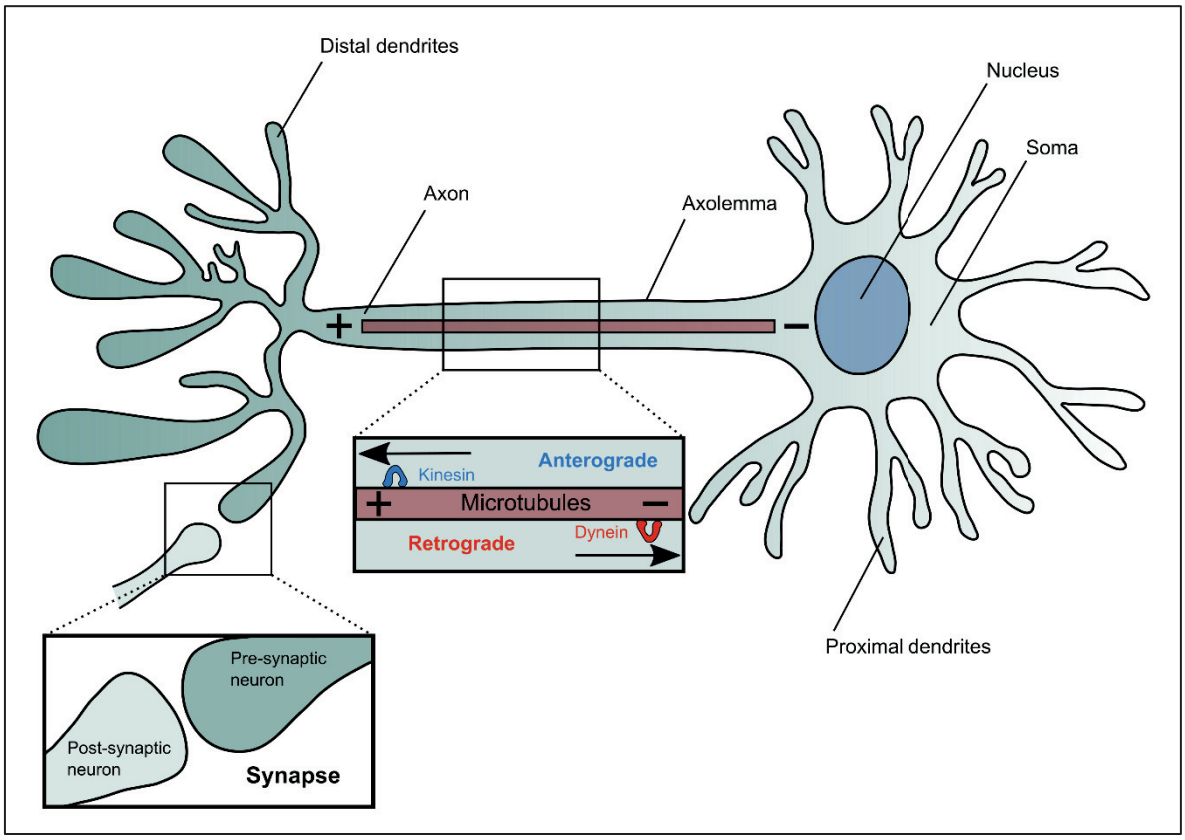

Fig. 6 Structure of a neuron. Neurons consist of soma, nucleus, axon as well as proximal and distal dendrites. The neuronal plasma membrane is designated as axolemma. Intraaxonal transport processes along microtubules are indicated. Microtubules are polar structures comprising two distinct ends (plus and minus). Herpesviruses reach post-synaptic neurons via anterograde transport mediated by kinesins from minus to plus end. Retrograde transport (plus to minus end) from synapses to the soma is mediated through dyneins. Adapted from Ekstrand *et al*. 2008.

The neural network of alphaherpesviral neuroinvasion

i) The trigeminal route

It is assumed that alphaherpesviruses are transported to the CNS via the trigeminal nerve (see Fig. 1 **paper II**) (Held and Derfuss 2011). This is supported by experimental evidence on HSV-1 from animal trials (Shukla *et al.* 2012, Mancini and Vidal 2018, Doll *et al.* 2019) and could be further demonstrated for PrV infection in mice (Babic *et al.* 1994, Klopfleisch *et al.* 2004, Klopfleisch *et al.* 2006). The trigeminal nerve is the fifth (V) of twelve cranial nerves and innervates the face via three branches: the sensory ophthalmic nerve (V1), the sensory maxillary nerve (V2) and the mixed sensory and motor mandibular nerve (V3) (Fehrenbach and Herring 2015).

The trigeminal nerve receives sensations of dermatomes of the face, the cornea, oro-nasal region including the oral and nasal mucosa, as well as parts of the ears, and carries sensory information such as touch, temperature, vibration or proprioception (Baumel 1974, Stewart 1989). Trigeminal nerve endings terminate in TG, where the cell bodies of the neurons accumulate in close association with glial satellite cells (Messlinger *et al.* 2020). These sensory trigeminal neurons in peripheral ganglia are referred to as first order neurons. From here, sensory fibers of V1, V2 and V3 then enter the middle cerebellar peduncle and reach into the pons located in the metencephalon and either project to synaptically connected principal sensory nucleus (Pr5) or to the spinal trigeminal nucleus (Sp5) lying in the met- and myelencephalon, respectively (Ezure *et al.* 2001). Together with the mesencephalic trigeminal nucleus (Me5), which directly receives signals bypassing the TG, the trigeminal nuclei are referred to as second order neurons, which have been shown to be infectable by PrV (Capra and Dessem 1992, Klopfleisch *et al.* 2004, Klopfleisch *et al.* 2006). Pr5 consists of two parts: the dorsomedial and ventrolateral division receiving particular fibers from different regions of the head (Patestas and Gartner 2016). Sp5 is the largest nucleus and consists of three sub-nuclei termed *pars oralis*, *pars interpolaris* and *pars caudalis*, which reflect sensory input in a somatotopic organization from three distinct regions of the face. Further, sensory fibers from other cranial nerves including the facial nerve (VII), the glossopharyngeal nerve (IX) and vagus nerve (X) project to Sp5 (Patel and Das 2020).

Originating from the trigeminal nuclei two distinct trigeminal tracts are formed: the spinal trigeminal tract and the trigeminothalamic tract. Sensory afferents from the head

join the spinal trigeminal tract and end up in Sp5, while the trigeminothalamic tract contains fibers from Sp5 and Pr5 which project to the ventral posteromedial nucleus of the thalamus (VPM) (Nash *et al.* 2010, Wilcox *et al.* 2015). Next, VPM third order neurons reach into the primary somatosensory cortex located in the parietal lobe (Corkin *et al.* 1970). The thalamus as well as the somatosensory cortex belong to higher brain areas, which have been shown to become infected by attenuated PrV strains after intranasal infection or intradermal administration of strain PrV-Kaplan (Klopfleisch *et al.* 2006, Maresch 2011). From VPM projections are send back to the reticular formation and from there towards the thalamus as well as to other parts of the cerebral cortex (Arbuthnott *et al.* 1990).

ii) The olfactory route

HSV-1 infection in humans as well as animal experiments suggest that alphaherpesviruses are also able to enter the CNS via the olfactory nerve since virus could be identified in the olfactory epithelium and olfactory cortex (Twomey *et al.* 1979, Esiri 1982, Shivkumar *et al.* 2013, Menendez and Carr 2017). Olfactory sensory neurons are bipolar neurons located in the olfactory neuroepithelium. With their long axons, these neurons penetrate the cribriform plate and synapse with mitral and tufted cells in the olfactory bulb (Durrant *et al.* 2016). From the olfactory bulb, they project to different areas of the brain including the piriform cortex as part of the traditional primary olfactory cortex as well as to the entorhinal cortex and amygdala lying in the temporal lobe (Sosulski *et al.* 2011). From the piriform cortex fibers reach into the thalamus and hypothalamus, hippocampus and frontal cortex (Illig and Wilson 2014, Yuan *et al.* 2014). For PrV, olfactory infection has been demonstrated in pigs of different ages (Kritas *et al.* 1994, Verpoest *et al.* 2017), but this has not been reliably proven in mice so far (Babic *et al.* 1994).

iii) The sympathetic and parasympathetic route

For PrV, infection of the autonomic nervous system has also been described (Babic *et al.* 1994, Klopfleisch *et al.* 2004, Klopfleisch *et al.* 2006). Especially the superior cervical ganglion (SCG) as part of the sympathetic system has been shown to be infected. In general, sympathetic preganglionic fibers originate in the thoracic spinal cord and ascend within the sympathetic trunk to the paravertebral superior cervical

ganglion (SCG) (Waxenbaum and Varacallo 2019). From here, postganglionic sympathetic nerves are sent to different locations of the head and join different cranial nerves including the trigeminal nerve (Kranzl and Kranzl 1976), and innervate different effector organs including nasal and salivary glands, the pharynx as well as glands of the oral and nasal cavity. Fibers from the SCG also project to different parts of the brain (Riganello *et al.* 2019). The head is further innervated by parasympathetic nerve endings which originate from cranial nerves III, VII, IX and X. Presynaptic fibers become postsynaptic fibers after passing different parasympathetic ganglia including the pterygopalatine and otic ganglion as well as the distal ganglion of cranial nerves IX and X or the geniculate ganglion of cranial nerve VII. Parasympathetic fibers innervate the glands of the head (Waxenbaum and Varacallo 2019). After PrV infection, in particular the pterygopalatine ganglion was positive for viral antigen (Babic *et al.* 1994, Klopfleisch *et al.* 2004, Maresch 2011).

3 Publications

Paper [I]

Roles of the Different Isoforms of the Pseudorabies Virus Protein Kinase pUS3 in Nuclear Egress

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VIRUS-CELL INTERACTIONS

Roles of the Different Isoforms of the Pseudorabies Virus Protein Kinase pUS3 in Nuclear Egress

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ABSTRACT Protein kinases homologous to the US3 gene product (pUS3) of herpes simplex virus (HSV) are conserved throughout the alphaherpesviruses but are absent from betaherpesviruses and gammaherpesviruses. pUS3 homologs are multifunctional and are involved in many processes, including modification of the cytoskeleton, inhibition of apoptosis, and immune evasion. pUS3 also plays a role in efficient nuclear egress of alphaherpesvirus nucleocapsids. In the absence of pUS3, primary enveloped virions accumulate in the perinuclear space (PNS) in large invaginations of the inner nuclear membrane (INM), pointing to a modulatory function for pUS3 during deenvelopment. The HSV and pseudorabies virus (PrV) US3 genes are transcribed into two mRNAs encoding two pUS3 isoforms, which have different aminoterminal sequences and abundances. To test whether the two isoforms in PrV serve different functions, we constructed mutant viruses expressing exclusively either the larger minor or the smaller major isoform, a mutant virus with decreased expression of the smaller isoform, or a mutant with impaired kinase function. Respective virus mutants were investigated in several cell lines. Our results show that absence of the larger pUS3 isoform has no detectable effect on viral replication in cell culture, while full expression of the smaller isoform and intact kinase activity is required for efficient nuclear egress. Absence of pUS3 resulted in only minor titer reduction in most cell lines tested but disclosed a more severe defect in Madin-Darby bovine kidney cells. However, accumulations of primary virions in the PNS do not account for the observed titer reduction in PrV.

IMPORTANCE A plethora of substrates and functions have been assigned to the alphaherpesviral pUS3 kinase, including a role in nuclear egress. In PrV, two different pUS3 isoforms are expressed, which differ in size, abundance, and intracellular localization. Their respective role in replication is unknown, however. Here, we show that efficient nuclear egress of PrV requires the smaller isoform and intact kinase activity, whereas absence of the larger isoform has no significant effect on viral replication. Thus, there is a clear distinction in function between the two US3 gene products of PrV.

KEYWORDS herpesvirus, pseudorabies virus, nuclear egress, pUS3 isoforms, pUS3 kinase

The serine/threonine kinase pUS3, encoded by the US3 gene, is conserved among the alphaherpesviruses. pUS3 is a multifunctional protein and is reported to be involved in many different steps of the viral replication cycle, including viral gene expression, inhibition of apoptosis, evasion of antiviral immune responses, modification of the cytoskeleton, enhancement of intercellular virus spread, modification of the nuclear lamina, and nuclear egress of herpesvirus capsids (1, 2). In accordance with its

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numerous functions, a plethora of viral and cellular proteins have been shown to be modified by pUS3 orthologues. These include the nuclear egress complex (NEC), which consists of pUL34 and pUL31, viral glycoproteins gB and gE, and components of the viral tegument (reviewed in references 1, 2).

Despite its variety of attributed functions and intensive interplay with viral and cellular proteins, pUS3 is dispensable for viral replication in standard cell lines, as well as in neuronal cultures (3–13). *In vivo*, pUS3 is reported to play a more pivotal role as a virulence factor. PrV-ΔUS3 is attenuated in pigs (14), while infection of rats or mice resulted in only a minor delay in disease progression, and all animals succumbed to death (12, 15).

During nuclear egress of herpesviruses, nucleocapsids bud through the inner nuclear membrane (INM), resulting in primary enveloped virions located in the perinuclear space (PNS). Subsequent fusion of the viral envelope with the outer nuclear membrane (ONM) releases the nucleocapsids into the cytoplasm (16–18). In the absence of pUS3, primary enveloped virions accumulate in the PNS in large invaginations of the INM (5, 10, 19, 20), indicating that pUS3 might play a role in efficient deenvelopment at the ONM. This function, however, is not essential, since titers of US3 deletion mutants are only reduced by up to 10-fold in most alphaherpesviruses and cell lines tested (3–5, 8, 10, 11).

For nuclear egress of herpesvirus capsids, the heterodimeric NEC, which consists of the conserved viral proteins designated pUL31 and pUL34 in the alphaherpesviruses HSV and PrV, is required. In line with this, pUL34 was the first substrate reported to be phosphorylated by pUS3 (21). Later, however, it was shown that modification by pUS3 of pUL31, rather than of pUL34, plays a regulatory role in nuclear egress (22, 23). Although pUS3 is not necessary for phosphorylation of pUL34 in PrV, nuclear envelope localization is more efficient in its presence (10). Furthermore, pUS3-mediated phosphorylation of the cytoplasmic tail in HSV-1 gB has been reported to be involved in efficient release of primary virions from the perinuclear space (24).

pUS3 is expressed early after infection (25) and is translated from two different transcripts into a longer and a shorter US3 gene product in HSV and PrV (26, 27). In PrV, the longer but minor transcript (ca. 5%) is translated into a 388-amino-acid-long polypeptide (pUS3-L), while the shorter transcript (ca. 95%) codes for a 334-amino-acid form designated pUS3-S, which is the major product present in infected cells and in purified virions (10, 27, 28). In contrast, in HSV-1 the larger translation product, pUS3, is the predominant form rather than the shorter pUS3.5, which lacks the 76 N-terminal amino acids (29). In HSV-1, both proteins are able to phosphorylate similar substrates, but only pUS3 appears to mediate efficient nuclear egress (30).

In transfected cells, PrV pUS3-S was detected mainly in the nucleus, while the 53-kDa pUS3-L localizes predominantly to the cytoplasm and the plasma membrane and was found associated with mitochondria (31). While both forms can elicit fundamental cytoskeletal rearrangements, only pUS3-S induces stress fiber breakdown (32). This effect seems to be due to differences in the localization of the respective isoforms and is dependent on the presence of sufficient amounts of nuclear pUS3 (33).

All pUS3 homologs, as well as cellular serine/threonine protein kinases, contain a highly conserved ATP-binding domain and a catalytically active site. The central lysine in the ATP-binding domain or the conserved aspartate in the catalytic triad are usually targeted to inactivate kinase activity (reviewed in reference 1). Unimpaired kinase function was found to be important for cytoskeletal remodeling in HSV-2 (34) and PrV (32) but, surprisingly, not in Marek's disease virus, where actin stress fiber breakdown was still efficient without intact pUS3 kinase function (20).

To determine whether the two pUS3 isoforms of PrV have different functions during the viral replication cycle, and to assay for importance of unimpaired kinase activity during nuclear egress, we isolated mutants expressing either pUS3-L or pUS3-S, a mutant with reduced pUS3-S expression, and a pUS3 kinase-dead mutant.

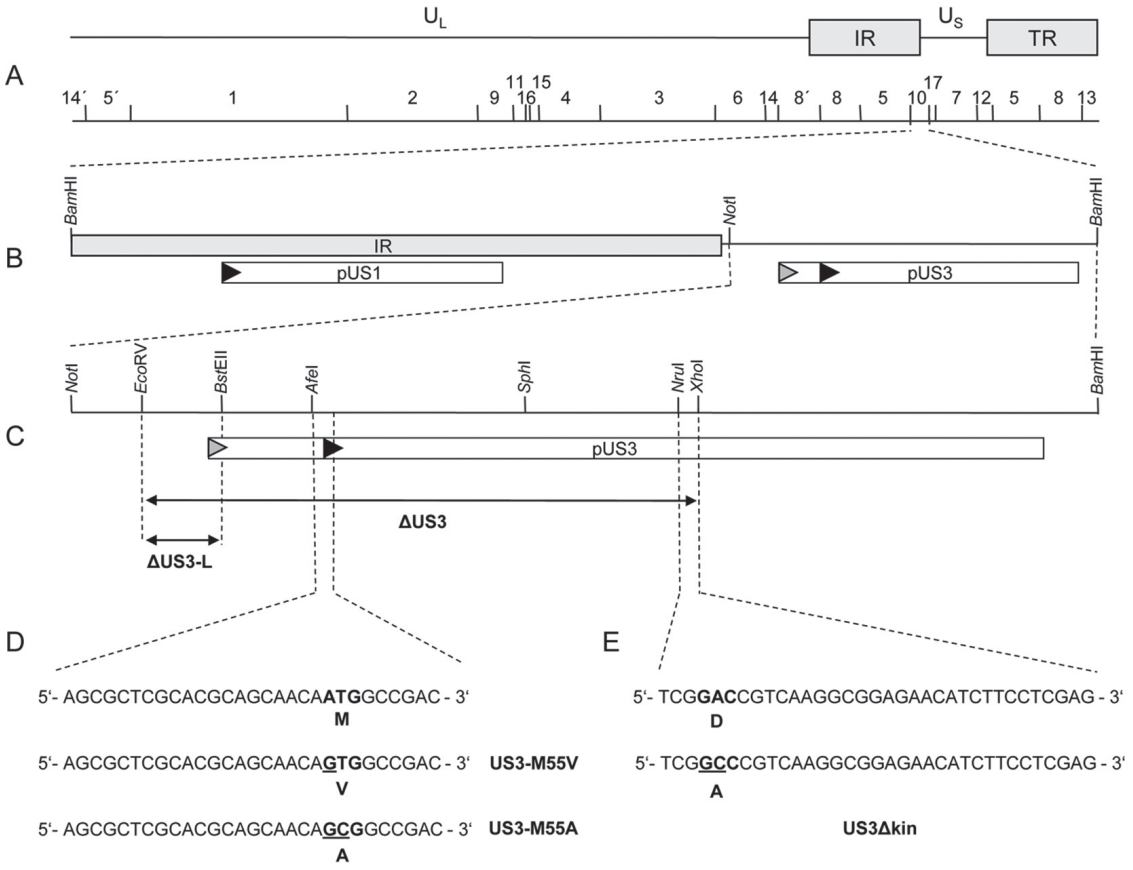

FIG 1 Construction of virus mutants. (A) Schematic map of the PrV genome, which contains of unique long (U_L) and unique short (U_S) regions, as well as internal (IR) and terminal (TR) inverted repeat sequences. The location of BamHI restriction fragments, which are numbered according to their size, is indicated. (B) Enlarged map of BamHI fragment 10. Coding regions for the US1 gene (which is contained within the IR sequences) and the US3 gene are indicated. Arrowheads mark transcriptional start sites and indicate transcriptional orientation. (C) Enlarged diagram of the US3 gene. Transcriptional start sites, as well as the deletion of 5′ regions abolishing expression of pUS3-L in mutant PrV-ΔUS3-L, and relevant restriction sites are indicated. (D and E) Genotypes of the constructed viral mutants. Underlined nucleotides indicate introduced point mutations (corresponding codons are shown in bold).

RESULTS

Generation of US3 mutants. The PrV US3 gene is located on BamHI fragment 10 in the genomic PrV DNA (Fig. 1). For generation of a mutant expressing only the major short isoform of pUS3 (PrV-ΔUS3-L), part of BamHI fragment 10 was deleted, thereby removing the start codon and upstream regulatory sequences of the larger transcript (Fig. 1C). For construction of a mutant expressing only the long isoform, the methionine start codon of pUS3-S was first mutated to valine (PrV-US3-M55V). Since expression of pUS3-S was still detectable, albeit at lower levels (Fig. 2), a second mutant was constructed in which the methionine codon was substituted by an alanine codon (PrV-US3-M55A) (Fig. 1D). To isolate a mutant virus with impaired kinase activity, the conserved aspartic acid in the catalytic center of the kinase domain (1) was substituted by alanine (PrV-US3Δkin) (Fig. 1E), as described earlier (13, 35).

Identification of the pUS3 isoforms in mutant viruses. For identification of the US3 gene products in the generated mutants, immunoblot analyses with lysates of PrV-ΔUS3-L, PrV-US3-M55A, PrV-US3-M55V, and PrV-US3Δkin and respective revertant virus-infected rabbit kidney (RK13) cells (Fig. 2) were performed. Blots were probed with the monospecific rabbit anti-pUS3 serum and with anti-pUL34 or anti-pUL38 serum as loading controls. As reported earlier (27), two proteins of approximately 41 kDa (pUS3-S) and 53 kDa (pUS3-L) were detected in lysates of cells infected with wild-type PrV (strain Kaplan, PrV-Ka), while no specific signal was present in cells infected with PrV-ΔUS3. Only the 41-kDa pUS3-S was detectable in PrV-ΔUS3-L-infected cells. In PrV-US3-M55V-infected cell lysates, both pUS3 species were still present, although

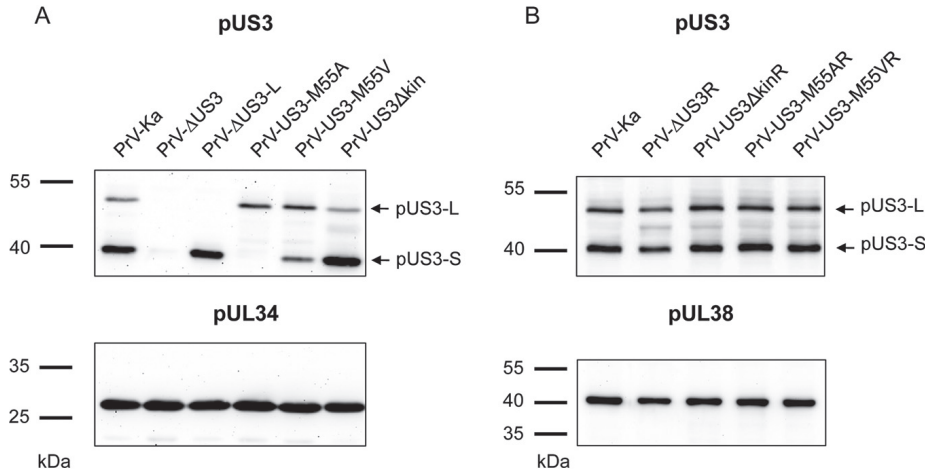

FIG 2 Immunoblots of mutant viruses. Lysates of RK13 cells infected with PrV-Ka, PrV-ΔUS3, PrV-ΔUS3-L, PrV-US3-M55A, PrV-US3-M55V, PrV-US3Δkin (A), and respective revertant viruses (B) were separated by electrophoresis in sodium dodecyl sulfate (SDS)-10% polyacrylamide gels. Thereafter, proteins were transferred to nitrocellulose filters and probed with antisera against pUS3 (upper panels), pUL34 (lower left) or pUL38 (lower right). Locations of the different proteins are indicated. Molecular masses of marker proteins are given on the left.

pUS3-S showed a weaker signal compared to that of PrV-Ka infected cell lysates, indicating that the exchange of methionine by valine led to a significantly decreased expression rate. In contrast, in lysates of PrV-US3-M55A-infected cells only the 53-kDa pUS3-L could be detected. In PrV-US3Δkin-infected cells, both isoforms of pUS3 were present in amounts comparable to that in PrV-Ka-infected cells. pUS3-S and pUS3-L were detectable in all revertant viruses (Fig. 2).

Localization of pUS3 isoforms in infected cells. Different localization patterns for the large and small PrV pUS3 isoforms have been reported in transfected cells (1). To test whether this is also evident during infection, RK13 and Madin-Darby bovine kidney (MDBK) cells were infected with the different mutants, as well as with wild-type PrV-Ka, and processed for confocal microscopy 17 hours postinfection (hpi) (Fig. 3). In PrV-Ka-infected cells, a diffuse pUS3-specific staining was detectable in the cytoplasm and in the nucleus. A comparable pattern was evident in cells infected with PrV-ΔUS3-L and PrV-US3-M55V, although expression levels were reduced in the latter (Fig. 2). In cells infected with PrV-US3-M55A, predominantly weak cytoplasmic staining was found. Reduced nuclear staining was also evident in PrV-US3Δkin-infected RK13 cells. Comparable results were obtained on MDBK cells (data not shown).

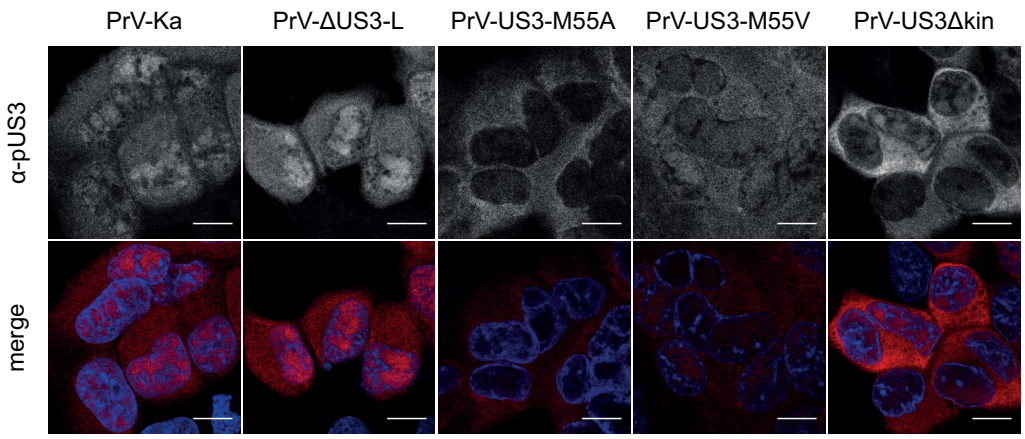

FIG 3 Localization of pUS3 in infected RK13 cells. RK13 cells infected with PrV-Ka, PrV-ΔUS3-L, PrV-US3-M55A, PrV-US3-M55V, and PrV-US3Δkin were stained 17 hpi with anti-pUS3 rabbit serum and Alexa Fluor 555 coupled secondary antibodies. Intensity levels were adjusted to better visualize cellular localization of pUS3-M55A and pUS3-M55V. DNA was visualized with 4′,6-diamidino-2-phenylindole (DAPI). Bar, 10 μm.

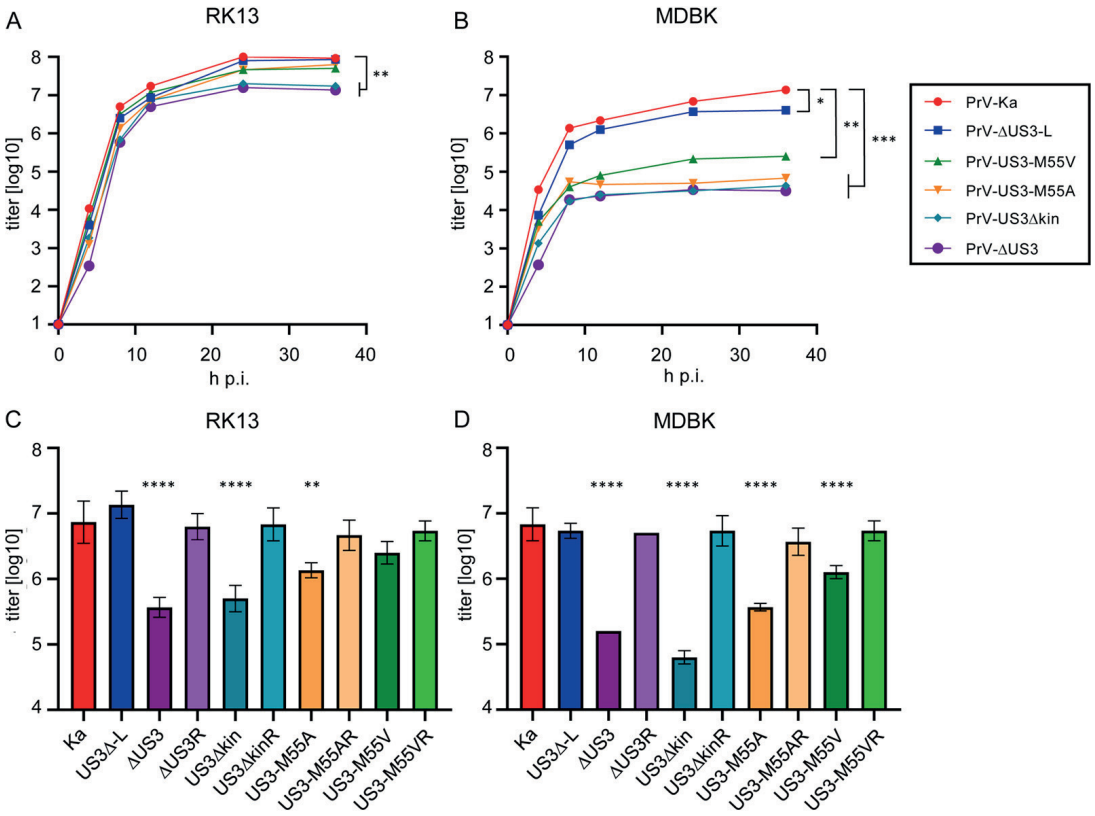

FIG 4 *In vitro* characterization of mutant viruses and revertants. (A) RK13 or (B) MDBK cells were infected with PrV-Ka and the respective mutants at a multiplicity of infection (MOI) of 10 and harvested at the indicated times after infection. (C and D) Comparative multistep growth (MOI = 0.03) of virus mutants and revertants 24 hpi on RK13 (C) or MDBK cells (D). Progeny viruses were titrated on RK13 cells. Average values and standard deviations from three independent experiments are shown. Asterisks indicate statistically significant differences of titers of virus mutants or revertants compared to that of PrV-Ka.

***In vitro* replication of PrV US3 mutants.** In single-step growth kinetics on RK13 cells (Fig. 4A) PrV-ΔUS3 replicated to 5- to 10-fold reduced titers, as reported earlier (10). Mutants expressing only the short or long isoform (PrV-ΔUS3-L and PrV-US3-M55A), as well as the mutant with reduced pUS3-S expression (PrV-US3-M55V), showed no significant difference in virus titers compared to that of PrV-Ka (Fig. 4A). PrV-US3Δkin replicated comparably to PrV-ΔUS3, indicating that the kinase activity is necessary for full replication competence in RK13 cells.

None of the tested mutants showed significant differences in one-step growth kinetics in Vero or embryonic pig kidney (SPEV) cells (data not shown). In contrast, all mutants with impaired expression of pUS3-S showed a severe defect on MDBK cells (Fig. 4B). Here, approximately 100-fold lower titers were found 8 to 36 h after infection with PrV-ΔUS3, PrV-US3-M55A, or PrV-US3Δkin. An intermediate phenotype was observed for PrV-US3-M55V that expressed reduced levels of pUS3-S with approximately 30-fold-lower final titers. Absence of pUS3-L (PrV-ΔUS3-L) resulted in final titers comparable to that of PrV-Ka in RK13 cells. However, a slight effect was observed in MDBK cells 36 hpi. Results from multistep growth kinetics analysis on RK13 (Fig. 4C) and MDBK (Fig. 4D) cells harvested 24 hpi generally confirmed the results from the one-step growth kinetics analysis, but differences became more obvious (Fig. 4C, D). All revertant viruses replicated to titers comparable to that of PrV-Ka.

Effect of PrV pUS3 on syncytium formation. Since PrV-ΔUS3 had been observed to produce syncytial plaques, we analyzed by immunofluorescence RK13 cells that were infected under plaque assay conditions with PrV-Ka, PrV-ΔUS3, PrV-ΔUS3-L, PrV-US3-M55A, PrV-US3-M55V, or PrV-US3Δkin. Whereas PrV-ΔUS3 and PrV-US3Δkin formed large syncytial plaques, this effect was less pronounced after infection with PrV-US3-

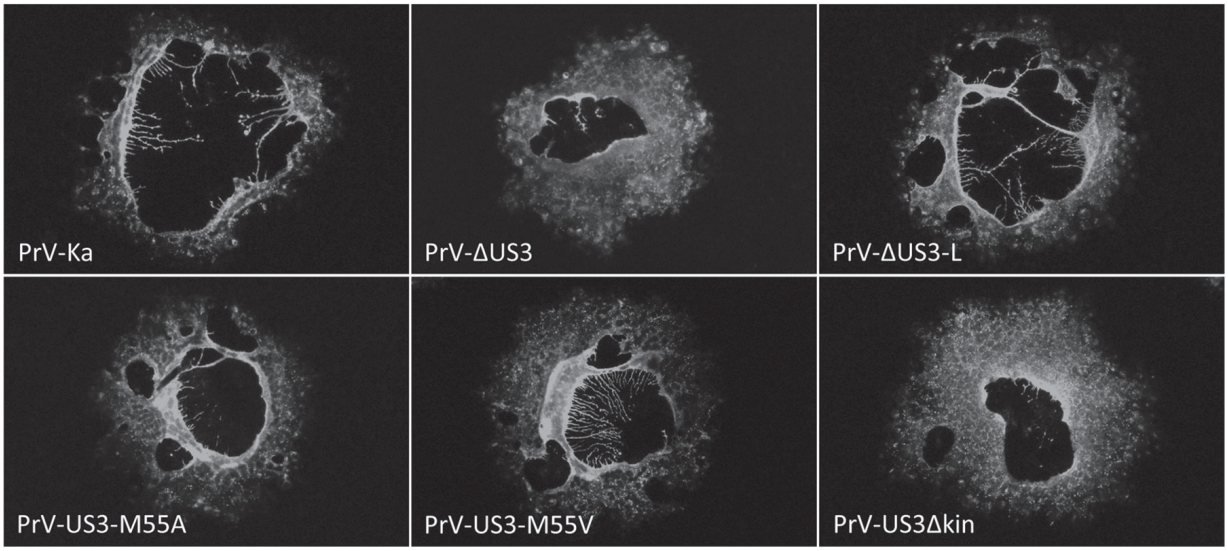

FIG 5 Plaque phenotype of virus mutants. RK13 cells were infected with PrV-Ka, PrV-ΔUS3, PrV-ΔUS3-L, PrV-US3-M55A, PrV-US3-M55V, and PrV-US3Δkin. Cells were fixed 24 hpi with ethanol and stained with a rabbit polyclonal antibody against glycoprotein B. Alexa Fluor 488 secondary antibody was used to visualize plaques.

M55A and PrV-US3-M55V. Plaques of PrV-ΔUS3-L were comparable to those of PrV-Ka (Fig. 5).

Effect of PrV pUS3 isoforms on nuclear egress. To examine the effects of the mutations on nuclear egress, RK13 and MDBK cells were infected with PrV-Ka, PrV-ΔUS3, PrV-ΔUS3-L, PrV-US3-M55A, PrV-US3-M55V, or PrV-US3Δkin and processed for electron microscopy 14 (RK13) or 17 hpi (MDBK). As reported previously, deletion of US3 resulted in accumulations of primary enveloped virus particles in the PNS within invaginations of the INM (Fig. 6B) (10). No or only few primary enveloped virus particles were found in RK13 cells infected with PrV-ΔUS3-L (Fig. 6C). Accumulations indistinguishable from those present in PrV-ΔUS3 infected cells, however, were found after infection with PrV-US3-M55A (Fig. 6D), PrV-US3-M55V (Fig. 6E), or PrV-US3Δkin (Fig. 6F). In MDBK cells (Fig. 7), similar accumulations were found in all mutants lacking active pUS3-S, despite the more drastically reduced virus titers compared to those in RK13 cells. Deletion of pUS3-L did not result in any obvious impairment in nuclear egress or virion formation (Fig. 7C).

DISCUSSION

HSV-1 and PrV pUS3 protein kinases exist in two isoforms, which are translated from two distinct transcripts. Both isoforms share the carboxyterminal sequences but differ in the aminoterminal part. So far, functions of the different isoforms have been tested in transient assays, but studies on the relevance of the different forms during infection is still incomplete (reviewed in reference 1).

We show here that, in PrV, absence of the large isoform, here designated pUS3-L, does not influence nuclear egress, whereas reduced expression, complete deletion, or inactivation of the kinase function of the small isoform, pUS3-S, resulted in accumulations of primary enveloped virions in the perinuclear cleft. This is in contrast to recent findings in HSV-1, in which only the larger isoform, pUS3, but not the smaller pUS3.5, has been shown to participate in nuclear egress (36). However, in both viruses, kinase activity of pUS3 is apparently critical for its role in nuclear egress.

To isolate a virus mutant that only expresses pUS3-S, the promoter sequences and the initiation codon for pUS3-L were deleted. As expected, this resulted in the abolishment of pUS3-L expression. The corresponding mutant PrV-ΔUS3-L replicated comparably to PrV-Ka in all cell lines tested, with no obvious impairments in nuclear egress or virion morphogenesis. Despite deletions of upstream sequences, expression of

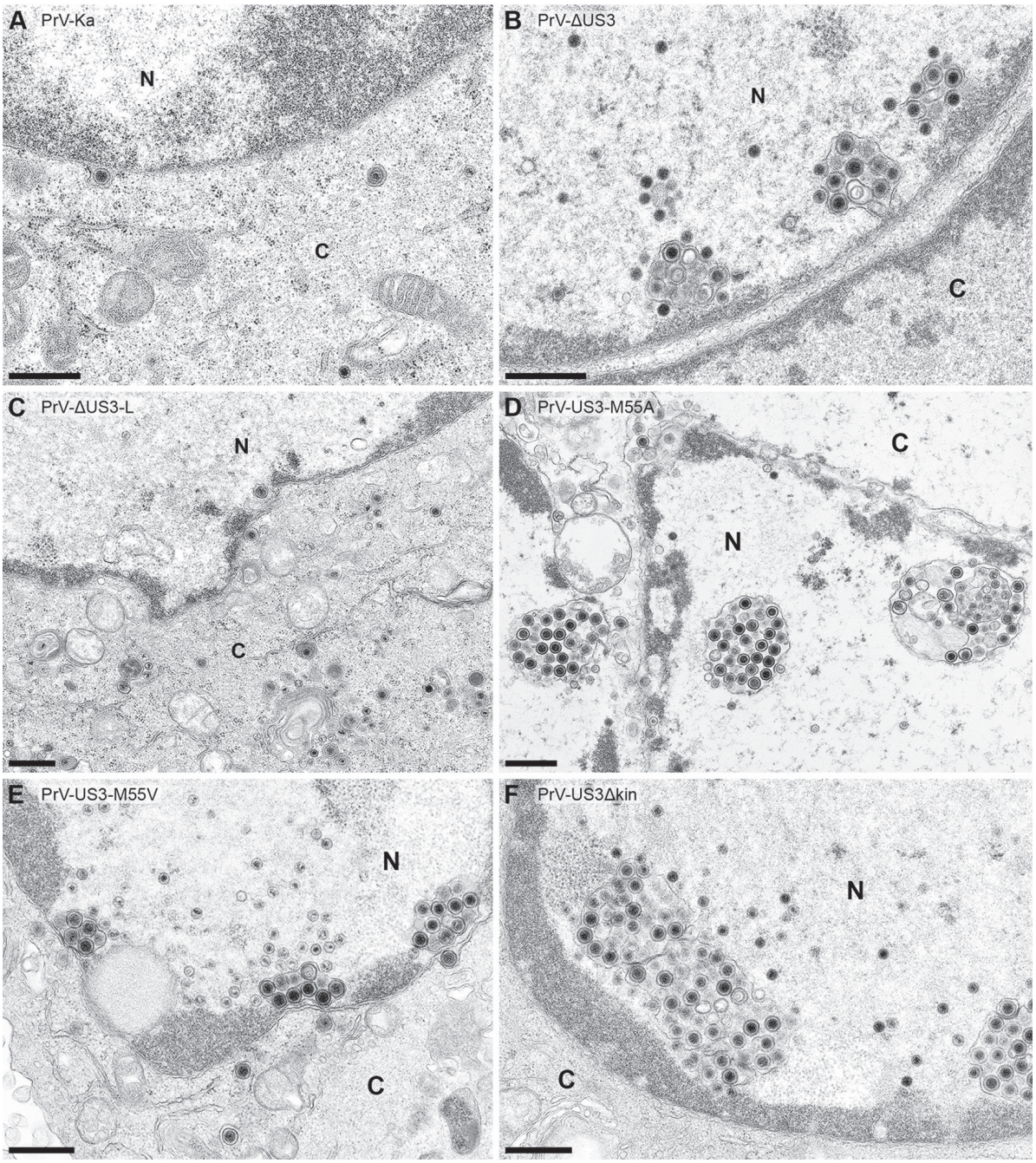

FIG 6 Electron microscopy of RK13 cells infected with PrV-Ka and US3 mutants. Cells were infected for 14 h at an MOI of 1 with PrV-Ka (A), PrV-ΔUS3 (B), PrV-ΔUS3-L (C), PrV-US3-M55A (D), PrV-US3-M55V (E), and PrV-US3Δkin (F). Bar, 600 nm. Nucleus (N) and cytoplasm (C) are indicated.

pUS3-S was not affected, and the staining pattern for pUS3 was comparable to the localization in PrV-Ka-infected cells.

When the initiation methionine of pUS3-S was altered to valine, expression of the smaller isoform was significantly reduced but not completely abolished. This was only achieved by changing the methionine codon to alanine. It has previously been shown that valine can substitute for methionine in mediating translation initiation, but only at 3 to 5% of the activity of methionine (37), which fits our observations. Thus, by isolating the corresponding virus mutant, we were also able to analyze the impact of the relative amount of the pUS3-S isoform.

The differences in the expression level were also obvious in immunofluorescence studies showing reduced pUS3-specific staining in cells infected with PrV-US3-M55V

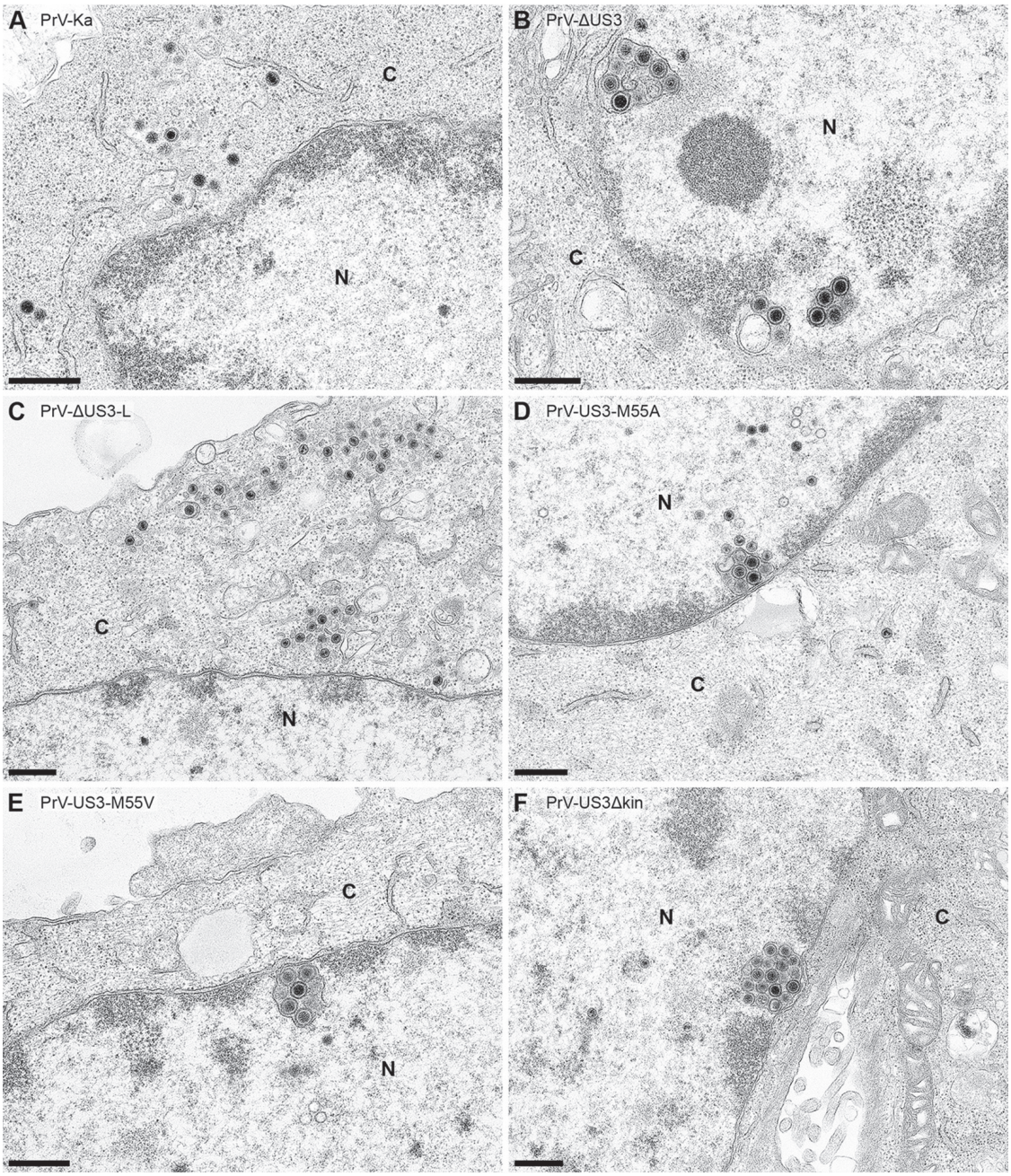

FIG 7 Electron microscopy of MDBK cells infected with PrV-Ka or US3 mutants. Cells were infected for 17 h at an MOI of 1 with PrV-Ka (A), PrV-ΔUS3 (B), PrV-ΔUS3-L (C), PrV-US3-M55A (D), PrV-US3-M55V (E), and PrV-US3Δkin (F). Bar, 500 nm. Nucleus (N) and cytoplasm (C) are indicated.

and PrV-US3-M55A. pUS3-L, the only pUS3 isoform present in PrV-US3-M55A, was found mainly in the cytoplasm. pUS3-L, with a molecular mass of approximately 53 kDa, is beyond the limit that allows passive diffusion into the nucleus through nuclear pore complexes (38). Predominant staining of the plasma membrane for pUS3-L, as reported for cells transfected with plasmids expressing the larger isoform (31), was not evident in PrV-US3-M55A-infected RK13 or MDBK cells. The low expression level of pUS3-L and/or its absence from the nucleus might account for the observed accumulations of primary enveloped virions in infected cells. Nevertheless, it appears that pUS3-L is sufficient to compensate for the cytoplasmic functions of pUS3 in RK13 cells, since titers of PrV-US3-M55A were only slightly reduced compared to those of PrV-Ka. In contrast, in MDBK cells, pUS3-L does not seem to be sufficient for unimpaired virus replication, since virus titers were similar to those produced by mutants completely lacking kinase-active pUS3.

Reduced expression of pUS3-S in the presence of pUS3-L, as in PrV-US3-M55V, resulted in the typical accumulations of primary virions in the PNS, indicating that sufficient nuclear pUS3 is necessary for efficient nuclear egress. A partial complementation of the cytoplasmic pUS3 function was observed in MDBK cells in which low levels of pUS3-S were present. Here, titers were only approximately 30-fold lower than those of PrV-Ka, compared to the 100-fold difference when pUS3 was absent.

In summary, the defect in nuclear egress resulting in accumulations of primary virions in the PNS does not obviously account for the observed titer reductions. It appears more likely that the cytoplasmic functions of pUS3 are responsible for the observed viral titer reduction. In HSV-1, pUS3 phosphorylates the cytoplasmic tail of glycoprotein gB, and mutation of the corresponding phosphorylation site was shown to impair efficient nuclear egress (24) but also resulted in enhanced cell surface expression of gB (39). However, the predicted phosphorylation site in the cytoplasmic tail of HSV-1 gB is not conserved in PrV gB and, consequently, we did not find an indication for PrV gB phosphorylation by pUS3.

The kinase-dead pUS3Δkin was found in the cytoplasm and, to a lesser extent, in the nucleus, in contrast to a kinase-dead mutant reported for HSV-2, which was absent from the nuclear compartment (40). Although PrV pUS3Δkin showed reduced nuclear staining, it is reasonable to speculate that unimpaired kinase activity and not a certain amount of nuclear pUS3 is required for efficient nuclear egress. Nuclear localization of pUS3 might be mediated by pUS3-dependent (auto)phosphorylation or by modification of another cytoplasmic factor.

It has previously been shown that deletion of PrV US3 resulted in a ca. 5- to 10-fold decrease in viral titers (5, 10). A similar impairment in viral replication was observed with the kinase-dead US3-mutant, indicating that, indeed, kinase activity is required for efficient viral replication. Both mutants, PrV-ΔUS3 and PrV-US3Δkin, showed similar accumulations of primary virions in the perinuclear cleft, demonstrating that the active kinase plays a critical role during nuclear egress of virus particles. However, those accumulations appear not to be responsible for the titer reduction observed in the cell lines tested. As shown in MDBK cells, despite the drastically reduced titers of mutants lacking the kinase-active pUS3-S, accumulations in the PNS were comparable to those found in RK13 cells where viral titers were not reduced (PrV-US3-M55A).

In summary, we show here that unimpaired expression of the catalytically active abundant smaller isoform pUS3-S is required for efficient nuclear egress of PrV. Mutation of the kinase domain, as well as abolishment or reduction of expression of pUS3-S, resulted in the accumulation of primary enveloped virions in the perinuclear space. However, these accumulations are not responsible for the observed titer reduction. Further studies are needed to pinpoint the cytoplasmic substrates of pUS3, which are required for full replication competence. In contrast to MDBK cells, viral replication in RK13, Vero, and SPEV cells is obviously less dependent on pUS3, indicating that cellular kinases might partially complement pUS3 function. It will be interesting to unravel the differences in cellular protein kinase activity in MDBK cells compared to that in other cell lines tested.

MATERIALS AND METHODS

Viruses and cells. All PrV mutants were derived from the laboratory strain Kaplan (PrV-Ka) (41). Viruses were grown on rabbit kidney (RK13) or Madin-Darby bovine kidney (MDBK) cells in Eagle's minimum essential medium supplemented with 10% fetal calf serum. Construction and characterization of the US3 deletion mutant PrV-ΔUS3 was described previously (10).

Plasmids. To generate a recombination plasmid for isolation of mutant PrV-ΔUS3-L, expressing only the short isoform of pUS3 (pUS3-S) (27), plasmid pBamHI-10, which contains the cloned BamHI fragment 10 of the PrV-Ka genome (Fig. 1), was cleaved with EcoRV and BstEII (Fig. 1C). After generation of blunt ends by treatment with Klenow polymerase, the plasmid was religated, thereby deleting the start codon, as well as the predicted TATA box, for the long isoform of pUS3 (27). For construction of recombination plasmids for mutants PrV-US3-M55V and PrV-US3-M55A in which the initiation methionine for pUS3-S was substituted either by a valine or an alanine codon, the region around the US3-S start codon was amplified by PCR using *Pfx* DNA polymerase (Invitrogen) with primers mutUS3k_for or mutUS3k_for2 and mutUS3k_rev (Table 1) on cloned BamHI fragment 10 as the template. Primer mutUS3k_for contains a single nucleotide mismatch, which results in the alteration of the methionine start codon to a valine

TABLE 1 Primers used for mutagenesis

Primer	Sequence[a]	Nucleotide position[b]	Mutant
mutUS3k_for	5′-CCAGCGCTCGCACGCAGCAACA**G**TGG-3′	118310–118335	PrV-US3-M55V
mutUS3k_for2	5′-AGCGCTCGCACGCAGCAACA**GC**GGCCG-3′	118312–118338	PrV-US3-M55A
mutUS3k_rev	5′-GCAGCAGCATGCCCTCCATCAGCGTCG-3′	118620–118594	PrV-US3-M55A/V
US3dk_for	5′-CG**C**CGTCAAGGCGGAGAACATCTTCC-3′	118829–118854	PrV-US3Δkin
US3dk_rev	5′-TCGAGGAAGATGTTCTCCGCCTTGACG**G**CG-3′	118858–118829	PrV-US3Δkin

[a]Introduced mutations are shown in bold.
[b]In GenBank accession number BK001744.1 (47).

codon (ATG → GTG). MutUS3k_for2 carries two nucleotide exchanges that lead to a substitution of the methionine codon by an alanine codon (ATG → GCG) (Fig. 1C). The PCR products were cleaved with AfeI and SphI and cloned into appropriately cleaved plasmid pBamHI-10. Mutant PrV-US3Δkin was constructed after hybridization of partially complementary oligomers US3dk_for and US3dk_rev (Table 1), comprising mismatches leading to the exchange of aspartic acid (amino acid [aa] 221 of pUS3-L and aa 167 of pUS3-S, respectively) in the catalytic center of the kinase domain by alanine (Fig. 1E). After annealing, the oligomers formed one blunt end and a 5′ overhang compatible with an XhoI restriction site. Plasmid pBamHI-10 was then cleaved with XhoI and NruI and ligated with the double-stranded oligomers. The inserted mutation resulted in deletion of the NruI restriction site and, simultaneously, in creation of a BglII site. Correct mutagenesis was verified by restriction enzyme digests and sequencing.

Isolation of US3 mutants and revertant viruses. Recombination plasmids containing the desired mutations were cotransfected with DNA of PrV-ΔUS3 expressing green fluorescent protein (GFP) (10) into RK13 cells by calcium phosphate coprecipitation (42). Plaques lacking green autofluorescence were picked and purified to homogeneity on RK13 cells. DNA of single-plaque isolates was prepared and controlled by restriction enzyme analysis, followed by Southern blotting and sequencing of the corresponding gene region (data not shown). Revertant viruses were generated by cotransfection of a plasmid containing the wild-type BamHI fragment 10 and viral DNA of the respective US3 mutants. Plaques with a wild-type-like phenotype were isolated, and restoration of wild-type sequences and correct US3 expression were verified by sequencing and Western blot analysis.

Western blotting. RK13 and MDBK cells were infected with PrV-Ka, the respective mutants, or revertants at a multiplicity of infection (MOI) of 5. Infected cell lysates were harvested 24 hpi by scraping cells into the medium. Cells were collected by centrifugation at 13,000 rpm for 2 min in an Eppendorf centrifuge. Thereafter, the pellet was washed twice with phosphate-buffered saline (PBS) and resuspended in 100 μl PBS and the same volume of sample buffer (0.13 M Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate [SDS], 20% glycerin; 0,01% bromophenol blue, and 10% β-mercaptoethanol). Cell lysate (15 μl) was separated by electrophoresis in SDS-10% polyacrylamide gels. Proteins were electrotransferred onto nitrocellulose membranes, which were subsequently blocked in 5% skim milk for 30 min. To detect pUS3 isoforms, blots were incubated overnight at room temperature with monospecific rabbit antisera against PrV pUS3 (1:50,000 [10]), pUL34 (1:50,000; [43]) or pUL38 (1:50,000; unpublished) as controls, followed by treatment with a horseradish peroxidase-conjugated secondary antibody (Dianova, Hamburg, Germany) for 1 h. Signals were detected by enhanced chemiluminescence (Clarity ECL Western blot substrate; Bio-Rad) and recorded in an imager (Bio-Rad).

Confocal microscopy. Cells were infected with PrV-Ka or the US3 mutants with approximately 200 PFU in 6-well dishes. At 17 hpi, cells were fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, and incubated with polyclonal rabbit anti-pUS3 (1:500). For detection, Alexa Fluor 555 goat anti-rabbit IgG (Invitrogen) was used. Images were acquired with a confocal laser scanning microscope (SP5; Leica, Germany) and processed using ImageJ (44). 4′,6-diamidino-2-phenylindole (DAPI) was used to visualize cellular DNA.

One-step and multistep growth analysis. RK13, MDBK, Vero, or SPEV cells were infected at an MOI of 10 with PrV-Ka, PrV-ΔUS3, PrV-ΔUS3-L, PrV-US3-M55A, PrV-US3-M55V, or PrV-US3Δkin and incubated for 1 h on ice. The inoculum was then replaced with prewarmed medium, and cells were incubated at 37°C for 1 h, allowing the virus to penetrate. Thereafter, remaining extracellular virus was inactivated by low-pH treatment (45). Cells and supernatant were harvested immediately (0 h) and after 4, 8, 12, 24, and 36 h. In a second experiment, the virus mutants and respective revertants PrV-US3R, PrV-US3-M55AR, PrV-US3-M55VR, and PrV-US3ΔkinR were analyzed accordingly at MOIs of 3 (data not shown) and 0.03 in RK13 and MDBK cells 24 hpi. Virus titers were determined on RK13 cells. Average values and standard deviations from three independent experiments were calculated.

GraphPad Prism was used for statistical analysis. All values of the first experiment were analyzed using an ordinary two-way analysis of variance (ANOVA) followed by corrected Dunnett's multiple-comparison test, by which the mean of each virus mutant and time point was compared to those of PrV-Ka as the control. An ordinary one-way ANOVA and Dunnett's *post hoc* test were performed on data from the second experiment. Statistical significance of $P \leq 0.05$ is indicated by an asterisk (*) in the graphs.

Determination of plaque phenotype. RK13 cells were incubated with virus suspension containing PrV-Ka, PrV-ΔUS3, PrV-ΔUS3-L, PrV-US3-M55A, PrV-US3-M55V, or PrV-US3Δkin. After 1 h, the inoculum was replaced by methylcellulose medium, and cells were incubated at 37°C for another 24 h. Cells were fixed with ethanol and stained with a polyclonal rabbit antiserum against PrV glycoprotein B (1:1,000 [46]) for 1 h at room temperature. Afterwards, a secondary goat anti-rabbit antibody-Alexa Fluor 488 stain (1:1,000) (Invitrogen) was used to visualize plaques.

Electron microscopy. For ultrathin sectioning, RK13 or MDBK cells were infected at an MOI of 1 with PrV-Ka, PrV-ΔUS3, PrV-ΔUS3-L, PrV-US3-M55A, PrV-US3-M55V, or PrV-US3Δkin and fixed at 14 or 17 hpi, respectively. Fixation, dehydration, and epoxy embedding were performed as described previously (43). The counterstained ultrathin sections were analyzed with an electron microscope (EM400T, Technai 12; Philips, Eindhoven, The Netherlands).

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Paper [II]

An improved animal model for Herpes Simplex Encephalitis in humans

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RESEARCH ARTICLE

An improved animal model for herpesvirus encephalitis in humans

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Abstract

Herpesviral encephalitis caused by Herpes Simplex Virus 1 (HSV-1) is one of the most devastating diseases in humans. Patients present with fever, mental status changes or seizures and when untreated, sequelae can be fatal. Herpes Simplex Encephalitis (HSE) is characterized by mainly unilateral necrotizing inflammation effacing the frontal and mesiotemporal lobes with rare involvement of the brainstem. HSV-1 is hypothesized to invade the CNS via the trigeminal or olfactory nerve, but viral tropism and the exact route of infection remain unclear. Several mouse models for HSE have been developed, but they mimic natural infection only inadequately. The porcine alphaherpesvirus Pseudorabies virus (PrV) is closely related to HSV-1 and Varicella Zoster Virus (VZV). While pigs can control productive infection, it is lethal in other susceptible animals associated with severe pruritus leading to automutilation. Here, we describe the first mutant PrV establishing productive infection in mice that the animals are able to control. After intranasal inoculation with a PrV mutant lacking tegument protein pUL21 and pUS3 kinase activity (PrV-ΔUL21/US3Δkin), nearly all mice survived despite extensive infection of the central nervous system. Neuroinvasion mainly occurred along the trigeminal pathway. Whereas trigeminal first and second order neurons and autonomic ganglia were positive early after intranasal infection, PrV-specific antigen was mainly detectable in the frontal, mesiotemporal and parietal lobes at later times, accompanied by a long lasting lymphohistiocytic meningoencephalitis. Despite this extensive infection, mice showed only mild to moderate clinical signs, developed alopecic skin lesions, or remained asymptomatic. Interestingly, most mice exhibited abnormalities in behavior and activity levels including slow movements, akinesia and stargazing. In summary, clinical signs, distribution of viral antigen and inflammatory pattern show striking analogies to human encephalitis caused by HSV-1 or VZV not observed in other animal models of disease.

Author summary

In developed countries, more than 50% of humans are seropositive for the neurotropic Herpes Simplex Virus 1 (HSV-1) and two to four million cases of Herpes simplex

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encephalitis (HSE) are reported per year worldwide. Primary infection with HSV-1 takes place via the skin or the oral mucosa followed by intraaxonal retrograde spread to sensory ganglia of the peripheral nervous system where HSV-1 usually establishes latency. Further spread to the central nervous system results in HSE, a necrotizing encephalitis effacing predominantly the temporal and frontal lobes of the brain. Mice infected with HSV-1 develop encephalitis, but do not show the typical lesions and exhibit high mortality rates. Here we demonstrate that mice infected with a mutant pseudorabies virus lacking the tegument protein pUL21 and an active viral kinase pUS3 were able to survive the productive infection but developed lymphohistiocytic encephalitis with viral antigen distribution, inflammation and associated behavioral changes comparable to HSE in humans. These striking analogies offer new perspectives to study herpesviral encephalitis in a suitable animal model.

Introduction

Herpes Simplex Virus-1 (HSV-1) infection is one of the most frequent causes of necrotizing encephalitis (HSE) in humans [1]. HSE is associated either with primary HSV-1 infection occurring mostly in children and adolescents, or reactivation of latent HSV-1 in adults. Mortality rates up to 70% have been reported when untreated [2–4]. Patients suffering from HSE mostly show fever, headache, lethargy, aphasia, disorientation, behavioral changes and seizures [2, 5]. Sequelae can include neurological deficits and dysfunctions such as behavioral and cognitive abnormalities, memory impairment, as well as seizures [1, 5, 6]. HSE mainly manifests in the frontal and mesiotemporal lobes including the limbic system, usually asymmetrically with relatively rare encephalitis in the brainstem [7]. Histopathologically, HSE is characterized by neuronal necrosis, mononuclear infiltrates and microgliosis [8, 9].

Varicella-Zoster Virus (VZV) is another neurotropic human pathogen, which causes Varicella (chickenpox) in young children after primary infection or herpes zoster (rash, shingles) primarily in elderly individuals [2, 10]. After primary infection, VZV establishes latency in dorsal root ganglia where it can reactivate from, and cause vesicular skin eruptions typically located unilaterally within a dermatome [11]. The rash can migrate to neighboring dermatomes and is associated with pain and extreme itching [10]. VZV can also affect other organs causing severe central nervous system (CNS) manifestations [12], but in contrast to HSV-1, vasculopathy is the key pathologic hallmark [13]. Meningoencephalitis occurs either after Varicella or Herpes Zoster in immunocompetent or immunosuppressed patients, representing as disseminated encephalomyelitis or inflammation with an unspecific distribution pattern [10]. As in HSV-1, clinical signs include alteration in mental status and focal neurological deficits, whereas the development of seizures is rare [14–16].

The herpesviruses HSV-1, VZV as well as Pseudorabies Virus (PrV) belong to the subfamily of the *Alphaherpesvirinae*. PrV is the causative agent of Aujeszky's disease in pigs but infects a variety of mammals ranging from ruminants to carnivores, rodents and lagomorphs. Horses and primates resist infection [17], although evidence for rare human infections have been reported recently [18, 19]. In adult pigs, the virus causes mostly respiratory illness, abortion or even subclinical infection, whereas piglets usually develop severe neurological signs. However, only pigs can survive a productive infection, while all other susceptible animals develop severe neurological signs and extreme pruritus, known as "mad-itch" syndrome, and succumb to death within a short time after infection [17].

In the past, different mechanisms have been discussed how alphaherpesviruses gain access to the CNS in humans [7]. Several mouse models with different age, genetic background,

inoculation routes, and virus strains of variable neurovirulence have been developed to investigate the pathogenesis of viral infections *in vivo* [20]. So far, mostly intranasal or intracerebral infection models have been used to study HSE in mice [21–23], whereas HSV-1 latency is established mainly by corneal scarification [21]. In general, after replication in the nasal mucosa and the trigeminal ganglion, HSV-1 has been detected in the brainstem (BS), cerebellum, thalamus (TH), hippocampus (HIP) or lateral ventricles, thus establishing a more diffuse infection pattern than observed in HSE patients [24]. Although viral antigen was present in the olfactory bulb, the virus did not spread to the temporal and frontal lobes, which are the typical locations in HSE in humans [22, 25]. Thus, the available models mimic HSE only insufficiently limiting experimental studies on the short- as well as long-term consequences of herpesviral infection.

Murine infection models of PrV have shown that the virus enters peripheral sensory neurons through free nerve endings. Via retrograde intraaxonal spread the virus is able to invade the CNS [26]. As shown in Fig 1, after intranasal infection, which mimics a natural route, PrV replicates initially in the respiratory epithelium of the nose (RE) [27–29]. The virus then travels along afferent trigeminal sensory fibers to neurons of the trigeminal ganglion (TG), an invasion route also used by HSV-1 [30]. First order neurons of the TG reach into the CNS through the pons located in the metencephalon (MET). These neurons are synaptically connected to three trigeminal nuclei present in the myelencephalon (spinal trigeminal nucleus, Sp5), MET (principal sensory trigeminal nucleus, Pr5) and mesencephalon (MES) (mesencephalic trigeminal nucleus, Me5). Second order neurons from the trigeminal nuclei project to the ventral posteromedial nucleus of the TH [31] whereas third order neurons originating from TH eventually reach the primary somatosensory cortex (SSC) in the parietal lobe (PL) [32]. For PrV, invasion of the CNS is also facilitated through sympathetic and parasympathetic nerve endings including their autonomic ganglia as well as through the facial nerve [27–29].

Since PrV also invades the CNS of pigs via the olfactory route [33], this alternative way of infection may also occur in mice and humans infected with HSV-1 [2, 7, 34–38], but still requires more in-depth investigation.

Intranasal PrV infection of mice resulted in a fulminant disease with animals succumbing 2–3 days after wild-type virus infection. At this stage, there is an abundance of infected neurons in the TG and trigeminal brainstem nuclei. Infection studies with a series of PrV deletion mutants resulted in extended survival times correlating with virus intrusion into higher areas of the CNS including the cerebral cortex [28, 29]. However, productive infection was always 100% fatal.

In contrast, a PrV mutant simultaneously deleted in genes encoding pUL21 and pUS3 was the only virus in our studies leading to a productive infection in mice, which the animals were able to control. Therefore we investigated in detail neuroinvasion of this virus as well as the inflammatory response to infection after construction of a novel mutant virus, PrV-ΔUL21/US3Δkin, which lacked the UL21 gene but contained only a specific mutation in pUS3 inactivating its kinase function.

The mean time to death (MTD), the clinical phenotype as well as kinetics of viral spread and inflammatory responses were compared to wildtype PrV strain Kaplan (PrV-Ka) and single mutant virus infected mice.

Results

In vitro replication properties

Replication of PrV-ΔUL21/US3Δkin was analyzed in rabbit kidney (RK13) cells and compared to infection with PrV wildtype strain Kaplan (PrV-Ka) and the single mutants PrV-ΔUL21

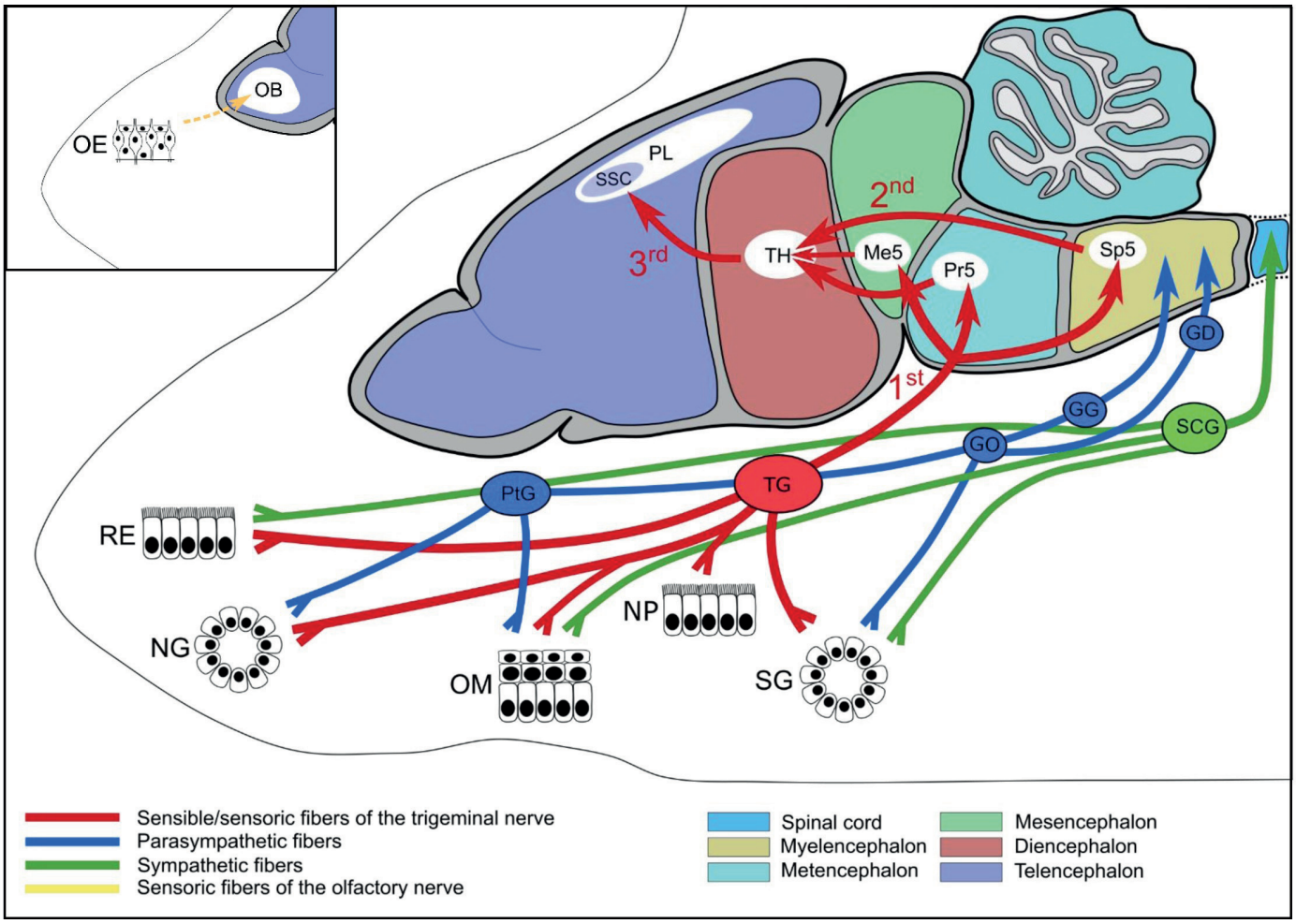

Fig 1. Schematic illustration of viral spread of PrV in mice after intranasal infection. After initial replication in the respiratory epithelium (RE) PrV primarily invades sensible/sensoric fibers of the trigeminal nerve (red) and is transported via 1st order neurons to trigeminal nuclei (Sp5, Pr5, Me5). From there, 2nd order neurons project to the thalamus (TH). Eventually, thalamic fibers end up as third order neurons in the somatosensory cortex (SSC) located in the parietal lobe (PL). PrV does also infect the nasal glands (NG), oral mucosa (OM), nasopharynx (NP), and salivary glands (SG) which are all innervated by the trigeminal nerve. Viral spread further occurs via sympathetic (green) or parasympathetic fibers (blue) and the corresponding autonomic ganglia such as the superior cervical ganglion (SCG) and pterygopalatine ganglion (PtG), otic ganglion (GO), geniculate ganglion (GG), and distal ganglion (GD), respectively. Although not yet reported in mice, herpesviral infection may be facilitated through the olfactory epithelium (OE) and the olfactory nerve (yellow) to finally infect the olfactory bulb (OB) (insert).

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[39] and PrV-US3Δkin [40]. Cells were infected at a MOI of 3, and harvested after 24 h. Progeny virus titers were determined on RK13 cells. As shown in Fig 2A, titers of the single mutants were approx. 5- to 10-fold lower compared to PrV-Ka as described earlier [39, 40], while PrV-ΔUL21/US3Δkin showed a more drastic effect with a 50- to 100-fold titer reduction.

Plaque diameters as a measure for cell-to-cell spread capability were determined 48 h after infection (Fig 2B). Plaque diameters reached by PrV-Ka were set as 100% and values of the single and double mutants were calculated accordingly. PrV-US3Δkin showed only slightly reduced plaque sizes as was shown for mutants lacking pUS3 completely (PrV-ΔUS3; [40] while PrV-ΔUL21 reached only 60% plaque size [39]). Only 50% plaque diameter was reached with PrV-ΔUL21/US3Δkin pointing to an additive effect of both mutations. However, despite the effects on viral replication and plaque sizes, PrV-ΔUL21/US3Δkin showed productive replication.

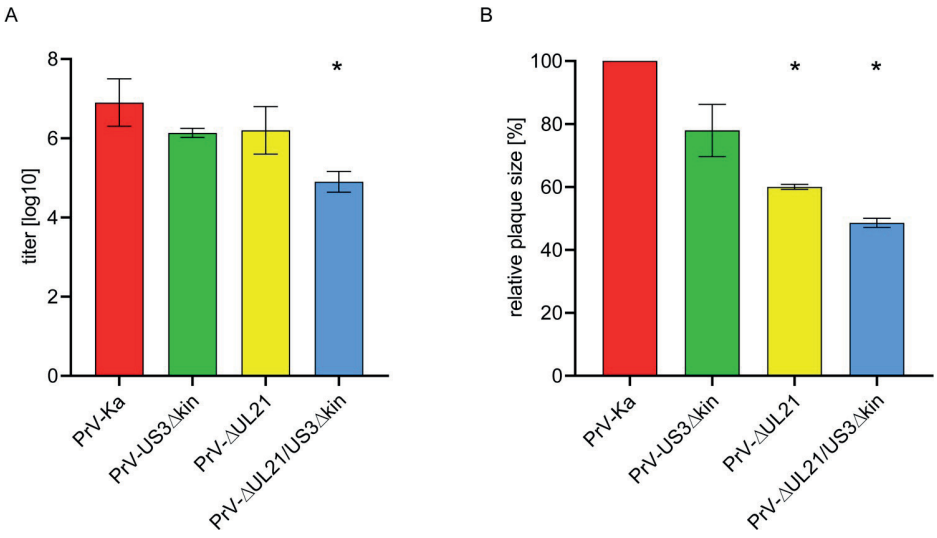

Fig 2. In vitro growth properties and cell-to-cell spread of mutant viruses. (A) RK13 cells were infected with PrV-Ka and the respective virus mutants at a MOI of 3, and harvested 24 h p.i. Plaque assays performed on RK13 cells were used to determine the titer of virus progeny. (B) Plaque diameters of the different virus mutants were assessed on RK13 cells. 20 plaques each per virus mutant were measured and compared to plaque diameter of PrV-Ka set as 100%. Average values and standard deviations of three independent experiments are shown. Significant differences of virus mutants compared to PrV-Ka are indicated by an asterisk (*).

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Mean time to death

To assess the MTD mice were infected intranasally with 1 x 10^4 PFU of PrV-ΔUL21/US3Δkin (n = 6) or PrV-Ka (n = 4), PrV-US3Δkin (n = 4), PrV-ΔUL21 (n = 4), and supernatant of non-infected cells (n = 2) as control. The animals were monitored daily, scored according to the scale given in S1 Table, and euthanized when the humane endpoint was reached. Relative survival rates are shown in Fig 3. On average, mice infected with PrV-Ka and PrV-US3Δkin reached the humane endpoint at 62 h p.i. and 63 h p.i., respectively (S2 Table). With 110 h, the MTD of mice infected with PrV-ΔUL21 was almost twice as long as in PrV-Ka-infected animals, but all animals succumbed to the infection. Mice inoculated with PrV-ΔUL21/US3Δkin, however, survived until the end of the experiment (450 h p.i.), except for one animal which had to be euthanized at 239 h p.i. (Fig 3; S2 Table).

Clinical evaluation

First clinical signs appeared at 47 and 51 h p.i. in PrV-Ka and PrV-US3Δkin-infected mice, respectively (S2 Table). PrV-ΔUL21-infected animals showed a delay in the onset of clinical signs starting 96 h p.i., and clinical signs developed slower but similar to PrV-Ka infection e.g. ruffled fur, hunched posture and mild pruritus. Subsequently, clinical signs aggravated and included mainly unilateral conjunctivitis, nasal bridge edema and multiple facial hemorrhagic skin erosions and ulcerations due to excessive pruritus and beginning auto mutilation. Mice were apathetic and showed phases of intermittent hyperactivity. In the final stage, some animals became dyspneic. These observations reflect previous data [28, 29], although mice in general survived slightly longer in the present study.

After infection with PrV-ΔUL21/US3Δkin, mice began to develop first clinical signs at 152 h p.i. on average. The animals showed mild pruritus starting at the head, but spreading to the whole body in the following days, particularly to the flank. This was further accompanied by

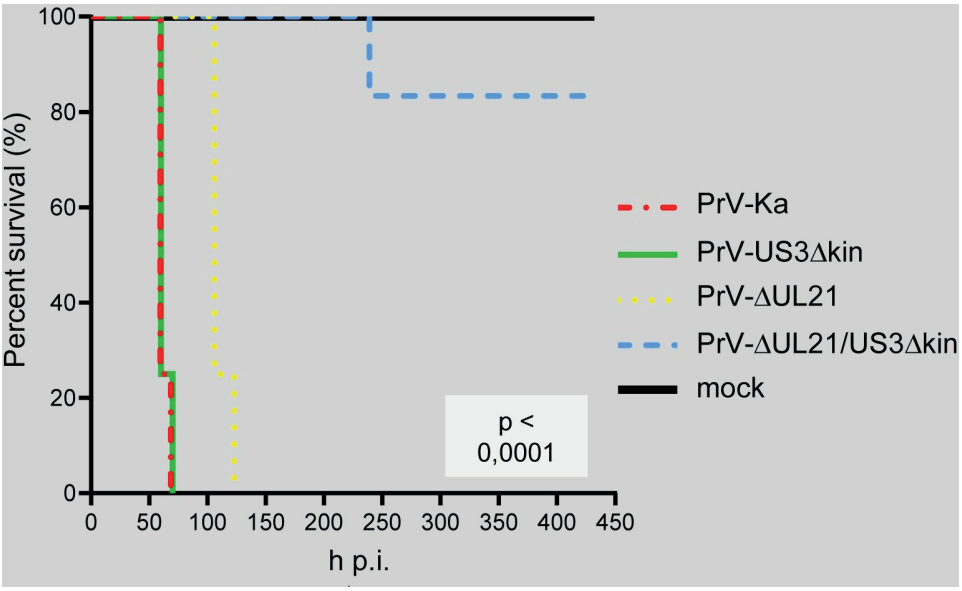

Fig 3. Kaplan-Meier curve showing the relative survival rates of mice after inoculation with PrV-Ka and mutants. Mice (PrV-Ka, n = 4; PrV-US3Δkin, n = 4; PrV-ΔUL21, n = 4; PrV-ΔUL21/US3Δkin, n = 6) were inoculated intranasally with a total of 10μl virus suspension containing $1x10^4$ PFU. The animals were daily monitored and sacrificed in the moribund stage. In contrast to mice infected with PrV-Ka, PrV-US3Δkin and PrV-ΔUL21, PrV-ΔUL21/US3Δkin-infected animals survived the infection except for one animal that had to be euthanized at 239 h p.i. The survival rates differ significantly (log-rank test, $p < 0,0001$) between the groups.

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phases of decelerated movements, retarded reactions to external stimuli, and reduced activity levels followed by phases of normal behavior. Moreover, signs of photophobia were present in individual animals. Of note, clinical signs reached their climax between 216 and 312 h after infection. As mentioned earlier, within this time one mouse had to be euthanized due to severe itching and bad condition. All other mice showed only mild to moderate clinical signs such as hunching, ruffled fur or even mild pruritus, until they recovered and survived the infection. Unlike PrV-Ka and single mutant infections, hemorrhagic erosions never appeared (S2 Table).

Kinetics of neuroinvasion

To investigate the clinical phenotype as well as the kinetics of viral neuroinvasion and inflammatory reaction in more detail, three mice each were inoculated with the respective viruses and sacrificed at different time points after infection (Fig 5). As negative controls, mock-infected mice were euthanized after 24, 168, 312 and 504 h.

Clinical phenotype. Prevalence and distribution of clinical scores of PrV-ΔUL21/US3Δkin infected animals compared to PrV-Ka and single mutant infected mice are presented in Fig 4. As observed previously, in PrV-Ka and PrV-US3Δkin infected animals clinical signs drastically increased very early after infection, although with a delay in PrV-ΔUL21 infected mice. Most animals showed severe clinical signs at the endpoint (Fig 4). In contrast, PrV-ΔUL21/US3Δkin-infected mice developed first mild clinical signs starting 144 h p.i. with a subsequent increase of the number of sick animals (Fig 4D). Most mice showed only moderate clinical signs including slowed movements and/or reduced reactions to external stimuli up to total inactivity with periods of akinesia and stargazing. Foci of alopecia mainly in the face, but also in other body regions, e.g. the neck, limbs, abdomen, back, flank and tail root occurred. Clinical signs reached their climax between 192 and 360 h p.i. Within this time period, some

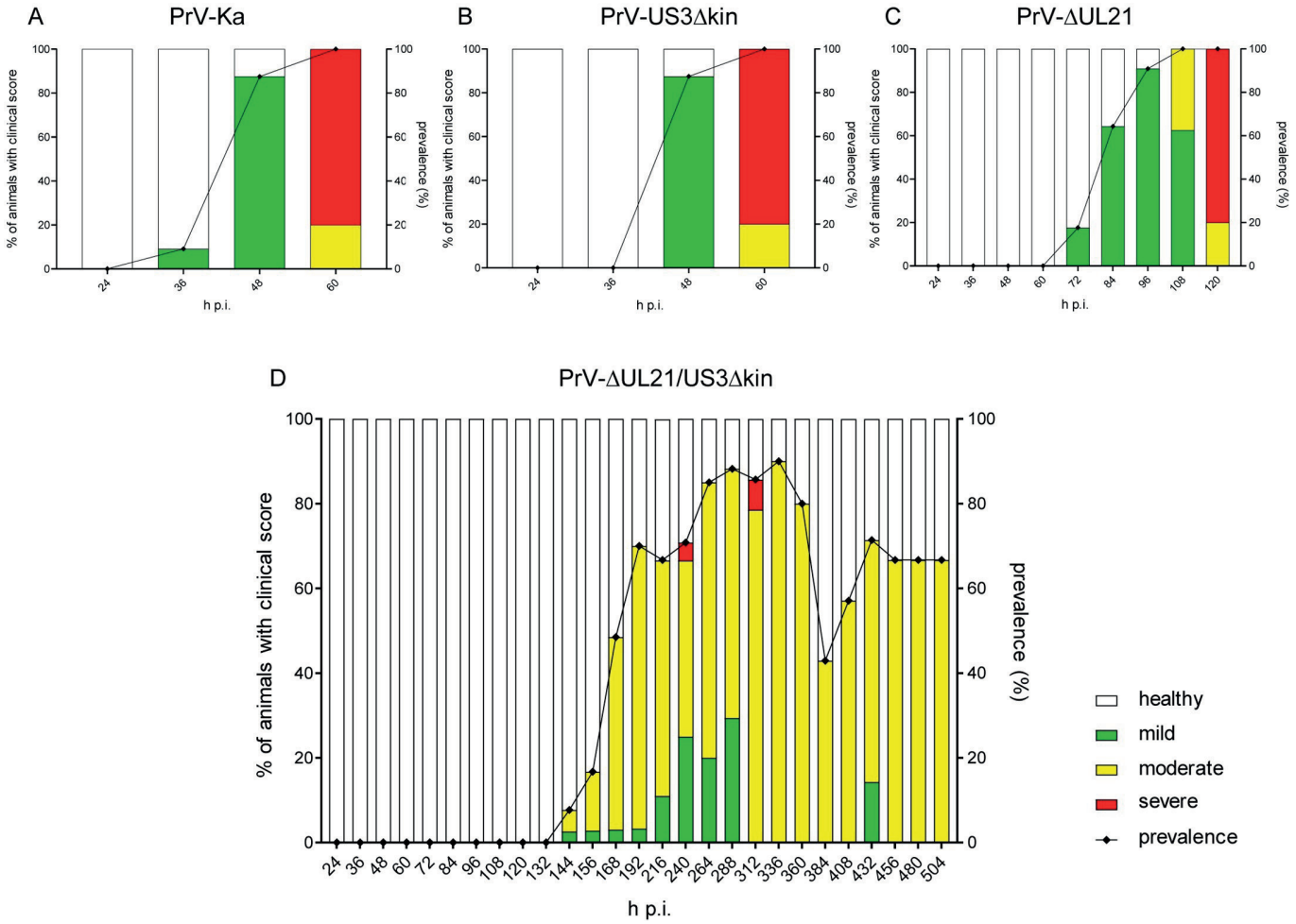

Fig 4. Prevalence of diseased animals after infection with PrV-Ka, PrV-US3Δkin, PrV-ΔUL21 and PrV-ΔUL21/US3Δkin in the kinetic trial. Starting 24 h p.i. at least 3 mice per indicated time point were sacrificed and submitted for pathohistological investigation. The frequency of sick animals infected with PrV-Ka (A), PrV-US3Δkin (B), PrV-ΔUL21 (C) or PrV-ΔUL21/US3Δkin (D) per time is indicated by bars. Based on a scoring system (S1 Table) animals were categorized into either mildly (green), moderately (yellow) or severely affected (red). Prevalence is given by the black line on the right Y-axis.

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animals were moderately or even severely affected. One mouse had to be euthanized 240 h p.i. and another one died unexpectedly at 301 h p.i., but the other animals survived beyond this point. Thus, the period between 216 and 336 h p.i. is the critical phase of PrV-ΔUL21/US3Δkin-infection, where the animals either exhibit only mild to moderate clinical signs leading to survival, or their condition deteriorated to a level requiring euthanasia or resulting in death.

Neuroinvasion and kinetics of viral spread. To track viral infection of the brain, 16 head sections per animal were analyzed by immunohistochemistry using an anti-PrV glycoprotein B (α-gB) serum [109].

In animals infected with PrV-Ka, viral antigen was first detected at 36 h p.i., and significantly increased from 48 to 60 h p.i. (Fig 5A). As shown in Fig 6 TG, second order neurons (Sp5), autonomic ganglia, nasal and oral epithelium and the medullary formatio reticularis (FR) with rostroventrolateral reticular nucleus (RVL) showed massive antigen staining. Compared to PrV-Ka, PrV-US3Δkin infection showed a slightly delayed spread but with comparable distribution of PrV antigen. At the final stage of PrV-Ka and PrV-US3Δkin infection (60 h

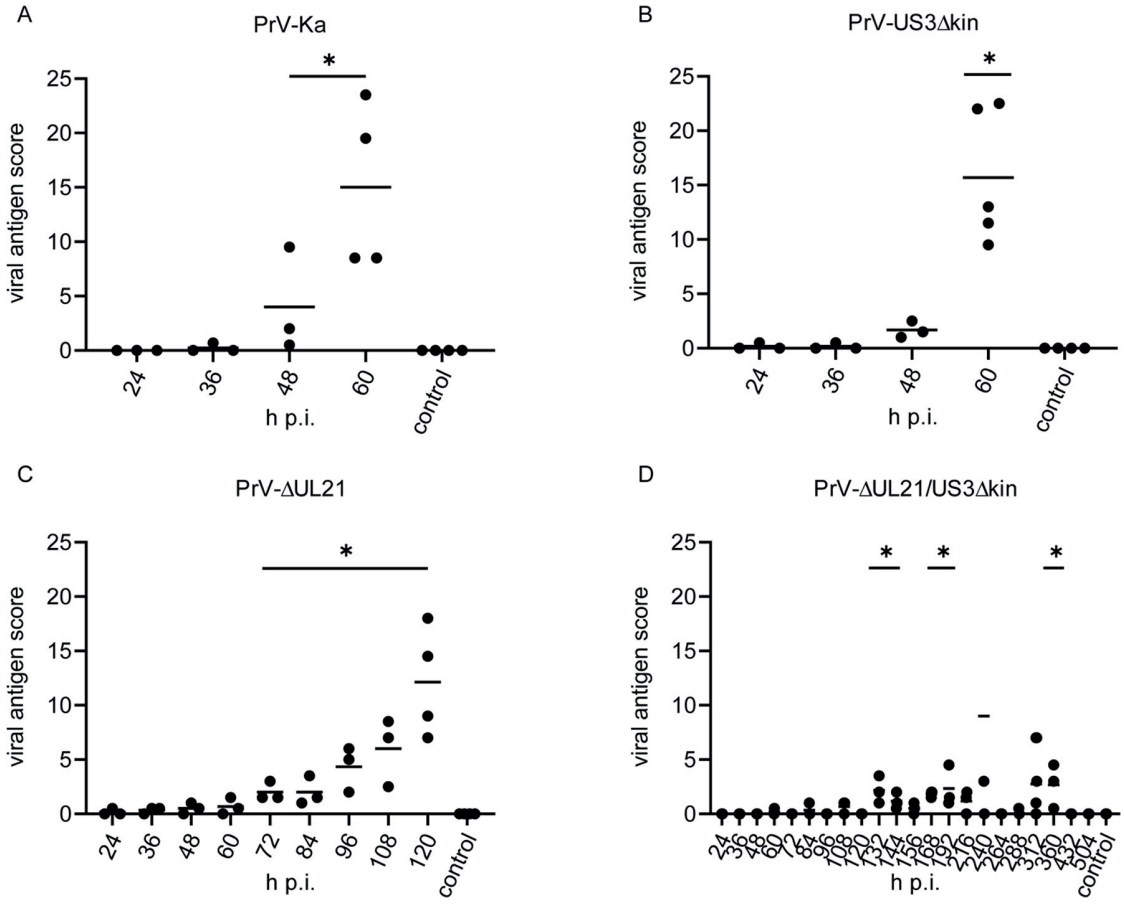

Fig 5. Viral antigen score in mice infected with mutant viruses. Histological specimen of mice infected with PrV-Ka (A), PrV-US3Δkin (B), PrV-ΔUL21 (C) and PrV-ΔUL21/US3Δkin (D) were scored for viral antigen distribution with negative (0), focal to oligofocal (1), multifocal (2) and diffuse (3). The final average value resulted from points given for each tissue section analyzed. Significant differences of mutant viruses compared to the mock-infected control group are indicated with an asterisk (*).

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p.i.), animals showed the highest viral antigen score (Fig 5A and 5B). PrV antigen was also found in different parts of the BS including the solitary tract (Sol) and FR with RVL, lateral reticular nucleus (LRt), parvicellular reticular nucleus (PCRt) and medullary reticular nucleus (dorsal part, MdD). The brain including the mes- (MES), di- (DI) and telencephalon (TEL), however, was free of viral antigen in both PrV-Ka and PrV-US3Δkin-infected mice. Details are given in S1 Fig. Although viral spread was consistent with previous studies [28, 29], infection of the FR had not been described yet. In PrV-ΔUL21-infected animals, viral antigen score increased more slowly compared to PrV-Ka-infected mice (Fig 5C). As shown in Fig 6, the distribution of viral antigen was similar compared to PrV-Ka and PrV-US3Δkin, but was in addition present in a few neurons of the inferior olive (IO), hypothalamus (HYPO) including the lateral hypothalamic area (LH), TH with ventromedial thalamic nucleus (VM) and mesiodorsal thalamic nucleus (lateral part), frontal lobe (FL) with agranular insular cortex (AI), parietal lobe (PL) with secondary auditory cortex (ventral area, AuV) and secondary somatosensory cortex (S2) and piriform lobe (PIR) at the time of euthanasia, indicating that longer survival results in more extensive neuronal spread towards the cerebral cortex.

In PrV-ΔUL21/US3Δkin-inoculated animals, viral antigen was detectable at varying amounts over the study period. The number of viral antigen positive cells was lower than in

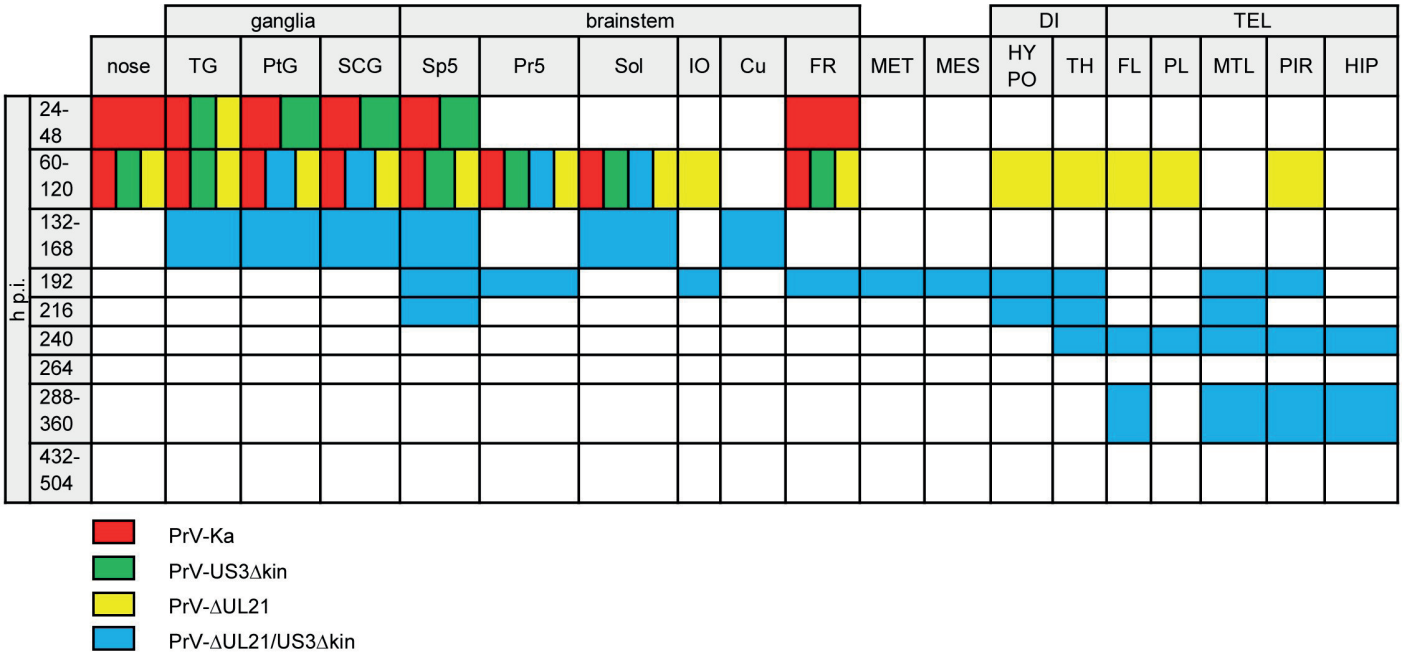

Fig 6. Antigen distribution. Colored boxes indicate viral antigen in the respective areas after infection with PrV-Ka (red), PrV-US3Δkin (green), PrV-ΔUL21 (yellow) and PrV-ΔUL21/US3Δkin (blue). TG = trigeminal ganglion, PtG = pterygopalatine ganglion, SCG = superior cervical ganglion, Sp5 = spinal trigeminal nucleus, Pr5 = principal sensory trigeminal nucleus, Sol = solitary tract, IO = inferior olive, Cu = cuneate nucleus, FR = reticular formation, MET = metencephalic regions others than Pr5, MES = mesencephalic regions others than Me5, DI = diencephalic regions, HYPO = hypothalamus, TH = thalamus, TEL = telencephalic regions, FL = frontal lobe, PL = parietal lobe, MTL = mesiotemporal lobe, PIR = piriform lobe, HIP = hippocampus.

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animals infected with PrV-Ka or single mutants (Fig 5D). The distribution of viral antigen is shown in Fig 6. Between 60 and 120 h p.i., as in PrV-Ka and single mutant infected mice, autonomic ganglia and parts of the BS including trigeminal second order neurons (Pr5) and Sol were positive. Between 132 to 168 h p.i. additionally TG, Sp5 and cuneate nucleus (Cu) became infected. The BS including IO and FR with PCRt and medullary reticular nulceus (ventral part) was antigen-positive at 192 h p.i. Viral antigen was further detectable in single neurons of the MET including the lateral parabrachial nucleus (central part, LPBC), in MES with lateral periaqueductal grey (LPAG), in TH, in HYPO with posterior hypothalamic area (PH) as well as in the TEL including the mesiotemporal lobe (MTL) with ectorhinal cortex (ECT) and lateral entorhinal cortex (LENT) and PIR. At 216 h p.i. viral antigen was detectable in second order neurons (Sp5), in HYPO with LH, in TH with dorsal lateral geniculate nucleus, and in MTL with LENT, the latter showing an increasing amount of PrV-gB positive neurons. At 240 h p.i., one animal had to be euthanized due to severe clinical signs. Histopathologically, this mouse showed a marked infection almost throughout the FL with primary motor cortex (M1), secondary motor cortex (M2), agranular insular cortex (posterior part, AIP), agranular insular cortex (dorsal part, AID), agranular insular cortex (ventral part, AIV), granular insular cortex (GI), dysgranular insular cortex (DIC), PL with primary somatosensory cortex (S1) and secondary somatosensory cortex (S2), MTL with ECT, LENT, perihinal cortex (PRH), temporal association cortex (TeA) and hippocampus (HIP), while all other animals had a low viral antigen load at that time in the respective areas as well as in TH and PIR. In brain sections of mice sacrificed at 264 h p.i., no viral antigen was detectable. Similar to the animal which had to be euthanized at 240 h p.i., in the mouse that died between 288 and 312 h p.i. the virus invaded cortical areas. Surprisingly, in animals sacrificed at 288 and 360 h p.i., viral antigen was again

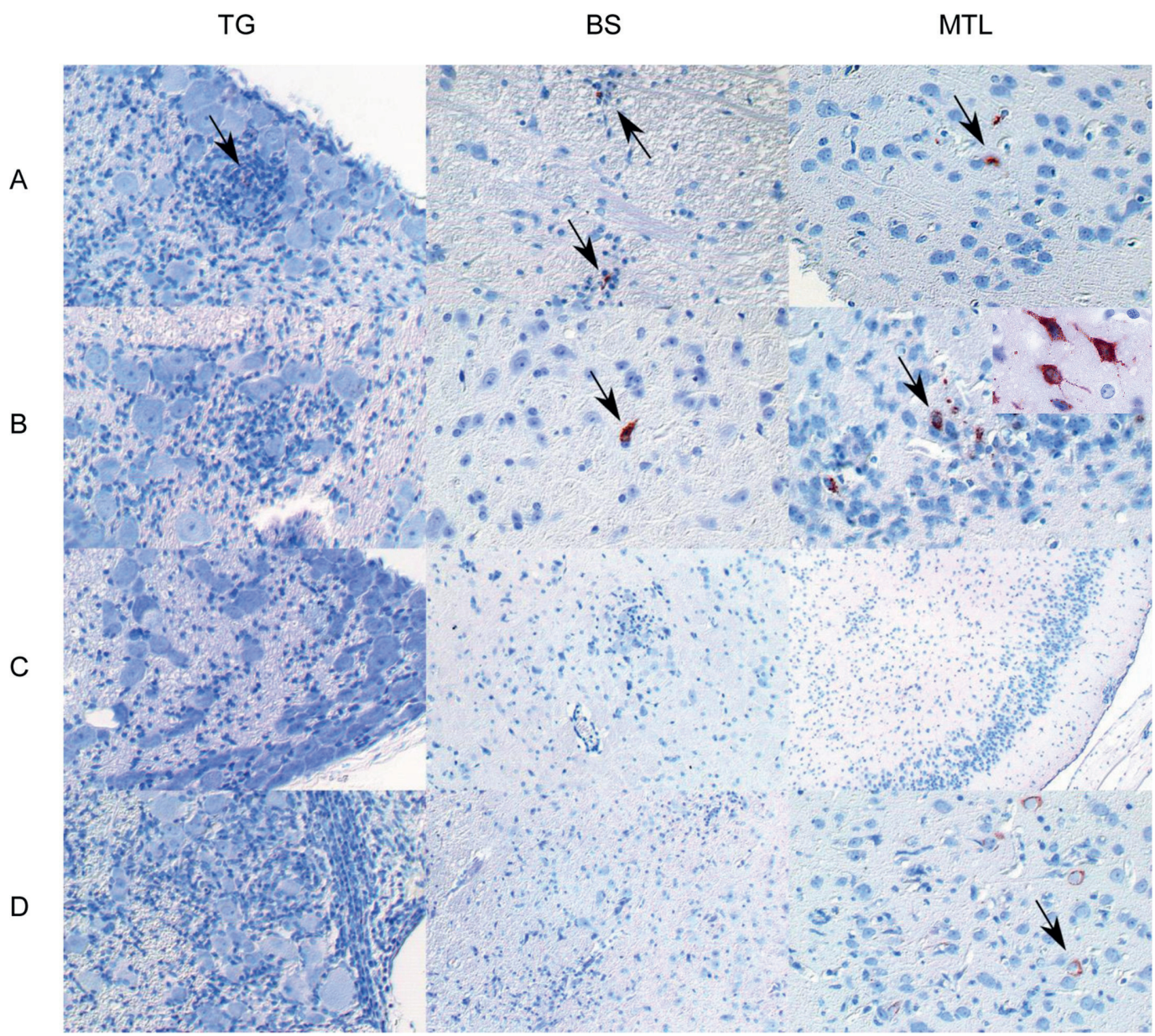

Fig 7. Detection of viral antigen in mice infected with PrV-ΔUL21/US3Δkin. Immunohistochemistry (anti PrV-gB) of the trigeminal ganglion (TG), brainstem (BS) and mesiotemporal lobe (MTL) are shown in A) 132–168 h p.i., B) 192–240 h p.i., C) 264 h p.i. and D) 288–360 h p.i. Positive staining is highlighted by arrows. A higher magnification of single infected neurons is shown in the inset in B) MTL.

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detectable in FL with M2 and agranular insular cortex (AI), MTL with ECT, LENT and PRH, PIR and HIP. In brain sections of mice analyzed at later time points (432 and 504 h p.i.) no antigen was detectable (summarized in S2 Fig). Representative examples of immunohistochemical analyses of TG, BS and MTL of animals infected with PrV-ΔUL21/US3Δkin for different times are shown in Fig 7.

Kinetics of the inflammatory response. Head sections were stained with hematoxylin/eosin and histopathologically analyzed. Inflammatory scoring results of mice infected with

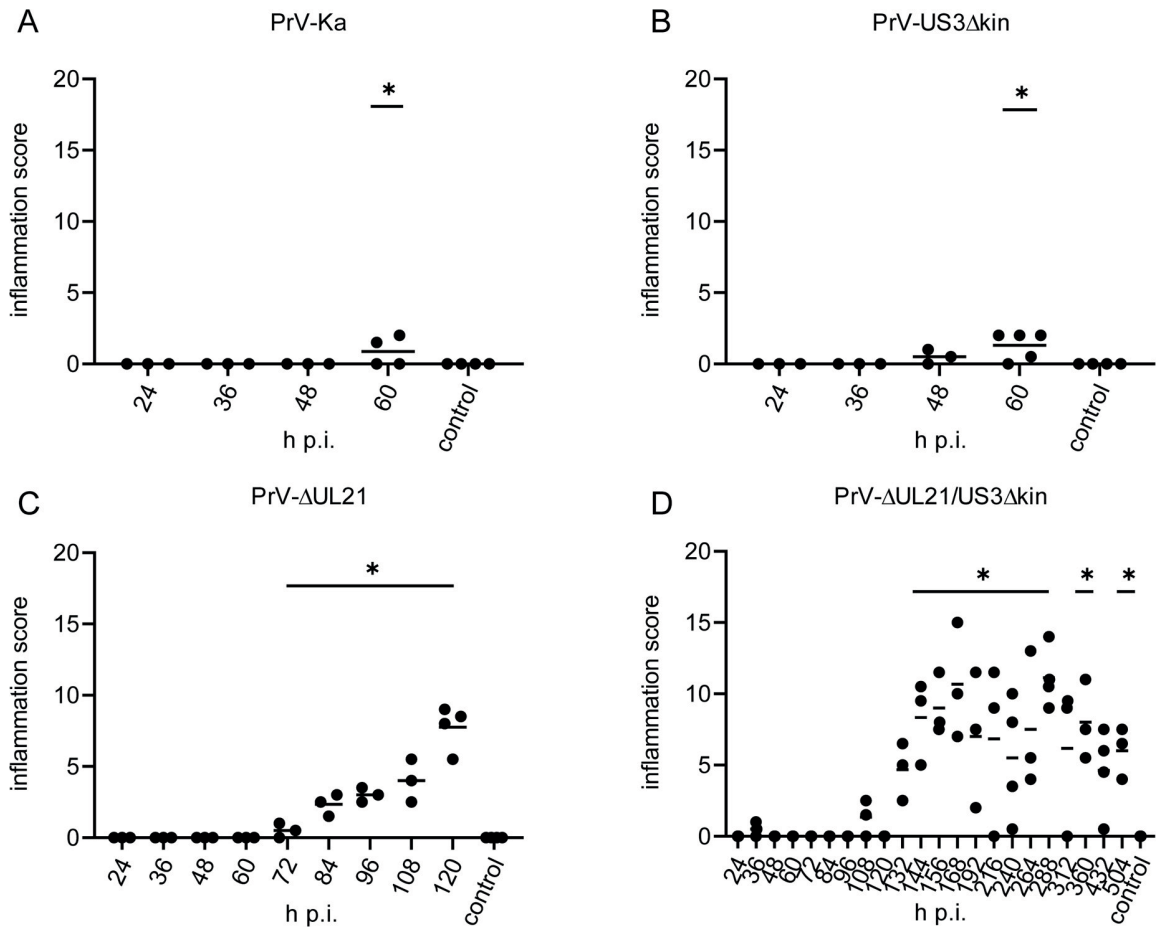

Fig 8. Inflammation score in infected mice. Histological specimen of mice infected with PrV-Ka (A), PrV-US3Δkin (B), PrV-ΔUL21 (C) and PrV-ΔUL21/US3Δkin (D) were scored for inflammation with 0 = no inflammation, 1 = mild inflammation, 2 = moderate inflammation, 3 = severe inflammation. The final average value resulted from points given for each tissue section analyzed. Significant differences of mutant viruses compared to the mock-infected control group are indicated with an asterisk (*).

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PrV-Ka, PrV-US3Δkin, PrV-ΔUL21 and PrV-ΔUL21/US3Δkin are shown in Fig 8 and the distribution of inflammatory reaction is indicated in Fig 9.

Only limited inflammation was observed in mice infected with PrV-Ka (Fig 8A) and PrV-US3Δkin (Fig 8B). Signs of inflammation were only present in the nose and in superior cervical ganglion (SCG) (Fig 9). In contrast, inflammatory response was significantly higher in mice infected with PrV-ΔUL21 (Fig 8C). Within 72 to 108 h p.i. mild to moderate inflammation of ganglia was present (Fig 9). Very mild lymphohistiocytic brainstem encephalitis occurred starting at 108 h p.i. Inflammatory changes were not detectable in MES, MET, DI and TEL.

Mice infected with PrV-ΔUL21/US3Δkin showed the highest inflammation score (Fig 8D). Besides an immediate inflammatory reaction at 36 h p.i. in the nose, inflammation was not detectable until 96 h p.i. At 108 h p.i. inflammatory response was found in the TG and in SCG, and subsequently inflammation scores significantly increased from 132h p.i. until the termination of the experiment (Fig 8). As presented in Fig 9, very mild inflammation in the nose was observed starting 36 h p.i., whereas after 108 h p.i. mild ganglionitis and ganglioneuritis in TG and autonomic ganglia was found. At 120 h p.i., no signs of inflammation were detectable, but

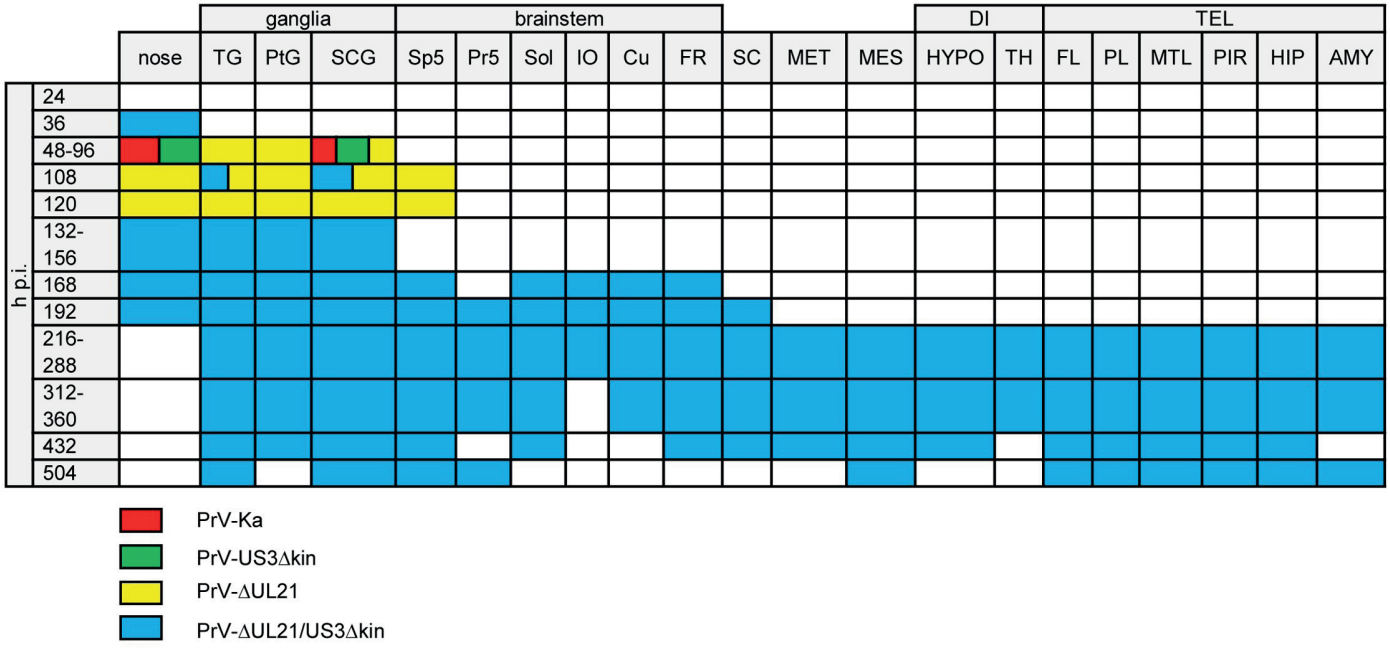

Fig 9. Sites of inflammatory response. Colored boxes indicate inflammation in the respective areas after infection with PrV-Ka (red), PrV-US3Δkin (green), PrV-ΔUL21 (yellow) and PrV-ΔUL21/US3Δkin (blue). TG = trigeminal ganglion, PtG = pterygopalatine ganglion, SCG = superior cervical ganglion, Sp5 = spinal trigeminal nucleus, Pr5 = principal sensory trigeminal nucleus, Sol = solitary tract, IO = inferior olive, CU = cuneate nucleus, FR = reticular formation, SC = spinal cord, MET = metencephalic regions others than Pr5, MES = mesencephalic regions others than Me5, DI = diencephalic regions, HYPO = hypothalamus, TH = thalamus, TEL = telencephalic regions, FL = frontal lobe, PL = parietal lobe, MTL = mesiotemporal lobe, PIR = piriform lobe, HIP = hippocampus, AMY = amygdala.

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ganglionitis aggravated thereafter. At 168 h p.i. inflammation was found in the brainstem including second order neurons (Sp5), Sol, IO, Cu and RF with PCRt, RVL, MdD, lateral paragigantocellular nucleus, intermediate reticular nucleus and LRt. At 192 h p.i. infiltrates were focally present in trigeminal second order neurons (Pr5) and in the cervical spinal cord. Between 216 and 240 h p.i., mild brainstem encephalitis turned to moderate and expanded to higher neuronal areas causing inflammation of the brain and meninges in MTL with LENT and PRH, PIR, amygdala (AMY) with posterolateral cortical amygdaloid nucleus as well as in MES with deep mesencephalic nucleus (DpMe) and ventral tegmental area (VTA), and in DI with substantia nigra reticular part, parasubthalamic nucleus, mamillothalamic tract, LH, PH, dorsomedial thalamic nucleus and ventromedial hypothalamic nucleus. At 264 to 288 h p.i, ganglia, BS and spinal cord were moderately affected, whereas MES with DpMe and DI with VM showed only few inflammatory infiltrates, but meningoencephalitis further spread to FL including AIP, M1, M2, lateral orbital cortex (LO), medial orbital cortex (MO), PL with S1, S2, and HIP. In animals investigated between 312 and 360 h p.i. meningoencephalitis progressed even further and included FL with AI, AIP, AID, LO, M1, M2, GI, DIC, AIV, LO, MO, ventral orbital cortex, dorsolateral orbital cortex, anterior olfactory nucleus (lateral part), anterior olfactory nucleus (posterior part), anterior olfactory nucleus (medial part) and infralimbic cortex, PL with S1, S2, primary auditory cortex, secondary auditory cortex (dorsal area) and AuV, MTL with LENT, PRH, ECT, TeA and dorsal endopiriform nucleus as well as PIR, HIP and AMY with lateral amygdaloid nucleus (ventrolateral part, ventromedial part), basomedial amygdaloid nucleus (posterior part) and basolateral amygdaloid nucleus (posterior part). At the end of the experiment at 504 h p.i., mild to moderate meningoencephalitis was still present in the mes- and telencephalic regions described above. Very mild inflammation in ganglia, BS,

and spinal cord was inconsistently observed at that time. In contrast, no signs of inflammation were found in the mock-infected control mice sacrificed in parallel.

Brain inflammation and neuronal degeneration

Histopathologically, meningoencephalitis was characterized by infiltration of lymphocytes and histiocytes with marked perivascular cuffing and neuronal degeneration followed by satellitosis, neuronophagy and reactive gliosis. Representative areas of inflammation at different time points in TG, BS and MTL are illustrated in Fig 10. Extensive neuronal necrosis was found in

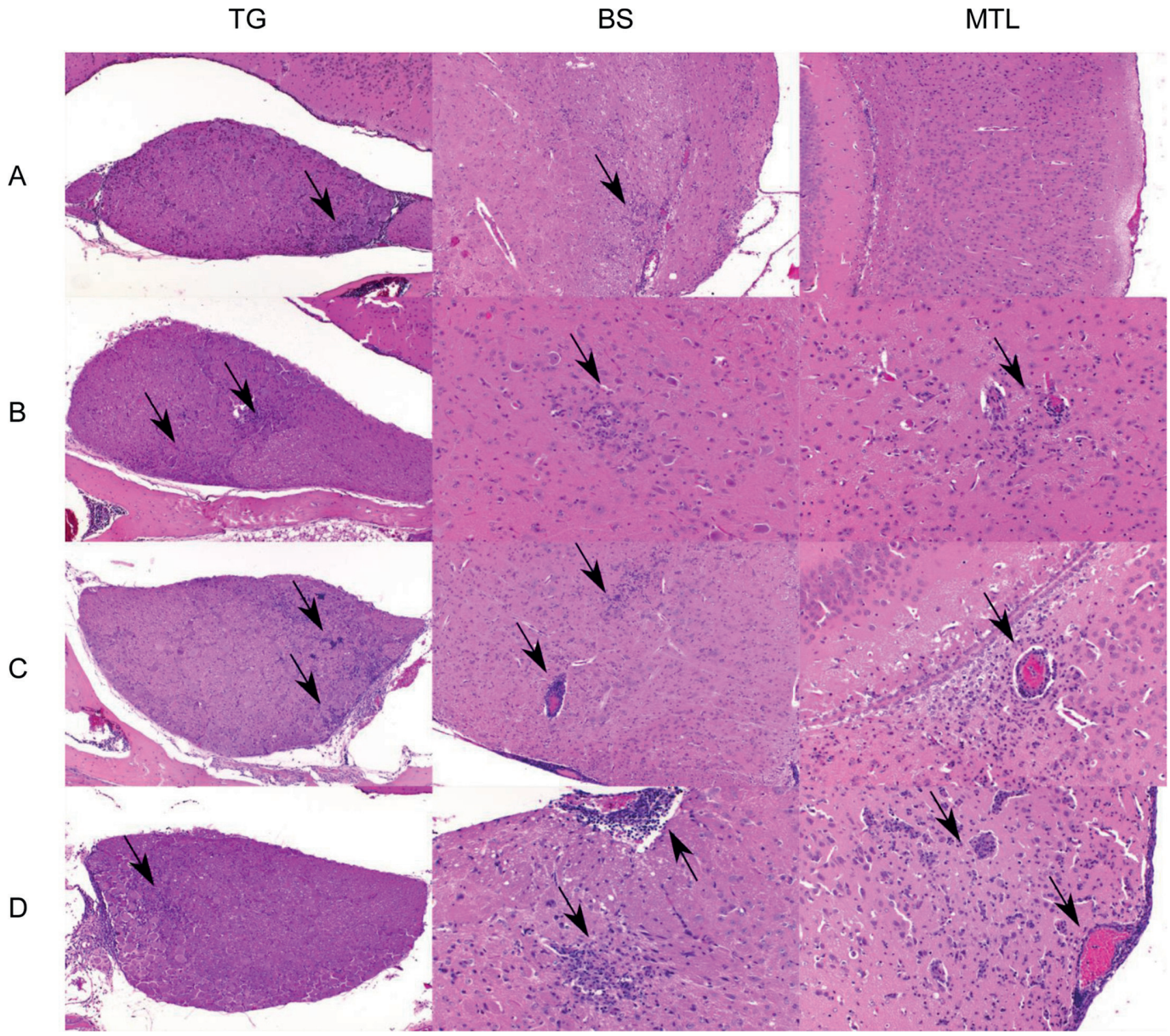

Fig 10. Detection of inflammation in mice infected with PrV-ΔUL21/US3Δkin. Hematoxylin and eosin stains of the trigeminal ganglion (TG), brainstem (BS) and mesiotemporal lobe (MTL) are shown in A) 132–168 h p.i., B) 192–240 h p.i., C) 264 h p.i. and D) 288–360 h p.i. Perivascular and parenchymal inflammatory infiltrates consisting of lymphocytes and histiocytes are highlighted by arrows.

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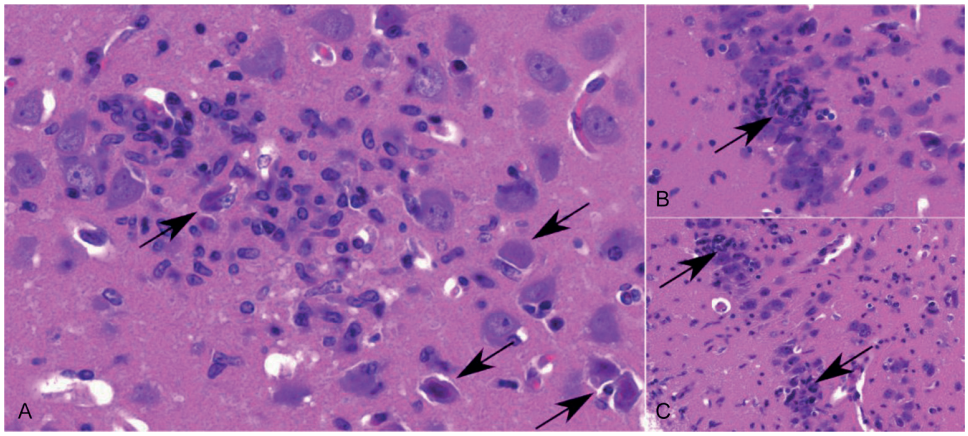

Fig 11. Inflammatory response in the mesiotemporal lobe. (A) Necrotic neurons (arrow) are surrounded by inflammatory cells and activated microglia. (B and C) Necrotic neurons in the cerebral cortical layer (arrow) with satellitosis and gliosis, as well as parenchymal infiltration of the adjacent neuropil (C).

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the cerebral cortex, which presented as hypereosinophilic, shrunken neurons with surrounding inflammatory cell infiltrates (Fig 11). To identify apoptotic cells, representative sections of the TG, BS and MTL of PrV-ΔUL21/US3Δkin infected mice were stained for active caspase-3. Apoptosis was mainly detected in areas without inflammation and was found in neurons and to a lesser extent in glial cells (S3 Fig). Only single caspase-3-positive cells were present in areas with inflammatory response.

Discussion

In this study, we characterized a PrV mutant simultaneously lacking the tegument protein pUL21 and the pUS3 kinase activity in cell culture and in mice. A mutant completely lacking both genes (PrV-ΔUL21/US3) was exceptional in previous *in vivo* testing of different PrV mutants [28, 29], since it was the only causing productive infection that most of the infected animals were able to control and survive. This exceptional finding prompted us to investigate infection with this virus mutant in more detail.

To test the influence of the pUS3 kinase activity, we generated a novel mutant, PrV-ΔUL21/US3Δkin, where only the kinase function was inactivated by a point mutation in the catalytic center, leaving the protein otherwise intact. Growth properties of PrV-ΔUS3 and PrV-US3Δkin were comparable as shown previously [40]. PrV-ΔUL21/US3Δkin displayed an approx. 50- to 100-fold titer reduction in cell culture although both proteins, pUL21 and pUS3, are dispensable for viral replication *in vitro* and *in vivo* of PrV [40–42], and HSV-1 [43, 44]. Cell-to-cell spread of PrV-ΔUL21/US3Δkin was significantly impaired and plaque diameters reached only approx. 50%. However, despite these impairments PrV-ΔUL21/US3Δkin was able to replicate productively in cell culture, a prerequisite for the subsequent animal studies. PrV-ΔUL21/US3Δkin was further investigated in our murine intranasal infection model and compared to PrV-Ka and the corresponding single mutants.

Animals infected with PrV-Ka and PrV-US3Δkin showed a similar MTD of 62 h and 63 h, respectively, and neuroinvasion of both viruses was mainly restricted to the trigeminal pathway including first and second order neurons. Mice infected with PrV-ΔUL21 showed an increased MTD of approximately 110 h, accompanied by a more widespread, but still limited invasion of the cerebral cortex. In contrast to PrV-Ka and PrV-US3Δkin infected animals, PrV-ΔUL21-infected mice showed fulminant ganglionitis as well as mild brainstem

encephalitis, but no inflammation in the cerebral cortex where only few neurons were infected. Both gene products, pUL21 and pUS3 are conserved among the *Alphaherpesvirinae* and dispensable for viral replication in PrV as well as in HSV-1 [39, 41, 44, 45]. pUS3 is a multifunctional protein which is involved in different processes during viral infection [46]. Whereas PrV lacking pUS3 is attenuated in pigs [45], only a slight delay in disease progression has been observed in mice [28]. Different mechanisms by which pUS3 modulates pathogenicity have been reported. Escape from the host immune response by downregulation of cell surface receptors including CD1d or major histocompatibility complex class I is induced by pUS3 [47–50] preventing efficient killing of virus-infected cells [48, 50]. pUS3 has been further demonstrated to affect T cell activity [51–53] and interferon signaling followed by impaired immune responses against viral infection resulting in longer survival times. Moreover, pUS3 has been reported to block virus-induced apoptosis by controlling a multitude of apoptosis-associated factors [54–61]. However, mice infected with PrV mutants lacking pUS3 completely or defective in kinase activity showed only a minimal delay in the onset of disease, and clinical signs were similar as in PrV-Ka infected animals. Like in PrV-Ka infected mice only very mild inflammatory infiltration was present in affected ganglia. In contrast, the functional role of pUL21 is still poorly understood. pUL21 has been implicated in different steps of the viral replication cycle in non-neuronal and neuronal cells [39, 62–67]. In particular, mutations in or complete absence of pUL21 affect retrograde transport processes in neurons [68, 69], and point mutations in the UL21 gene of live-attenuated vaccine strain PrV-Bartha contribute to its avirulence in pigs [42]. Repair of the UL21 locus in PrV-Bartha has been shown to enhance retrograde intraaxonal and transneuronal spread [69]. The delay in retrograde transport of UL21-null PrV has been linked to an interaction of the carboxyl terminus of pUL21 with Roadblock-1, a dynein light chain belonging to the dynein motor complex important for retrograde transport along microtubules [68]. Mice infected intraocularly or intranasally with a UL21-deleted PrV showed prolonged survival times compared to mice infected with wildtype virus [68] confirming previous results that pUL21 has an impact on neuroinvasion and spread [28]. As shown in the present study infection with PrV lacking pUL21 resulted in extended times until death compared to PrV-Ka. However, all mice developed severe clinical signs and had to be euthanized, although the disease developed slower. In the light of decelerated axonal spread [68, 69] and subsequent delayed infection of higher areas an immune response is beginning to develop in PrV-ΔUL21-infected mice, but not effective enough to eliminate the virus. In summary, these data confirm that pUL21 contributes to neuroinvasion, but elucidation of the molecular function requires more in-depth investigation.

Severe clinical signs as seen in PrV-Ka and single mutant infected animals seem not to correlate with the presence or absence of inflammation, but rather be associated with brainstem infection, particularly with damage of neurons in the formatio reticularis, which are important to preserve cardiovascular functions. Sudden death of infected neurons of the FR cause cardiorespiratory collapse as observed in our experiment when the animals developed dyspnea, which was also reported after Enterovirus 71 infection of mice leading to severe encephalomyelitis [70].

In contrast to mice infected with PrV-Ka or the single mutants, mice infected with PrV-ΔUL21/US3Δkin survived, except for one animal, which had to be euthanized 239 h p.i. The phenotype observed after simultaneous deletion of pUL21 and pUS3 kinase *in vivo* and *in vitro* might be either based on additive effects of usually separate functions or explained by a functional relationship between these two proteins. In line with this, previous data indicated that in the absence of pUL21 less pUS3 is incorporated into progeny virions [65, 71] which could result in the amplification of effects observed with the single deletion mutants. Aside from the mutations infection with PrV-ΔUL21/US3Δkin mimics herpesviral encephalitis very well in different ways.

Infection with PrV-ΔUL21/US3Δkin caused severe meningoencephalitis with extensive neuronal necrosis, meningeal and perivascular infiltrates of lymphocytes and histiocytes as well as glial activation, which is fully consistent with lesions described for HSE patients [8, 72–75]. Histopathological changes including viral antigen detection and inflammatory response in mice infected with PrV-ΔUL21/US3Δkin were mainly observed unilateral in FL, MTL and PIR as well as in HIP as reported for patients who died of HSE [74, 76, 77]. Apoptotic cells were identified mainly in areas without inflammatory reaction indicating increased neuronal death in the context of virus-induced neuronal damage [78–80]. So far, PrV-ΔUL21/US3Δkin is the only PrV mutant that establishes a productive infection in mice, which the animals are able to control and survive despite widespread neuroinvasion towards the cerebral cortex and a concomitant severe inflammatory response particularly in the frontal and mesiotemporal lobes. While studies in mice showed that HSV-1 rather causes a diffuse inflammation throughout the brain or only in the brainstem depending on the inoculation route used [21–23], in our experiments the typical focal lesions in the mesiotemporal and frontal lobes as observed in human patients were prevalent. Thus, our model authentically reproduces the pattern of herpesviral encephalitis in humans. Since details on the tropism of HSV-1 in mice and humans are still unknown the use of our mouse model offers new perspectives to investigate how alphaherpesviruses gain access to the brain and why infection is established preferably in the temporal and frontal lobes. Although speculative, neurons may differ in their susceptibility to infection, which in turn may result in infection of more vulnerable neuronal subtypes. Unique innate immune responses have been described for granule cell neurons of the cerebellum and cortical cerebral neurons upon West Nile virus infection [81], but little is known in the context of alphaherpesvirus infections [82].

In the present study, we were able to trace the immune response against herpesvirus infection over a long period. Due to the extended survival time of mice infected with PrV-ΔUL21/US3Δkin, it will be feasible to investigate long-term pathobiological mechanisms for development and during herpesviral encephalitis, their contribution to disease severity and associated immunopathological processes as described for HSE [83–85].This was not possible in mice infected with virulent HSV-1, regardless of intranasal or intracerebral infection [86–89], since animals invariably succumbed to the infection.

Behavioral changes including stargazing which occurred with increasing inflammation in the brain indicate impairments, which represent analogies to neurophysiological phenomena in HSE or VZV patients, which are mostly related to damages in the limbic system including HIP [90, 91]. Behavioral as well cognitive impairment may well be even more pronounced beyond the 21 days of our infection experiment, where all mice still showed brain inflammation and exhibited signs of abnormality. Problems with memory and cognitive failure as well as development of epilepsy have been discussed in association with HSV-1 infection [92]. Whether mice infected with PrV-ΔUL21/US3Δkin develop similar clinical signs remains to be tested in long-term studies and analyzed by appropriate learning and memory tests. However, to our knowledge, this is the first description of behavioral failure in mice in the course of herpesviral encephalitis, which offers new perspectives to study behavioral disorders associated with herpesviral infection.

Herpesviruses establish latency after infection and viral DNA was found not only in the peripheral nervous system, but also in the CNS in HSE patients [93, 94]. In our study, viral antigen was not detectable by immunohistochemistry beyond 360 h p.i., which might be due to the low amount of viral antigen in the tissue section analyzed [95] or explained by early virus clearance leading to latent infection. This parallels HSE where viral antigen could be detected only within 3 weeks of onset of the disease in HSE patients, but was absent afterwards [74]. Experiments to test for latent DNA of PrV-ΔUL21/US3Δkin are planned for the future.

In HSE patients, involvement of BS as seen in mice infected with PrV-ΔUL21/US3Δkin has been reported but thought to be a special form of manifestation of HSV-1 encephalitis [96–98]. Involvement of BS may point to the trigeminal pathway of neuroinvasion. However, in PrV-ΔUL21/US3Δkin infected mice viral antigen and brain inflammatory response were first present in cortical areas such as MTL and PIR, which receive projections from the olfactory bulb indicating the possibility of infection via the olfactory route as discussed in the development of HSE.

In mice infected with PrV-ΔUL21/US3Δkin, myelitis as well as zosteriform alopecic skin lesions which are typical pathologic hallmarks of infection primarily with VZV [10, 99] were observed. In VZV, segmental rash occurs during virus reactivation in dorsal root ganglia, which are associated with a corresponding dermatome, but, in contrast to our finding, carry viral antigen [10, 100]. The pathogenesis of myelitis is still unknown, but it is suspected that the virus spreads from infected dorsal root ganglia to the spinal cord [10]. However, our data also indicate that PrV-ΔUL21/US3Δkin may has been transported along the spinal cord resulting in lesions distant from the head. In this respect, virus-induced lesions in peripheral and central sensory tracts may be the reason for the pruritus observed in different body regions of mice after infection with PrV-ΔUL21/US3Δkin as well as in herpesvirus-infected humans. As reported in humans the trigeminal trophic syndrome (TTS) caused by herpesviral infections also results in painless itch of the facial skin caused by injury of the trigeminal nerve, TG and even parts of the brain including TH [101–103].

Ganglionitis as well as ganglioneuritis as seen in our model are usually not observed in patients suffering from HSV-1 associated encephalitis, except for one case report of cervical dorsal root ganglionitis without encephalitis [104]. However, for VZV patients such cases have been repeatedly described [16, 105, 106]. For HSV-1 and VZV, viral genomes have been detected independently in sensory and autonomic ganglia (e.g. SCG) of the human head and neck [11].

In summary, mice infected with PrV lacking pUL21 and functional pUS3 kinase were able to survive infection despite extensive neuroinvasion and severe meningoencephalitis. The animal model presented here reflects important aspects of herpesviral encephalitis in humans including the characteristic distribution of histopathological changes and behavioral abnormalities. Thus, we suggest that this animal model is highly suitable for further investigations towards understanding the pathogenesis of herpesviral encephalitis.

Material and methods

Viruses and cells

All virus mutants used were derived from PrV wildtype strain Kaplan (PrV-Ka) [107]. PrV-Ka, PrV-ΔUL21 [39] and PrV-US3Δkin [40] were propagated in rabbit kidney cells (RK13), an established cell line for PrV infection in our lab, and grown at 37˚C in minimum essential medium (MEM) (Invitrogen) supplemented with 10% fetal calf serum.

Generation of PrV-ΔUL21/US3Δkin

PrV-ΔUL21/ΔUS3gfp lacking US3-specific sequences but expressing green-fluorescent protein was generated after co-transfection of PrV-ΔUL21 viral DNA [39] with plasmid pΔUS3gfpII [41] into RK13 cells. Green fluorescing plaques were purified to homogeneity and absence of US3 was verified by Southern and Western blotting. PrV-ΔUL21/US3Δkin was isolated after co-transfection of plasmid pUC-US3Δkin [40] with PrV-ΔUL21/US3gfp genomic DNA and purification of non-fluorescing plaques. Immunofluorescence and immunoblotting using a rabbit anti-pUS3 (1:50 000) [41] and anti-pUL21 (1:20 000) [39] serum were used to verify the

absence of the UL21 protein and presence of pUS3. Presence of the mutation in the catalytic domain of pUS3 was confirmed by sequence analysis.

In vitro replication studies

One-step growth kinetics were established by infection of RK13 cells with PrV-Ka, PrV-U-S3Δkin, PrV-ΔUL21, and PrV-ΔUL21/US3Δkin at a multiplicity of infection (MOI) of 3 as described [108]. Plaque assays were used to determine the titer of virus progeny on RK13 cells. Three independent experiments were performed to assess the mean viral titers. In addition, plaque diameters of 20 plaques per virus in three independent assays were measured to determine characteristics of cell-to-cell spread of the respective virus mutants.

Ethics statement

Animal experiments were approved by the State Office for Agriculture, Food Safety and Fishery in Mecklenburg-Western Pomerania (LALFF M-V) with reference number 7221.3-1-064/17.

Animal experiments

In the present study, we used 6–8 weeks old female CD-1 mice as our standardized animal model for PrV infection [27–29]. Female animals were used to facilitate group housing. The animals were purchased from Charles River Laboratories and housed in groups of maximal five animals per cage at the animal facilities of the Friedrich-Loeffler-Institut, Greifswald-Insel Riems. Animals were kept under controlled conditions of 12 h light: 12 h dark with free access to food and water. After one week of acclimatization, mice were deeply anesthetized with 200 μl of a mixture of ketamine (60mg/kg) and xylazine (3mg/kg) dissolved in 0.9% sodium chloride, which was administered intraperitoneally. Afterwards a total volume of 5 μl each of the corresponding virus suspension was inoculated in both nostrils ($1x10^4$ plaque forming units). Mock mice were treated with cell culture supernatant from RK13 cells (MEM + 5% FCS) accordingly.

Determination of the mean time to death and clinical evaluation. Mean time to death (MTD) was established after inoculation of mice with PrV-Ka (n = 4), PrV-US3Δkin (n = 4), PrV-ΔUL21 (n = 4) and PrV-ΔUL21/US3Δkin (n = 6) and cell culture supernatant (n = 2) as control. Following intranasal inoculation, animals were monitored twice daily every 12 hours over a period of maximal 21 days. Mice were evaluated based on a predefined scoring system with the following three categories: (I) external appearance, (II) behavior and activity and (III) body weight (S1 Table). For each category, a score ranging from 0 to 3 was determined. Score 3 in one category or score 2 in all three categories was defined as the humane endpoint on which the animal was euthanized.

Determination of the kinetics of viral spread and inflammatory changes. To assess the kinetics of viral spread and inflammatory changes after inoculation with PrV-Ka, PrV-U-S3Δkin, PrV-ΔUL21 and PrV-ΔUL21/US3Δkin a minimum of three mice was sacrificed and examined at each time point. For analysis of PrV-Ka and PrV-US3Δkin animals were sacrificed after inoculation at the following time points: 24, 36, 48, 60 h. For investigation of PrV-ΔUL21 mice were sacrificed at 24, 36, 48, 60, 72, 84, 96, 108 and 120 h. Animals inoculated with PrV-ΔUL21/US3Δkin were sacrificed at 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156, 168, 192, 216, 240, 264, 288, 312, 336, 360, 432 and 504 h. After 24, 168, 312 and 504 h one mock-infected mouse each was sacrificed.

Euthanasia and tissue sample collection. Animals were deeply anesthetized with isoflurane, and subsequently sacrificed through cardiac bleeding with a 21-gauge needle. The head

was removed at the level of the first or second cervical vertebrae. The skull was taken off to allow accurate fixation and subsequent evaluation of all anatomical-physiological structures of the head of all mice. In addition, the spinal column including the spinal cord as well as affected skin tissue were collected from several mice. All tissue specimens were fixed in 10% neutral buffered formalin for at least one week.

Histopathology and immunohistochemistry. Following fixation, heads and spinal column were decalcified for at least three days in Formical 2000 (Decal, Tallman, N.Y.). Heads were subsequently sliced from caudal to cranial to obtain eight coronal sections of 2–3 mm thickness each. The spinal column was cut into 2–3 mm sections at the cervical (3–4 sections), thoracical (7–8 sections), lumbal (4–5 sections), and sacral (3 sections) level. Tissue specimen of the head, spinal cord and skin were then embedded in paraffin wax and cut at 3 μm thick slices. Two slices per each head section separated by 100 μm were collected to increase the amount of tissue for investigation. Therefore, 16 head sections of each animal were analyzed histopathologically and immunohistochemically, respectively. For light microscopical investigation, the sections were mounted on Super-Frost-Plus-Slides (Carl Roth GmbH, Karlsruhe, Germany) and stained with hematoxylin-eosin.

For immunohistochemistry, sections were dewaxed and rehydrated. Intrinsic peroxidase activity was blocked through 3% of hydrogen peroxide (Merck, Darmstadt, Germany) treatment for 10 min. Sections were then incubated with undiluted normal goat serum for 30 min to block unspecific binding sites before incubation with the respective primary antibody. After washing with Tris-buffered saline (TBS) the rabbit glycoprotein B-specific antiserum (1:2000, diluted in TBS, 60 min, [109]) was used to detect infected cells. To investigate apoptosis a rabbit antiserum against active caspase-3 (Promega, Madison, USA; 1:200, diluted in TBS) was used. Sections were rinsed and subsequently incubated with a biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA; diluted 1:200 in TBS, 30 min), followed by an incubation with ABC (Vector) diluted 1:10 in TBS for 30 min, providing the conjugated horseradish peroxidase. Positive reactions were visualized with AEC-substrate (DAKO, Hamburg, Germany). After washing with deionized water, the sections were counterstained with Mayer's Hemalaun for 2 min and mounted with Aquatex (Merck). To verify positive viral antigen staining, selected sections were treated with a rabbit antiserum against the major capsid protein pUL19 [110] and further examined with RNAScope using a probe against the same epitope. Comparable results were obtained for all staining techniques.

Semi quantitative analysis of histological specimens and scoring of viral antigen distribution. Light microscopical examination was conducted using a Zeiss Axio Scope.A1 microscope equipped with 5x, 10x, 20x, and 40x N-ACHROPLAN objectives (Carl Zeiss Microscopy GmbH, Jena, Germany).

Each of the immunohistochemically stained head and spinal cord as well as skin sections were examined for positive anti-PrV-glycoprotein B reactions. For identification of viral antigen positive neural structures in the brain the mouse brain atlas was used, and distribution of viral antigen positive cells in the respective sections were scored as follows: 0 = negative, 1 = focal to oligofocal, 2 = multifocal, 3 = diffuse. Points given for each section were added up to obtain a total sum for the whole head, which was then taken into analysis. Since each of the eight head main sections was investigated twice, the total amount of the first sectional plane and the second sectional plane, respectively, was averaged and statistically analyzed.

Scoring of inflammation. Tissue sections were stained with hematoxylin and eosin, investigated for histopathological changes and analyzed as described above. Evaluation was based on the following scoring system: 0 = no inflammation, 1 = mild inflammation, 2 = moderate inflammation, 3 = severe inflammation. Along with the inflammatory response, infiltration of immune cells as well as reactive changes (e.g. neuronal necrosis, degeneration and loss,

gliosis, satellitosis, neuronophagy) were additionally characterized at each day of investigation. Total values were analyzed as described above (see scoring of viral antigen distribution).

Statistical analysis. Statistical analysis and graphical presentation were performed by using GraphPad Prism. A Kaplan-Meier curve was generated to illustrate the relative survival rate after inoculation with the respective viruses followed by survival analysis using the log-rank test. All values were analyzed using the standard error of the mean (SEM). All animal groups examined on 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156, 168, 192, 216, 240, 264, 288, 312, 336, 360, 432 and 504 h compared to mock-infected mice (control) were analyzed by the nonparametric Kruskal-Wallis test followed by pairwise uncorrected Dunn's *post hoc* tests. Growth of virus mutants in cell culture were analyzed with an ordinary one-way ANOVA followed by Dunnet's multiple comparison test. Average values and standard deviations from three independent experiments were calculated, and the mean of each virus mutant and time point was compared to PrV-Ka as control. P-values with a significance limit of ≤ 0.05 were considered and indicated by an asterisk (*) in the graphs.

Supporting information

S1 Table. Scoring system for mice infected with PrV. Three categories including (I) external appearance, (II) behavior and activity and (III) body weight were assessed daily and utilized to group mice into either mildly (max. score 1 in three out of three categories), moderate (max. score 2 in two out of three categories) and severely affected (max. score 2 in all categories or max. score 3 in one out of three categories).
(DOCX)

S2 Table. Clinical signs and mean time to death (MTD). The onset of disease, MTD as well as typical clinical signs, which have been observed in mice infected with different virus mutants are listed.— = not present, ✓ = present, (✓) = occasionally observed.
(DOCX)

S1 Fig. Viral antigen distribution in infected with PrV-Ka. Immunohistochemistry using an anti-glycoprotein-B-antibody was performed to detect PrV in the respective tissues. A) nasal respiratory epithelium (RE), B) trigeminal ganglion (TG), C) spinal trigeminal nucleus (Sp5), D) nasopharynx (NP), E) salivary glands (SG), F) pterygopalatine ganglion (PtG), G) superior cervical ganglion (SCG) and H) cerebral cortex. Viral antigen has not been detected in the cerebral cortex.
(TIF)

S2 Fig. Schematic illustration of spread of PrV-ΔUL21/US3Δkin in mice after intranasal infection. Localization of viral antigen detection is shown for different times p.i. No viral antigen was detectable later than 360 h p.i. RE = respiratory epithelium, NG = nasal gland, OM = oral mucosa, NP = nasopharynx, SG = salivary gland, TG = trigeminal ganglion, PtG = pterygopalatine ganglion, SCG = superior cervical ganglion, Cu = cuneate nucleus, Sol = solitary tract, Sp5 = spinal trigeminal nucleus, FR = reticular formation, IO = inferior olive, LPBC = lateral parabrachial nucleus, Pr5 = principal sensory trigeminal nucleus, Me5 = mesencephalic trigeminal nucleus, PAG = periaqueductal grey, VTA = ventral tegmental area, TH = thalamus, HYPO = hypothalamus, OB = olfactory bulb, FL = frontal lobe, PL = parietal lobe, PIR = piriform lobe, MTL = mesiotemporal lobe, HIP = hippocampus, AMY = amygdala.
(TIF)

S3 Fig. Immunohistochemistry showing active caspase-3. The trigeminal ganglion (TG), brainstem (BS) and mesiotemporal lobe (MTL) were examined for apoptosis. A, C and E)

Caspase-3-positive cells only appeared occasionally in regions adjacent to inflammatory spots (asterisk) and included both neuronal and glial cells (arrowhead). B, D and F) Apoptosis of neurons or glial cells was mainly detectable in non-inflamed areas which are indicated by arrows.
(TIF)

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4 Contribution to publications

[I] Sehl J, Pörtner S, Klupp BG, Granzow H, Franzke K, Teifke JP, Mettenleiter TC. 2020. Roles of the Different Isoforms of the Pseudorabies Virus Protein Kinase pUS3 in Nuclear Egress. J Virol. **94**(7), doi: 10.1128/JVI.02029-19.

Julia Sehl	Generation and isolation of revertant viruses; Validation of virus mutants and revertants by sequencing and western blot analysis; Characterization of virus mutants and revertants by western blot analysis, growth kinetics, plaque assays and laser scanning confocal microscopy; Interpretation of data; Visualization of data; Writing and correction of the manuscript
Sandy Pörtner	Generation of recombination plasmids expressing different pUS3 isoforms; Isolation and sequencing of mutant viruses
Barbara G. Klupp	Conceptualization of the study; Provision of mutant PrV-ΔUS3; Interpretation of data; Preparation, correction and review of the manuscript
Harald Granzow	Electron microscopical investigation of virus mutants
Kati Franzke	Electron microscopical investigation of virus mutants
Jens P. Teifke	Interpretation of data; Preparation, correction and review of the manuscript
Thomas C. Mettenleiter	Conceptualization of the study; Interpretation of data; Preparation, correction and review of the manuscript; Corresponding authorship

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[II] Sehl J, Hölper JE, Klupp BG, Teifke JP, Mettenleiter TC. 2020. An Improved Animal Model for Herpes Simplex Encephalitis in Humans. PLoS Pathog. **16**(3), doi: 10.1371/journal.ppat.1008445

Julia Sehl	Conceptualization of the study; Generation of virus mutant PrV-ΔUL21/US3Δkin; Mutant characterization in cell culture by growth kinetics, plaque assays and western blot analysis; Conduction of animal experiments; Histological preparation of mouse organs including the brain, spinal cord and skin; Pathohistological examination of virus spread and inflammation using hematoxylin and eosin stain as well as immunohistochemistry; Interpretation of data; Visualization of data; Writing and correction of the manuscript
Julia E. Hölper	Participation in animal experiments; Visualization of data
Barbara G. Klupp	Provision of mutants PrV-US3Δkin and PrV-ΔUL21; Interpretation of data; Preparation, correction and review of the manuscript
Christina Baumbach	Conduction of animal experiments with PrV-ΔUL21/US3
Jens P. Teifke	Conceptualization of the study; Interpretation of data; Preparation, correction and review of the manuscript
Thomas C. Mettenleiter	Conceptualization of the study, Interpretation of data; Preparation, correction and review of the manuscript; Corresponding authorship

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T. C. Mettenleiter

5 Results and discussion

Herpesviruses are associated with severe diseases in humans and animals including HSE or the mad-itch syndrome associated with PrV. VZV can also lead to devastating NS manifestations in humans (Steiner and Benninger 2018). Patients who have incurred herpes encephalitis almost always have severe long-term consequences or suffer from recurrent inflammation. Why and how infection with these common human viruses, which usually only cause mild to moderate clinical signs, can lead to life-threatening diseases is still not understood. Even mechanisms of pathogenesis or temporal processes of infection and neuroinvasion are not yet completely clear. Animal models established so far do not mimic HSE satisfactorily emphasizing the need for improvement (Kollias *et al.* 2015, Mancini and Vidal 2018). Despite its strong neurotropism, PrV infection of non-natural hosts such as mice is not primarily linked to CNS disorders, but is rather exclusively associated with intense itching, automutilation and rapid death (Mettenleiter *et al.* 2019). *In vivo* testing of different PrV deletion mutants identified one exceptional mutant, designated PrV-ΔUL21/US3. Mice infected with this mutant were able to survive (Maresch 2011).

In the present thesis, a novel PrV mutant was constructed and investigated in detail lacking the UL21 gene but defective only for the pUS3 kinase activity (PrV-ΔUL21/US3Δkin). The corresponding single deletion mutant PrV-ΔUL21 had been previously characterized in cell culture in and mice (Klupp *et al.* 2005, Klopfleisch *et al.* 2006), whereas PrV-US3Δkin has not. Therefore, PrV-US3Δkin was investigated in cell culture and compared to a US3 deletion mutant lacking the complete open reading frame, thereby separating the structural and enzymatic functions of pUS3. In addition, the role of the two isoforms of PrV pUS3 during virus replication was studied in cell culture (**paper I**). These data formed the basis for the second study testing PrV-US3Δkin as well as PrV-ΔUL21/US3Δkin in cell culture and in mice. Results achieved with PrV-ΔUL21/US3Δkin after infection of mice showed striking homologies to the clinical picture of HSE in humans. Based on this, an improved animal model for HSE was proposed which would be helpful for better understanding and possibly treatment of this life-threatening disease (**paper II**).

5.1 Characterization of the pUS3 kinase and its role in nuclear egress (paper I)

For **paper I** pUS3 kinase function as well as the role of the two PrV pUS3 isoforms were investigated. pUS3 is involved in nuclear egress of capsids, a crucial step in the herpesviral replication cycle. Although pUS3 is not essential for this process, absence of pUS3 results in accumulations of primary virions within large invaginations of the INM (Klupp *et al.* 2001, Wagenaar *et al.* 2001, Reynolds *et al.* 2002), indicating that fusion with the ONM is affected. So far, it has not been studied how the two isoforms (pUS3-S and pUS3-L), which differ in size, abundance, and subcellular localization, function upon viral infection, particularly in nuclear egress. To address this and to test the impact on viral propagation different cell lines were infected with pUS3 mutants which either expressed exclusively the long isoform (PrV-US3-M55A) or the short isoform (PrV-ΔUS3-L), with reduced expression of the short isoform (PrV-US3-M55V), completely lacking pUS3 (PrV-ΔUS3), or only defective in the kinase activity (PrV-US3Δkin).

Of all mutants tested, only PrV-ΔUS3 and PrV-US3Δkin showed slightly impaired viral replication on rabbit kidney (RK13) cells with up to 10-fold reduced viral titers, which is in line with earlier studies for the US3-deletion mutant (Wagenaar *et al.* 1995, Klupp *et al.* 2001). On Madin-Darby bovine kidney (MDBK) cells, the effect was more pronounced and all mutants lacking sufficient amount of enzymatically active pUS3-S replicated to significantly lower viral titers (100- to 1000-fold reduction). The mutant defective for the long isoform showed no phenotype on RK13 cells and only a marginal drop in titers on MDBK cells indicating that this isoform does not play an important role during viral replication in cell culture. In contrast, the enzymatic activity of pUS3 is the major determinant as demonstrated by the highly similar phenotypes of PrV-ΔUS3 and PrV-US3Δkin.

pUS3-L was not sufficient to compensate for the lack of the short isoform. This might be due to either the low expression level or a different subcellular localization. Localization was investigated by confocal laser scanning microscopy showing that pUS3-L, which is the only isoform expressed in PrV-US3-M55A infected cells, predominantly localized in the cytoplasm. In contrast, pUS3-S as expressed by PrV-ΔUS3-L was found in the nucleus and in the cytoplasm. This reflects earlier results on localization of pUS3-L and pUS3-S in cells transfected with expression plasmids (Calton *et al.* 2004). However, targeting of pUS3-L to mitochondria as detected in

transfected cells (Calton *et al.* 2004) was not evident in infected cells. Inactivation of the kinase function in PrV pUS3 however, did not alter the staining pattern for pUS3, which is in contrast to data shown for HSV-2 (Finnen *et al.* 2010).

pUS3-L, which does not contain a classical nuclear localization signal, might be too large (53kDa) for nuclear import which appears a prerequisite to mediate efficient nuclear egress. In line with this finding, accumulations of primary virions in the PNSp in large invaginations of the INM were found in all US3 mutants except for mutant PrV-ΔUS3-L lacking the long isoform. Surprisingly, accumulations of primary enveloped virions were undistinguishable for all pUS3-S mutants and cell lines, and did not correlate with the observed titer reduction. These results clearly demonstrate that sufficient amounts of pUS3-S and unimpaired kinase activity are required for efficient release of primary virions form the PNS.

Viral replication was more impaired in MDBK than in RK13 cells. A huge variety of cell lines is permissive for PrV including rabbit kidney cells (RK13) cells, porcine kidney cells (SPEV, PK15, SK6), monkey kidney cells (Vero) as well as bovine kidney cells (MDBK). However, few comparative studies in different cell lines have been performed showing that the outcome after PrV infection may differ. It was demonstrated that PrV downregulated the MHC-I receptor in PK15 cells, but not in MDBK cells (Ambagala *et al.* 2000). PrV infection was shown to inhibit autophagy in Vero and PK15 cells, but less in mouse embryonic fibroblasts (3T3 cells) which has been linked to the pUS3 kinase activity which might seems to impact autophagy in the non-human cells (Sun *et al.* 2017).

The drastic effect observed in MDBK cells after infection with the different pUS3 mutants was striking. None of the other cell lines tested (Vero, SPEV) showed a similar drop in viral titers. While at early times after infection, the difference in virus titers was small, infectious virus propagation appeared to cease after the eight-hour time point indicating the shortage of a cellular factor, most likely a kinase, compensating for the pUS3 defect in MDKB cells. To test this the amount of cellular kinases should be investigated as reported recently (Smolko and Janes 2019). This as well as proteomic analyses of the two cell lines infected with PrV mutants could help to shed light on this striking phenotype. In addition, proteomics could be very useful to identify cellular or viral substrates, which are modified by pUS3. In an earlier study, the cellular response to PrV infection in MDBK cells was analyzed using quantitative proteomics in cells

infected with PrV-Kaplan versus PrV-ΔUS3 (Skiba *et al.* 2010). The absence of pUS3 affected protein levels of pUL29 (major DNA-binding protein), pUL39 (ribonucleotide-diphosphate reductase large subunit), and pUL42 (DNA polymerase processivity factor). However, so far functional interaction between pUS3 and the identified viral proteins have not been studied further and have not been compared to other cell lines such as RK13 cells.

In humans, more than one third of proteins, primarily involved in signal transduction pathways, are posttranslationally modified by kinases (Manning *et al.* 2002). Particularly protein kinase A (PKA) modulates a variety of substrates involved in different physiological cellular pathways (Shabb 2001). For HSV-1, it has been shown that the phosphorylation target site specificity of pUS3 is similar to that of PKA and PKB (Benetti and Roizman 2004, Chuluunbaatar *et al.* 2010). Although this has not been shown for PrV pUS3 it might be reasonable to compare PKA/B levels in RK13 and MDBK cells.

It was reported recently that pUS3 induces tunneling nanotubes which facilitate virus spread between cells (Jansens *et al.* 2017). In addition, PKA was shown to regulate tubular transport (Tenorio *et al.* 2015). Although very speculative, the reduced viral titer could be explained by impaired transport capacity, which is usually modulated by pUS3. Thus, in RK13 cells protein kinase A might be able to complement pUS3 function while MDBK cells fail to do so.

In summary, the data shown in **paper I** revealed that unimpaired pUS3 kinase activity is required for efficient nuclear egress and, thus, is important for full replication competence. However, impaired nuclear egress is not responsible for the reduced viral titers. Finally, it could be demonstrated that the absence of pUS3 kinase activity is comparable with PrV completely lacking pUS3, which was an important prerequisite to start with characterization of PrV-ΔUL21/US3Δkin (**paper II**).

5.2 Characterization of mutant PrV-ΔUL21/US3Δkin *in vitro* and *in vivo* (paper II)

Preceding the animal experiment, PrV-ΔUL21/US3Δkin was characterized *in vitro* and compared to PrV-Kaplan and the corresponding single mutants. PrV-ΔUL21/US3Δkin was able to replicate productively in cell culture, although the mutant showed a significant titer reduction of 50- to 100-fold in RK13 cells, while the single mutants

displayed only slightly reduced titers of up to 5- to 10-fold as shown previously (**paper I** (Klupp *et al.* 2005)). A small plaque phenotype was evident in the mutants lacking UL21 pointing to a role of this protein in efficient virus spread.

In the animal infection model, PrV-ΔUL21/US3 was the only mutant tested so far, which surprisingly did not kill 100% of the infected mice despite productive infection (Klopfleisch *et al.* 2004, Klopfleisch *et al.* 2006, Maresch 2011). Comparing the MTD, PrV-ΔUL21/US3Δkin revealed very similar results as PrV-ΔUL21/US3, indicating that the phenotype observed in combination with deletion of UL21 is due to dysfunctional pUS3 kinase activity. Only one out of six mice infected with PrV-ΔUL21/US3Δkin had to be euthanized due to severe clinical signs while the others survived the infection showing only mild to moderate symptoms. In contrast, mice infected with PrV-Kaplan or PrV-US3Δkin showed a MTD of 62 and 63h p.i., respectively, whereas PrV-ΔUL21 infected mice lived almost twice as long. Even though all mice in general survived slightly longer, data were highly comparable to previous findings (Klopfleisch *et al.* 2006).

Neither the *in vitro* data nor the animal experiment allowed a clear conclusion how pUS3 and pUL21 contribute to the striking effect in mice, and whether there is a direct or indirect interaction between pUL21 and pUS3. Although both proteins are known to be virulence determinants in pigs (Kimman *et al.* 1994, Klupp *et al.* 1995), the molecular basis for this attenuation remains largely unclear.

For further characterization, a kinetic experiment was conducted to monitor clinical signs, virus spread and induction of an inflammatory response to infection with PrV-Kaplan, the single mutants and PrV-ΔUL21/US3Δkin at different time points (**paper II**). Also in this thorough investigation, infection with PrV-Kaplan and PrV-US3Δkin were comparable, even though a slight delay in the onset of symptoms was observed in PrV-US3Δkin infected mice. This is in line with a study that reported a delay in the onset of clinical signs in rats after PrV-ΔUS3 infection. This delay has been linked to defects in cell-to-neuron spread since the velocity of intraaxonal virion transport was not affected (Olsen *et al.* 2006).

In PrV-Kaplan and PrV-US3Δkin infected mice clinical signs were severe, and first and second order neurons as well as autonomic ganglia and some brainstem areas showed a high viral antigen load. No or very mild inflammation in the ganglia or in the nose was

detectable as shown in earlier studies (Klopfleisch *et al.* 2006). PrV-Kaplan and PrV-US3Δkin infected mice had to be euthanized two to three days after infection, which is why an immune reaction might not have been developed (Janeway Jr *et al.* 2001). The comparable phenotypes in PrV-Kaplan and PrV-US3Δkin infected mice implicates that defective kinase activity or absence of the complete gene (Klopfleisch *et al.* 2006) has no major impact on neuroinvasion and spread in the mouse. In contrast, a significant impact on neurovirulence had been reported for HSV-1 pUS3 (Morimoto *et al.* 2009). Here, the lethal dose 50% was 680-fold higher than for wildtype HSV-1.

In PrV-ΔUL21 infected mice, light to moderate ganglionitis as well as mild focal brainstem inflammation were detectable. This mutant was not only able to infect first and second order neurons, but spread to the cerebral cortex where few neurons were found positive for viral antigen. However, an inflammatory response was not detectable in higher areas than the brainstem. pUL21 has been reported to be important for neuroinvasion affecting retrograde intraaxonal transport processes (Curanovic *et al.* 2009, Yan *et al.* 2019). Here, we could confirm a delayed neuroinvasion accompanied by a prolonged survival time for PrV-ΔUL21 infected mice. Due to longer survival, the immune system in the infected mice was able to react with the development of an innate antiviral defense. Immunomodulatory properties of pUL21 have been suggested (Sarfo *et al.* 2017). This would mean that the immune system might react more efficiently against PrV infection in the absence of pUL21. However, the immune response in PrV-ΔUL21 infected mice was not sufficient to eliminate the virus, and all animals had to be euthanized due to severe clinical signs.

A pronounced inflammatory response was detectable in mice infected with PrV-ΔUL21/US3Δkin after 9 to 10 days post infection (d p.i.). The replication defect of this mutant as observed in cell culture was also evident in infected mice since the amount of viral antigen was lower compared to the single mutants. In the early phase of infection, viral antigen was only sparsely detectable in the ganglia and brainstem, but 8 d p.i. higher brain areas, especially neurons of the temporal and frontal lobe were positive by immunohistochemistry. Viral antigen was no longer detectable than 15 d p.i.

Compared to PrV-Kaplan and the single mutants, PrV-ΔUL21/US3Δkin infected mice showed a significant delay in the onset of clinical signs, viral antigen detection and

anti-inflammatory reaction, which indicates a delayed neuroinvasion allowing the immune system to react against the infection.

Herpesviruses including HSV-1 and PrV have evolved different strategies to escape from the host's immune response (Suazo *et al.* 2015, Yang *et al.* 2019). The multifunctional pUS3 is involved in this process (Kato and Kawaguchi 2018), which might explain the effects on neurovirulence after infection with PrV-ΔUL21/US3Δkin. pUS3 has been shown to downregulate essential receptors on the surface of immune cells including the major histocompatibility complex I (MHC-I) on cytotoxic CD8 T cells or CD1d on natural killer T cells (Cartier and Masucci 2004, Rao *et al.* 2011, Imai *et al.* 2013, Xiong *et al.* 2015, Rao *et al.* 2018), thus promoting prolonged survival of herpesvirus-infected cells. pUS3 also inhibits TLR 2 signaling (Sen *et al.* 2013) and further impairs the activity of T cells by interacting with different downstream effector molecules in the T cell or interferon receptor signaling cascade (Yang *et al.* 2015, Qin *et al.* 2019). In the same way, several anti (B-cell lymphoma 2 (Bcl-2))- and proapoptotic (Bcl-2 agoinst of cell death (Bad), Bcl-2-like protein 11 (Bim), BH3 interacting-domain death agonist (Bid)) factors have been shown to be upregulated or inactivated by pUS3, thereby suppressing death of infected cells (You *et al.* 2017). Upon infection with PrV-ΔUL21/US3Δkin, this would mean that in the absence of enzymatically active pUS3 virus mediated immune evasion was impaired, while the delayed neuroinvasion due to the absence of pUL21 obviously resulted in an effective inflammatory reaction.

In PrV-ΔUL21/US3Δkin infected animals inflammation was first detected in ganglia and brainstem, whereas the telencephalon including the temporal and frontal lobes were infiltrated starting at 9 to 10 d p.i. Inflammatory response was detectable until the end of the experiment at day 21. In contrast to PrV-ΔUL21 infected animals, mice did not die from infection with PrV-ΔUL21/US3Δkin and only two mice had to be euthanized. Inflammation mainly consisted of lymphohistiocytic infiltration of the meninges and brain parenchyma, neuronal death and glial activation.

Particularly activated microglia, the resident macrophages of the brain, play a pivotal role in the innate antiviral defense (Kim and Joh 2006). As classically activated M1 macrophages, they produce pro-inflammatory cytokines, thereby activating and recruiting other inflammatory cells to the brain lesion. If overactivated, microglia can

be also neurotoxic and cause severe neuronal damage. Like alternatively activated M2 macrophages, microglia also has anti-inflammatory properties.

In PrV-ΔUL21/US3Δkin infected mice it appeared that the immune system could control virus replication, probably through efficient activation of microglia, which recruited other immune cells like lymphocytes, and eventually through the induction of immunoregulatory effects since mice recovered. In two out of 69 mice, however, an excessive, probably neurotoxic immune response seemed to be present leading to death of the animals between 9 and 14 d p.i. Whether the induction of M1 or M2 mircroglia is modulated by pUL21 and/or pUS3 remains to be tested by comparative immunological studies.

The ability of mice to survive an infection with PrV-ΔUL21/US3Δkin indicates either a functional and/or physical interaction of a protein complex, which has not been described so far, or an additional effect of distinct independent functions of pUL21 and the pUS3 kinase. However, the simultaneous absence of pUL21 and pUS3 kinase activity significantly decreased neurovirulence in the mouse. In the pig, this mutant is most likely avirulent as it was shown for PrV-Bartha (Klupp *et al.* 1995). PrV-Bartha contains several mutations in the UL21 gene and incorporates less pUS3 into virions (Lyman *et al.* 2003, Michael *et al.* 2007). However, PrV-Bartha was not significantly attenuated in mice (Brittle *et al.* 2004, Klopfleisch 2005, Laval *et al.* 2018) in contrast to PrV-ΔUL21/US3Δkin, which most mice are able to survive. This points to a specific role of functional pUL21 and pUS3 kinase at the same time. It would be very interesting how PrV-ΔUL21/US3Δkin behaves in other highly susceptible animals such as dogs since a vaccine for this species is still not available (Lin *et al.* 2019).

Evaluation of PrV-ΔUL21/US3Δkin infected mice as a model for Herpes Simplex Encephalitis

The in-depth evaluation of the data generated with PrV-ΔUL21/US3Δkin infected mice disclosed a striking analogy to HSE in humans (**paper II**) (Bradshaw and Venkatesan 2016). As described for patients dying of HSE, viral antigen and inflammatory reaction in PrV-ΔUL21/US3Δkin infected mice were mainly present unilaterally in the temporal and frontal lobes. To a lesser extent, inflammatory foci were also identified in the ganglia, brainstem and other parts of the brain sections, which is consistent with data published in several case reports of uncommon cases of HSE (Hamilton *et al.* 1995,

Wasay *et al.* 2005, Arita *et al.* 2010, Livorsi *et al.* 2010). In addition, with increasing inflammation in the brain mice exhibited distinct behavioral changes such as slow movement, akinesia and intermitting phases of star gazing which potentially resemble mental status changes observed in patients with HSV-1 or VZV induced meningoencephalitis (Steiner and Benninger 2018). These changes are linked to lesions in the limbic system including the hippocampus located in the mesiotemporal lobe (Squire *et al.* 2004, Jonker *et al.* 2014) which was also affected in mice infected with PrV-ΔUL21/US3Δkin. Not primarily associated with HSV-1, but commonly part of VZV infections asymptomatic myelitis, zosteriform skin lesions as well as ganglionitis (Haanpaa *et al.* 1998, Skripuletz *et al.* 2018, Steiner and Benninger 2018) were observed in PrV-ΔUL21/US3Δkin infected mice.

To date, various mouse models have been used to study the pathogenesis of HSE (Mancini and Vidal 2018). Nevertheless, these models do not sufficiently reflect the disease in humans. PrV-ΔUL21/US3Δkin infected mice, however, present a model, which obviously combines the lesion-associated disease pattern and pathohistological features of HSE. Here, meningoencephalitis was mainly associated with the temporal and frontal lobes, and therefore, this model can be utilized to analyze area-dependent tropism of brain infection. Alphaherpesviruses might have a restricted subneuronal cell tropism which could explain the lesion pattern of HSE, but which has not been investigated in detail yet (Bloom and Stevens 1994, Rosato and Leib 2015, Cabrera *et al.* 2018). Differences in the subneuronal cell tropism, permissivity and mechanisms of viral and immune-mediated neuronal injury have been already described for flaviviruses such as West Nile Virus or Japanese Encephalitis Virus infections (Shrestha *et al.* 2003, Xiao *et al.* 2015).

It is also still unclear how HSV-1 reaches its target regions. From infection experiments in mice and rats, it is known that HSV-1 can be transported via the trigeminal and the olfactory nerve (Lundberg *et al.* 2008, Shivkumar *et al.* 2013, Boukhvalova *et al.* 2019). However, viral antigen was present either in the brainstem or TG, but showed no signal in the cerebral cortex including the temporal and frontal lobe in infected mice. In contrast, in mice infected with PrV-ΔUL21/US3Δkin the way of infection was clearly traceable, and indicated infection either via the trigeminal or via the olfactory pathway. However, also here further detailed analyses are required. For instance, stereotactic virus injection into defined brain areas would help to analyze the way of viral spread

towards the temporal and frontal lobes (Mcsweeney and Mao 2015). By this, a Cre recombinase-based virus/mouse system (Dutia *et al.* 2009) or an *in vivo* live imaging approach (Granstedt *et al.* 2013) could significantly contribute to the identification of infected cells, which would increase the knowledge of either the neuroanatomical pathway or cell-specific tropism of herpesviruses.

The results of the present study indicate that behavioral changes are linked to lesions in the mesiotemporal lobe. Thus, detailed behavioral analyses combined with magnetic resonance tomography as done in mice infected with HSV-1 (Hafezi and Hoerr 2013), could be useful to monitor the development of meningoencephalitis and associated cognitive alterations. Since the mesiotemporal lobe is particularly important for memory (Urgolites *et al.* 2017) and the most affected area in PrV-ΔUL21/US3Δkin infected mice cognitive tests should be applied to test different aspects of learning and memory (Levin and Buccafusco 2006). This would not only make an important contribution to elucidate the pathogenesis of HSE, but could also unravel basic behavioral mechanisms.

To better understand the pathogenesis of HSE and to further characterize our PrV-based mouse model for HSE in-depth immunological analyses of infected animals would be important. A follow-up study performed recently revealed first insights into the immune response of mice infected with PrV-ΔUL21/US3Δkin. In addition to histopathological data, immune cell infiltration as well as cytokine levels were investigated. We found that infiltration of T cells peaked between 288 to 360 h p.i. and decreased in the following, which fully correlates with histopathology from the kinetic trial (unpublished data).

We further analyzed different cytokines and found that CXCL10, CCL5, CCL2, CXCL1, IFN-γ and TNF-α were highest 288 h p.i. and decreased thereafter (unpublished data). Human microglia as well as mice infected with HSV-1 showed similar upregulation (Lokensgard *et al.* 2001, Marques *et al.* 2008). As a first response against HSV-1, microglia initially produced pro-inflammatory cytokines such as IFN-I, IL-6, IL-1β as well as IFN-γ, and TFN-α which disrupted the blood brain barrier and promoted entry of immune cells (Aravalli *et al.* 2005, Weiser *et al.* 2007). Subsequently, chemokine production by CNS resident cells was upregulated, particularly in a TLR-2 dependent manner (Aravalli *et al.* 2005, Wang *et al.* 2012). Among others, these chemoattractants include CCL2 and CCL5 recruiting monocytes and neutrophils to the site of infection,

which in turn produced high amounts of CXCL10, CCL5 and TNF-α to attract other immune cells. CXCL1 drives neutrophil recruitment. In particular, CCL2 and CXCL10 are thought to play the most important role in the innate immune response against HSV-1 (Wuest and Carr 2008). The preliminary immunological study in PrV-ΔUL21/US3Δkin infected mice clearly reflects histopathological results and correlates with the critical phase of infection, where most of the animals have shown moderate clinical signs. This study also demonstrates that encephalitis caused by PrV-ΔUL21/US3Δkin is comparable to HSV-1 encephalitis in mice, and supports that this model, in addition to brain lesions and behavioral changes, also better reflects human disease immunologically.

To further characterize PrV-ΔUL21/US3Δkin encephalitis, long-term experiments should be carried out to investigate consequences including behavioral alterations, and to assess and evaluate the development of brain lesions, the immunological status and the establishment of latency. In line with this, in patients with HSE, infected and activated microglia was found to persist for more than one year in the brain, even after antiviral therapy (Cagnin *et al.* 2001). This led to chronic inflammation and neuronal cell death due to the production of proinflammatory cytokines such as TFN-α (Lokensgard *et al.* 2001). TNF-α has been reported to stimulate astrocytes leading to altered astrocyte-neuron-signaling in the hippocampus which impairs cognitive performance (Habbas *et al.* 2015). Preliminary data of a first long term study in PrV-ΔUL21/US3Δkin infected mice revealed that behavioral changes either remained at the same level, worsened or even occured as late as after 21 days (unpublished data). Histopathologically, mainly mild focal inflammation of brain parenchyma and meninges was detected in the temporal and frontal lobes even until six months after the infection, which supports the chronic course of HSE. Seizures, which were usually only present in severe cases and appeared in the critical phase between 10 and 14 d p.i., also occurred in several mice months after infection. HSE is associated with severe long-term sequelae such as epilepsy, which can appear in the acute, chronic or in the relapsing form, and is attributed to lesions in the temporal lobe (Misra *et al.* 2008, Liu and Zhou 2019). Although seizures were only rarely observed in mice infected with PrV-ΔUL21/US3Δkin this still supports the suitability of this animal model.

As shown in **paper II**, some mice infected with PrV-ΔUL21/US3Δkin did not show any clinical signs over the whole study period or presented only alopecic skin lesions. Skin

lesions and mainly mild pruritus were observed in different body regions of PrV-ΔUL21/US3Δkin infected mice. PrV-ΔUL21/US3Δkin was probably transported along the spinal cord since an inflammatory response was focally present in the spinal cord. As known for VZV (Zerboni *et al.* 2014) a subsequent infection of spinal nerves innervating discrete dermatomes possibly resulted in PrV-ΔUL21/US3Δkin replication in the skin and led to the observed skin lesions. However, detection of viral antigen in these lesions was not successful. This could be due to the fact that mice were examined relatively late after the skin lesions appeared and that the virus had already been cleared. Pruritus-induced licking might be the reason for the hairless skin lesions. Following shingles VZV patients often suffer from neuropathic itch which describes pruritus in the absence of pruritogenic stimuli (Hachisuka *et al.* 2018). After reactivation of VZV in DRG, the virus causes painful rash in the corresponding dermatome. Afferent nerve fibers remain damaged for months which resulted in postherpetic neuralgia or itch of the innervated skin (Oaklander 2008). Very recently, PrV infected mice had been proposed as a putative model to study VZV-induced peripheral neuropathies since the molecular reason of postherpetic itch is not well understood (Laval and Enquist 2020). For this purpose, our mouse model for herpesviral encephalitis using PrV-ΔUL21/US3Δkin would also be very useful.

In conclusion, mice infected with PrV-ΔUL21/US3Δkin provide an excellent model to analyze crucial steps in herpesviral neuroinvasion and to answer open questions including: Which neuroanatomical pathway does the virus use? How does the host immune system respond to infection? How does inflammation correlate with behavioral alterations? What contributes to a chronic course of herpesvirus induced encephalitis? How is latency established in mice? How and why does the virus primarily affect the temporal and frontal lobes?

6 Summary

Alphaherpesviruses are neuroinvasive viruses with the ability to cause severe diseases in humans and animals. HSV-1 is a human pathogen known to sporadically induce fronto-temporal meningoencephalitis, which is associated with high mortality rates and long lasting sequelae such as chronic or relapsing inflammation, cognitive alterations or epilepsy. PrV is an animal pathogen causing CNS disorders primarily in piglets. In non-porcine species PrV infection leads to severe itching followed by automutilation and death of the infected and diseased animal. After primary replication in epithelial cells, HSV-1 and PrV infect sensory neurons in which they are transported to peripheral ganglia and towards the CNS. In the past, the function of different PrV proteins for neuroinvasion has been studied in mice using corresponding viral gene deletion mutants. These studies revealed that infection with a PrV mutant lacking the tegument protein pUL21 and the pUS3 kinase led not only to a significantly prolonged survival time but unlike to infection with PrV wildtype or other mutants, which was always fatal, most mice survived the infection.

In this thesis, this phenotype was studied in detail. To test whether the effect resulted from the absence of the pUS3 kinase function of or from the absence as virion structural component, virus mutants PrV-US3Δkin and PrV-ΔUL21/US3Δkin were constructed and compared to PrV-ΔUS3 and PrV-ΔUL21/US3. In addition to the influence of the kinase-active pUS3, the function of the two described isoforms, pUS3-S and pUS3-L, was analyzed. In vitro data showed that while pUS3-L plays no major role in the viral replication cycle, the abundant, catalytically active short isoform of pUS3 is required for efficient nuclear egress and production of infectious progeny. However, the accumulations of primary virions in huge herniations of the INM described for mutants lacking US3 are not the cause for the observed drop in virus titers. PrV-ΔUS3, lacking the open reading frame completely, or PrV-US3Δkin, carrying only a single nucleotide exchange resulting in inactivation of the kinase activity, showed comparable phenotypes not only in cell culture but also in the mouse indicating that the enzymatic function but not pUS3 as structural virion component are important. Corresponding phenotypes in cell culture and for survival times in mice were also evident for the mutants lacking in addition UL21 (PrV-ΔUL21/ΔUS3, PrV-ΔUL21/US3Δkin).

Based on these data, PrV-ΔUL21/US3Δkin was used to analyze the pathobiological mechanism in comparison to PrV-Kaplan and the corresponding single mutants (PrV-US3Δkin, PrV-ΔUL21) in mice. As reported before for PrV-ΔUL21/US3 (Maresch 2011), and in contrast to mice infected with wildtype PrV or the single mutants, nearly all PrV-ΔUL21/US3Δkin infected animals survived.

In an extended experiment, mice infected with PrV-ΔUL21/US3Δkin and the control viruses were euthanized and thoroughly studied at different time points after infection. Viral spread and inflammatory response were assessed histologically and correlated to the clinical status. Besides a slight delay in the onset of clinical signs and neuroinvasion in PrV-US3Δkin infected mice, no difference to mice infected with PrV-Kaplan was evident indicating that the pUS3 kinase obviously has no role in neuroinvasion or pathogenicity. PrV-Kaplan and PrV-US3Δkin showed fast neuronal spread and occasionally induced only very mild inflammatory reaction, which might be due to the short time between infection and death of the animals. Infection with PrV-ΔUL21 resulted in delayed, but more widespread neuroinvasion and pronounced inflammation in the ganglia and mild inflammatory response in the brainstem, which correlates with the extended survival time. However, all mice infected with PrV-ΔUL21 developed severe clinical signs and had to be euthanized. PrV-ΔUL21/US3Δkin spread even slower than PrV-ΔUL21, but invaded the cerebral cortex, mainly the frontal and temporal lobe starting 8 d p.i. A fulminant lymphohistiocytic reaction developed, which was first detected in the ganglia and brainstem, but was more pronounced in the telencephalon affecting the temporal and frontal lobe starting 9 d p.i. With increasing inflammation, also behavioral abnormalities occurred in mice, which culminated between 9 to 14 d p.i., whereas mild pruritus and skin lesions developed after 6 d p.i. While viral antigen was no longer detectable in mice sacrificed later than 15 d p.i., meningoencephalitis was present in mice examined at the end of the experiment at day 21 p.i. Despite widespread neuroinvasion and concomitant meningoencephalitis mice were able to survive an infection with PrV ΔUL21/US3Δkin, which had not been reported for any other PrV mutant.

Although the mode of interplay between pUL21 and the kinase activity of pUS3 remained enigmatic, the most important result of this study is that PrV-ΔUL21/US3Δkin infected mice showed unexpected but striking analogies to human HSE including fronto-temporal, lesion-associated virus tropism combined with behavioral

abnormalities, which in combination could not be achieved in other animal models. Furthermore, skin lesions and the presence of myelitis in PrV-ΔUL21/US3Δkin infected mice partially resembled the disease caused by VZV in humans.

With PrV-ΔUL21/US3Δkin infection in mice as model, detailed investigations on herpesvirus neuroinvasion, neuronal spread, and immune reaction in the brain but also advanced neuronal tracing is possible that will significantly contribute to our understanding not only of herpesvirus pathobiology but also of neuroanatomical networks.

6 Zusammenfassung

Alphaherpesviren sind neuroinvasive Viren, die schwerwiegende Erkrankungen bei Menschen und Tieren auslösen können. Das humanpathogene HSV-1 führt in sporadischen Fällen zu einer fronto-temporalen Meningoenzephalitis, die mit einer hohen Mortalitätsrate und mit schwerwiegenden Langzeitfolgen wie chronischer oder rezidivierender Entzündung, kognitiver Beeinträchtigung oder Epilepsie assoziiert ist. PrV ist ein porzines Herpesvirus, das vorwiegend bei Ferkeln zu zentralnervösen Symptomen führt. In nicht-porzinen Spezies führt die PrV-Infektion zu schwerwiegendem Juckreiz, zu Automutilation und letztendlich zum Tod des infizierten und erkrankten Tieres. HSV-1 und PrV infizieren nach der primären Replikation in Epithelzellen sensorische Nervenzellen, in welchen sie über periphere Ganglien zum ZNS transportiert werden. In der Vergangenheit konnte die Funktion von verschiedenen PrV Proteinen für die Neuroinvasion bei Mäusen mit Hilfe von viralen Gendeletionsmutanten untersucht werden. Diese Studien zeigten, dass eine Infektion mit der PrV-Doppelmutante PrV-ΔUL21/US3, nicht nur in einer signifikant verlängerten Überlebenszeit resultierte, sondern dass im Gegensatz zur tödlichen Infektion mit dem Wildtyp oder anderen Mutanten, die meisten Mäuse überlebten.

In der vorliegenden Dissertation wurde dieser Phänotyp weitergehend untersucht. Um zu testen, ob die Eigenschaft auf die fehlende Kinasefunktion oder das Fehlen des pUS3 als Virion-Strukturkomponente zurückzuführen ist, wurden die Virusmutanten PrV-US3Δkin und PrV-ΔUL21/US3Δkin, bei denen nur die Enzymaktivität durch eine Punktmutation inaktiviert wurde, das Protein aber weiterhin exprimiert wird, generiert und mit den US3-Deletionsmutanten PrV-ΔUS3 und PrV-ΔUL21/ΔUS3 verglichen. Neben dem Einfluss der Kinaseaktivität wurde zusätzlich die Funktion der beiden beschriebenen unterschiedlich langen Isoformen, pUS3-S (*short*) und pUS3-L (*long*), getestet. Die In-vitro-Untersuchungen zeigten, dass pUS3-L keine wesentliche Rolle im viralen Replikationszyklus spielt, jedoch eine genügende Menge des katalytisch-aktiven pUS3-S für einen effizienten Austritt der Nukleokapside aus dem Kern und für die Produktion infektiöser Virusnachkommen vorhanden sein muss. Die großen Akkumulationen von primären Virionen in Hernien der inneren Kernmembran, die für pUS3-Mutanten typisch sind, sind jedoch nicht der Grund für den beobachteten Abfall des Virustiters. Die Mutante PrV-ΔUS3, der der offene Leserahmen vollständig fehlt und die Mutante PrV-US3Δkin mit inaktivierter Kinaseaktivität, zeigten nicht nur in der

Zellkultur vergleichbare Phänotypen, sondern auch in der Maus. Dies deutet darauf hin, dass die enzymatische Aktivität die entscheidende Rolle für den Phänotyp spielt. Entsprechende Ergebnisse zeigten sich auch beim Vergleich der Mutanten, denen zusätzlich UL21 fehlte (PrV-ΔUL21/ΔUS3, PrV-ΔUL21/US3Δkin). Basierend auf diesen Daten wurde PrV-ΔUL21/US3Δkin genutzt, um den pathobiologischen Mechanismus im Vergleich zu PrV-Kaplan und den entsprechenden Einzelmutanten (PrV-US3Δkin, PrV-ΔUL21) bei Mäusen zu testen. Wie zuvor für PrV-ΔUL21/US3Δkin (Maresch, 2011) gezeigt, jedoch im Gegensatz zu Mäusen, die mit PrV-Wildtyp oder den Einzelmutanten infiziert waren, überlebten fast alle PrV-ΔUL21/US3Δkin infizierten Tiere.

In einem umfassenden Experiment wurden die mit PrV-ΔUL21/US3Δkin und den Kontrollviren infizierten Mäuse zu verschiedenen Zeitpunkten nach der Infektion euthanasiert und untersucht. Die Ausbreitung der Viren und die Entzündungsreaktion wurden histologisch beurteilt und mit dem klinischen Status der Tiere korreliert. Abgesehen von einer leichten Verzögerung des Auftretens klinischer Erscheinungen und der Neuroinvasion bei den mit PrV-US3Δkin infizierten Mäusen war kein Unterschied im Vergleich zu PrV-Kaplan erkennbar. Dies weist daraufhin, dass die pUS3-Kinase offensichtlich keine Rolle bei der Neuroinvasion oder für die Pathogenität in diesem System spielt. PrV-Kaplan und PrV-US3Δkin zeigten eine schnelle neuronale Ausbreitung und induzierten in wenigen Tieren eine nur sehr milde Entzündungsreaktion, was auf die kurze Zeit zwischen der Infektion und dem Tod der Tiere zurückzuführen ist. Eine Infektion mit PrV-ΔUL21 führte zu einer deutlich verzögerten, aber ausgedehnteren Neuroinvasion und zu einer ausgeprägten Entzündung der Ganglien sowie einer leichten Entzündungsreaktion im Hirnstamm, was möglicherweise auf die verlängerte Überlebenszeit der Tiere zurückzuführen ist. Alle mit PrV-ΔUL21 infizierten Mäuse entwickelten jedoch schwere klinische Symptome und mussten euthanasiert werden. PrV-ΔUL21/US3Δkin breitete sich noch langsamer als PrV-ΔUL21 aus, drang jedoch ab 8 d p.i. in die Großhirnrinde, hauptsächlich in den Frontal- und Temporallappen, vor. Es entwickelte sich eine fulminante lymphohistiozytäre Entzündungsreaktion, die zuerst in den Ganglien und im Hirnstamm festgestellt wurde, die jedoch im Telenzephalon, besonders im temporalen und frontalen Lappen ab 9 d p.i. deutlicher wurde. Mit zunehmender Entzündung traten auch Verhaltensstörungen bei den Tieren auf, die zwischen 9 und 14 d p.i. ihren Höhepunkt erreichten, während leichter Juckreiz und Hautläsionen

schon nach 6 d p.i. beobachtet wurden. Obwohl virales Antigen bei Mäusen, die nach Tag 15 getötet wurden, nicht mehr nachweisbar war, konnte eine Meningoenzephalitis auch bei Tieren nachgewiesen werden, die am Ende des Experiments (Tag 21) getötet wurden. Trotz dieser starken Neuroinvasion und der damit einhergehenden Meningoenzephalitis überlebten die Mäuse eine Infektion. Dies wurde bisher für keine andere PrV-Mutante berichtet.

Obwohl die Art des Zusammenspiels zwischen pUL21 und der Kinaseaktivität von pUS3 unklar blieb, ist das herausragende Ergebnis dieser Studie, dass mit PrV-ΔUL21/US3Δkin infizierte Mäuse unerwartete, aber sehr auffällige Analogien zur HSE im Menschen zeigten. Dazu gehörte der fronto-temporale, läsionsassoziierte Virustropismus, der in Kombination mit den beobachteten Verhaltensstörungen so in anderen Tiermodellen bisher nicht reproduziert werden konnte. Ferner ähnelten die Hautläsionen und das Vorhandensein einer Myelitis bei mit PrV-ΔUL21/US3Δkin infizierten Mäusen VZV-induzierten Krankheitsbildern beim Menschen.

Mit dem Modell der PrV-ΔUL21/US3Δkin-Infektion von Mäusen sind detaillierte Untersuchungen zur herpesviralen Neuroinvasion, zur neuronalen Ausbreitung und zur Immunreaktion im Gehirn sowie innovatives neuronales Tracing möglich, die nicht nur zum Verständnis von herpesviraler Pathobiologie, sondern auch zur Aufklärung neuroanatomischer Netzwerke beitragen könnten.

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8 Appendix

8.1 Eigenständigkeitserklärung

"Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der "Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis" niedergelegt sind, eingehalten."

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Julia Sehl

8.2 Publications

Sehl J, Pörtner S, Klupp BG, Granzow H, Franzke K, Teifke JP, Mettenleiter TC. 2020. Roles of the Different Isoforms of the Pseudorabies Virus Protein Kinase pUS3 in Nuclear Egress. J Virol. **94**(7), doi: 10.1128/JVI.02029-19.

Sehl J, Hölper JE, Klupp BG, Teifke JP, Mettenleiter TC. 2020. An Improved Animal Model for Herpes Simplex Encephalitis in Humans. PLoS Pathog. **16**(3), doi: 10.1371/journal.ppat.1008445

8.3 Oral and poster presentations

09/2019	**Sehl, J.**; Hölper, J.; Teifke, J.P.; Klupp, B.G.; Mettenleiter, T.C. (2019), Talk. An animal model for herpes simplex virus induced encephalitis. Junior Scientist Symposium, Friedrich-Loeffler-Institut, Jena, 25.-27.09.2019
09/2019	**Sehl, J.**; Schwaiger, T.; Hölper, J.; Teifke, J.P.; Klupp, B.G.; Mettenleiter, T.C. (2019), Poster. PrV-ΔUL21/US3Δkin in mice – an appropriate animal model for herpes simplex virus induced encephalitis?, Mini-Herpesvirus Workshop, Helmholtz Centre for Infection Research, Braunschweig, 20.09.2019
03/2019	**Sehl, J.**; Teifke, J.P.; Hölper, J.; Klupp, B.G.; Mettenleiter, T.C. (2019), Talk. Simultaneous absence of two viral proteins in the Alphaherpesvirus Pseudorabies virus results in a drastically reduced mortality in mice despite widespread neuroinvasion. 29th Annual meeting of the society for virology, Düsseldorf, 20.-23.03.2019
03/2019	**Sehl, J.**; Teifke, J.P.; Hölper, J.; Klupp, B.G.; Mettenleiter, T.C. (2019), Talk. Funktionelle Charakterisierung von zwei Pseudorabiesvirus-Proteinen während der neuronalen Invasion in der Maus. 62. Jahrestagung der Fachgruppe Pathologie der Deutschen Veterinärmedizinischen Gesellschaft, Fulda, 01.-03.03.2019.
10/2018	**Sehl, J.**; Teifke, J.P.; Hölper, J.; Klupp, B.G.; Mettenleiter, T.C. (2018), Talk. Function of Pseudorabies Virus proteins pUL21 and pUS3 in neural invasion and propagation in the mouse, Mini-Herpesvirus Workshop, Heinrich-Pette-Institut, Hamburg, 05.10.2018.
09/2018	**Sehl, J.**; Teifke, J.P.; Hölper, J.; Klupp, B.G.; Mettenleiter, T.C. (2018), Poster. Characterization of Pseudorabies Virus proteins pUL21 and pUS3 in neural invasion and propagation *in vitro* and *in vivo*, Junior Scientist Symposium, Friedrich-Loeffler-Institut, Greifswald, 24.-26.09.2018

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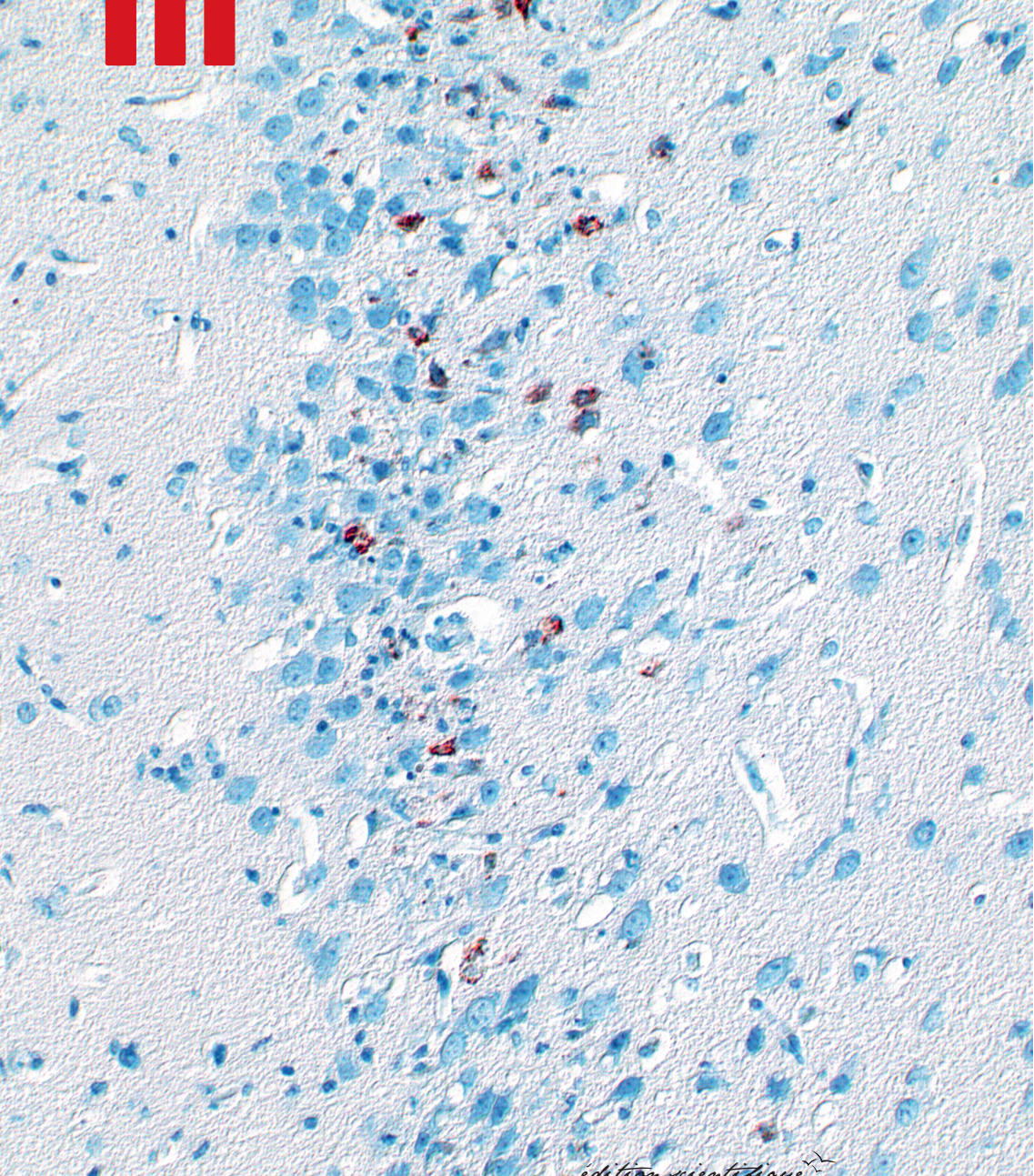

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