Development and evaluation of antiviral immunoglobulin single variable domains for prophylaxis of rabies in mice

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Dutch translation of the title:

Ontwikkeling en evaluatie van antivirale enkelvoudige variabele domeinen van immunoglobulinen voor de behandeling van rabiës bij muizen


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# Table of contents

List of abbreviations ......................................................................................................................... i

1 Introduction ........................................................................................................................................ 1

1.1 Rabies virus .................................................................................................................................. 1

1.1.1 History ...................................................................................................................................... 2

1.1.2 Epidemiology and transmission ................................................................................................. 2

1.1.3 Classification ............................................................................................................................... 4

1.1.4 Rabies virus biology ...................................................................................................................... 6

1.1.5 Pathogenesis ............................................................................................................................... 11

1.2 Prevention and treatment of rabies ............................................................................................. 16

1.2.1 Prevention of rabies before exposure (pre-exposure prophylaxis) ............................................ 16

1.2.2 Prevention of rabies after exposure (post-exposure prophylaxis) ............................................. 17

1.2.3 Treatment of clinical rabies infection ......................................................................................... 23

1.2.4 Alternatives for Human and Equine Rabies Immunoglobulins .............................................. 24

1.3 Immunoglobulin single variable domain (VHH) or Nanobodies® ............................................ 27

1.3.1 Camelid antibodies and VHH .................................................................................................... 27

1.3.2 Diagnostic applications of VHH ................................................................................................ 28

1.3.3 Therapeutic applications of VHH .............................................................................................. 29

2 Aims .................................................................................................................................................. 41

3 Validation of the intranasal virus inoculation model ...................................................................... 43

4 Development of anti-rabies VHH and validation of their antiviral activity .................................... 59

4.1 VHH in the treatment of rabies virus disease ................................................................................. 60

4.2 The effect of half-life extension of VHH on the in vivo efficacy .................................................. 76

5 VHH linked to neurotropic peptides ................................................................................................. 97

6 Post-exposure prophylaxis with half-life extended VHH and vaccine in mice ............................ 127

7 General Discussion ......................................................................................................................... 147

8 Summary – Samenvatting ................................................................................................................. 163

Dankwoord ........................................................................................................................................ 173

Curriculum vitae ................................................................................................................................. 175
List of abbreviations

A Avidin
ATTC American Type Culture Collection
AUC Area Under Curve
BBB Blood-Brain Barrier
BDNF Brain-Derived Neurotropic Factor
BHK-21 Baby Hamster Kidney Cells-21
BoNT Botulinum Neurotoxin
CB-1 Chien Beersel-1
CCID<sub>50</sub> 50 % Cell Culture Infectious Dose
CDC Centres for Disease Control and Prevention
CDR Complementary Determining Region
CNS Central Nervous System
CNT Clostridium Neurotoxin
Ct Cycle Threshold
CVS-11 Challenge Virus Standard-11
DMEM Dulbecco’s Modified Eagle Medium
DPI Days Post Inoculation
EDC 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride
ELISA Enzyme-Linked Immuno Sorbent Assay
ERIG Equine Rabies Immunoglobulins
FAT Fluorescent Antibody Test
FcRN Neonatal Fc-Receptor
FITC Fluorescein isothiocyanate
G Rabies virus Glycoprotein
GS Glycine-Serine Linker
HC Heavy Chain
HcAbs Heavy Chain-only Antibodies
H<sub>cc</sub> C-terminal Part of the Heavy Chain
HDCV Human Diploid Cell Vaccine
HLE Half-Life Extension
HRIG Human Rabies Immunoglobulins
HRP Horse Radish Peroxidase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>IC</td>
<td>Intracranial</td>
</tr>
<tr>
<td>ID</td>
<td>Intradermal</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IGR</td>
<td>Intergenic Region</td>
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<tr>
<td>IM</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>IMAC</td>
<td>Immobilized Metal Affinity Column</td>
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<tr>
<td>IN</td>
<td>Intranasal</td>
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<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IPH</td>
<td>Scientific Institute of Public Health-Brussels</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-1-thiogalactopyranoside</td>
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<tr>
<td>IU</td>
<td>International Units</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>L</td>
<td>Large Polymerase</td>
</tr>
<tr>
<td>LC</td>
<td>Light Chain</td>
</tr>
<tr>
<td>M</td>
<td>Matrix Protein</td>
</tr>
<tr>
<td>Mab</td>
<td>Monoclonal Antibody</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>N</td>
<td>Nucleocapsid</td>
</tr>
<tr>
<td>N2a</td>
<td>Neuroblastoma 2a</td>
</tr>
<tr>
<td>nAChR</td>
<td>Nicotinic Acetylcholine Receptor</td>
</tr>
<tr>
<td>NCAM</td>
<td>Neuronal Cell Adhesion Molecule</td>
</tr>
<tr>
<td>NS</td>
<td>Nervous System</td>
</tr>
<tr>
<td>P</td>
<td>Phosphoprotein</td>
</tr>
<tr>
<td>P75NTR</td>
<td>p75 Neurotrophin Receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCEV</td>
<td>Purified Chick Embryo Vaccine</td>
</tr>
<tr>
<td>PDEV</td>
<td>Purified Duck Embryo Vaccine</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
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<tr>
<td>PEP</td>
<td>Post-Exposure Prophylaxis</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonylfluoride</td>
</tr>
<tr>
<td>PrEP</td>
<td>Pre-Exposure Prophylaxis</td>
</tr>
<tr>
<td>PVRV</td>
<td>Purified Vero Cell Rabies Vaccine</td>
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<tr>
<td>RFFIT</td>
<td>Rapid Fluorescent Focus Inhibition Test</td>
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<tr>
<td>RIG</td>
<td>Rabies Immunoglobulins</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory Syncytial Virus</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Real-Time Reverse Transcriptase quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RVG</td>
<td>Rabies Virus Glycoprotein</td>
</tr>
<tr>
<td>S</td>
<td>Streptavidin</td>
</tr>
<tr>
<td>siRNA</td>
<td>Silencing Ribonucleic Acid</td>
</tr>
<tr>
<td>t1/2</td>
<td>Half-Life</td>
</tr>
<tr>
<td>TeNT</td>
<td>Tetanus Neurotoxin</td>
</tr>
<tr>
<td>TIS</td>
<td>Transcription Initiation Signals</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3’5,5’-Tetramethylbenzidine</td>
</tr>
<tr>
<td>TTP</td>
<td>Transcription Termination Polyadenylation</td>
</tr>
<tr>
<td>VHH</td>
<td>Variable domain of heavy-chain antibody</td>
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<td>WHO</td>
<td>World Health Organisation</td>
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</tbody>
</table>
1.1 Rabies virus

Rabies is an invariable fatal infectious disease caused by the rabies virus. Each year, more than a million people are exposed to the virus of which over 59,000 die, mainly in Africa and Asia where post-exposure prophylaxis is unavailable or too expensive [1]. Transmission occurs mainly via (feral) dogs, but nearly all mammals can get infected with the virus.
1.1.1 History

Rabies is one of the oldest recognized infectious diseases affecting humans. Ancient civilisations were already familiar with rabies, or more specifically with the consequences of being bitten by ‘mad’ dogs, as can be seen in early writings from Mesopotamia and Egypt. *Hydrophobia*, Greek for ‘fear of water’, one of the key symptoms of infection, was already described in Chinese medical texts of about 500 BC. In 1804, Zinke showed that saliva from a rabid animal could be used to transmit the disease. Since then, several techniques were used for the prevention of rabies after exposure to mad dogs, like eating the liver from a mad dog, eating crayfish eyes and carrying sacred talismans. In 1885, the first person being bitten by a rabid dog survived after treatment with a vaccine developed by Louis Pasteur. At that moment the causative agent was even not yet identified to be a virus [2,3].

Pasteur’s research on the vaccine started with his discovery that the properties of the virus could be changed by adapting the virus to laboratory animals, generating what is now called ‘fixed’ viruses. These viruses were used in vaccination trials in rabbits, and from the spinal cords of these animals the first vaccine was developed. Although this vaccine became the accepted post-exposure rabies prophylaxis, problems remained due to improperly inactivated virus in these vaccines and allergic reactions induced by brain tissue. Most importantly, it was not very effective in the case of severe bites. In 1889 Babès introduced an addition to this protocol by simultaneous administration of an anti-rabies serum with the vaccine. Despite its effectiveness, especially in the case of severe bites, it only became generally accepted when the World Health Organization (WHO) recommended the combined therapy as standard prophylaxis for human rabies exposure in 1954. In the 1960s, production of the virus in human diploid cells resulted in safe and efficacious vaccines without the problems associated with vaccines produced in nervous tissues [2,3]. However, even today we do not yet have an effective treatment for acute rabies virus infection. Once the symptoms are apparent, the patient is condemned to die.

1.1.2 Epidemiology and transmission

Rabies is present on all continents except Antarctica (Figure 1.1). Depending on the part of the world, different mammals serve as the major host [2]. Although virtually all mammals may be infected with the virus, the susceptibility to infection differs greatly from one species to another. Canines are the main reservoir and vector. Cats are very effective vectors, but do not seem to serve as reservoir hosts. Opossums are rather refractory to infection, while humans are generally dead-end hosts [2,4]. Since dogs are the major source of infection in humans, control of canine rabies could lead to almost complete prevention of human rabies. In Europe and the Americas, the combination of mass vaccination of dogs and cats, and the elimination of terrestrial sylvatic rabies by
oral vaccination of wild reservoirs, like foxes and racoons, has led to a significantly reduced prevalence of the disease (Figure 1.2) [5].

![Rabies distribution map](image)

**Figure 1.1:** Rabies distribution map which shows no, low, medium and high risk areas for rabies transmission in humans. From WHO.

![Oral vaccine and fox](image)

**Figure 1.2:** Oral vaccine used for wildlife vaccination and young fox playing with oral vaccine.

Despite the availability of effective prophylaxis for rabies virus infection, rabies causes the highest case-fatality rate among any infectious disease. The estimation of 59,000 fatal cases each year is likely to be an underestimate because infections occur mainly in resource-poor countries, which often lack proper surveillance, diagnostic capacity and/or disease registries [1]. On average, one person dies from the disease every ten minutes, and more than 300 are exposed. Children under the age of 15 are mainly at risk and 95 % of all rabies victims live in Africa or Asia [2,6–8].
Another major source of rabies are bats. Due to the small lesions associated with bat bites, these often go unnoticed, leaving the victims unaware of potential exposure to the virus. Mainly in the Americas and the Caribbean islands, rabies exposure via bats is important, but bats are primary reservoirs of rabies on all inhabited continents [2,4,8].

1.1.3 Classification

Classification The Mononegavirales order is composed of four families: Bornaviridae, Filoviridae, Paramyxoviridae and Rhabdoviridae. All viruses contained in this order are enveloped and have linear, nonsegmented, negative sense, single stranded RNA genomes. In addition, they all share a similar gene order and helical nucleocapsids. Although these ribonucleocapsids are infectious, naked RNA is not, because RNA-dependent-RNA polymerase is associated with the ribonucleocapsid. Viruses within the family of Rhabdoviridae are characterized by typical bullet-like particles (Rhabdos – Latin for ‘rod’) and consist of six genera, initially based on serological and antigenic cross-reactivity (Table 1.1) [9]. The genus of Lyssavirus was named after the Greek goddess of rage, fury, raging madness and frenzy, and has the rabies virus as prototype virus. The genus is divided into 12 species, harboured within at least two different phylogroups based on phylogenetic reconstruction (Table 1.1, Figure 1.3) [9].

Whereas viruses from phylogroup I show high homology on amino acid level, viruses from phylogroup II have greater divergence, found mainly in the glycoprotein G [3,9–12]. Vaccines derived from classical rabies virus strains have been shown to confer little or no protection against members of other phylogroups in experimental studies. Similarly, no serum cross-neutralization was observed between viruses from phylogroup I and II [13]. Initially it was presumed that phylogroup II viruses were less pathogenic than phylogroup I viruses, but more elaborate pathogenicity studies with viruses of phylogroup II showed that this hypothesis should not be generalized. A number of phylogroup II isolates are potentially less pathogenic, but some other are likely more pathogenic than the classical rabies virus, at least in mice. However, this reduced pathogenicity might also be related to the fact that these viruses are less adapted to mice [12,14]. In contrast to phylogroup I viruses, which show a high degree of sequence homology, phylogroup II viruses have greater divergence at amino acid level [12]. A third phylogroup currently only includes West Caucasian bat virus [13].
### Introduction

Table 1.1: Mononegavirales taxonomy with species from the Lyssavirus genus. Adapted from Lyles et al. [3].

<table>
<thead>
<tr>
<th>Order Mononegavirales</th>
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<tbody>
<tr>
<td>• Family Bornaviridae</td>
</tr>
<tr>
<td>• Family Filoviridae</td>
</tr>
<tr>
<td>• Family Paramyxoviridae</td>
</tr>
<tr>
<td>• Family Rhabdoviridae</td>
</tr>
<tr>
<td>o Genus Lyssavirus</td>
</tr>
<tr>
<td>▪ Phylogroup I</td>
</tr>
<tr>
<td>– Rabies virus (RABV)</td>
</tr>
<tr>
<td>– European bat lyssavirus 1 (EBLV-1)</td>
</tr>
<tr>
<td>– European bat lyssavirus 2 (EBLV-2)</td>
</tr>
<tr>
<td>– Australian bat lyssavirus (ABLV)</td>
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<tr>
<td>– Duvenhage virus (DUVV)</td>
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<tr>
<td>– Irkut virus (IRKV)</td>
</tr>
<tr>
<td>– Khujand virus (KHUV)</td>
</tr>
<tr>
<td>– Aravan virus (ARAV)</td>
</tr>
<tr>
<td>▪ Phylogroup II</td>
</tr>
<tr>
<td>– Lagos bat virus (LBV)</td>
</tr>
<tr>
<td>– Mokola virus (MOKV)</td>
</tr>
<tr>
<td>– Shimoni bat virus (SHBV)</td>
</tr>
<tr>
<td>▪ Phylogroup III</td>
</tr>
<tr>
<td>– West Caucasian bat virus (WCBV)</td>
</tr>
<tr>
<td>o Genus Vesiculovirus</td>
</tr>
<tr>
<td>o Genus Ephemerovirus</td>
</tr>
<tr>
<td>o Genus Novirhabdovirus</td>
</tr>
<tr>
<td>o Genus Cytorhabdovirus</td>
</tr>
<tr>
<td>o Genus Nucleorhabdovirus</td>
</tr>
<tr>
<td>o Genus Dichorhabdovirus</td>
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</table>

**Species** The genus of Lyssavirus can be divided into three different phylogroups containing in total 12 different species. With the exception of Mokola virus, all of the species have been isolated from bats, suggesting that the virus most likely originated from bats. Whereas the classic rabies virus species persists predominantly in a variety of wild carnivores, the most likely reservoir of the other species are bats. The rabies virus (RABV) is responsible for the majority of recorded infections and deaths in humans, and thus the best studied [13].

The different rabies virus species can be subdivided into different types of strains depending on their characteristics. The term street strain is used to refer to isolates from naturally infected animals, whereas viral strains multiplied within cell cultures or laboratory animals are referred to as fixed strains, since their incubation period and virulence has been stabilized after multiple passages. Among the fixed strains, vaccinal, virulent and non-virulent isolates can be found. Virulent rabies strains are typically highly neurotropic and cause a lethal infection of the central nervous system (CNS), whereas non-virulent strains may also infect other cell types [11].
Chapter 1

1.1.4 Rabies virus biology

1.1.4.1 Structural biology

Rabies virus particles share the same structural characteristics as other rhabdoviruses being enveloped, bullet-like virions, with a diameter of 75 nm and a length of 100-300 nm. The nucleocapsid (N) proteins packages the negative single-stranded RNA genome into a helical nucleocapsid and form the ribonucleoprotein (RNP) by binding to the phosphoprotein (P) and to a lesser extent the large polymerase protein (L), the active component for transcription and replication. In turn, this RNP is associated with the matrix protein (M) which links it to the lipid bilayer of the viral envelope. This envelope contains spike-like projections composed of a single type of viral glycoprotein (G) (Figure 1.4) [15].
Figure 1.4: Electron microscopy image of the rabies virus (Pasteur Institute Brabant) and (B) schematic overview of the rabies virus particle. Adapted from Albertini et al. [15].

The genome of rhabdoviruses consists of a single stranded, non-segmented, negative RNA molecule of about 12 kilobases. The genome encodes the five viral proteins in the order of 3′-N-P-M-G-L-5′ (Figure 1.5). Each gene is flanked by transcription initiation signals (TIS) and transcription termination polyadenylation (TTP) signals. They modulate the activity of the polymerase during transcription and are responsible for the addition of the polyadenylated tail. The genes are separated from each other by short (2-6 nucleotides) non-transcribed intergenic regions (IGR). Between the G and the L gene a long untranslated region can be found (508 - 560 nucleotides) which may be responsible for the reduced efficiency of L transcription [16]. The beginning and the end of the genome are characterized by the presence of respectively a leader and a trailer sequence responsible for the initiation and termination of genome transcription and replication [15,17]. The first and last nine nucleotides of the genome are inversely complementary and contain sequences
that serve as promoters for transcription and signals for encapsidation. So far, no evidence of a hairpin structure in this region has been obtained [18]. The sequential transcription of the genome is considered to be a stop-start mechanism, producing the six consecutive transcripts: the leader RNA and then the five successive mRNAs coding for the N, P, M, G and L protein [15,17,18].

![Schematic overview of the rabies virus genome organisation.](image)

Figure 1.5: Schematic overview of the rabies virus genome organisation. Transcription initiation signals (TIS) and termination signals and polyadenylation signals (TTP) are indicated. The leader and trailer respectively initiates and terminates transcription and replication. The intergenic region (IGR) between the G and L gene is larger and contains a pseudogene (Ψ). The sequences (9 nucleotides) at the genomic termini are inversely complementary, so far it has not been shown that they form a hairpin structure. From Albertini et al. [15].

The nucleoprotein or N protein is the most conserved protein among Lyssaviruses [16]. This two-domain protein is wrapped around the viral RNA, each protein binding exactly nine nucleotides. The fact that the N protein completely covers the viral RNA, indicates that this conformation is a sort of storage state in which the RNA is hidden from cellular RNases or factors that might trigger interferon production. However, since the L protein cannot access the RNA this state, a conformational change in the N protein is needed for transcription and replication [15]. In addition the N protein also plays a role in evasion of the innate immune responses against the rabies virus and thereby aiding the spread of the virus in the brain [19].

The phosphoprotein or P protein forms dimers and is bound to the C-terminal domain of the N protein. It is responsible for binding the L protein to the nucleocapsid and interacts with the cytoplasmic dynein light chain, and plays therefore a role in the axonal transport of the RNP [20]. By binding to the N protein, the P protein plays a major role in genome replication, and similar to the N protein it also interferes with the host innate immune responses, more specifically in the STAT signalling pathway [21,22]. It has also been shown that this protein enhances virus replication in muscle cells by counteracting the interferon response and thereby promoting the invasiveness into peripheral nerves by the virus [23].

The matrix protein or M protein forms the link between the helical RNP and the lipid bilayer envelope. By condensing the RNP into a tightly coiled nucleocapsid-M protein complex, it gives the
virion its typical bullet-like shape. On top of this, the M protein plays a central role in budding and in viral assembly [24].

The glycoprotein or G protein is probably the best studied rabies virus protein. This transmembrane protein forms trimers, also referred to as spikes, on the viral surface. These play a major role in rabies pathogenesis. They are required for virus entry into cells, by interacting with cell receptors, and promoting virus and cell membrane fusion. Most likely, the receptors are mainly found in the nervous system (NS). G protein therefore contributes to the neurotropism and neuroinvasiveness of the virus. Since they are the only surface proteins on the virus particles, they are also the major antigens for the induction of virus-neutralizing, and thus protective, antibodies (VNA) [3,20,25]. In addition, the cytoplasmic part of the G protein seems to play a role in the inhibition of apoptosis in rabies virus infected cells, at least in vitro [26].

The large polymerase protein or L protein is an RNA-dependent-RNA polymerase incorporated into the RNP. It is capable of producing positive-strand RNA molecules from the viral genome as soon as the RNP is liberated into the cytoplasm, without the synthesis of viral proteins or additional host proteins. In general, it only recognizes RNA in a matrix with N and it requires P to form a functional polymerase complex. This large protein occupies more than half of the viral genome and shows sequence homology with L proteins found in other members of the Mononegavirales, insinuating a common ancestor. Little is known about the large protein as no easy in vitro models for transcription and replication exist [15,17].

1.1.4.2 Virus receptors and life cycle

Although considerable evidence is available about what types of cells are preferentially infected by the rabies virus, the receptors used by the virus for cell entry still remain a mystery. Considerable evidence shows that the virus can enter motor neurons via the neuromuscular junctions, but it can also enter the sensory neurons via nerve spindles [27]. The Nicotinic Acetylcholine Receptor (nAChR) was the first receptor identified to play a role in rabies virus entry [28]. Although a number of in vitro experiments clearly show the role of nAChR for rabies virus entry into susceptible cells, the role of this receptor in vivo is less clear. Surprisingly, this receptor was located at the postsynaptic muscle membrane, rather than the presynaptic nerve membrane. This indicates that the receptor might play a role in muscle infection rather than infection of the nerves. A second receptor that was identified as playing a role in rabies virus infection was the neuronal cell adhesion molecule or NCAM [27]. It was shown that this cell adhesion molecule, present at the presynaptic membrane of the neuromuscular junctions, was present on cell lines susceptible to rabies virus infection, whereas
it was absent in cell lines resistant to infection. However, as was seen for the nAChR receptor, the role of NCAM remains uncertain in vivo since NCAM-deficient mice still remain susceptible to infection, albeit brain invasion in NCAM-deficient mice was less efficient compared to wild-type mice [27,29]. This implies that NCAM most likely plays a role in infection, but that other receptors might also play a role. A third receptor that so-far has been identified as potential receptor is the neurotropin p75NTR receptor [27]. Interestingly, in vitro experiments showed that the presence of this receptor rendered cell lines susceptible only to a number of lyssavirus species, but not all of them [27]. In vivo data from mice lacking the extracellular domain of p75NTR showed that this receptor is not indispensable for viral infection. However, since virus particles are able to bind to this receptor, the receptor might play a role in rabies pathogenesis besides viral entry [30].

The rabies virus life cycle (Figure 1.6) begins with binding and entry into the host cell, followed by transport to the cell body. Once the virus is uncoated into the cell cytoplasm, protein production and virus replication takes place. Eventually, the virus is assembled and released. As mentioned above, the rabies virus glycoprotein plays a major role in receptor binding and so far it remains unclear which receptors are responsible for rabies virus binding [11,27]. Once the virus has bound to the receptor or receptors, the virus is internalized via clatherin-mediated endocytosis [31]. Since the virus is internalized in the axon which does not provide the correct biochemical environment for protein and RNA synthesis, the virus is transported to the neuronal body for replication and transcription. Although it has been of discussion for a long time, recent research suggests that the virion is most likely transported within the endosomes rather than as ribonucleocapsid [31,32]. Once the endosome containing the rabies virus has reached the cell body, the endosomes becomes more acidic as they mature which induces a conformational change in the glycoprotein. The conformational change in the glycoprotein allows the fusion with the membrane and the release of the ribonucleocapsid in the cytoplasm. Transcription and translation of the viral genome is highly regulated and takes place in inclusion bodies called Negri Bodies. The highly regulated gene expression and genome replication avoids the induction of an early antiviral responses that might result in neuronal death [11,15]. Transcription of rabies virus is regulated by the use of the stop-start model in which the transcription holds at conserved signal sequences within the genome. Since reinitiation of transcription does not always occur, transcription is attenuated from the 3’ to 5’ end resulting in a transcription gradient over the genome. Eventually budding takes place at the plasma membrane, although it is not yet known how the ribonucleocapsid reaches the site of budding. The G protein plays a major role in virus entry, but it also plays a role in budding as in the absence of the glycoprotein virus release is reduced. However, the main role in budding is played by the matrix protein M, as its absence leads to a 500,000 fold reduction in virus release [11,24].
Introduction

1.1.5 Pathogenesis

As already mentioned above, rabies virus is usually transmitted in the saliva of a rabid animal (Figure 1.7). Each year 59,000 people die from rabies, and millions of people receive post-exposure prophylaxis, although most of them receive vaccine-only prophylactic treatment [1,8,33]. This equals to one person dying from the disease every 10 minutes and hundreds being exposed.

Figure 1.6: Simplified rabies virus life cycle. The life cycle can be divided into different steps. The first steps involve the binding of the virus to the host cell and entry via endocytosis. The virus is then (most likely) transported to the cell body within the endosome. In the cell body the viral membrane fuses with the endosome and the virus is uncoated into the cytoplasm. The viral components are produced and eventually all viral components are assembled and virions bud from the cell.
Clinical features Rabies is a central nervous system (CNS) disease that is almost invariably fatal without proper post-exposure prophylaxis. The virus infects almost exclusively neurons [34]. The incubation period can vary according to the site of exposure (highly enervated tissue results in faster progression of the disease) and severity of the bite, and can range from 1-12 weeks to several years. Intriguingly, this seems independent of the distance between the bite location and the nervous system (NS) [35–37]. The clinical stages of rabies are: incubation, prodrome, acute neurological disorder, coma and death [37]. Because the majority of the physicians are not familiar with the disease, it is often misdiagnosed or diagnosed only late in the clinical course [4]. Prodromal stages are characterized by non-specific flu-like symptoms like fever, anorexia, headache and sore throat. By the time the patient develops these symptoms, the virus is already widely disseminated throughout the CNS [35,37]. The disease then progresses either to the “furious” form (70 % of the cases) or the less common “dumb” form (30 % of the cases). The “furious” form is the classical form of the disease and is characterized by a progressively deteriorating neurological status with
symptoms like aerophobia, hydrophobia (spasms of swallowing muscles by the sight, sound or perception of water), disorientation, hyperreactivity and aggressive behaviour. The ‘dumb’ or paralytic form, is characterized by loss of sensation, weakness, pain and progressive flaccid paralysis, making the diagnosis very difficult [4,35,37].

**Biological features** Typically, the rabies virus is transmitted by animal bites, although some cases of aerosol transmission are also described. The saliva of infected animals contains high doses of virus, which are introduced into the (wounded) muscular tissue or dermis upon biting [27]. Although the virus infects almost exclusively neurons, the virus can also replicate in non-neuronal cells at the entry sites. Studies in skunks showed that rabies virus antigen and genome could be detected in the muscle at the inoculation site up to two months after inoculation [34,38,39]. This may explain the long incubation period seen in some cases of rabies virus infection. Although the precise receptors used by the virus to enter the NS are not known, the virus most likely enters the NS via (a) receptor(s) at the neuromuscular junctions, but might also be able to infect sensory neurons [27,40]. When the bite is located on the limbs or torso, the virus spreads via retrograde transport in peripheral nerves to the dorsal root ganglia, which are infected about four days after virus contamination/inoculation. The virus then spreads further to infect the brainstem and higher-order neurons of the CNS [37]. When the bite is located in the face area, it can be presumed that the virus enters the NS via the cranial nerves. This virus spread from the site of contamination to the brain is referred to as centripetal spread. Soon after infection of the neurons (approximately 2 days), the virus starts a centrifugal (anterograde) propagation via the ventral and dorsal roots throughout the body leading to infection of the muscle spindles, skin and immune and visceral organs. Anterograde spread from the brainstem leads to infection of the salivary glands, heart and blood vessels. It is thought that this route of transport is less efficient than retrograde transport, and therefore it can take weeks before distant organs can be reached [37]. As soon as the virus has infected the cells of the salivary glands, it produces and sheds large amounts of virions in the saliva [34].

In contrast to the dramatic and severe clinical signs, only mild histopathological lesions are typically found under natural conditions [41,42]. This implies that neuron dysfunction, rather than neuronal death, is responsible for the fatal outcome of the disease [6]. Magnetic resonance imaging of the brain of patients does not show consistent patterns of abnormalities. Apoptosis or inflammation is rarely seen in patients probably since this would affect viral transport in the neurons. The extent of apoptosis correlates inversely with the pathogenicity of rabies strains: in general non-lethal infections produce a higher rate of apoptosis compared to virulent infections. This implies that apoptosis might rather be a protective rather than a pathogenic mechanism.
[39,42,43]. High rate of apoptosis, linked to non-lethal infections, is generally associated with higher rates of glycoprotein production. Low viral replication might also be a key factor in rabies pathogenesis, since lower replication levels conserve neuronal structure better and are probably also contributing to evasion of the host’s immune responses [43].

Studies have investigated abnormalities in neurotransmitters, electroencephalographic changes and ion channel dysfunction during rabies, but so far none of these have explained the dysfunction shown by the clinical features [6,42]. Electroencephalographic recordings showed alterations in sleep stages in infected mice and that all electrical activity ended about half an hour prior to cardiac arrest. Host gene expression is likely not inhibited in rabies virus infection until the late stages of infection and similar effects are seen on neurotransmitter release [42]. A recent study showed that structural changes in neurons may play a clinical role in the disease. These changes include the swelling of mitochondria and the Golgi apparatus [41]. More studies are needed to fully comprehend the extent of neuronal changes upon rabies virus infection and this may hopefully allow more targeted symptomatic therapy.

**The Blood-brain barrier** The blood-brain barrier (BBB) is a neurovascular filtering system that segregates the blood and the brain via firmly sealed endothelial cells. Tight junctions between the endothelial cells make this barrier relatively impermeable, providing a natural defence mechanism to protect the brain from toxic and infectious agents in the blood. Besides the limited permeability of the barrier due to the close association of the cells, endothelial cells making up the barrier also possess few transport pathways and express high levels of efflux transport pathways, thus molecules must interact with specific transporters or receptors to be able to cross the BBB [44,45]. However, a number of pathogens have found a way to circumvent the BBB and infect the CNS. Rabies virus bypasses the BBB by travelling through the neuronal cell network [44].

Whereas rabies virus can be quickly cleared from the periphery when an immune response is mounted, the immune system fails to clear the infection from the CNS, despite the presence of immune effectors in the blood. However, studies with attenuated rabies virus strains have shown that these strains can effectively be controlled in the CNS [46,47]. This control seems to be associated with changes in BBB permeability, indicating that it is not the absence of an effective immune response, but rather the inability to increase the BBB permeability and reach the brain, that probably differentiates a lethal outcome from survival [48]. Indeed, experiments have shown that there are no significant differences between the immune responses in reaction to infection with a virulent or an attenuated strain, and transfer of lymphocytes from mice infected with virulent strains were able to protect immunodepressed mice from infection with attenuated strains, indicating that
these cells are capable of controlling infection. However, they failed to rescue these mice from infection with virulent strains [46,49,50]. This hypothesis was further supported by the fact that opening the BBB upon infection with the lethal silver-haired bat rabies virus led to increased survival in infected animals [51]. Although further research is needed, experiments indicate that rabies virus infection reduces the production of steroid hormones, which are also known to influence BBB permeability [51].

Since the BBB limits the entry of antibodies from the circulation into the CNS, the administration of rabies immunoglobulins is poorly effective once the virus has reached the CNS. These results also suggests that opening of the BBB might have therapeutic benefits for individuals presenting with early signs of rabies [49]. However, the induction of CNS inflammation to open up the BBB can be harmful, and it remains to be determined how the BBB can be opened without putting patients at risk [51].
1.2 Prevention and treatment of rabies

Rabies can be prevented either by vaccinating people at risk before a potential exposure, or by treating exposed individuals soon after contact with a (potentially) rabid animal. Preventive treatment, often referred to as pre-exposure prophylaxis, consists of a vaccination schedule with booster vaccinations and will be discussed in the first part of this section (1.2.1). Afterwards, post-exposure prophylaxis, the treatment schedule administered to rabies virus exposed individuals, will be discussed (1.2.2). Since post-exposure prophylaxis requires both an active immune response as passive immunity, this part also discusses the production of rabies immunoglobulins used for passive immunotherapy, as well as the limitations of this product. In the following part the treatment options for people who develop clinical disease will be discussed (1.2.3) and a final part will discuss the current research for alternatives (1.2.4).

1.2.1 Prevention of rabies before exposure (pre-exposure prophylaxis)

Pre-exposure prophylaxis (PrEP) for rabies is a safe and effective technique to prevent infection in individuals that, due to their profession (e.g. veterinarians, laboratory workers, ...) or travel, could come in contact with the virus or infected animals [31]. So far, no rabies deaths have been reported in people that received PrEP, followed by two booster vaccinations after exposure [6,33,52]. The standard PrEP regimen consists of three doses of vaccine administered intramuscularly on days 0, 7 and 28 (or 21). The antibody response is prolonged and increased by a booster vaccination after one year, although it is presumed that vaccination establishes an immunological memory that persists for life [6,8]. Due to occasional shortages of vaccine, alternative routes of administration have been investigated and are also used. Administration of the vaccine via the intradermal route limits the used volume to 0.1 ml per site following the same schedule. This reduces the necessary vaccine by 60-80 %, but since vaccine formulations come in vials of 0.5-1 ml and can only be kept up to six hours once reconstituted, it might result in more loss in areas where vaccination is not frequent [8]. The Belgian Scientific Institute of Public Health (IPH), together with the Queen Astrid Military Hospital and the Dutch Academic Medical Center, is currently conducting a number of studies investigating the efficacy of a shortened intradermal vaccination schedules. Currently three different (accelerated) schedules are under investigation in which the number of injections is limited to one or two days and a booster vaccination one to three years later. These studies are currently under analysis.

Rabies vaccines have come a long way since the first successful vaccination by Pasteur in 1885. The first vaccines consisted of air-dried infected rabbit spinal cord. Whereas nowadays the vaccines
Within one schedule are identical, Pasteur’s team gradually administered spinal cord suspensions that were dried for shorter and shorter periods of time to eventually end with freshly prepared rabies virus-infected rabbit spinal cord, thus essentially virulent rabies virus was administered to the patient at the end of the vaccination period. This caused severe criticism at the time, as some patients died following vaccination. In addition, the vaccine was also difficult to preserve for longer time periods as it was a mix of live and inactivated virus. In order to overcome this, Fermi and Semple proposed some simple modifications to the protocol: addition of phenol to Pasteur’s vaccine not only inactivates the live virus, but also served as a preservative to prevent contamination. Although Semple and Fermi vaccines respectively used sheep or goat brain tissue, all three vaccines used adult mammal nervous tissue, which can induce auto-immune encephalitis after administration. Even the use of neonatal (rodent) brain, containing less myelin responsible for the adverse reactions, could not completely rule out adverse reactions upon vaccination [53]. This led the WHO to recommend to discontinue the use and production of these so-called nerve-tissue vaccines. Despite this, nerve tissue vaccines are still used in a number of developing countries [8].

Adaptation of human viruses, including the rabies virus, to embryonated eggs created a new tool for vaccine production. Although original chick and duck-embryo vaccines induced poor antigenic responses, improvements have led to the licensing of purified duck and chick embryo cell vaccines (PDECV and PCECV respectively) in Europe since 1985. In addition to embryonated egg vaccines, two other types of vaccines are currently in use. The human diploid cell vaccine (HDCV), recommended by the WHO as gold standard reference vaccine, is produced on human diploid cells and has only caused few severe adverse effects since it was licensed in 1974. However, the diploid cell strains used for the production of HDCV have a limited capacity to divide and thus a finite life span. This makes it difficult to scale-up the production process and increases the costs associated with the production of the vaccine, making it often unavailable in developing countries. This led to the use of immortalized cell lines for vaccine production; resulting in a fourth licensed anti-rabies vaccine produced in Vero cells and hence referred to as the purified Vero cell rabies vaccine (PVRV) [53].

### 1.2.2 Prevention of rabies after exposure (post-exposure prophylaxis)

Post-exposure prophylaxis (PEP) for rabies consists of three different components. Wound cleansing is indispensable after rabies exposure, even if the person has previously been vaccinated against rabies. The wound should be thoroughly flushed and washed with soap and water, detergent and disinfected with povidone iodine or other substances with anti-viral activity as soon as possible.
Chapter 1

Suturing should be avoided or delayed if possible [52]. Experiments have shown that proper wound care could prevent rabies disease in up to 50% of the cases [54].

A second aspect of PEP is passive immunotherapy: the administration of rabies immunoglobulin (RIG) administered locally. These passively administered antibodies serve to neutralize the virus locally and prevent the spread of the virus, especially inhibiting the entry into the NS, and serves to span the period needed by the immune system to mount a proper immune response against the virus. Administration of RIG away from the wound, e.g. intramuscularly, leads to low doses in circulation and is unable to (sufficiently) neutralize the virus. Since an active immune response is typically detectable only seven to ten days after vaccination, no vaccine regimen can substitute for the administration of RIG. Under certain circumstances the administration of RIG is unnecessary or redundant. Administration of RIG is useless when the individual has already developed an active immune response, as would be the case if the person was vaccinated previously or more than seven days before the administration of RIG. In case of low risk exposure, as determined by the WHO and depicted in Table 1.2, administration of RIG is not required. The rationale behind the division of exposure into different categories depends mainly on the rationing of RIG, which is a scarce and expensive product, rather than real redundancy of RIG administration [4,33,52,55,56].

The third aspect is vaccination to obtain an active immune response against the virus to confer prolonged protection. Active immunization after exposure uses the same vaccines as those used for PrEP, but a different more pushed schedule is applied. As is the case for RIG, the vaccination should start as soon as possible. Currently the WHO has recognized three different PEP schedules: the Essen, Zagreb and Thai Red Cross regimens. The Essen regimen is the gold standard and consists of one vaccine dose administered intramuscularly on days 0, 3, 7, 14 and 28. It thus consumes five ampoules of vaccine and requires an equal amount of clinic visits, which is very time consuming and often too expensive for people living in low-income countries. This has led to the development of the Zagreb regimen which still consumes four vaccine doses, but only requires three clinic visits, as two intramuscular vaccinations are given on day 0, and one dose on day 7 and 21. A major disadvantage of this regimen is that the patient is not seen by a doctor on day three, at which early signs of bacterial wound infection usually appear. Whereas these two regimens use intramuscular injections, the Thai Red Cross regimen is the only intradermal vaccination schedule. As mentioned above, intradermal vaccination reduces the vaccine dose needed by 60-80%. Since this schedule requires two intradermal injections on day 0, 3, 7 and 28, it requires more clinic visits than the Zagreb regimen [56]. Recent studies in Thailand have tried to shorten the intradermal schedule, further reducing the number clinic visits and vaccine costs. This new regimen consists of intradermal
injections at four different sites on day 0, 3 and 7. Although this regimen looks promising additional studies are required to confirm the results [57]. All four regimens are summarized in Table 1.3.

Table 1.2: WHO post-exposure prophylaxis guidelines based on the category of exposure to a rabid animal (confirmed or suspected).

<table>
<thead>
<tr>
<th>Category</th>
<th>Types of contact</th>
<th>PEP</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Touching or feeding animals; licks on skin</td>
<td>None</td>
</tr>
<tr>
<td>II</td>
<td>Nibbling of uncovered skin; minor scratches or abrasions without bleeding; licks on broken skin</td>
<td>Wound cleansing and vaccination</td>
</tr>
<tr>
<td>III</td>
<td>Single or multiple transdermal bites or scratches; contamination of mucous membrane with saliva from licks; exposure to bat bites or scratches, even if not evident</td>
<td>Wound cleansing, vaccination and (local) administration of rabies immunoglobulins</td>
</tr>
</tbody>
</table>

Table 1.3: Summary of the different rabies post-exposure prophylaxis vaccination schedules. The Essen, Zagreb and Thai Red Cross regimen are WHO-approved, whereas the 1-week ID PEP is still under investigation. Adapted from Shantavainkul et al [58]. Two studies currently running in the Scientific Institute of Public Health (IPH-Brussels) were added to this table.

<table>
<thead>
<tr>
<th>Schedule</th>
<th>Route of administration</th>
<th>Dosing</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHO approved</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESSEN</td>
<td>Intramuscular</td>
<td>1 dose on days 0, 3, 7, 14 and 28</td>
</tr>
<tr>
<td>Zagreb</td>
<td>Intramuscular</td>
<td>2 doses at different sites on day 0, 1 dose on day 14 and 28</td>
</tr>
<tr>
<td>Thai Red Cross</td>
<td>Intradermal</td>
<td>2 doses at different sites on days 0, 3, 7 and 28</td>
</tr>
<tr>
<td>1-week ID PEP (IPH-Brussels study)</td>
<td>Intradermal</td>
<td>4 doses at different sites on days 0, 3 and 7</td>
</tr>
</tbody>
</table>

Under investigation |                          |                                             |
| 1-week ID PEP (IPH-Brussels study) | Intradermal           | 1 dose on day 0 and 7 (booster vaccination after 1, 2 or 3 years) |
| 1-day ID PEP (IPH-Brussels Study) | Intradermal           | 2 doses on day 0 (booster vaccination after 1 year) |

1.2.2.1 **Rabies immunoglobulins (RIG)**

Rabies immunoglobulins (RIG) are an indispensable part of rabies post-exposure prophylaxis. The effectiveness of a combination of vaccination and administration of anti-rabies serum was first
Chapter 1

shown by Babès & Lepp in 1889, but experiments soon after by other groups failed to reproduce its effectiveness in vivo. It was not until 1930 that more and more experimental data accumulated supporting the original data of Babès. This eventually led to the decision of the WHO to approve a trial to test the effectiveness of this approach in humans [57]. An attack of a rabid wolf on an Iranian village in 1954 proved to be the ideal setting for a trial. The 29 villagers attacked by the animal were first divided into different groups depending on the nature of their injuries and within these groups, victims were randomly divided into groups receiving either vaccination alone or vaccination in combination with anti-rabies serum. The results of the trial were impressive, especially in victims with severe injuries to the head. In those cases, treatment with one dose of anti-rabies serum decreased mortality from 40 % to 14 %, or even completely avoided mortality when two doses of anti-serum were administered [58]. Figure 1.8 shows pictures of victims of this attack. Although it was previously shown that local administration of antiserum was important, because the virus tends to persist at the site of inoculation, antiserum was administered peripherally in the Iranian trial, which might explain why incomplete protection was observed [57]. Indeed, later research stressed the importance of local wound treatment, not only by thorough washing of the wound, but also by local infiltration of the wound with immunoglobulins to allow virus neutralization in situ [54].

Although this first trial was promising, a lot of questions remained unanswered. In the years following this first trial, Atanasiu et al. published a series of trials in non-exposed individuals in order to optimize the post-exposure prophylaxis schedule [59–62]. The main rationale at the start of these trials was an attempt to reduce the quantity of vaccine used or even eliminate the use of vaccine, since at that time the available nerve tissue vaccines were associated with severe paralytic events and failures. Since rabies causes a lethal infection, it was unthinkable to experiment with the existing treatment protocol in exposed individuals, and thus protection was measured indirectly by the detection of antibodies in the blood [59]. Although exact anti-rabies serum titers were not determined in these trials, and doses of rabies immune serum administered were often not calculated or mentioned, some important conclusions could be drawn from these trials, which set the basis of current rabies post-exposure prophylaxis. Already after the first trial, it was clear that immune serum interfered with vaccine-induced immunity. This set the basis of the elaborated post-exposure vaccination schedule, in which administration of booster vaccines were to overcome the interference [60,63].
Originally the antiserum used was of animal origin (rabbit or equine), which soon showed to hold a risk of immunogenicity. Although reaction rates with equine serum were lower compared to rabbit serum, up to 46% of the treated individuals developed serum sickness with varying intensity [64,65]. Serum sickness is a systemic type III hypersensitivity reaction, occurring in a number of people upon the injection of large quantities of foreign proteins, like antibodies. As it is caused by the induction of an immune response against the foreign protein, the disease follows the same course as the mounting of a typical immune response and can be characterized by chills, fever and arthritis [66]. The question therefore rose whether antiserum from human origin could not be produced.

In order to avoid the potentially severe side effects of immune serum without losing the advantages, attempts were undertaken to produce immune serum from human origin or human rabies immunoglobulin (HRIG) [64,67]. A first batch of HRIG was produced and proven effective in 1969. It could even been shown that homologous serum is often more efficient than heterologous serum, most likely due to the longer half-life of homologous antibodies as compared to heterologous
antibodies [68,69]. Remarkably this higher efficacy also resulted in a higher interference with rabies vaccination, indicating the need for lower dosing [55,69]. Archer et al. [70] further investigated the nature of the interference and were able to show that homologous serum interfered more than serum of heterologous origin and suggested that the recommended dose has to be lowered when homologous serum is administered [70]. HRIG can be purified from human plasma in a similar way that equine rabies immunoglobulins (ERIG) is purified from equine plasma. But since it requires human donors with high antibody titers (≥ 15 IU/ml), the product is both scarce and expensive. Often human donors need to be boosted several times to obtain sufficiently high titers, which increases the costs [71]. Besides the costs associated with vaccination of donors and purification of the immunoglobulins, the donations have to be tested and treated to minimalize the risk of transmission of blood-borne pathogens [72]. These high costs make HRIG often unavailable for people in developing countries who are most at risk. The development of F(ab')2 by pepsin digestion of ERIG and extra purification steps to eliminate albumin from equine serum decreased the risks of serum sickness upon administration and serves as a cheaper alternative. Pepsin digestion of antibodies removes the Fc part of the antibodies, making them smaller and decreases their serum half-life in circulation (Figure 1.9). Thus, this requires administration of an increased dosage, but decreases the incidence of adverse effects from 46 % to 0.82-6.16 %, depending on the purification methods [2,56,73].

Besides the high costs associated with HRIG, both ERIG and HRIG use polyclonal sera and are therefore subject to a number of limitations. They may contain also non-neutralizing virus-specific antibodies and, more importantly, they are subject to batch to batch variations [33,74].

![Production of ERIG](image)

**Figure 1.9: Production of ERIG.** ERIG is produced after the pepsin digestion of conventional antibodies, this separates the Fc part of the antibody and results in two variable fragments linked by a disulphide bridge. Adapted from Saerens et al. [75].

1.2.2.2 **Failures of post-exposure prophylaxis**

PEP failure is rare and in most cases associated with an incorrect or incomplete PEP schedule. In the majority of the cases, PEP fails due to the absence or incorrect administration of RIG and/or
inadequate wound care. In some rarer cases, the vaccine or RIG potency was too low, or an exceptionally large viral load or an atypical strain, not neutralized by RIG, was involved [76]. Surveys have shown that the high costs associated with RIG is the main reason why less than 2% of the exposed individuals receive RIG in combination with vaccination in developing countries [8,77]. Although ERIG is about a quarter of the price of HRIG, it is still unaffordable in large parts of the world, hampering the efforts to reduce the rabies death toll [33].

Incorrect administration of RIG not only implies peripheral administration rather than local, but also includes cases in which administration of RIG was delayed or not all wounds were infiltrated. Even when the wounds are irrigated with RIG, suturing should be delayed as it has also been shown to be linked to failure of PEP. Many of these failures can be avoided when clinicians in rabies endemic regions are properly trained [78].

Besides these failures due to protocol errors, a number of ‘true’ PEP failures have been described. In these cases, patients received the complete PEP schedule soon after exposure according to the correct protocol (prompt and appropriate wound care, RIG infiltrations into the wound and a complete vaccination regimen) but still developed rabies. It should be noted that so far all of the reported cases were associated with wounds to the head or hands and rabies developed soon after exposure. Although it is unlikely that protocols were misreported or biologicals were of low potency in these cases, it is impossible to exclude minor errors in management like small transdermal puncture wounds that might not have been cleansed and/or infiltrated with RIG [78,79]. This stresses again the importance of RIG for local virus neutralization.

### 1.2.3  Treatment of clinical rabies infection

Rabies can be very effectively prevented if vaccines and RIG are available, but there is still no therapy for a patient with clinical rabies [80,81]. In general the management of patients with rabies is palliative with barrier nursing to protect health care workers and family from potential exposure. However, so far no transmission from human patients to health care workers has been documented.

An aggressive therapeutic approach might be considered with patients presenting at a very early stage of the disease or patients that received an incomplete rabies vaccination schedule (either in pre- or post-exposure prophylaxis) before the clinical onset and are considered to be in good health. Importantly, relatives should understand and accept the high probability of an unsuccessful outcome and the possibility of disability upon survival [81].

In analogy with treatment of other infectious diseases, combination of therapies are likely to be more effective than a single approach. Such therapies might include rabies vaccine and anti-rabies antibodies in combination with antivirals like ribavirin and ketamine. The use of corticosteroids
should be avoided as it increased mortality in animal models [81]. In 2003 this advice was used for the treatment of a 15-year old girl bitten by a bat. She had not received PEP and upon admission was treated with ketamine, ribavirin and amantadine, in combination with an induced therapeutic coma. The rationale of this protocol was to reduce brain excitotoxicity, which is the damage and killing of neuronal cells by excessive stimulation by neurotransmitters, by administering various sedatives inducing a coma [39,82]. Although she survived with mild neurological deficits, it is still unclear why she survived. It is very likely that good medical care in a critical care unit was beneficial, but the role played by any specific therapy remains unclear. The induced coma is the most controversial part of the protocol now referred to as the Milwaukee protocol [82]. So far, of the 43 attempts of the Milwaukee protocol, only five survived (excluding the first attempt) [83]. Of these five survivors, it is also possible that some of these might not have had rabies, have received PrEP or died later after surviving the most acute phase of the disease [84]. At the moment, the use of the Milwaukee protocol is no longer recommended as it includes some components that cause immunosuppression, and a general acceptance of this protocol might hinder the development of better therapies [82].

At the moment, there are ten known survivors of rabies and only three of them have mild neurological sequelae or have made a complete recovery [84]. Most of them had received (incomplete) vaccination regimens prior to onset of disease, but none had received RIG for PEP. In none of the patients the virus could be isolated, and indication of rabies was often indirect by the presence of anti-rabies immunoglobulins in cerebrospinal fluid and/or serum [85–89]. Out of the three survivors that made a nearly complete recovery, two were presumable exposed to bat rabies variants, which might be less neurovirulent than the canine variants [84,90].

To develop new therapies for clinical rabies, better understanding of the pathogenesis of the disease is critical, but currently little basic research is done on rabies pathogenesis [84]. Therefore, treatment is mainly focussed on the comfort of the patients, rather than on survival. Even though rabies is not always fatal, it is impossible to predict which patient is more likely to recover. Sedation is desirable as patients remain conscious and are often aware of their situation as disease progresses, the fact that the disease itself often leads to agitation, underlines its importance [37].

1.2.4 Alternatives for Human and Equine Rabies Immunoglobulins

Because HRIG is short in supply and more and more international manufacturers of ERIG discontinue production, the WHO has called for the development of (cheaper) alternatives [91,92]. In this context, monoclonal antibodies (Mab) might serve as an attractive alternative which would be safer (no risk of transmission of blood-borne pathogens) and show less variability in specificity and potency [33].
Although the focus lays mainly on monoclonal antibodies (cocktails) to serve as alternatives for RIG, other antibody derivates may also be useful as alternative for RIG.

1.2.4.1 Monoclonal antibody cocktails

Monoclonal antibodies (Mabs) are attractive as therapeutic candidates because of their stability, tolerance, functionality and amenability for engineering to enhance various desirable characteristic such as reduced immunogenicity, longer half-lives, higher affinity, and better effector functions. Today, more than 25 monoclonal antibodies are licensed for human use to treat various conditions and many more are in different stages of clinical trials. The main advantage of monoclonal antibodies, compared to polyclonal serum, is that they provide a more potent product with better activity. Because of their homogenous nature, there is no variability with regards to isotype or epitope and they show relatively low batch-to-batch variations. Probably even more important, the production of Mabs does not depend on suitable hyperimmune donors [93,94]. However, Mabs also have some disadvantages. Because of their mono-specificity, they may not cover all naturally circulating strains of a virus and partial neutralization might generate escape mutants. This is especially the case for RNA viruses that have polymerases without proofreading capacity and thus are highly prone to mutations. The use of Mabs cocktails instead of single antibody clones can overcome this problem [74,93,95]. Main limitations of Mabs are the high production costs and their limited tissue penetration. Because of their size and complexity, Mabs require eukaryotic cells for production and yields are typically low (5-25 kg antibody/1-2 weeks) [96], production costs are high, despite the low costs associated with the raw materials, which limits the wide use of these drugs. Although their size provides them with a relatively long circulatory half-life, it also limits tissue penetration of these molecules [97].

The effectiveness of Mabs in vivo depends also on their ability to interact with the immune system. This requires the interaction of Mabs via their Fc part with Fc receptors in the patient. Since these receptors show a high degree of polymorphisms, the ability of Mabs to induce an immune reaction will depend on their isotype and the affinity with which Mabs can interact with the Fc receptor [98].

Koprowski and Wiktor were the first to show the in vivo potential of monoclonal antibodies in rabies. They implanted hybridomas producing rabies-specific Mabs in mice prior to intracranial virus challenge. Upon challenge, 90-100 % of the mice that had hybridomas secreting monoclonal antibodies implanted, survived virus challenge [99]. Schumacher et al. further investigated the potential of Mabs and developed a cocktail that could protect hamsters in both a pre- and post-
exposure setting [100]. Because these Mabs are from murine origin, they can induce side effects similar to serum sickness when used in humans. To avoid this, they should be ‘humanized’ [94,100].

Human Mabs can be produced in different expression systems which all have their own advantages and disadvantages. Human Mabs can be produced in transgenic mice or in human x mouse heterohybridomas, but the Mab production levels are rather low. Phage display technology is a quick method for screening candidates, but results in antibody fragments rather than full-length Mabs. These might be less stable. The most optimal expression system is most likely eukaryotic systems, predominately mammalian cell lines. These allow correct folding, assembly, glycosylation and secretion of the Mabs. Before these Mabs can be produced, the gene of a potent Mab must be cloned into the expressing cell line and to avoid instability of expression, a selective pressure must be present [94].

Because of the worldwide shortage of rabies immunoglobulins, the WHO has recommended to develop and validate Mab cocktails containing at least two Mabs for post-exposure prophylaxis. Mabs used in these cocktails also have to fulfil a number of criteria, like in vitro cross-reactivity over a variety of lyssavirusses isolated from different geographic areas [92]. A number of anti-rabies Mabs are currently in clinical trials, despite the difficulties with human clinical trials for rabies. A Mab cocktail developed by Crucell in collaboration with the US Centres for Disease Control and Prevention (CDC) successfully passed through phase I and II clinical trials, and phase III clinical trials are currently prepared [94]. Phase I studies are promising as no adverse effects were reported by the subjects and antibody titres obtained when a combination of Mab and vaccine was administered where protective (≥ 0.5 IU/ml) [101]. SII Rmab (Serum Institute of India Ltd.) is another Mab currently undergoing clinical trials. Pre-clinical and phase I study results showed that this Mab neutralized rabies strains from different geographic areas and had comparable tolerability and safety compared to HRIG (CTRI/2009/091/000465). As was shown for CL 184 Mab cocktail from Crucell, this Mab also induces protective antibody titres when administered in combination with the vaccine (CTRI/2011/10/002060) [102]. A number of monoclonal antibody cocktails are currently under investigation in clinical trials. So far, only one of these (SII Rmab, Serum Institute of India, Ltd.) has been tested in a phase II/III trial in which potentially rabies virus-exposed individuals were included (CTRI/2012/05/002709). The results of this trial are currently being analysed and are expected to be published soon (Prasad Kulkarni, personal communication) [103].
1.3 Immunoglobulin single variable domain (VHH) or Nanobodies®

1.3.1 Camelid antibodies and VHH

Typical immunoglobulins are composed of two heavy and two light chains, which can functionally be divided into variable domains which bind antigens and constant domains responsible for effector functions. It is thought that for each existing molecule a specific immunoglobulin can be generated by the vertebrate immune system [104]. It was long time thought that all antibodies were composed according to this fixed design, except in the case of ‘heavy chain’ disease in which non-functional heavy chain antibodies are produced. In 1989 this view changed with the discovery of a new class of antibodies consisting only of heavy chain dimers in the camel (Figure 1.10). Heavy chain antibodies were discovered by chance by the research group of Hamers-Casterman at the university of Brussels (VUB) [105]. In contrast to those found in ‘heavy chain’ diseases, these heavy chain-only antibodies (HcAbs) are fully functional. Between 45 and 75 % of the antibodies produced in camelids are HcAbs, but despite this abundance and the presence of different isotypes of HcAbs, their role in immunity remains unclear [105,106]. Besides lacking the light chains, HcAbs also lack the first constant domain, thus their antigen-binding domain is formed by a single domain directly linked to the Fc-domain [107]. This antigen-binding domain is referred to as VHH or Nanobody® (Figure 1.10) [106].

![Conventional antibody, Heavy chain antibody, VHH formats](image)

**Figure 1.10: Schematic representation of conventional antibodies, heavy chain antibodies, single domain antibodies and their recombinant derivatives (VHH formats). From Saerens et al. [75].**

Compared to the variable domain of conventional antibodies (VH), VHH have some unique biophysical and biochemical features that create new possibilities. A major advantage of these VHH is that their naturally hydrophilic nature allows relatively easy production with high yield. They also have been shown to be more stable and are better at recognizing small cavities than conventional antibodies [106,108]. VHH against a high range of targets have been reported, and despite their monomeric nature they have affinities in the same range as conventional bivalent antibodies. In contrast to antibodies that typically have concave or flat antigen binding sites, VHH have a convex conformation resulting in CDR loops that are highly exposed. This, combined with their small size (15 kDa), makes that VHH can bind to epitopes which are hidden from conventional antibodies. As they...
are highly stable and consist only of a single domain they can also easily be formatted into bivalent, trivalent and even decavalent molecules [106]. The combination of these features makes them easy-to-produce alternatives to conventional antibodies, and whereas stability and storage of conventional antibodies are often susceptible to environmental factors, VHH are remarkably stable under extreme conditions, like high temperatures and even the harsh condition of the gastrointestinal tract [106]. However, their small size also comes with a disadvantage: being only 15 kDa, they are smaller than the pore size of the renal barrier and are rapidly cleared by renal filtration, resulting in a short half-life in circulation.

VHH can be produced with high yields in a broad range of hosts. The majority of VHH are produced in bacteria, mostly *Escherichia coli*. Using a signal peptide they can be produced in the periplasma which allows easy purification of soluble and correctly folded VHH from periplasmic extracts or even direct testing of the periplasmic product before purification. Typical yields are 1 to 10 mg/ml, with a few VHH reaching a yield of 50-70 mg/ml. VHH can also be produced in yeasts, mainly *Pichia pastoris* and *Saccharomyces cerevisiae*. This production system often results in higher yields than those that can be obtained using bacteria, reaching over 100 mg/ml. Thus the choice of the best production system may vary from one VHH to another. The use of mammalian cells has not yet been evaluated for the production of VHH. Plant cell lines have not proven to be good production systems, as only low yields can be obtained. The yields can be increased by fusing the VHH to stabilising partners. The major advantages of production of VHH in plants are the possibility of oral administration without purification by consumption of the seeds and the absence of possible contamination with human pathogens, a potential hazard with the use of mammalian cell lines [109].

1.3.2 Diagnostic applications of VHH

VHH are attractive tools for diagnostics. Their small size and convex antigen-binding domain allows them to bind epitopes that are inaccessible for conventional antibodies. These cryptic epitopes are often enzymatic clefts or ligand binding pockets, but can also be small epitopes that allow the differentiation between different species from the same genus. An example hereof is the use of VHH for the species-specific detection of *Taenia solium* in pigs which is impossible using monoclonal antibodies. Identification of the species is of particular concern for human health. A similar example was found in the case of trypanasomiasis. The parasite has adopted an antigenic variation strategy which allows constant evasion of the host immune response. This limits the development of pan-reactive antibodies binding *Trypanosoma* spp., as large antibodies are unable to bind the small conserved epitopes, which are still accessible for VHH [109,110].
Another inherent feature of VHH is the ease at which an immune library can be generated and the easy production of VHH. Theoretically, this would allow the production of VHH specific for an antigen, which could be used for diagnostic applications without prior knowledge about the pathogen. This immunization of camelid animals, followed by the production of a cDNA library expressed in phage and finally phage panning against the selected pathogen or antigens. These VHH could replace monoclonal antibodies in ELISA tests, but could also be further developed towards dipstick diagnostic tests which would be especially useful in developing countries were diagnosis using sophisticated equipment is not always an option [110].

VHH can also be used for in vivo imaging. The use of monoclonal antibodies raised against the so-called tumor-associated proteins and labelled with radionuclides allows the tracing in vivo, but is also associated with some disadvantages inherent to antibodies. The long circulatory half-life of antibodies results in a peak contrast between target and surrounding tissue only two to four days post-injection, requiring the use of long-lived radionuclides which is unfavourable in terms of dosimetry and radioprotection. Poor tumor penetration and considerable degree of non-specific uptake are other disadvantages of antibodies for imaging. The short circulatory half-life of VHH allows imaging within a few hours after administration with low background due to rapid renal clearance of excess tracer and their small size allows for better tumor penetration than monoclonal antibodies. Because of their rapid renal clearance, VHH tend to accumulate in the kidney which complicates the imaging in tissues close to the kidneys. This aspecific signal can be reduced by alternative labelling reactions [109,111].

1.3.3 Therapeutic applications of VHH

Besides their diagnostic applications, VHH can also be used as therapeutics. As already mentioned, their small size makes them particularly suitable as therapeutics against antigens in isolated locations, like tumor tissue, inaccessible to antibodies [111]. Since VHH can be selected for resistance to extremes of pH and proteolytic stability, they are very suitable for oral therapy. The major disadvantage of VHH from a therapeutic view point is their short serum half-life. This rapid renal clearance limits the efficacy of VHH in many parenteral applications. To prolong their half-life, VHH can be generated with an additional binding site that recognizes normally long-lived serum proteins such as albumin or immunoglobulin. The formulation of bispecific VHH recognizing both the therapeutic target and serum proteins, increases their half-life to that of the selected serum protein they recognize. Alternatively, half-life extension can be obtained by addition of poly-ethylene glycol or PEGylation [112].
In contrast to conventional antibodies, VHH lack the Fc part, disabling them to exert Fc effector functions. VHH might therefore prove ineffective in vivo against pathogens that absolutely require those functions for protection. Nevertheless, many diseases could potentially successfully be treated with VHH [112].

So far a number of antiviral VHH have been developed. Anti-influenza virus VHH, when in a bivalent construction, were able to protect mice when administered one day prior to lethal challenge. The same VHH were also able to significantly delay death and morbidity in mice when administered in a therapeutic setting [113]. VHH directed against food-and-mouth disease virus proved to be very effect in vitro, but were unable to allow full protection in vivo, most likely because they lack the Fc part, but were able to reduce transmission between animals [114,115]. Ablynx recently developed an anti-respiratory syncytial virus (RSV) VHH. This VHH inhibited more efficiently cell-to-cell fusion than antibodies currently on the market, probably because the VHH recognizes epitopes that are not recognized by antibodies. Intranasal administration of these VHH significantly reduced the viral load in the respiratory tract of mice and was able to protect them from lethal challenge [116]. Ablynx is currently developing this VHH for the treatment of RSV in young children using an inhalation system. It was well tolerated in multiple phase I studies and is currently undergoing a phase IIa study in infants.


Aims

Even in the 21st century, rabies remains one of the most feared and important threats to public health. Although effective pre- and post-exposure prophylaxis exists, rabies still kills more than 59,000 people each year, mainly in developing countries. Passive immunisation is a vital component of post-exposure prophylaxis, as it can neutralize the virus before entry into the nervous system. In addition most post-exposure prophylaxis failures are associated with incorrect application or inability to administer rabies immunoglobulins. Currently rabies immunoglobulins are very scarce and expensive, making them unavailable for people with limited income in developing countries. Moreover, even in the Western world, including Belgium, this product is scarce with periodical shortages leading to occasional rationing at the Rabies Centre of the Scientific Institute of Public Health. Therefore, the World Health Organisation urges the scientific community to develop alternatives for this expensive product.

VHH are the antigen-binding domains of heavy chain antibodies found in Camelids. Since they lack a light chain, this small domain is hydrophilic and can easily be produced at high yields. In addition, VHH show high thermostability and are better at recognizing small cavities than conventional antibodies.

The general aim of this thesis was to develop and validate several types of anti-rabies VHH for rabies prophylaxis in mice. We investigated whether VHH can be used for neutralisation of rabies virus and prevent infection and disease in mice. We attempted to determine the advantages and limitations of VHH in this model and investigated a number of modifications that could be applied to improve the therapeutic efficacy of anti-rabies VHH.
The **specific objectives** of this thesis were to:

- Validate the intranasal challenge model to test preventive or therapeutic interventions against rabies virus infection in mice. Therefore we performed a thorough (retrospective) analysis of the reproducibility of the clinical and virological outcome of the intranasal rabies virus infection model (Chapter 3)
- Compare monovalent, homo-bivalent and hetero-bivalent anti-rabies VHH *in vitro* and *in vivo* and assess their potency to delay infection and disease, or even rescue mice, by post-exposure treatment after a lethal intranasal virus challenge (Chapter 4.1)
- Assess the effect of half-life extension to improve the prophylactic effect of anti-rabies VHH (Chapter 4.2)
- Examine whether addition of neurotropic peptides, which can potentially cross the blood-brain barrier or specifically bind to neurons, might enhance the prophylactic/therapeutic activity of antiviral VHH (Chapter 5)
- Examine whether the combined treatment of mice with half-life extended anti-rabies VHH and vaccine after virus exposure has added value compared to single treatment with either of the compounds (Chapter 6)
Validation of the intranasal virus inoculation model

This chapter addresses the first objective of the thesis: validation of the intranasal inoculation model to test preventive or therapeutic intervention against rabies virus infection. Since intranasal virus inoculation is not the standard route of administration of the rabies virus, this chapter gives an overview of the literature on intranasal inoculation and compares this route with the more typical intramuscular or the direct intracranial administration methods. The data used in this chapter are obtained from different infection experiments performed between 2009 and 2015.
3.1 Abstract

It is generally accepted that natural rabies virus infection occurs via inoculation into the muscular tissue upon a bite from a rabid animal. After a variable incubation period in the muscle, the virus gains access to the central nervous system (CNS) via the neuromuscular junctions or sensory nerves endings. Once the virus has gained access to the CNS, it spreads retrogradely to the brain were it causes encephalitis. It is therefore not surprising that the intramuscular virus inoculation is considered the gold standard for rabies virus research, however previous experiments in our lab have shown that this method shows high variability and relatively high survival rates for a supposedly lethal infection. To circumvent these drawbacks, unnatural high doses of virus are needed, posing a safety risk for people handling the virus and making the analysis of potential therapies unnecessarily difficult.

Our group recently published a detailed description of the method for virus inoculation using the intranasal route. This allows a quick infection of the olfactory nerves, resulting in a short incubation period and nearly 100 % mortality rate, with little variation in survival time [1]. In this chapter, we present a retrospective analysis of the outcome of the intranasal infection model using data from mock-treated or untreated infection control groups from the different experiments performed between 2009-2015.

We observed that the intranasal inoculation technique resulted in very reproducible survival times and a nearly 100 % mortality rate. This in contrast with the intramuscular inoculation model, which showed high variation in mortality rates. Survival times and mortality rates upon intracranial virus inoculations were similar to those obtained upon intranasal virus inoculation, but this technique is highly invasive and showed higher ‘interoperator’ variation, a phenomenon that was not observed upon intranasal virus inoculation. In conclusion, intranasal virus inoculation is a safe, needle-free technique with low ‘interoperator’ variation, stable incubation period and a high mortality rate.
3.2 Introduction

Olfactory receptor neurons are unique in the sense that they are directly exposed to the external environment via their cilia, which are dendritic nerve terminals. Via this way, external macromolecules can be absorbed by these neurons, and transported to the central nervous system [2,3].

The nose is designed in such a way that inspired air is directed to the olfactory epithelium located at the top of the nasal cavity. This way odorants are immediately brought in close contact with their receptors. Whereas the cilia of the olfactory neurons are embedded in the nasal mucosa, their axons project across the cribriform plate entering the olfactory bulb (Figure 3.1). Here, they synapse with the neurons of the central olfactory nervous system. The neuroepithelium consist only of a limited number of cells. Sustentacular cells serve as supporting cells and play a role in detoxification, besides the olfactory neurons also basal cells can be found. The latter are multipotent progenitor cells that can give rise to new olfactory neurons, a unique aspect of the olfactory system. Chemosensation in the nose is mediated by both the olfactory and the trigeminal nerves [2,4]. Neurotropic viruses can take advantage of this exposure of the olfactory system and use it as a shortcut to enter the CNS. The most obvious examples are viruses that are naturally associated with infections of the CNS like herpes simplex virus and Borna virus. In rare cases, the herpes simplex virus can cause encephalitis, an infection associated with high mortality and significant neurological sequelae, but even when the infection occurs asymptomatically, the virus remains in a latent form in the pheripheral ganglions [2,5].
Rabies virus is a highly neurotropic virus that, despite the generally accepted transmission via bite wounds, also has been shown to be transmitted via aerosol. This transmission can occur in caves with a high density of bats, which carry the virus [6], or when high quantities of virus are aerosolized in laboratory settings [7]. In these settings, it is most likely that infection occurs via the intranasal route as the nasal cavity is more enervated than the lungs or stomach. It seems that the ‘fixed’ rabies strains, which are strains that are highly adapted to laboratory animals resulting in a fixed incubation period, are highly effective at infecting mice through intranasal exposure [8]. Indeed, Lafay et al. showed that soon upon intranasal instillation, both the olfactory epithelium and the trigeminal nerve are infected by the virus. Via retrograde and anterograde transport, the virus eventually infects the whole brain quickly after inoculation [9]. Other studies confirmed that the
virus effectively infects the animals via the olfactory pathway rather than via the lungs or stomach [10]. These findings led to the publication of a detailed technical description of the procedure for intranasal virus inoculation by our research group [1]. Several experiments were performed to further optimize the anaesthesia, viral dose and volume, which allowed routine application of this technique. The technique is now used as the standard technique for rabies virus inoculation in our laboratory. It is a needle-free technique that requires a low viral dose compared to the dose administered via the intramuscular route (Figure 3.2).

Figure 3.2: Intranasal inoculation as performed by our group. Briefly, a droplet of virus is administered on the nostrils of a (lightly) anesthetized mouse which inhales the virus via the nose.

The aim of this first chapter was to retrospectively validate the technique of intranasal rabies virus inoculation as performed by our research group. Since its fine-tuning, the technique has been used for the majority of experimental infections at our rabies laboratory [1]. This allowed us to analyse the variation in average survival over time, evaluate the variation between different operators and compare these results with other inoculation techniques used in our laboratory. In addition, we determined the kinetic profile of the viral spread throughout the brain upon intranasal virus inoculation.

3.3 Material and methods

3.3.1 Rabies virus

Challenge Virus Standard (CVS)-11 is a virulent classical rabies virus obtained from the American Type Culture Collection (ATCC reference VR959) and was grown in baby hamster kidney (BHK)-21 cells. Chien Beersel-1 (CB-1) is a street strain isolated by our laboratory from a rabid dog illegally imported in Belgium in 2009. Challenge Virus Standard (CVS)-27 is a virulent classical rabies virus obtained from the American Type Culture Collection (ATCC reference VR321). For intranasal
(IN), intracranial (IC) and intramuscular (IM) virus inoculation in mice, a dose of 1.26x10⁵, 5x10³ and 2x10⁶ 50 % cell culture infectious doses (CCID₅₀) per milliliter of CVS-11 was used respectively.

### 3.3.2 Mouse inoculation experiments

All experimental procedures were in compliance with the European (2010/63/EU) and Belgian (C-2013/24221) legislation concerning laboratory animal welfare. Briefly, mice were kept in filter top cages and exposed to a natural day/night cycle. Feed and water was provided at libitum and cage enrichment was provided for all animals. Mice were observed at least once daily. The presence of clinical signs was recorded in a register. Humane endpoints were used for euthanasia of sick animals, presenting signs of severe disease, such as hind limb paralysis. All experimental procedures were approved by the Ethical Committee of the institute (advice number 070515-05). Six-to-eight weeks old female Swiss outbred mice (Charles River, France) were used for experimental procedures. The intracranial and intranasal inoculation procedures were described by Rosseels et al. [1]. Prior to intranasal or intracranial virus inoculation, mice were anesthetised using isoflurane gas anesthesia. As soon as mice lost consciousness, they were inoculated either by placing a small volume of viral suspension on both nostrils (intranasal virus inoculation) or by injection a small volume of virus suspension directly into the brain using insulin syringes. Intramuscular inoculation was performed in the quadriceps muscle of the hind leg. For IC, IN and IM inoculations volumes of respectively 20, 25 and 50 µl of virus suspension were used. All experimental interventions were performed by trained, rabies-vaccinated staff in a biosafety level 3 laboratory.

Prior to IN and IC inoculations, mice were briefly anesthetized using isoflurane gas (IsoFlo, Abbott Laboratories Ltd., United Kingdom).

### 3.3.3 Determination of viral kinetics in the brain

The viral RNA load was determined using real-time reverse transcription polymerase chain reaction (RT-qPCR), as described by Rosseels et al. [1], in the olfactory bulbs, mid (cerebrum and diencephalon) and anterior (hindbrain and cerebellum) parts of the brain. Primers used are targeted against the nucleocapsid gene [11]. Brain samples were homogenized using a tissue homogenizer (Bullet Blender, Next Advance, USA) and 5 mm stainless steel beads in 350-1,000 µl lysis buffer (RLT buffer, as supplied with the Qiagen RNeasy kit, with 1 % β-mercaptoethanol). Total RNA was extracted using Qiagen RNeasy kit (Qiagen, Germany), according to manufacturer’s instructions. RNA was quantified with the NanoVue spectrophotometer (GE Healthcare, United Kingdom). A CVS-11 standard curve was established for each plate by adding serial dilutions of RNA extracted from a well-defined virus stock. Ribosomal 18S was used as a reference gene for standardization. Delta cycle thresholds (Δ Ct) values were calculated using the following formula: Δ Ct = Ct_ref – Ct, with Ct_ref equal to 45, which is the number of cycles in this qPCR program.
3.3.4 Clinical follow up

Mice were observed daily for signs of disease throughout the experiment until maximum 35 days post inoculation (DPI) (experimental end point). A score was attributed to weight loss (0 = absent, 1 ≥ 10 %), depression (0 = absent, 1 = lower (re)activity), hunched back (0 = absent, 1 = present), wasp waist (0 = absent, 1 = present), roughed hair coat (0 = absent, 1 = present); motoric incoordination (0 = absent, 1 = present), paresis (0 = absent, 1 = present) and paralysis of the hind legs (0 = absent, 1 = present). A cumulative daily clinical score per mouse was calculated. Mice that reached score 6 or 7 were euthanized.

3.3.5 Statistical analysis

GraphPad Prism was used for statistical analysis of all data. Differences in average survival times and differences in Δ Ct values were tested using a student t-test after normalization to an housekeeping gene.

3.4 Results

3.4.1 Comparison of the survival time of mice after different inoculation techniques

Data of all the experimental procedures performed since 2009 were used to analyse the survival times and survival percentages after different inoculation techniques, including the intranasal, intracranial and intramuscular routes. Five independent experiments were performed using the intramuscular inoculation, containing a total of 40 mice. For the intracranial and intranasal route respectively 4 and 28 independent experiments were performed resulting in a total of respectively 34 and 251 mice used.

Comparison of the average survival times after inoculation via the intranasal, intramuscular or intracranial route, showed that survival was slightly prolonged when mice were inoculated intranasally compared to the two other routes (Figure 3.3). Average survival time after intranasal inoculation was \(8.47 \pm 1.36\) days in contrast to \(7.04 \pm 2.28\) days with intramuscular inoculation or \(6.37 \pm 0.83\) days upon intracranial inoculations. However, these results were calculated solely based on diseased mice. Calculations of the survival percentage showed that 32.50 % and 5.71 % of the mice survived respectively intramuscular or intracranial virus challenge, whereas only 1.59 % of the mice survived intranasal virus inoculation.

We also compared the outcome of intranasal inoculation with different viruses. With the exception of the CB-1 strain, a street strain isolated from a rabid dog, all strains induced similar clinical symptoms and survival times. The CB-1 strain resulted in a 90 % survival rate, most likely because it is not adapted to mice (Figure 3.4).
Figure 3.3: Comparison of the average survival time of mice infected with CVS-11 via different inoculation routes. Although survival times were slightly longer after intranasal virus inoculation, less variation is seen compared to intramuscular inoculations. N represents the number of dead mice over the total of inoculated mice.

Figure 3.4: Comparison of the survival times of different rabies strains upon intranasal virus inoculation. Both CVS-11 and CVS-27 had similar median survival times upon intranasal administration, whereas CB-1, a strain not adapted to mice, had a 90% survival rate.
3.4.2 Characterization of intranasal inoculation technique

In order to further characterize the intranasal inoculation technique, we tested the reproducibility of the technique by comparing the average survival time after virus inoculation between different operators and by investigating the variation of the average survival time in experiments performed over the years.

Evolution of the average survival time throughout the years showed that in 2010 intranasal inoculation showed higher variability. This was the period at which the method was first used on a regular basis and is thus likely linked to the variability often seen when a model has to be acquired for routine practice. More specifically, we observed in this period that when more than 10 mice were inoculated with a same aliquot of virus, higher survival rates could be observed. This is most likely linked to the (partial) inactivation of the virus during handling. This led to the decision to limit the number of mice inoculated with the same aliquot to 10. Since 2011, the average survival times shows only slight variations (Figure 3.5).

A similar effect can be observed when comparing experiments performed by different operators. Operator 1 was responsible for the majority of the experiments in 2009 – 2011, including the optimization of the protocol (Figure 3.6). We observed in a significantly higher average survival time after virus inoculation by operator 1 compared to operator 2 (p<0.001). When excluding the data from 2009-2010 we observed that the differences are no longer significant between the two operators.

Figure 3.5: Evolution of the average survival time over time. High variability is seen in 2010, the moment at which the method was first being used on a regular basis. No significant differences were observed in the median survival time starting from 2011.
3.4.3 Characteristics of rabies virus infection and disease after intranasal virus inoculation

Upon intranasal virus inoculation first disease signs appear at 6.06 ± 0.59 days post inoculation (DPI) and severe nervous disease, requiring euthanasia, is observed at 8.47 ± 1.36 days. Mortality is nearly 100%. Disease progression follows a typical pattern, allowing us to develop a progressive scoring system typical for each stage of the disease (Figure 3.7).
The intranasal inoculation model

Figure 3.7: Disease progression in mice. Typical nesting behaviour (A) is absent in diseased mice (B). As the disease progresses, mice lose their typical curiosity (C), have rough hair coat, hunched back and are unresponsive to stimuli (D). Other typical symptoms are burying of the head in bedding material (E) and isolation from the group (F).

The evolution of the viral RNA load in the brain upon intranasal virus inoculation was monitored from 1 to 7 DPI, the time point at which clinical disease becomes severe (Figure 3.8). Mice were intranasally inoculated with rabies virus and sacrificed at varies time points after inoculation (7-10 mice per time point). Viral RNA could be detected starting from 1 DPI in the olfactory bulbs (of 3/10 mice), and in that of all mice from 2 DPI onwards. The virus then spreads from the front to the back of the brain in a matter of days. In the cerebrum and diencephalon, viral RNA can be detected as
soon as 2 DPI in 4/7 mice and from 3 DPI onwards in those of all mice. In the hindbrain and cerebellum, viral RNA can be detected as soon as 3 DPI in 2/7 mice and in those of all mice from 4 DPI onwards. Peak viral RNA levels (Δ Ct ≥ 25) are observed from 6 DPI onwards, and precede the occurrence of severe nervous disease (score ≥ 6) by 1 day. In contrast to intracerebral inoculation, IN inoculation leaves the brain mechanically intact, and yields a highly reproducible brain infection and disease outcome with little variation in the median survival time. The intranasal inoculation of rabies virus provides thus an excellent infection model to study antiviral treatment in the brain.

![Image](image1.png)

Figure 3.8: Progression of virus infection after intranasal virus inoculation. The graph presents the load of viral RNA in different parts of the brain (illustrated on the left of the graph) upon intranasal virus inoculation (n=55, 3 mice on day 0, 10 mice on day 1, 7 mice/time point at later time points).

### 3.5 Discussion

It is generally accepted that rabies virus infection occurs initially via inoculation of the virus into muscle tissue, which allows virus replication prior to entry into the central nervous system. The virus may reside at the site of inoculation for a long time, possibly explaining the long incubation period, and potentially the existence of abortive rabies virus infections [12,13]. It is therefore self-evident that most experimental inoculations use the intramuscular route. However, previous experiments in our laboratory showed that this experimental procedure resulted in high variation and a high and variable survival rate after viral challenge (on average 32.50 %), which poses difficulties to perform statistical analysis on the data, especially when testing anti-rabies therapeutics. It is important to remark that experimental CVS-11 infection in mice is an ‘all-or-nothing’ model in which mice either develop symptoms, eventually requiring euthanasia or mice do not develop any symptoms and survive the infection. A survival rate of 32.50 % thus means that 32.50 % of the mice never developed any disease symptoms.

In 2009, our research group optimized an alternative, needle-free, inoculation technique using the intranasal route [1]. This method requires a relatively low dose of virus, but results in a highly reproducible (short) incubation period and a nearly 100 % mortality rate. Once the technique was
optimized, it was used for nearly all experimental infections by our research group resulting in a huge amount of data, allowing a retrospective validation of this method.

We observed the lowest level of variation on the average survival time when mice were inoculated via the intracranial route, closely followed by intranasal inoculation and eventually intramuscular inoculation. Intracranial inoculation places the virus directly in contact with the tissues in which the virus replicates best, but is also a highly invasive technique. Upon inoculation, delicate brain tissue gets damaged, and this most likely induces an inflammatory response which can influence virus infection and disease progression [14]. Moreover, studies have shown that only a very small fraction of the inoculum can be found in the brain (5-10%), while most of it is absorbed into the circulation [15].

More importantly for the interpretation and statistical analysis of the experiments, disease and mortality rates upon intranasal virus inoculation are significantly higher compared to intramuscular or intracranial inoculation. Whereas over all experimental procedures only 1.59% of the animals survived infection after intranasal virus inoculation, respectively 32.50% and 5.71% survived upon intramuscular or intracranial inoculations. Both the limited variation in average survival times, as the high disease and mortality rates facilitate the interpretation and analysis of experimental intervention and treatment protocols. Despite the large variation in survival rates, the incubation periods are comparable between the different routes of inoculation. This might suggest that upon intramuscular inoculation, the virus did not take the natural route via the motoric or sensory nerves, but rather entered the brain via a faster route. In our model we used a high viral dose (10^5 CCID_{50}/mouse) for intramuscular inoculation and therefore it is not unthinkable that the virus entered the brain via the blood. Indeed, in a number of brain regions the blood-brain barrier does not exist allowing transport of proteins and small organic molecules from the blood to the brain [16].

Remarkably, the use of different rabies virus strains resulted in only little variation in average survival time and resulted in a comparable symptomatology, with the exception of the CB-1 street strain. The fact that this strain is not yet adapted to laboratory animals, most likely explains the lower mortality rate. Once the technique is mastered by the different operators, only small differences in average survival times were observed. Confirming that this technique is highly reproducible, even when different operators are performing the experimental procedure. From our experience ‘inter-operator’ variation is higher for intracranial inoculation as that technique is more sensitive to small variations in inoculation technique, and thus more difficult to master.

Using quantitative real-time PCR (qRT-PCR) analysis, we were able to follow the path of the virus through the brain upon intranasal inoculation. This showed that in a number of mice the neurons of the olfactory bulbs are infected as soon as 1 DPI. At 2 DPI the olfactory bulbs were infected in all
mice, and from there on the virus steadily spreads to the brain leading to the infection of all tested parts of the brain four days after virus inoculation. This is in line with the finding of Lafay et al. which showed that virulent rabies strains quickly infect the olfactory epithelium [9]. Surprisingly, even though all parts of the brain are infected as soon as 4 DPI, with relatively high viral titers, the first symptoms in mice do not appear before 6 DPI.

Although the incubation time in humans can be highly variable and span several months to years, severe bites or bites to highly enervated regions like the face, neck and hand are generally associated with a short incubation period of one week or less [17]. The rare cases of PEP failure often also involve (severe) bites to these regions and short incubation periods [18–20]. The intranasal inoculation is a model with a very short incubation time, making it a useful model to study this type of cases. In addition, intranasal inoculation is performed without the use of needles, reducing not only the risk for the operators, but also unnecessary stress and injury in laboratory animals.
Reference List


Development of anti-rabies VHH and validation of their antiviral activity

This chapter bundles the research results, addressing the second and third objective of the thesis; assessment of the efficacy of VHH for the neutralization and prophylactic treatment of rabies virus disease in mice. In the first part (Chapter 4.1), the protective effect of different VHH constructs was evaluated in vitro and in vivo and in chapter 4.2 the effect of half-life extension on the antiviral potency was investigated.

4.1 VHH in the treatment of rabies virus disease

4.1.1 Abstract

Rabies virus causes a lethal brain infection in about 59,000 people yearly. Each year, tens of thousands of people receive anti-rabies prophylaxis with plasma-derived immunoglobulins and vaccine soon after exposure. Anti-rabies immunoglobulins are however expensive and have limited availability. VHH are the smallest antigen-binding functional fragments of camelid heavy chain antibodies, also called Nanobodies®. The therapeutic potential of anti-rabies VHH was examined in a mouse model using intranasal challenge with a lethal dose of rabies virus. Anti-rabies VHH were administered directly into the brain or systemically, by intraperitoneal injection, 24 hours after virus challenge. Anti-rabies VHH were able to significantly prolong survival or even completely rescue mice from disease. The therapeutic effect depended on the dose, route of administration and time of treatment. Increasing the affinity by combining two VHH with a glycine-serine linker into bivalent constructs, increased the neutralizing potency to the picomolar range. Upon direct intracerebral administration, a dose as low as 33 μg of the hetero-bivalent Rab-E8/H7 was still able to establish an anti-rabies effect. Systemic administration of the VHH could only delay disease by one day, indicating that systemic administration of VHH limits its potential to reach and protect the brain. These observations show that VHH are able to block or delay virus infectivity in vivo, but that effectiveness of treatment depends on timing and route of administration.

4.1.2 Introduction

Rabies virus (Family Rhabdoviridae, Genus Lyssavirus) is a model neurotropic RNA virus, which causes an aggressive and lethal infection in the brains of humans and other mammals [1]. Once the virus enters the peripheral nerves or neurons, the virus replicates quickly in the neuronal cytoplasm and progeny virus is transported through the neuronal network by crossing interneuronal synapses [2,3].

Nanobodies® (a trade-name by Ablynx) or VHH are the smallest functional portions (15 kDa) of heavy chain-only antibodies, naturally occurring in Camelidae, and represent the antigen-binding variable domain. VHH consist of a single antigen-binding variable domain that does not require hydrophobic interaction with a light chain, leading to high solubility, physiochemical stability and high-yield production in Escherichia coli and yeast. Their small size allows easy formatting by genetic fusion into multimeric constructs with multiple specificities [4–6].

Previously, we developed a number of rabies virus-specific VHH directed against the rabies virus spike G protein [7]. In vitro, these VHH were fully able to neutralize the rabies virus infectivity in
neuroblastoma and baby hamster kidney cells-21 (BHK-21) and could neutralize a wide spectrum of Lyssavirus species. The neutralizing potency increased massively when two VHH were combined with a glycine-serine linker into homo- or hetero-bivalent constructs [7].

Other research groups have also developed antiviral VHH against a number of viruses [6]. For foot-and-mouth disease virus, rotavirus, respiratory syncytial virus and influenza virus, specific antiviral VHH were also tested in animal models. For these viruses, preventive treatment with VHH could delay or prevent disease upon challenge. In general, administration of VHH after infection had limited effect on viral load or animal-to-animal transmission [8–11]. In the case of rotavirus, preventive treatment with VHH and continued administration until day 7 of infection was able to completely protect pigs from diarrhea, resulting in an asymptomatic infection and the development of a humoral immune response [11].

Anti-rabies antibodies are able to protect mice upon preventive administration and offer partial protection against disease and mortality upon early administration in a post exposure setting [12–14]. Antibody fragments, such as VHH or F(ab’)$_2$, lack Fc domains, which render them incapable of exerting Fc effector functions, such as complement activation or interaction with Fc receptors on phagocytes. To what extent these effector functions contribute in control and clearance of infection, seems to depend on the virus [15,16]. For example in the case of human immunodeficiency virus, the loss of Fc-Y-receptor binding function greatly increased the risk of infection upon pre-exposure treatment [17].

F(ab’)$_2$ fragments, obtained after pepsin digestion of whole antibodies, have reduced activity against rabies virus in mice [14]. Still, F(ab’)$_2$ fragments derived from equine immunoglobulins are used in post-exposure prophylaxis in humans as a cheaper substitute for human anti-rabies immunoglobulins [18]. Recommended doses for equine F(ab’)$_2$ (40 IU/kg) are two-fold higher than for human rabies immunoglobulins (20 IU/kg).

To date, little is known concerning the potential of VHH to neutralize rabies virus in vivo or to treat rabies virus infection. Viral receptors present in vivo are most likely different from the receptors responsible for virus uptake in cell lines [19]. Previously, Dietzschold et al. [20] found that the neutralizing potency of conventional antibodies determined in cells lines can differ substantially from the in vivo potency. Since VHH lack the Fc fragment of conventional antibodies, the in vivo antiviral activity might be compromised. A recent paper by the group of Boruah et al. [21] showed that pentameric constructs of anti-rabies VHH were able to partially neutralize rabies virus when co-injected with virus in the hindleg of mice. Constructs were composed of five homologous single domain anti-rabies virus antibodies fused to a coiled-coil peptide. The used dose was however
relatively low (0.2 - 1.6 IU/ml). Also, the effect of treatment after virus challenge, as would occur under natural circumstances, was not examined.

In the current work we wanted to further explore the protective effect of anti-rabies VHH in vitro and in vivo using constructs with high antiviral potencies. Two homologous (homo-bivalent) or heterologous (hetero-bivalent) VHH were genetically fused with glycine-serine linkers to increase potency.

The aims of this study were to compare the neutralizing potency of distinct monovalent, homo-bivalent and hetero-bivalent anti-rabies VHH both in vitro and in vivo and assess the efficacy of anti-rabies VHH in delaying infection and disease, or even to rescue mice, by post-exposure treatment after lethal intranasal virus challenge.

4.1.3 Materials and methods

4.1.3.1 VHH and antibody

VHH directed against the rabies virus glycoprotein G were generated previously [7]. Briefly, llamas were vaccinated with the inactivated rabies Human Diploid Cell Vaccine (HDCV, Sanofi, France) and RNA was extracted from peripheral blood lymphocytes. VHH genes were amplified from a cDNA library. Anti-rabies virus VHH were selected by panning phage libraries on plates coated with the native G protein. For the generation of multivalent VHH constructs, monovalent VHH were genetically fused into dimeric VHH constructs using flexible glycine-serine (GS) linkers. Homo-bivalent VHH contained two identical VHH monomer clones, whereas hetero-bivalent VHH contained two different VHH clones. RSV117, a bivalent respiratory syncytial virus (RSV)-specific VHH was used as a negative irrelevant control. For initial characterization, all VHH were produced with C-terminal cMyc-His6 tags in Escherichia coli. For subsequent in vivo experiments Rab-E8/H7 was recloned to Pichia pastoris expression vectors for production in the X-33 strain (Invitrogen, cat n° C18000) as tagless proteins. All VHH were purified to endotoxin levels < 5 EU/mg.

4.1.3.2 Rabies virus

Challenge Virus Standard (CVS)-11 is a virulent classical rabies virus obtained from the American Type Culture Collection (ATCC reference VR959) and was grown in baby hamster kidney (BHK)-21 cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). For virus inoculation in mice, a dose of $10^{2.5}$ 50 % cell culture infectious doses (CCID_{50}) of CVS-11 was used.
**4.1.3.3 In vitro virus neutralization**

The virus-neutralizing potency was titrated with the rapid fluorescent focus inhibition test (RFFIT) according to the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Office International des Epizooties, 2008). Briefly, a standard dose of virus was pre-incubated with serially diluted VHH/antibody for 90 minutes at 37 °C. BHK-21 cells were then added to the mix and co-incubated for 24 hours. Infected BHK-21 cells were stained with fluorescent anti-nucleocapsid antibody and foci of infected cells were counted under the fluorescent microscope. The dilution that yielded 50 % inhibition of infected foci was determined. The neutralizing potency is expressed in international units (IU)/ml in reference to “The Second International Standard for Anti-Rabies Immunoglobulin”, purchased from the United Kingdom National Institute for Biological Standards and Control.

**4.1.3.4 Mouse inoculation experiments**

Six-to-eight weeks old female Swiss outbred mice (Charles River, France) were used for all experimental procedures. The experimental procedures were approved by the local ethical committee of the institute (advice number 070515-05). Mice were kept in filter top cages, water and feed provided ad libitum and exposed to a natural day/night light cycle. The intracranial (IC) and intranasal (IN) inoculation procedures are described in detail by Rosseels et al. [22]. For IC, IN and intraperitoneal (IP) injection volumes of respectively 20, 25 and 1,000 µl were used.

The intranasal inoculation of rabies virus is an excellent technique to study antiviral treatment in the brain, since it leaves the brain mechanically intact, in contrast to intracranial inoculation, and yields a highly reproducible brain infection and disease outcome with little variation in the median survival time. This inoculation route has been used before for the evaluation of post exposure prophylaxis of rabies in mice [14].

Prior to IN or IC virus or VHH administration, mice were briefly anesthetized using isoflurane gas (IsoFlo, Abbott laboratories Ltd., Queensborough, Kent, United Kingdom).

**4.1.3.5 Clinical follow-up**

Mice were observed daily for signs of disease until 35 days post virus inoculation. Signs of disease were evaluated as follows: weight loss (0 = absent, 1 = visible), depression (0 = absent, 1 = lower (re)activity), hunched back (0 = absent, 1 = present), motoric incoordination (0 = absent, 1 = present), rough hair coat (0 = absent, 1 = present), paralysis of the hind legs (0 = absent, 1 = present) and conjunctivitis (0 = absent, 1 = present). The cumulative daily clinical score per mouse was calculated as the sum of the scores for each parameter. Disease progression was represented by plotting the cumulative daily score in function of the days post inoculation (DPI). The cumulative daily score per mouse ranged from 0 (no disease) to 7 (severe nervous disease). In our experience,
mice with a disease score of 6 or more die within one day [22]. Therefore, mice were euthanized by cervical dislocation when they reached a disease score of ≥ 6. Results were expressed as Kaplan-Meier survival curves. GraphPad Prism was used for statistical analyses of in vivo data. Differences in survival times were tested using the Log-Rank test with a Bonferroni post-test, differences in ΔCt values were tested using a student t-test after normalization to the house-keeping gene.

4.1.3.6 Determination of viral load in the brain

The viral RNA load was determined using real-time reverse transcription polymerase chain reaction (RT-qPCR), as described by Rosseels et al. [1], in the olfactory bulbs, mid (cerebrum and diencephalon) and anterior (hindbrain and cerebellum) parts of the brain. The primers were targeted against the nucleocapsid gene [11]. Brain samples were homogenized using a tissue homogenizer (Bullet Blender, Next Advance, USA) and 5 mm stainless steel beads in 350-1,000 μl lysis buffer (RLT buffer, as supplied with the Qiagen RNeasy kit, with 1 % β-mercaptoethanol). Total RNA was extracted using Qiagen RNeasy kit (Qiagen, Germany), according to the manufacturer’s instructions. RNA was quantified with the NanoVue spectrophotometer (GE Healthcare, United Kingdom). A CVS-11 standard curve was established for each plate by adding serial dilutions of RNA extracted from a well-defined virus stock. Ribosomal 18S was used as a reference gene for standardization. Delta cycle thresholds (Δ Ct) values were calculated using the following formula: ΔCt = Ct_ref − Ct, with Ct_ref equal to 45, which is the number of cycles in this qPCR program.

4.1.3.7 Statistical analysis

GraphPad Prism was used for statistical analyses of in vivo data. Differences in survival times were tested using the Log-Rank test with a Bonferroni post-test, difference in ΔCt values were tested using a student t-test after normalization to a house-keeping gene.

4.1.4 Results

4.1.4.1 Neutralizing potency of different anti-rabies VHH constructs in vitro and in vivo

In previous work, we described the generation of different anti-rabies G protein VHH constructs [7]. The virus-neutralization capacity of different monovalent, homo-bivalent and hetero-bivalent VHH constructs in vitro and in mice was compared (Table 4.1.1). Low doses of anti-rabies VHH (1 IU) were pre-incubated for 30 minutes at 37 °C with the rabies virus, prior to administration to either BHK-21 cells or to different virus-receptive body compartments of the mouse (intranasal IN, intracerebral IC and intramuscular IM).
All VHH constructs showed *in vitro* virus neutralization with IC$_{50}$ values ranging from 0.1-15 nM whereas an irrelevant VHH construct was not active, confirming the specificity of the neutralization effect. The *in vitro* neutralization experiments showed that the overall potency of the VHH constructs increased significantly from monovalent to homo-bivalent and finally to the hetero-bivalent constructs, with the latter having a comparable or higher potency as compared to a rabies monoclonal antibody.

*In vivo*, most homo-bivalent and hetero-bivalent constructs offered full protection in all the body compartment tested. Monovalent VHH could partially protect mice when the mix was administered intranasally, but not when administered directly into the brain. Remarkably, both monovalent and homo-bivalent Rab-C12 VHH, while highly neutralizing *in vitro*, protected less well *in vivo*. The hetero-bivalent Rab-E8/H7 proved most potent *in vitro* and *in vivo*, and was thus selected for further *in vivo* studies.

In addition, the neutralizing potency of the Rab-E8/H7 was further investigated by determination of the viral load in the brain after co-administration of the VHH and the virus. Figure 4.1.1 shows that Rab-E8/H7 VHH efficiently inhibits the infectivity of rabies virus in the brain, as no viral antigen could be detected and only minimal levels of viral RNA at 7 DPI. The viral RNA load was significantly lower compared to that in the brain of control mice treated with irrelevant VHH (p<0.001, t-test). In conclusion, most homo- and hetero-bivalent anti-rabies VHH could completely protect mice from disease upon inoculation of a pre-incubated mix of VHH and a lethal dose of rabies virus in different body compartments.

In addition to the co-administration experiments, the most potent rabies VHH, Rab-E8/H7 was tested for efficacy upon preventive treatment. Pre-exposure treatment with a low dose of Rab-E8/H7 (0.12 μg, 1 IU) applied in the target organ (brain, IC) was followed by virus challenge in the nose 24 hours after VHH treatment. Complete protection was obtained against later IN virus challenge (p<0.01, Log-Rank test, Bonferroni post-test). This indicates that a sufficiently high level of VHH remains present in the brain one day after intercerebral inoculation to neutralize incoming virus. We cannot exclude that part of the VHH diffused from the brain to the nose and was capable to neutralize the virus in the nose.
Table 4.1.1: Comparison of the neutralizing potency of different anti-rabies VHH constructs *in vitro* and *in vivo*

<table>
<thead>
<tr>
<th>Compound</th>
<th><em>In vitro</em> virus-neutralization (RFFIT assay in BHK-21 cells)</th>
<th><em>In vivo</em> virus-neutralization: % mortality in mice (upon inoculation of a mix of 1 IU VHH and virus)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IU/μM</td>
<td>IC₅₀⁰ nM</td>
</tr>
<tr>
<td>Phosphate-buffered saline</td>
<td>&lt; 0.5</td>
<td>&gt; 5,881</td>
</tr>
<tr>
<td>Rabies monoclonal antibody RV1C5</td>
<td>193,500</td>
<td>0.17</td>
</tr>
<tr>
<td>Irrelevant VHH</td>
<td>RSV-D3</td>
<td>&lt; 0.5</td>
</tr>
<tr>
<td>Anti-rabies VHH</td>
<td>Rab-C12</td>
<td>4.60</td>
</tr>
<tr>
<td></td>
<td>Rab-E6</td>
<td>2.54</td>
</tr>
<tr>
<td></td>
<td>Rab-E8</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>Rab-E7</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>Rab-C12/C12</td>
<td>C12-15GS⁻C12</td>
</tr>
<tr>
<td></td>
<td>Rab-E8/E8</td>
<td>E8-15GS-E8</td>
</tr>
<tr>
<td></td>
<td>Rab-H7/H7</td>
<td>H7-15GS-H7</td>
</tr>
<tr>
<td></td>
<td>Rab-E8/H7</td>
<td>E8-15GS-H7</td>
</tr>
<tr>
<td></td>
<td>Rab-E8/C12</td>
<td>E8-15GS-C12</td>
</tr>
<tr>
<td></td>
<td>Rab-E6/C12</td>
<td>E6-15GS-C12</td>
</tr>
<tr>
<td></td>
<td>Rab-E6/H7</td>
<td>E6-15GS-H7</td>
</tr>
</tbody>
</table>

⁰ IC₅₀: 50% inhibitory concentration

ⁱ nd: not determined

⁰ GS: glycine-serine
Development and validation of anti-rabies VHH

4.1.4.2 Post exposure treatment by direct intracerebral administration of Rab-E8/H7

4.1.4.2.1 Administration of increasing doses at day one after virus inoculation

To determine the minimal effective dose to obtain protection against virus-induced mortality, mice were treated in two independent experiments with different doses of Rab-E8/H7 administered directly to the brain at one day after virus challenge. Table 4.1.2 summarizes the set-up of these experiments. In a first experiment, mice were treated with 1 µg (4.63 IU), 10 µg (46.3 IU) or 100 µg (463 IU) at 24 hours after virus inoculation (Figure 4.1.2A). Treatment with 1 µg of Rab-E8/H7 gave no significant delay in the median survival time compared to animals treated with irrelevant VHH (9 days). A non-significant effect was observed at a dose of 10 µg Rab-E8/H7 and significant protection at a dose of 100 µg (p<0.01). To determine the effective dose more precisely, a second experiment
was performed using two extra doses between 10 and 100 µg (Figure 4.1.2B). Significant protection was observed starting from a dose of 33 µg (p<0.01 for 33 µg, 67 µg and 100 µg, Log-Rank test, Bonferroni post-test). One third to more than half of the mice that were treated with a dose of 33 µg or higher survived the infection. There was no straightforward dose-response relationship in the second experiment, as the 33 µg dose performed better than the 67 and 100 µg doses. This inconsistency was probably due to experimental variation. Figure 4.1.3A shows that Rab-E8/H7 treatment had significantly reduced the spread of the virus from the frontal to the posterior parts of the brain at day 7 after virus inoculation (t-test, ** p<0.01 for olfactory bulbs and cerebrum/diencephalon, *** p<0.0001 for hindbrain/cerebellum).

Table 4.1.2: Set-up of post-exposure treatment experiments at 1 day after virus inoculation.

<table>
<thead>
<tr>
<th>Group</th>
<th>Intervention at day…</th>
<th>0</th>
<th>1</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td>Viruses</td>
<td>VHH IC</td>
<td>End observation - euthanasia</td>
</tr>
<tr>
<td>1 µg Rab-E8/H7 (n=7)</td>
<td>Virus challenge</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 µg Rab-E8/H7 (n=7)</td>
<td>Virus challenge</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 µg Rab-E8/H7 (n=7)</td>
<td>Virus challenge</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 µg irrelevant VHH (n=7)</td>
<td>Virus challenge</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td>Viruses</td>
<td>VHH IC</td>
<td>End observation - euthanasia</td>
</tr>
<tr>
<td>33 µg Rab-E8/H7 (n = 7)</td>
<td>Virus challenge</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>67 µg Rab-E8/H7 (n = 7)</td>
<td>Virus challenge</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 µg Rab-E8/H7e (n = 7)</td>
<td>Virus challenge</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 µg irrelevant VHH (n=7)</td>
<td>Virus challenge</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The long-term antiviral effect of intracerebrally injected Rab-E8/H7 (treatment before virus challenge: 0.12 µg, 1 IU or after: 100 µg, 463 IU) was also examined in survivor mice. Figure 4.1.3B shows the residual viral RNA load in the whole brain of survivor mice at the convalescent phase of infection (day 35). The survivor mice, which were treated either one day before or one day after virus inoculation, had only minimal amounts of viral RNA (ΔCt 5 ± 2.9) in the brain at 35 DPI (** p<0.0001), which demonstrates that they had successfully overcome the acute infection. Mice treated with an irrelevant VHH had to be euthanized around day 7-9 of infection and always contained high levels of viral RNA in the brain (ΔCt ≥ 28).

---

**Figure 4.1.2:** Dose-dependent efficacy of anti-rabies Rab-E8/H7 upon intracerebral post-exposure treatment at one day after intranasal virus inoculation. Two independent experiments were performed. In the first experiment (A), doses of 1, 10 and 100 µg Rab-E8/H7 were tested and in the second experiment (B), two additional doses of 33 µg and 67 µg were included. Significant protection was observed starting from a dose of 33 µg (p<0.01). One third to more than half of the mice that were treated at this or a higher dose survived the infection.

**Figure 4.1.3:** Viral RNA load in the brain after anti-rabies Rab-E8/H7 treatment. (A) Mice were treated with Rab-E8/H7 (100 µg) by intracerebral injection (IC) 24 hours after intranasal virus inoculation and sacrificed at 7 DPI to assess the viral RNA loads in different brain parts. Rab-E8/H7 VHH treatment significantly reduced the spread of the virus from the front to the posterior parts of the brain (t-test, ** p<0.01, *** p<0.0001). (B) Mice were treated with Rab-E8/H7 at 24 hours before (0.12 µg) or after (100 µg) intranasal virus inoculation. Control mice were mock-treated with irrelevant VHH before virus inoculation. Viral RNA loads were measured at 35 DPI in the brain of survivor mice. Four out of five survivor mice, treated after the virus inoculation, showed residual traces of viral RNA in the brain (ΔCt 5 ± 2.9; *** p<0.0001). These mice had however never developed signs of disease. All mock-treated mice had to be euthanized at 7-9 DPI, because of serious disease, which coincided with high viral RNA loads in their brains (ΔCt ≥ 28).
4.1.4.2.2 Administration at increasing time points after virus inoculation

Given the protective nature of Rab-E8/H7 VHH treatment when given 1 day post-exposure, a follow-up experiment was designed to elucidate the time-course of protection by Rab-E8/H7. The set-up of this experiment is given in Table 4.1.3. Mice were treated with an intracerebral dose of 100 µg (463 IU) anti-rabies VHH at 1, 3 or 5 days after intranasal virus inoculation. The protective effect of Rab-E8/H7 VHH diminished progressively when treatment was initiated at later stages of infection (Figure 4.1.4). Median survival times were 13, 11 and 10.5 days upon starting Rab-E8/H7 VHH treatment at respectively day 1, 3 and 5 post virus inoculation, compared to a median survival time of 9 days in mock-treated mice. The prolongation of the median survival time was significant for treatment at day 1 or 3 (p<0.01, LogRank test, Bonferroni post-test), but not for treatment at day 5. Thus, 3 DPI was identified as the latest treatment point to result in significant protection.

Table 4.1.3: Set-up of post-exposure treatment experiments at different days after virus inoculation.

<table>
<thead>
<tr>
<th>Group</th>
<th>Intervention at day...</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
</tr>
<tr>
<td>100 µg Rab-E8/H7 (n=6)</td>
<td>Virus challenge VHH IC</td>
</tr>
<tr>
<td>100 µg Rab-E8/H7 (n=6)</td>
<td></td>
</tr>
<tr>
<td>100 µg Rab-E8/H7 (n=6)</td>
<td>Virus challenge VHH IC</td>
</tr>
<tr>
<td>100 µg irrelevant VHH (n=7)</td>
<td>Virus challenge VHH IC</td>
</tr>
</tbody>
</table>

In two previous experiments (Figure 4.1.2A and B), 43-71 % of mice treated IC with 100 µg Rab-E8/H7 at 1 DPI survived. In the third experiment (Figure 4.1.4), no mouse survived, albeit the median survival time was still significantly prolonged. This experimental variation may be explained because of small variation in the site of intracerebral injection.
Figure 4.1.4: Post-exposure treatment by intracerebral injection at different time points of infection. Mice were treated with a single dose of 100 µg (463 IU) Rab-E8/H7 at increasing time points of infection. The protective effect of anti-rabies VHH diminished progressively when treatment was initiated at later stages of infection.

4.1.4.3 Post-exposure treatment by peripheral administration of Rab-E8/H7

Mice were also treated with a high doses of Rab-E8/H7 administered peripherally (intranasally and intraperitoneally) one day after intranasal virus challenge. The set-up of these experiments is given in Table 4.1.4. Administration of Rab-E8/H7 intranasally soon after virus inoculation (24 hours), failed to delay disease onset or decrease mortality, despite the high dose administered to the animals (4 to 20 times higher than the dose administered intracerebrally) (Figure 4.1.5A). Similarly, a high dose of anti-rabies VHH administered intraperitoneally 24 hours after virus inoculation, was unable to stop the virus from infecting the brain and causing lethal infection. A bolus administration of 10 mg Rab-E8/H7 administered 24 hours after virus inoculation could significantly delay disease onset, but only by one day (p<0.001) (Figure 4.1.5B).
### Table 4.1.4: Set-up of post-exposure treatment experiments at different days after virus inoculation.

<table>
<thead>
<tr>
<th>Group</th>
<th>Intervention at day...</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>400 µg Rab-E8/H7 (n=7)</td>
</tr>
<tr>
<td></td>
<td>2 mg Rab-E8/H7 (n=7)</td>
</tr>
<tr>
<td></td>
<td>2 mg Rab-E8/H7 (n=7)</td>
</tr>
<tr>
<td></td>
<td>5 mg Rab-E8/H7 (n=7)</td>
</tr>
<tr>
<td></td>
<td>2 mg irrelevant VHH (n=7)</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>10 mg Rab-E8/H7 (n = 7)</td>
</tr>
<tr>
<td></td>
<td>10 mg irrelevant VHH (n = 7)</td>
</tr>
</tbody>
</table>

Administration of this high dose resulted in a significant delay in both disease onset as in mortality by one day (p<0.001). Although this effect was significant, this high dose was unable to finally protect mice from lethal infection.

![Graph showing survival rates over DPI](image-url)
4.1.5 **Discussion**

In this study, the antiviral effect of different anti-rabies VHH constructs, targeted against the surface glycoprotein of the rabies virus, was examined in a brain infection model in mice. Monovalent, homo-bivalent, hetero-bivalent and half-life extended anti-rabies VHH were first compared *in vitro*. Then, a step-wise approach was used for extending the *in vitro* neutralization results to *in vivo* neutralization, starting with a pre-exposure setting and then testing the VHH in a prophylactic-therapeutic setting. Pre-exposure treatment and virus-VHH co-administration were primarily performed to proof the concept in a model with highest chance of success.

Despite the absence of Fc effector functions and the small size, homo- and hetero-bivalent anti-rabies VHH are able to significantly prolong survival or even completely rescue mice from disease. The therapeutic effect depends on the time of treatment and dose of the VHH construct administered.

Increasing the affinity by combining two VHH with a glycine-serine linker into bivalent constructs, increased the *in vitro* neutralizing potency to the picomolar range. The potency of the different hetero-bivalent constructs seemed to be higher than that of the monovalent or homo-bivalent VHH, which has also been reported for other VHH directed against membrane receptors, such as CXCR4 and EGFR, where conformational changes play a role in the receptor activity [23,24].

---

**Figure 4.1.5:** Post-exposure treatment by intranasal or intraperitoneal injection 24 hours after intranasal virus inoculation. (A) Mice were treated with different doses of Rab-E8/H7 via intranasal or intraperitoneal injection 24 hours after intranasal virus inoculation. Despite the high dose of Rab-E8/H7 administered via those routes, mice were not protected from lethal disease. (B) Mice were treated with a single dose of 10 mg Rab-E8/H7 1 day after virus inoculation. In contrast to the lower doses administered, this treatment significantly prolonged survival by one day (p<0.001).
The inhibitory effect of the anti-rabies VHH might be due to steric hindrance, blocking the viral G protein and cell receptor interactions, or impairment of conformational changes in the G protein. Typically, hetero-bivalent VHH are better in preventing conformation changes. However, the precise mechanism of VHH-mediated virus neutralization remains unknown.

At one day of virus infection, direct intracerebral administration of the hetero-bivalent Rab-E8/H7 at a dose as low as 33 μg was able to establish a significant anti-rabies effect. This is remarkable since the brain is extremely sensitive to infection and, in essence, only one infectious particle is sufficient to induce lethal infection in the absence of immunity [25].

Surprisingly, both monovalent and homo-bivalent Rab-C12 VHH were highly neutralizing in vitro, but protected less well in vivo. Previously, we found that Rab-C12 recognizes a different epitope than Rab-E8 and Rab-H7 [7]. We did not map epitopes, but possibly the Rab-C12 epitope is less important for neutralization in vivo. Correspondingly, Dietzschold et al. [20] already described that the neutralizing potency of antibodies can differ significantly in vitro and in vivo. Possibly, the virus uses different receptors for binding and uptake in vitro than in vivo.

Boruah et al [21] reported that their pentavalent anti-rabies VHH constructs were able to partially (40-50 %) protect mice against infection upon co-administration with virus in the hindleg. Our results confirm their observations, albeit that both our monovalent and homo/hetero-bivalent VHH constructs offered complete protection upon co-administration.

Obviously, when sufficient amounts of VHH are introduced in the brain at an early phase of infections (day 1), the further spread of the virus slows down to such an extent that complete rescue of mice becomes feasible. Most likely, in survivor mice, the viral load never reached the critical threshold to induce disease. In our experiments, a viral RNA load corresponding with a ΔCt of 28 or higher is associated with the appearance of severe nervous system disease. A delay in the build-up of virus, probably allows the immune response to kick in and clear our or control the virus infection. Indeed, upon post-exposure treatment, survivor mice still harboured small amounts of residual virus in their brain (Figure 4.1.3A), but appeared in perfect health and had mounted neutralizing antibodies in their blood (data not shown).

The efficacy of post-exposure treatment diminishes progressively when initiated at advanced stages of infection, varying from significant protection (treatment at day 1) to no protection (treatment at day 5) (Figure 4.1.4). At day 1, the spread of the infection is still limited to neurons of the olfactory bulbs (Figure 3.8), which agrees with previous studies [25,26]. At this early stage, treatment can still prevent or delay spread to the rest of the brain. At later stages, the virus has spread over larger parts of the brain. We assume that anti-rabies VHH are able to intercept
Development and validation of anti-rabies VHH

intracellular virus spread, but cannot diminish or clear out intracellular virus, which limits the effect of VHH treatment at more advanced stage of infection.

Compared to direct intracerebral treatment (33 µg), much higher doses (10 mg) are needed to delay disease or protect mice upon systemic treatment. The relative weaker performance of systemic treatment can easily be explained by the fact that only a small fraction of Rab-E8/H7 eventually reaches the brain.

4.1.6 Acknowledgements

Marie-Louise Blondiau is great-fully acknowledged for performing the RFFIT with utmost reliability. Sophie Lamoral and Aurélie Francart are respectively acknowledged for performing RT-qPCR and mouse inoculations with great precision and care.
4.2 The effect of half-life extension of VHH on the \textit{in vivo} efficacy

4.2.1 Abstract

In the previous chapter, we showed that VHH were able to neutralize the rabies virus both \textit{in vitro} and \textit{in vivo}. The best \textit{in vivo} results were obtained when the VHH was administered directly into the brain soon after virus inoculation. The short half-life of VHH might make it difficult to reach the site of infection at sufficient quantities to block infection when administered peripherally. Hence, the purpose of this investigation was to see if half-life extension (HLE) of the VHH increases the systemic availability of VHH enough to improve treatment of infection. By comparing different methods of half-life extension and comparing HLE VHH with conventional antibodies, we could conclude that addition of a third VHH directed to (human) serum albumin gave the best results \textit{in vivo}. We showed that addition of the anti-albumin VHH to Rab-E8/H7 increased the circulatory half-life from 1.16 to 30.5 hours, resulting in a higher exposure of the brain to anti-rabies VHH. Up to 71% of the mice could be rescued when this VHH was administered soon after virus inoculation. In contrast, PEGylated VHH could delay disease onset by four days, but was unable to rescue animals from lethal infection. Treatment with a high dose of human anti-rabies immunoglobulins could only prolong survival by one day, despite the significantly longer half-life \textit{in vivo}.

Multiple dosing at consecutive days did not result in an increased survival as compared to one bolus administration of the VHH soon after virus exposure. This indicated that most of the protective effect is obtained during the very early stage of infection.

4.2.2 Introduction

VHH are the smallest antigen-binding fragments of antibodies. Their small size allows them to be easily produced and access epitopes inaccessible to conventional antibodies, but also has a downside effect since they are rapidly cleared by renal filtration.

Since VHH have a molecular mass of less than 60 kDa they have a short half-life in circulation. This means that they need to be dosed at high frequency to maintain a therapeutic effect [27]. The glomerular filtration is responsible for the renal clearance of molecules in the range below 40-50 kDa. Since filtration is not only regulated via pores, but also by an anionic barrier, not only the size, but also the physicochemical properties play a role in half-life. In general, there are three approaches that can be used to extend the half-life of protein therapeutics: increase in hydrodynamic volume, neonatal Fc receptor recycling and modulation of the protein-receptor stability [28].
The hydrodynamic volume of a protein can be increased by addition of highly flexible, hydrophilic molecules like polyethylene glycol and carbohydrates. An example of this is PEGylation, in which the protein is chemically coupled to polyethylene glycol (PEG). However, the number and size of the PEG, as well as the site of PEGylation must be carefully chosen not to interfere with the activity of the protein. Besides increasing the hydrodynamic size of the molecule and thus increasing the half-life in circulation, addition of PEG also alters the physicochemical properties of the molecule. These can be differences in receptor binding and biological activity, but also reduced immunogenicity or toxicity. In general it is considered a safe technique that is well tolerated, and currently several PEGylated protein drugs are approved for human use [28,29].

The half-life of small proteins like VHH can also be increased by incorporation of components or fragments that allow neonatal Fc-receptor (FcRn) recycling. Immunoglobulins typically have long half-lives in the range of three to four weeks, mainly mediated by recycling through FcRn. FcRn is a receptor that is widely distributed in many tissues and cell types including the brain endothelia. It plays a major role in IgG homeostasis and transcytosis, but only recently it was shown that it also plays a role in the half-life extension of serum albumin. In both cases, the FcRn rescues the proteins from degradation by binding them at low pH in the acid endosome and thus diverting these from the lysosomal pathway. The half-life of VHH can therefore also be extended by addition of an Fc fragment or a fragment that allows Fc binding, serum albumin or a fragment binding to albumin [27,28].

Here, we assumed that increasing the circulatory half-life, will allow a prolonged contact of the VHH with the virus and increase its therapeutic potential. Firstly, we examined the pharmacokinetic properties of this VHH. Then, we assess the impact of half-life extension on the protective effect in mice. Finally, we compared the therapeutic potential of these HLE VHH to that of PEGylated VHH and human rabies immunoglobulins.

### 4.2.3 Materials and methods

#### 4.2.3.1 VHH and antibody

The Rab-E8/H7 VHH, as described above (4.1.3.1), was genetically fused with a VHH directed against mouse serum albumin to extend the half-life, resulting in HLE Rab-E8/H7-ALB11. The activity of this VHH was tested in the virus-neutralization assay to confirm that the fusion did not impact the rabies neutralization. In order to pegylate the Rab-E8/H7 VHH, constructs were generated whereby a GGC-sequence was added to the C-terminus. Constructs were expressed in *Pichia pastoris* and purified via mercapto-ethyl-pyridine (MEP HyperCel) mixed mode chromatography, followed by ion
exchange in the presence of 5 mM dithiothreitol (DTT). The VHH were thoroughly reduced and ligated to PEG40 and finally polished by size exclusion chromatography.

Human rabies immunoglobulins (HRIG) (Imogam®, Sanofi Pasteur SA, France) are gammaglobulins purified from plasma of vaccinated human donors. HRIG are used worldwide for post-exposure prophylaxis in humans at a dose of 20 IU/kg, preferably infiltrated at the site of virus entry, in combination with a series of 5 vaccine doses. Post-exposure prophylaxis with immunoglobulins is a medical urgency and is recommended for risk category 3 exposure, such as transdermal bite or scratch, a lick on broken skin or contamination of mucous membrane with saliva from a rabid animals or after close contact with a bat [35].

4.2.3.2 Rabies virus

Challenge Virus Standard (CVS)-11 is a virulent classical rabies virus obtained from the American Type Culture Collection (ATCC reference VR959) and was grown in baby hamster kidney (BHK)-21 cells (Deutsche Sammlung von Mikroorganismen un Zellkulturen GmbH, Braunschweig, Germany). For virus inoculation in mice, a dose of $10^{1.5}$ 50 % cell culture infectious doses (CCID50) of CVS-11 was used.

4.2.3.3 In vitro virus neutralization

The virus-neutralizing potency was titrated with the rapid fluorescent focus inhibition test (RFFIT) according to the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Office International des Epizooties, 2008). Briefly, a standard dose of virus was pre-incubated with serially diluted VHH/antibody for 90 minutes at 37 °C. BHK-21 cells were then added to the mix and co-incubated for 24 hours. Infected BHK-21 cells were stained with fluorescent anti-nucleocapsid antibody and foci of infected cells were counted under the fluorescent microscope. The dilution that yielded 50 % inhibition of infected foci was determined. The neutralizing potency is expressed in international units (IU)/ml in reference to “The Second International Standard for Anti-Rabies Immunoglobulin”, purchased from the United Kingdom National Institute for Biological Standards and Control.

4.2.3.4 Rabies ELISA

The Platelia TM Rabies II kit from Bio-Rad (cat n° 3551180) was used for assessing the functional activity of different VHH constructs. A dilution series (in R6 buffer provided with the kit) of the anti-rabies VHH was incubated on the wells precoated with rabies virus glycoprotein for 1 hour at 37 °C. After washing, bound VHH was incubated for 1 hour at 37 °C with anti-VHH antibodies (in-house produced, R345 Rabbit anti-VHH polyclonal antibodies, 1/2,500) followed by an incubation with an anti-rabbit-HRP antibody (BETHYL, Cat A120-201P, 1/1,000) for 1 hour at 37 °C. TMB (es(Hs)TMB,
Pierce) substrate was added and incubated in the dark at room temperature, the reaction was stopped after 30 minutes by addition of 1 M HCl. Read-out was done at 450-620 nm.

4.2.3.5 Mouse inoculation experiments

Six-to-eight weeks old female Swiss outbred mice (Charles River, France) were used for all experimental procedures, approved by the local ethical committee of the institute (advice number 070515-05). Mice were kept in filter top cages, water and feed provided ad libitum and exposed to a natural day/night light cycle. The intracranial (IC) and intranasal (IN) inoculation procedures are described in detail by Rosseels et al. [22]. For IC, IN and intraperitoneal (IP) injection volumes of respectively 20, 25 and 1,000 µl were used.

Prior to administration of the virus or VHH, mice were briefly anesthetized using isoflurane gas (IsoFlo, Abbott laboratories Ltd., United Kingdom).

4.2.3.6 Pharmacokinetic study

The plasma and brain disposition upon systemic administration of the Rab-E8/H7 VHH and its albumin-binding counterpart was investigated in a pharmacokinetic study. To this end, 48 mice were treated with a single intraperitoneal injection of 10 mg Rab-E8/H7 VHH or 5 mg HLE Rab-E8/H7-ALB11. Three mice were sacrificed per sampling point. Immediately before euthanasia, each mouse received a transcardial perfusion with phosphate-buffered saline (PBS) [30]. Briefly, mice were injected intraperitoneally with a mixture of xylazine (9.9 mg/kg, Rompun 2 %, Bayer Healthcare, Germany) and ketamine (100 mg/kg, Ceva, Belgium) to induce deep anaesthesia. Upon the disappearance of the eye lid and motor reflexes, the thorax was opened to expose the heart. An incision was made in the right heart chamber to drain out the blood from circulatory system. Twenty ml of PBS (37 °C) was injected directly into the left ventricle of the heart at a steady perfusion rate of 10 ml/min. After perfusion, both brain halves were collected and snap frozen in dry ice.

One brain half was homogenized in ice cold PBS supplemented with 1 mM phenylmethanesulfonylfluoride (PMSF) and protease inhibitor cocktail (VWR international) using 5 mm stainless steel beads in a tissue homogenizer (Bullet Blender, Next Advance, New York, USA). For homogenization of the brain samples a fixed volume of 1.25 ml was used (approximately a 1:10 ratio of tissue: lysis buffer). Homogenates were subsequently centrifuged for 20 minutes at 13,000 g and supernatant was transferred to a new tube and stored at −80 °C until quantification. The total amount of protein present in the homogenates was determined using the Bradford method. For the calibration curve a dilution series of BSA was used ranging from 1.0 to 0.063 mg/ml. 20 µl of standard and samples (1/30 dilution) was mixed with 300 µl Bradford Ultra Reagent (Expedeon, CatBFU1L). Absorbance at 595 nm was measured and concentration of samples was interpolated from the standard curve.
For ELISA analysis, total brain amounts were calculated and were normalized for a theoretical brain weight of 0.5 g (source www.mbl.org/atlas170) per mouse brain. A density of 1 g/ml was assumed to calculate VHH brain concentration [31]. Undiluted brain lysate or plasma samples that resulted in signals below the assay’s limit of quantification (0.1 ng/ml) were considered as missing. Pharmacokinetic parameters were estimated by non-compartmental analysis (Plasma Model Type 200, sparse sampling) using WinNonLin software version 6.3 (Phoenix Pharsight, Mountain View, CA).

The average maximum concentration in plasma ($C_{\text{max}}$) and corresponding mean time ($t_{\text{max}}$) were directly derived from the plasma concentration-time profiles. The area under the plasma concentration-time curve from the time of dosing to the time of the last measurable concentration ($\text{AUC}(0-t)$) was calculated by the linear trapezoidal rule and extrapolated to infinity ($\text{AUC}_{\text{infty}}$) as $\text{AUC}(0-t)+C/t/z$, in which $\lambda z$, the first order rate constant associated with the terminal elimination phase, was estimated by linear regression of time versus log concentration. The half-life ($t_{1/2}$) of the terminal elimination phase was calculated as $\ln(2)/\lambda z$.

### 4.2.3.7 Quantification of anti-rabies VHH in plasma and brain homogenates

To quantify the amount of anti-rabies VHH in the homogenized brain tissues and plasma, the samples were tested in a virus neutralization assay (RFFIT) or in an ELISA using the Platelia TM Rabies II Kit from Bio-Rad (both as described above). For the standard curve, a serial 1.7 dilution series of the anti-rabies VHH ranging from 250 to 0.05 ng/ml in 20 % brain matrix was used. Brain samples were measured at 1/5 dilution, plasma samples at dilutions ranging from 1/25 to 1/900, all in duplicate. Samples (50 µl/well) were transferred to the plate provided by the kit. The linear range of the standard curve was determined using 4PL analysis (GraphPad Prism), which ranged from 0.1 to 10 ng/ml. The assay’s range and accuracy was confirmed with spiked VHH controls of 1, 2 and 5 ng/ml. Concentrations of unknown samples, the reported concentration was derived from averaging the values of the different dilutions.

### 4.2.3.8 Clinical follow-up

Mice were observed daily for signs of disease until 35 days post virus inoculation. Signs of disease were evaluated as follows: weight loss (0 = absent, 1 = visible), depression (0 = absent, 1 = lower (re)activity), hunched back (0 = absent, 1 = present), motoric incoordination (0 = absent, 1 = present), rough hair coat (0 = absent, 1 = present), paralysis of the hind legs (0 = absent, 1 = present) and conjunctivitis (0 = absent, 1 = present). The cumulative daily clinical score per mouse was calculated as the sum of the scores for each parameter. Disease progression was represented by plotting the cumulative daily score in function of the days post inoculation (DPI). The cumulative daily score per mouse ranged from 0 (no disease) to 7 (severe nervous disease). In our experience,
mice with a disease score of 6 or more die within one day [22]. Therefore, mice were euthanized by cervical dislocation when they reached a disease score of ≥ 6. Results were expressed as Kaplan-Meier survival curves. GraphPad Prism was used for statistical analyses of in vivo data. Differences in survival times were tested using the Log-Rank test with a Bonferroni post-test, differences in ΔCt values were tested using a student t-test after normalization to the house-keeping gene.

**4.2.3.9 Statistical analysis**

GraphPad Prism was used for statistical analyses of in vivo data. Differences in survival times were tested using the Log-Rank test with a Bonferroni post-test, difference in ΔCt values were tested using a student t-test after normalization to a house-keeping gene.

**4.2.4 Results**

**4.2.4.1 Pharmacokinetic characteristics of anti-rabies VHH**

The short half-life of VHH possibly limits the therapeutic possibilities of these antibody fragments. In this study, the half-life of Rab-E8/H7 was increased by the addition of a third VHH directed to serum albumin. To determine the extent of half-life extension (HLE) by addition of this VHH, a pharmacokinetic experiment was designed to determine the plasma and brain exposure following a single intraperitoneal (IP) administration of Rab-E8/H7. In this experiment, Rab-E8/H7 was compared with a trivalent form of this VHH containing and extra anti-albumin VHH. Addition of this VHH is supposed to increase the half-life in circulation by binding to serum albumin. Both VHH were administered in different doses (5 mg Rab-E8/H7-ALB11 and 10 mg Rab-E8/H7). Serum versus time concentration profiles displayed a monophasic pharmacokinetic profile for both VHH constructs, but with an obvious rapid decline for Rab-E8/H7, which is likely explained by the rapid renal filtration (Figure 4.2.1). Table 4.2.1 shows an overview of the mean calculated pharmacokinetic parameter estimates. Mean serum levels peaked at 0.08 h for Rab-E8/H7 and at 4 h for Rab-E8/H7-ALB11, confirming the half-life extension (HLE) by addition of an anti-albumin VHH. We therefore refer to this VHH as HLE Rab-E8/H7. Maximum average brain levels were attained respectively at 0.5 h and 8 h after dose administration, revealing a fairly rapid influx into the brain, which is indicative of a fast equilibrium between the blood and the brain. Due to the albumin-binding capacity of HLE Rab-E8/H7, a substantially higher systemic exposure (approximately hundred-fold upon dose normalization) was attained for the half-life extended VHH. Similarly to what was seen in the blood, the brain was exposed to markedly higher VHH concentrations after dosing with the HLE Rab-E8/H7. Elimination of the VHH from the brain followed the same exponential disposition as in serum with no apparent accumulation in the brain. This was observed for both VHH constructs although slopes
were steeper for the Rab-E8/H7 because of higher clearance rates. When differences in average systemic exposure were accounted for, both VHH constructs displayed a similar mean "area under curve (AUC)"-base brain/serum ratio of approximately 0.1 %, despite their differences in size and clearance rates. Also, mean brain/serum concentration ratios stayed fairly constant over time (Figure 4.2.2).

Figure 4.2.1: Mean brain and serum concentration of Rab-E8/H7 and HLE Rab-E8/H7. Individual brain (circles) and serum (triangles) concentrations and mean values (lines) of Rab-E8/H7 (A) or HLE Rab-E8/H7 (B) upon intraperitoneal injection of 5 mg HLE Rab-E8/H7 or 10 mg Rab-E8/H7.

Figure 4.2.2: Mean brain/serum concentration ratio. Mean brain/serum concentration ratio over time for HLE Rab-E8/H7 and Rab-E8/H7 upon intraperitoneal injection of 5 mg HLE Rab-E8/H7 or 10 mg Rab-E8/H7 in mice.
Table 4.2.1: Overview of average pharmacokinetic parameter values. Values were estimated by non-compartmental analysis (sparse sampling, WinNonLin version 6.3, Phoenix Pharsight) for Rab-E8/H7 and HLE Rab-E8/H7 VHH in the serum and brain tissue. Brain concentrations were normalized for a theoretical brain weight of 0.5 g per animal and a density of 1 g/ml was assumed.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Serum</th>
<th>Brain tissue</th>
<th>Serum</th>
<th>Brain tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax (µg/mL)</td>
<td>162</td>
<td>0.23</td>
<td>539</td>
<td>0.241</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>0.08</td>
<td>0.50</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>1.16</td>
<td>2.06</td>
<td>30.5</td>
<td>41.7</td>
</tr>
<tr>
<td>AUC(_\text{infinity}) (h*µg/mL)</td>
<td>493</td>
<td>0.58</td>
<td>24,613</td>
<td>26</td>
</tr>
<tr>
<td>AUC(_\text{infinity}/DOSE) (h*µg/mL/µg)</td>
<td>0.0493</td>
<td>0.000058</td>
<td>4.93</td>
<td>0.0052</td>
</tr>
</tbody>
</table>

4.2.4.2 Neutralizing potency of different half-life extended anti-rabies VHH in vitro

Virus-neutralization of different half-life extended and the non-half-life extended Rab-E8/H7 was compared in cell culture (Table 4.2.2). The in vitro neutralization experiments showed a lowered neutralization of the HLE Rab-E8/H7 compared to the non-HLE Rab-E8/H7.

Table 4.2.2: Comparison of the neutralizing potency of different HLE anti-rabies VHH constructs and non-HLE VHH in vitro.

<table>
<thead>
<tr>
<th>In vitro virus-neutralization in BHK-21 cells</th>
<th>IU/µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate-buffered saline</td>
<td>&lt; 0.5</td>
</tr>
<tr>
<td>Non half-life extended Rab-E8/H7</td>
<td>230,000</td>
</tr>
<tr>
<td>Half-life extended Rab-E8/H7-PEG40</td>
<td>102,720</td>
</tr>
<tr>
<td>Half-life extended Rab-E8/H7-ALB11</td>
<td>54,388</td>
</tr>
</tbody>
</table>

4.2.4.3 Assessment of the effect of half-life extension by addition of an anti-albumin VHH to Rab-E8/H7 on rabies virus neutralization in vivo

To assess the efficacy of the HLE Rab-E8/H7 (t1/2 = 30.5 h), mice were treated by intraperitoneal injection with an equimolar dose (15 mg, 25,050 IU) or lower doses (5 mg, 8,350 IU and 1.5 mg, 2,505 IU) of HLE Rab-E8/H7 at 24 hours after virus inoculation (Table 4.2.3, Figure 4.2.3). Half-life extension of Rab-E8/H7 strongly enhanced the protective effect against rabies virus-induced mortality. In comparison to a one-day survival benefit seen with systemically delivered Rab-E8/H7, the introduction of half-life extension into the Rab-E8/H7 VHH improved the median survival time by
several weeks and resulted in a high percentage of animals being completely protected from lethal rabies challenge \((p<0.001)\) for all doses. We also observed a dose-dependence on overall survival \((5/7, 4/7 \text{ and } 3/7 \text{ mice surviving, respectively for 15 mg, 5 mg and 1.5 mg})\) and an increased median survival time \((20 \text{ days, } 26 \text{ days and } > 26 \text{ days, respectively})\).

Table 4.2.3: Set-up of post-exposure treatment experiments with half-life extended VHH one day after virus inoculation.

<table>
<thead>
<tr>
<th>Group</th>
<th>Intervention at day...</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
</tr>
<tr>
<td><strong>10 mg Rab-E8/H7 (n=7)</strong></td>
<td>Virus challenge</td>
</tr>
<tr>
<td><strong>15 mg HLE Rab-E8/H7-ALB11 (n=7)</strong></td>
<td>Virus challenge</td>
</tr>
<tr>
<td><strong>5 mg HLE Rab-E8/H7-ALB11 (n=7)</strong></td>
<td>Virus challenge</td>
</tr>
<tr>
<td><strong>1.5 mg HLE Rab-E8/H7-ALB11 (n=7)</strong></td>
<td>Virus challenge</td>
</tr>
<tr>
<td><strong>10 mg irrelevant VHH (n=7)</strong></td>
<td>Virus challenge</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
</tr>
<tr>
<td><strong>5 mg HLE Rab-E8/H7-ALB11 (n=7)</strong></td>
<td>Virus challenge</td>
</tr>
<tr>
<td><strong>1.5 mg HLE Rab-E8/H7-ALB11 (n=7)</strong></td>
<td>Virus challenge</td>
</tr>
<tr>
<td><strong>0.5 mg HLE Rab-E8/H7-ALB11 (n=7)</strong></td>
<td>Virus challenge</td>
</tr>
<tr>
<td><strong>0.15 mg HLE Rab-E8/H7-ALB11 (n=7)</strong></td>
<td>Virus challenge</td>
</tr>
<tr>
<td><strong>5 mg irrelevant HLE VHH (n=7)</strong></td>
<td>Virus challenge</td>
</tr>
</tbody>
</table>
Development and validation of anti-rabies VHH

Figure 4.2.3: Post-exposure treatment with anti-rabies Rab-E8/H7 with or without half-life extension (HLE). Half-life extension was accomplished by adding a third anti-albumin VHH to Rab-E8/H7. Mice were treated intraperitoneally 24 hours after intranasal virus inoculation. The clinical effect of Rab-E8/H7 was significantly improved by the half-life extension. The median survival time was prolonged by 6 to more than 26 days (p<0.01), depending on the dose. More than 70% of the mice were completely protected against disease upon treatment with 15 mg HLE Rab-E8/H7.

In a further experiment, the minimal effective dose of HLE Rab-E8/H7 to obtain protection against virus-induced mortality was determined. Mice were treated with IP doses ranging from 0.15 to 5 mg HLE Rab-E8/H7 24 hours after IN virus inoculation (Figure 4.2.4). The median survival time following administration of an irrelevant VHH was 8 days. Treatment with 0.15 mg of HLE Rab-E8/H7 gave no significant delay of the median survival time compared to mock-treated controls (9 days). A protective effect was observed starting from a dose of 0.5 mg HLE Rab-E8/H7 (p<0.05). In the case of 5 mg of HLE Rab-E8/H7, some mice had longer incubation periods up to 28 days (instead of 9 days). These mice also showed slower progression to severe nervous disease once first symptoms had set in. In contrast to the usual development of severe nervous disease within 24 hours after the appearance of first symptoms, mice with longer incubation periods developed severe nervous disease 36 hours after the first symptoms appeared.

Based on the pharmacokinetic studies, the enhanced efficacy and potency of the HLE Rab-E8/H7 can likely be explained by higher brain exposure secondary to prolonged plasma exposure, rather than to enhanced brain uptake.
Figure 4.2.4: Survival curves of mice treated with irrelevant or HLE Rab-E8/H7 VHH containing ALB11 VHH. The median survival times of mice treated with HLE Rab-E8/H7 were always significantly longer than the one of the irrelevant ALB11 control except for those treated with 0.15 mg Rab-E8/H7-ALB11 VHH. At 31 DPI the last mouse of the group treated with 5 mg E8-H7-ALB11 VHH died.

4.2.4.4 Comparison with PEGylation for half-life extension and human rabies immunoglobulins

In order to benchmark the efficacy seen with the HLE Rab-E8/H7 VHH, similar experiments were conducted using a PEGylated Rab-E8/H7 and commercially available human rabies immunoglobulins (HRIG) purified from plasma of vaccinated human donors (Imogam, Sanofi Pasteur SA, Lyon, France). Table 4.2.4 summarizes the set-up of the different experiments.

Addition of PEG40 to Rab-E8/H7 would theoretically increase the half-life to a similar extent as addition of the anti-albumin VHH. PEGylated Rab-E8/H7 and HLE Rab-E8/H7 were therefore administered intraperitoneally 24 hours after intranasal virus inoculation in equimolar doses. Although the half-life of both constructs was theoretically similar, HLE Rab-E8/H7 was more effective in postponing clinical disease and survival time (Figure 4.2.5).
Table 4.2.4: Set-up of post-exposure treatment experiments with PEGylated VHH or human rabies immunoglobulins (HRIG) one day after virus inoculation.

<table>
<thead>
<tr>
<th>Group</th>
<th>Intervention at day...</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>Experiment 1</td>
<td></td>
</tr>
<tr>
<td>4 mg HLE Rab-E8/H7-ALB11 (n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>2.6 mg Rab-E8/H7 PEG40 (n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>2 mg irrelevant VHH(n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
</tr>
<tr>
<td>65 mg HRIG (n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>2 mg irrelevant VHH(n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>Experiment 3</td>
<td></td>
</tr>
<tr>
<td>65 mg HRIG (n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>2 mg irrelevant VHH(n=7)</td>
<td>Virus challenge</td>
</tr>
</tbody>
</table>

Figure 4.2.5: Comparison of the effect of equimolar doses of PEGylated Rab-E8/H7 or HLE Rab-E8/H7 on the survival of rabies infected mice. Addition of PEG40 to the Rab-E8/H7 resulted in a similar half-life extension as addition of an anti-albumin VHH, however survival was not prolonged to a similar extend when Rab-E8/H7-PEG40 was administered (Log-Rank test, Bonferroni post-test).

In contrast to HLE Rab-E8/H7, human anti-rabies immunoglobulins have an even longer theoretical plasma half-life in mice (up to 8 days) [38]. Intraperitoneal treatment with the maximal feasible dose of HRIG (1 ml intraperitoneally, 65 mg (111 IU)/mouse) was able to prolong the median survival time by two days (p<0.05) (Figure 4.2.6). All mice still developed serious neurological disease. The used dose of immunoglobulins was 308 times higher than the prescribed dose for
humans (5,550 IU/kg compared to 20 IU/kg) and represented the highest feasible dose which could be administered to mice, respecting a maximum IP injection volume of 1 ml.

Figure 4.2.6: Post-exposure treatment with human anti-rabies immunoglobulins (Imogam®). Mice were treated intraperitoneally with 65 mg (111 IU, 1 ml) of human rabies immunoglobulins at 24 hours after intranasal virus inoculation in two independent experiments (A and B). The median survival time was prolonged by 2 days, but all mice developed serious nervous disease, requiring euthanasia.

4.2.4.5 Multiple dosing of HLE Rab-E8/H7

Based on previously obtained results, we reasoned that protection could possibly be obtained when plasma levels of HLE Rab-E8/H7 were higher for a longer period of time, resulting in a longer exposure of the brain to HLE Rab-E8/H7. In order to achieve this, mice received multiple doses of HLE Rab-E8/H7 at three different time points after virus inoculation: 5 mg at 24 hours, at 4 DPI and at 7 DPI. These time points were chosen based on the pharmacokinetic models to guarantee continuous exposure to neutralizing VHH levels in the brain (>0.1 µg/ml) for at least 10 days.
However, despite the multiple dosing, nor disease onset, nor survival was prolonged as compared to a single dose of 5 mg HLE Rab-E8/H7 at 24 hours after virus inoculation. (Figure 4.2.7; see above: Figure 4.2.4).

![Graph showing percent survival vs DPI](image)

Figure 4.2.7: Post-exposure treatment with 3 doses of 5 mg HLE Rab-E8/H7 at 24 hour, 4 days and 7 days after virus inoculation.

### 4.2.5 Discussion

Although the small size of VHH is advantageous for some purposes, a disadvantage is that it implies a relatively short half-life in vivo. The molecular weight of a single VHH domain is only 15 kDa and is thus below the threshold for renal filtration. Therefore, VHH are rapidly cleared from the blood. They can however easily be coupled with another VHH that can bind serum albumin or IgG, thus increasing the half-life to an extent similar to that of the target proteins [32,33].

Here, we investigated the impact of half-life extension on the antiviral effect of anti-rabies VHH. First, we investigated the role of addition of an anti-serum albumin VHH. This significantly prolonged survival, with a dose-dependent effect. To further investigate this effect, we replaced the anti-albumin VHH by PEGylation, which is supposed to increase the circulatory half-life of small proteins by addition of flexible, hydrophilic molecules of polyethylene glycol [28]. Both techniques can increase the hydrodynamic volume of a VHH in circulation, resulting in a prolonged half-life. In addition, albumin is also recycled by the neonatal Fc receptor (FcRn) [27]. In our experiments we observed that despite a similar theoretical half-life of PEGylated VHH and HLE Rab-E8/H7, results differed. PEGylated VHH only slightly prolonged the survival in mice, but not as long as an equimolar dose of anti-albumin VHH and was unable to rescue any mice from disease. Likely, the voluminous PEG group might hinder the penetration of the PEGylated VHH into the brain, with other words, it might pass less efficiently the blood-brain barrier than the HLE Rab-E8/H7. This might explain the
superior effect of VHH in which half-life was extended by anti-albumin binding as compared to those that were PEGylated.

We also compared the effect of human rabies immunoglobulins, with that of the HLE Rab-E8/H7. We tested commercially available human anti-rabies immunoglobulins (HRIG) purified from plasma from vaccinated donors (Imogam®). Despite the administration of the highest feasible dose, which is 308 times higher than the prescribed dose for exposed humans, the median survival time was only prolonged by two days and HRIG was unable to rescue any of the mice. It is likely that the more effective treatment with HLE Rab-E8/H7 is due to the higher neutralizing dose achieved with HLE Rab-E8/H7 as compared to that obtained with Imogam®. But it should be noted that the half-life of HLE Rab-E8/H7 is still significantly shorter than that of human immunoglobulins (30.5 h versus 8 days respectively) [34]. In contrast to VHH, which lack the Fc part, antibodies have species-specific characteristics. Interaction with Fc receptors does not only increase the half-life of antibodies in circulation, but also enables specific effector functions [15].

Our pharmacokinetic data on systemic administration support that the therapeutic benefit depends on the time of brain exposure and the plasma half-life of the used VHH construct. Indeed, half-life extension (HLE) of Rab-E8/H7, by adding a third VHH targeting serum albumin, considerably prolonged the VHH formulation half-life in circulation and improved the therapeutic effect. Systemic administration of HLE Rab-E8/H7 resulted in a prolonged survival of at least six days and complete protection from disease in part of the mice (43 - 71 %) in a dose-dependent manner (Figure 4.2.3). Upon intraperitoneal administration, the brain levels of Rab-E8/H7 peaked 0.5 h after injection and Rab-E8/H7 was almost completely cleared from the brain within one day, whereas HLE Rab-E8/H7 peaked 8 h after injection and remained clearly detectable for ten days. This may explain why the delay in disease onset with Rab-E8/H7 was only one day, but was six days with HLE Rab-E8/H7.

The enhanced exposure in the brain of HLE Rab-E8/H7 seems mainly due to the prolonged retention in the systemic circulation. We found no evidence for active blood-brain barrier crossing with the tested (HLE) anti-rabies VHH, as the brain levels followed the systemic exposure. The brain levels of (HLE) VHH represented 0.1% of the plasma levels, suggesting a limited diffusion of the anti-rabies VHH to the brain, corresponding with distribution data also described for antibodies [35,36]. It has been reported that certain VHH, directed against glial fibrillary acidic protein, that contain many positively charged residues, show enhanced crossing of the blood-brain barrier, presumably via an adsorption-mediated uptake mechanism [37]. In this respect, it should be noted that the VHH building blocks of Rab-E8/H7 each have isoelectric point (pl) values around 7 and hence are neutrally charged. Our pharmacokinetic results do not indicate that the rabies VHH constructs used in our study can cross the blood-brain barrier to a higher extent than reported for antibodies, despite their
smaller size. Modification of VHH by adding blood-brain barrier targeting peptides might be an interesting future strategy to increase their therapeutic potential [38].

Despite the pharmacokinetic data obtained, multiple dosing of the HLE Rab-E8/H7 failed to protect mice from lethal challenge and was not significantly superior to a single dose at one day after infection. This stresses the importance of a high bolus early in infection.


This chapter groups the research results addressing the fourth objective of this thesis: to examine whether linking VHH to neurotropic peptides or proteins increases the capacity of VHH to protect from the disease.
5.1 Abstract

The central nervous system is one of the few so-called immune privileged sites of the body. Since the brain is encased in a non-elastic skull, swelling associated with an inflammatory reaction can have potentially deleterious effects on its function. The blood-brain barrier protects the delicate brain tissue from potentially harmful immune reactions. Unfortunately, pathogens, like the rabies virus, can take advantage of this, resulting in an infection that is difficult to control. Some pathogens have developed mechanisms to by-pass the blood-brain barrier. Proteins or peptides derived from these pathogens might serve as tools to deliver molecules to the central nervous system.

Previously, we showed that anti-rabies VHH were able to protect mice from lethal infection when VHH were administered intracerebrally soon after infection. We hypothesized that VHH might become more effective if they are linked to neurotropic peptides/proteins that better target them to the brain. We therefore selected a number of neurotropic proteins/peptides (RVG, tet1 or tetanus anatoxin) and linked these, either by (strept)avidin-biotin linkage or by genetic linkage, to anti-rabies Rab-E8/H7 VHH. First, we investigated the neutralizing potency and the potency to bind to neuronal cells in vitro. Secondly, the constructs were used to treat mice infected with rabies virus in a head-to-head comparison with control VHH. Linkage to the neurotropic peptides/proteins did not abolish the neutralizing potency of the VHH, albeit that the potency was somewhat lower compared to ‘naked’ VHH. Immunofluorescence microscopy demonstrated clear binding to or uptake in neuroblastoma cells of RVG, tet1 or tetanus anatoxin-linked VHH, which was not observed with control VHH. Intranasal administration of RVG-linked horse radish peroxidase also led to quick penetration of the olfactory bulbs, but unexpectedly, the same was also true for unlinked horse radish peroxidase. Large proteins can apparently easily migrate from the nose into the olfactory bulbs. In mice, treatment with VHH-neurotropic peptide/protein constructs did not result in a superior protection compared to treatment with ‘naked’ VHH. It remains unclear why RVG/tet1/anatoxin-linkage did not improve efficacy of VHH in vivo, compared to unlinked VHH.

We can conclude that the addition of neurotropic peptides/proteins to anti-rabies VHH did not have an added value in our model at the tested doses. It is possible that higher doses of the neurotropic constructs are needed to achieve better antiviral activity in the brain.

5.2 Introduction

The central nervous system (CNS) is considered to be an immune privileged site, implying that there is a poor stimulation of the immune response and systemic immune effectors have difficulties to reach the brain. The blood-brain barrier (BBB) plays a major role in this immune ‘isolation’ of the CNS [1]. The BBB is a functional barrier between the brain interstitial fluid and the blood. The BBB is not an absolute barrier, but the endothelial cells making up the BBB are so tightly joined to one
another that it is almost impermeable for large hydrophilic molecules. In addition, CNS endothelial cells lack a number of transport pathways and express high levels of active efflux transport proteins. This prevents the entry of the majority of blood proteins to the CNS, apart from some small molecules with appropriate lipophilicity, molecular weight (< 500 Da) and charge, which are able to pass the BBB. The only way for larger molecules to reach the brain from the blood is by interaction with specific receptors and specific transporters on the BBB (Figure 5.1) [2].

![Diagram of the blood-brain barrier](image)

**Figure 5.1:** Schematic representation of the blood-brain barrier (A) and potential routes for transport across the blood-brain barrier (B). (A) Tight junctions between the endothelial cells make the blood vessels impermeable, shielding the brain from molecules in the blood. (B) A number of pathways exist that allow transport across the blood-brain barrier. Small hydrophilic molecules can cross between cells, called the paracellular pathway, whereas small lipophilic molecules can pass the blood-brain barrier by passively diffusing through the cell membrane (transcellular lipophilic pathway). Larger molecules must use either receptor-mediated transcytosis by interaction with specific receptors or modulate tight junctions. Adapted from Vidu et al.[3].

Another approach to reach the brain is by entering peripheral neuronal projections and travelling through the neuronal network. Indeed, neuronal projections functionally connect peripheral tissues and organs with the brain. A wide range of pathogens and toxins use receptors present at these nerve terminals to enter the neurons and be transported throughout the network. Different pathogens use different strategies to enter the neuronal network, therefore peripheral
virus entry is not limited to sensory and motor neurons in skin or muscle, but also the olfactory nerves can serve as a gateway [4,5]. Following their entry, a number of pathogens and toxins use the axonal transport system to move within the neuron, either towards the cell body (retrograde) or towards the synapses (anterograde). Once within the neurons they can use different mechanisms to spread from one cell to another [4,5]. These characteristics make neurotropic pathogens and toxins an attractive tool to target therapeutics to the brain. Indeed, a number of peptides and proteins derived from neurotropic pathogens have been used for directing RNA, peptides and even proteins to the CNS [6–13].

The rabies virus G protein is a major determinant for pathogenicity of the virus, since it is the structure responsible for receptor binding on neuronal cells [14]. Lentz et al. discovered that a very specific region of the G protein is responsible for receptor binding. This 29 amino acid long peptide from the G protein competed effectively with the binding of the rabies virus to immobilized acetylcholine receptor, indicating its importance in receptor binding. Although it cannot be excluded that the intact glycoprotein also binds to other receptors on neuronal cells [15,16]. This peptide, referred to as RVG29, was shown to specifically bind to neuronal cells and deliver small interfering RNA (siRNA) to these cells both in vitro as in vivo [6]. Since its discovery, the peptide has been used for the targeted delivery of siRNA or plasmid DNA to the brain, for example for the treatment of Japanese encephalitis virus in mice using antiviral siRNA linked to the peptide. Although, this treatment was able to prevent lethal disease in mice, other groups were unable to show an accumulation in CNS of RNA or DNA attached to nanoparticles labelled with the peptide, despite the successful delivery to the brain [6–11,17]. Rohn et al. and Fu et al. respectively described the successful delivery of cdk4 siRNA in the brain, resulting in a reduction of cdk4 protein production and the therapeutic delivery of brain-derived neurotropic factor (BDNF) for the treatment of stroke [11,17]. Liu et al. (2009) were the first to determine the uptake pathway. RVG29 labelled nanoparticles are taken up by brain capillary endothelial cells in vitro. This uptake is temperature-dependent and occurs via a clathrin- and caveolae-mediated endocytotic pathway, possibly initiated by binding to the GABA(B) receptor [18].

Clostridium neurotoxins (CNT), like the botulinum (BoNT) and tetanus neurotoxin (TeNT), are examples of toxins targeting the CNS which can also be used as a carrier for neuronal transport. CNT are synthetized as single-chain polypeptides and are subsequently cleaved into a light (LC) and a heavy chain (HC), which are linked with a disulphide bond. Whereas the light chain is the enzymatic domain responsible for the devastating effects of the illness, the heavy chain consists of four functional domains: one of the four is responsible for binding to susceptible cells and a second domain that creates pores in the endocytic vesicles allowing the translocation of the light chain (Figure 5.2) [19]. Despite the fact that they have similar targets and entry sites, TeNT and BoNT cause
different clinical diseases. BoNT remain in the periphery, blocking neurotransmitter release at the neuromuscular junctions. TeNT enters neuronal cells via clathrin-dependent endocytosis upon binding and uses the axonal retrograde transport pathway in endocytic vesicles to reach the soma of the motor neurons located in the spinal cord, where it blocks neuroexocytosis [12,20]. The localised effect of BoNT allows its use as a therapeutic agent. Its paralysing effects are used to induce localized paralysis of muscle groups to relieve conditions like strabismus and hemifacial spasms, but also a number of disorders that do not have a neuromuscular basis like the treatment of hyperhidrosis and migraines [19]. The best known commercial products containing BoNT is Botox. TeNT is currently not used for therapeutic applications in humans, despite the positive results in laboratory animals. Devoid of the light chain, TeNT is no longer toxic and more specifically the 50 kDa C-terminal part of the heavy chain (HCC) has a natural capacity to travel via retrograde transport [21]. Proteins as large as 150 kDa have been shown to be successfully internalized and transported when linked to HCC. Evidently, only the non-toxic heavy chain can be used or more specifically the C-terminal part of the heavy chain (HCC) which is responsible for binding to motor neurons and retrograde axonal transport [12].

Figure 5.2: Schematic and crystallographic overview of the clostridial toxins. The red ribbon represents the 50 kDa light chain (LC) linked by a disulphide bond to the heavy chain (HC). The heavy chain can be subdivided into four domains: a belt domain, a translocation domain (Hm, green) and the binding domain (Ht) consisting of a N-terminal (Htn, light blue) and a C-terminal part (Hcc, dark blue), which is responsible for binding to the gangliosides. From Binz et al. [22].

In 2005, Liu et al. used phage display to identify a peptide with the same binding characteristics as the tetanus toxin. They used a random 12 amino acid peptide phage library in different biopanning rounds against trisialogangliosides, the known receptor of the tetanus toxin. Throughout the different rounds of panning, more stringent elutions were performed, increasing the chances to isolate strong binders. After four rounds of panning they isolated 42 phage clones, which after sequencing could be grouped into four different peptide sequences. The majority of them contained the sequence hereafter referred to as tet1. Competition assays with the tetanus toxin confirmed that both tet1 and the tetanus toxin bind to the same site on the gangliosides, despite the absence
of sequence homology with the tetanus toxin [23]. Rabies virus has also been shown to bind to gangliosides [24]. Tet1 was shown to bind to neuronal cell lines in vitro [23].

Since its discovery, tet1 has been used for a number of in vitro and in vivo applications. The group of Pun showed that polyplexes containing tet1 and DNA plasmid could successfully deliver DNA to neuronal cells in vitro and in vivo in cells of the subventricular zone after direct intraventricular injection [13, 25]. These results showed that tet1 can be used for CNS delivery. Previously we showed that anti-rabies VHH can efficiently neutralize the rabies virus both in vitro as in vivo. However, possibly the effectiveness of systemic VHH is limited because of the isolation of the CNS from the circulation. We attempted to increase the potency of anti-rabies VHH by addition of neurotropic peptides, which may facilitate entry and retention in the CNS.

First, a selection of neurotropic proteins and peptides was made based on a number of criteria like availability to the laboratory, possibility to cross the BBB and binding to neurons. The proteins/peptides mentioned here above all fulfilled a number of these criteria. Most importantly all proteins/peptides were easily available, either because of published sequences or because of a connection with the producing laboratories. RVG29 was shown to be able to selectively bind to neurons and travel within the neuronal network [6]. In addition, since it is part of the rabies virus glycoprotein, it most likely follows the same entry mechanism as the virus. The tetanus anatoxin and tet1 also both bind the neuronal cells, and although the anatoxin can travel within the neuronal network, this remains uncertain for tet1.

Secondly, we developed different strategies to link the selected proteins/peptides with anti-rabies VHH previously developed. Two main strategies were followed: on the one hand a (strept)avidin-biotin linker system and on the other hand genetic fusion using recombinant plasmid technology. The first strategy allows high versatility in the proteins and peptides which can be linked and uptake of these molecules can easily be measured by using horse radish peroxidase (HRP) or fluorescein isothiocyanate (FITC) labelled (strept)avidin. The disadvantage of this technique however is that the stoichiometry of these molecules is dependent on probability. On the other hand, genetic fusion allows constructs with a lower molecular weight and a fixed stoichiometry.

Thirdly, the functional characteristics (virus neutralizing capacity and capacity to bind to or enter neuronal cells) were tested using different assays. Virus neutralization was investigated using two different tests. The first assay, a seroneutralization assay (RFFIT), tests the ability of the construct to neutralize free virus in culture medium by mixing the virus and the construct prior to addition to susceptible cells. A second infection assay, allowed us to investigate whether cells become refractory to infection when the constructs are added to the cells, followed by washing and virus inoculation. In a third in vitro assay, we used fluorescently labelled VHH constructs to visualize
binding or entry of the constructs in cells. We also examined the possibility of (strept)avidin constructs to enter the brain upon intranasal administration.

Finally, we examined whether the use of these constructs, combing the anti-rabies activity of VHH with the documented neurotropism of a set of proteins/peptides, improves the protection of mice upon treatment, before or early after virus exposure, in a head-to-head comparison with naked VHH.

5.3 Material and methods

5.3.1 VHH

Rab-E8/H7 VHH were produced by the Nanobody Service Facility (NSF, VIB, Brussels, Belgium) from plasmids produced by Ablynx as described by Hultberg et al. [26].

VHH were biotinylated using EZ-Link-PEG₂-biotin (cat n° 21346, Thermo Scientific, Belgium) according to the manufacturer’s instructions. Briefly, a 100-fold molar excess of biotinylation reagent amine-PEG₂-biotin and a 10-fold molar excess of EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride, cat n° 22980, Thermo Scientific, Belgium) was mixed with VHH and incubated with gentle agitation for 2 hours at room temperature. After the incubation, the non-reacted biotinylation reagent and the EDC by-products were removed by desalting using Zeba Spin Desalting columns (cat n° 89891, Thermo Scientific, Belgium).

Genetically fused peptide constructs obtained by linking either tet1 or RVG29 to the C-terminal of the VHH, were obtained via the Nanobody Service Facility of the VIB (Belgium). Briefly, after codon optimization, sequences were cloned into *Escherichia coli* WK6. VHH expression was induced using IPTG (isopropyl β-D-1-thiogalactopyranoside). Histidine-tagged VHH were purified from the periplasmic extract using immobilized metal affinity columns (IMAC) and then by size exclusion chromatography using and AKTAXpress. Neutralizing activity of the genetically linked constructs was determined using RFFIT.

5.3.2 Neurotropic peptides, proteins and their linkers

RVG29 is a 29-amino acid (YTIWMPENPRPGTPCDIFTNSRGK) peptide that binds selectively to neuronal cells and was derived from the rabies virus glycoprotein. RVMAT (MNLLRKIVKNRRDEDTQKSSPASPLDDG) is a control peptide of the same length, derived from the rabies virus matrix protein, and is unable to selectively bind to neuronal cells [6]. Tet1 is a short 13 amino acid peptide (HLNLDLYLKYYR) that was selected via phage display and binds the same receptor as the tetanus toxin [23].

RVG29, tet1 and RVMAT peptides were synthesized by and purchased from Genecust (Luxemburg), all peptides contain a C-terminal biotin. All peptides were synthesized using solid-
phase synthesis. Briefly, the C-terminal amino acid is attached to an insoluble support via its carboxyl group and an excess of the second amino acid is introduced and allowed to be coupled to the first amino acid, excess reagents are removed by washing and this process is repeated until the desired peptide sequence is assembled. During the process the N-terminal part is bound to a protective group to avoid undesired reactions. Once the peptide is synthesized, the peptide is purified using HPLC (high-performance liquid chromatography) to remove peptides lacking one or more amino acids and to obtain a purity of over 95 %. Mass spectrometry analysis verifies the peptide composition and reversed phase HPLC confirms its purity.

Biotinylated tetanus anatoxin was kindly given to us by the service of Foodborne Pathogens of the Scientific Institute of Public Health (WIV-ISP, Belgium). Briefly, *Clostridium tetani* is cultured in Mueller and Miller medium and toxin production is obtained by reducing iron levels in the culture medium [27]. After 6 to 9 days, the toxin is purified by filtration and the toxicity is determined via mouse inoculations. The toxin is detoxified using formaldehyde and incubation at 40 °C for three weeks. The anatoxin (detoxified toxin) is purified using ultrafiltration and precipitation using ammonium sulphate. Absence of residual toxicity is confirmed by *in vivo* tests. Anatoxin was biotinylated as described above using EZ-Link-PEG₂-biotin (cat n° 21346, Thermo Scientific, Belgium) and successful biotinylation was confirmed using ELISA.

Linking of the biotinylated peptides/proteins with the VHH was performed using either avidin (cat n° 21128, Thermo Fisher Scientific, Belgium), horse radish peroxidase (HRP) conjugated avidin (cat n° 21125, Thermo Fisher Scientific, Belgium) or FITC conjugated streptavidin (Streptavidin Alexa fluor ® 488 conjugate, cat n° S-32354, Thermo Fisher Scientific, Belgium). Table 5.1 gives an overview of all the available doses of the differente proteins/peptides and genetically fused constructs.

<table>
<thead>
<tr>
<th>Peptide or VHH</th>
<th>Available quantity (mg)</th>
<th>Concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RVG29</td>
<td>15.1</td>
<td>5</td>
</tr>
<tr>
<td>tet1</td>
<td>15.2</td>
<td>5</td>
</tr>
<tr>
<td>RVMAT</td>
<td>15.2</td>
<td>5</td>
</tr>
<tr>
<td>Tetanus anatoxin</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>Rab-E8-H7-RVG</td>
<td>2</td>
<td>0.2</td>
</tr>
<tr>
<td>Rab-E8-H7-tet1</td>
<td>0.8</td>
<td>0.1</td>
</tr>
<tr>
<td>Rab-E8-H7-RVMAT</td>
<td>Production failed</td>
<td></td>
</tr>
</tbody>
</table>
5.3.4 Cells and coloration

For all in vitro cell binding/entry assays, mouse neuroblastoma N2a cells (ATCC CCL-131) were used. Cell culture medium consisted of Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10 % Fetal Calf Serum, antibiotics and antifungicides (100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin). All incubations were performed at 37 °C, 5 % CO₂ and cell fixations were performed with 100 % methanol for 15 minutes at – 20 °C. Nuclear staining was performed using DAPI (cat n° P-36931, Thermo Fisher Scientific, Belgium).

5.3.5 Microscopy and imaging

Cell cultures were observed and photographed using a fluorescent microscope (IX73, Olympus). This microscope has a 10x ocular and pictures were taken using either 40x or 60x phase contrast objectives and a CCD monochrome camera (XM10, Olympus).

5.3.6 Rabies virus

Challenge Virus Standard (CVS)-11 is a virulent classical rabies virus obtained from the American Type Culture Collection (ATCC reference VR959) and was grown in baby hamster kidney (BHK)-21 cells. For virus inoculation in mice, a dose of 10².⁵ 50 % cell culture infectious doses (CCID₅₀) of CVS-11 was used. For in vitro tests a viral dose of 100-150 TCID₅₀ was used.

5.3.7 Transcardial perfusion

Mice received transcardial perfusion with phosphate-buffered saline (PBS) to eliminate all blood from the brain [28]. Briefly, mice were injected intraperitoneally with a mixture of xylazine (9.9 mg/kg, Rompun 2 %, Bayer Healthcare, Germany) and ketamine (100 mg/kg, Ceva, Belgium) to induce deep terminal anaesthesia. Upon the disappearance of the eye lid and motor reflexes, the thorax was opened to expose the heart. An incision was made in the right heart chamber to drain out the blood from the circulatory system. At a steady rate of 10 ml/min, twenty ml of PBS of 37 °C was injected directly into the left ventricle of the heart. After perfusion, both brain halves were collected and snap frozen in dry ice.

5.3.8 Colorimetric assay to measure level of the HRP activity (brain uptake assay)

Mice were sacrificed and received transcardial perfusion to wash out the blood at different time points after intranasal administration of horse-radish peroxidase (HRP) labelled avidin to determine the entry of HRP-avidin and HRP-avidin-RVG in the brain. Brain tissue was collected and homogenized in 1 ml phosphate buffered saline (PBS) using 5 mm stainless steel beads in a tissue homogenizer (Bullet Blender, Next Advance, USA). Homogenates were subsequently centrifuged for 20 minutes at 2,500 rpm, 4 °C. Supernatant was collected and substrate (3,3’5,5’-Tetramethylbenzidine, TMB, cat n° 34028, Thermo Fisher Scientific, Belgium) was added at a 1:1
ratio. The mixture was centrifuged for 4 minutes at 1,500 rpm, room temperature. The colorimetric reaction was measured after 30 minutes at 620 nm.

5.3.9 RFFIT

The virus-neutralizing potency was titrated with the rapid fluorescent focus inhibition test (RFFIT) according to the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Office International des Epizooties, 2008). Briefly, a standard dose of virus was pre-incubated with serially diluted VHH/antibody for 90 min at 37 °C. BHK-21 cells were then added to the mix and co-incubated for 24 hours. Infected BHK-21 cells were stained with fluorescent anti-nucleocapsid antibody and foci of infected cells were counted under the fluorescence microscope. The dilution that yielded 50% inhibition of infected foci was determined. The neutralizing potency is expressed in international units (IU)/ml in reference to "The Second International Standard for Anti-Rabies Immunoglobulin", purchased from the United Kingdom National Institute for Biological Standards and Control.

5.3.10 Mouse inoculations

Six-to-eight weeks old female Swiss outbred mice (Charles River, France) were used for all experimental procedures, approved by the local ethical committee of the institute (advice number 070515-05). Mice were kept in filter top cages, water and feed provided ad libitum, and exposed to a natural day/night light cycle. The intranasal (IN) and intracerebral (IC) inoculation procedures are described in detail by Rosseels et al. [29]. For intracerebral (IC), intranasal (IN) and intraperitoneal (IP) injections volumes of respectively 20, 25 and 1,000 µl were used.

The intranasal inoculation of rabies virus is an excellent technique to study antiviral treatment in the brain, since it leaves the brain mechanically intact, in contrast to intracranial inoculation, and yields a highly reproducible brain infection and disease outcome with little variation in the median survival time. This inoculation route has been used before for the evaluation of post exposure prophylaxis of rabies in mice [30].

Prior to IN and IC injections, mice were anesthetized using isoflurane gas (IsoFlo, Abbott laboratories Ltd., United Kingdom).

5.3.11 Clinical follow-up

Mice were observed daily for signs of disease throughout the experiment until maximum 35 days post inoculation (DPI) (experimental end point). A score was attributed to weight loss (0 = absent, 1 ≥ 10 %), depression (0 = absent, 1 = lower (re)activity), hunched back (0 = absent, 1 = present), wasp waist (0 = absent, 1 = present), rouged hair coat (0 = absent, 1 = present), motoric incoordination (0 = absent, 1 = present), paresis (0 = absent, 1 = present) and paralysis of the hind legs (0 = absent, 1 = present).
Disease progression was represented by plotting the cumulative daily score in function of the days post inoculation (DPI). This score per mouse ranged from 0 (no disease) to 7 (severe nervous disease). In our experience, mice with a disease score of 6 or more die within 24 hours. Therefore, mice were euthanized by cervical dislocation when they reached a score of ≥ 6. Results were expressed as Kaplan-Meier survival curves.

5.3.12 Statistical analysis

GraphPad Prism was used for statistical analyses of in vivo data. Differences in survival times were tested using the Log-Rank test with a Bonferroni post-test, difference in ΔCt values were tested using a student t-test after normalization to a house-keeping gene.

5.4 Results

5.4.1 Linkage of VHH with peptides/proteins

Two different strategies were used to link the VHH to the different peptides/proteins: linking using a biotin-(strept)avidin system or genetic fusion by cloning both VHH and peptide genes into an expression vector. For the latter, cloning and expression was done by the Nanobody Service Facility at the VIB.

For the Biotin-(Strept)Avidin Linkage, VHH were biotinylated as described in materials and methods. Biotinylated VHH can be linked to the biotinylated peptides/proteins by mixing them with (strept)avidin. To obtain an equal distribution of VHH and peptide/protein per (strept)avidin, VHH and peptide/protein were mixed in a 1:1 molar ratio, before adding this mix to (strept)avidin, resulting in a final ratio of 2:2:1 VHH:peptide/protein:(strept)avidin. Table 5.2 gives an overview of the available constructs and their use in the different experiments. For the ease of visualization FITC-labelled streptavidin was used for the cell binding/entry assays, for other experimental procedures avidin was used as a linker.

5.4.2 In vitro virus neutralizing capacity

In vitro virus neutralizing was determined using two different methods. Firstly, all constructs were tested in the rapid fluorescent focus inhibition test (RFFIT) which determines the neutralizing capacity against free viruses. In a second test, neuroblastoma cells were pre-incubated with VHH constructs to examine if they become refractory to infection.

The RFFIT showed that all constructs were able to neutralize the virus, albeit that the neutralizing potential was slightly reduced as compared to the naked VHH. More specifically,
neutralizing capacity was about 1.5 to 2 times weaker for the constructs as compared to the ‘naked’ VHH. Results are shown in Table 5.3.

Table 5.2: Overview of the available constructs and their use.

<table>
<thead>
<tr>
<th>Construct name</th>
<th>VHH</th>
<th>Linker</th>
<th>Peptide/protein</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>VHH</td>
<td>Rab-E8/H7</td>
<td>none</td>
<td>none</td>
<td>Cell binding/entry assay</td>
</tr>
<tr>
<td>A-HRP</td>
<td>none</td>
<td>Avidin-HRP</td>
<td>None</td>
<td>Measurement of brain uptake</td>
</tr>
<tr>
<td>RVG-A-HRP</td>
<td>none</td>
<td>Avidin-HRP</td>
<td>RVG</td>
<td>Measurement of brain uptake</td>
</tr>
<tr>
<td>VHH-S-RVG</td>
<td>Rab-E8/H7</td>
<td>Streptavidin-FITC</td>
<td>RVG</td>
<td>Cell binding/entry assay</td>
</tr>
<tr>
<td>VHH-S-tet1</td>
<td>Rab-E8/H7</td>
<td>Streptavidin-FITC</td>
<td>tet1</td>
<td>Cell binding/entry assay</td>
</tr>
<tr>
<td>VHH-S-anatoxin</td>
<td>Rab-E8/H7</td>
<td>Streptavidin-FITC</td>
<td>Tetanus anatoxin</td>
<td>Cell binding/entry assay</td>
</tr>
<tr>
<td>VHH-S-RVMAT</td>
<td>Rab-E8/H7</td>
<td>Streptavidin-FITC</td>
<td>RVMAT</td>
<td>Cell binding/entry assay</td>
</tr>
<tr>
<td>VHH-A-RVG</td>
<td>Rab-E8/H7</td>
<td>Avidin</td>
<td>RVG</td>
<td>In vitro neutralization tests, in vivo treatment</td>
</tr>
<tr>
<td>VHH-A-tet1</td>
<td>Rab-E8/H7</td>
<td>Avidin</td>
<td>tet1</td>
<td>In vitro neutralization test, in vivo treatment</td>
</tr>
<tr>
<td>VHH-A-anatoxin</td>
<td>Rab-E8/H7</td>
<td>Avidin</td>
<td>Tetanus anatoxin</td>
<td>In vitro neutralization tests, in vivo treatment</td>
</tr>
<tr>
<td>VHH-A-RVMAT</td>
<td>Rab-E8/H7</td>
<td>Avidin</td>
<td>RVMAT</td>
<td>In vitro neutralization tests, in vivo treatment</td>
</tr>
<tr>
<td>VHH-RVG</td>
<td>Rab-E8/H7</td>
<td>Genetically fused</td>
<td>RVG</td>
<td>In vitro neutralization tests, in vivo treatment</td>
</tr>
<tr>
<td>VHH-tet1</td>
<td>Rab-E8/H7</td>
<td>Genetically fused</td>
<td>tet1</td>
<td>In vitro neutralization tests, in vivo treatment</td>
</tr>
</tbody>
</table>
Table 5.3: Summary of neutralizing capacity of different constructs that were linked using avidin or genetically linked. All constructs were able to neutralize the virus, but showed decreased neutralisation in RFFIT compared to ‘naked’ VHH. The genetically fused construct containing tet1 had a significant lower neutralizing capacity compared to the other constructs.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Neutralization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free virus (RFFIT) (IU/nmol)</td>
</tr>
<tr>
<td>PBS</td>
<td>&gt; 0.5</td>
</tr>
<tr>
<td>VHH</td>
<td>68.43</td>
</tr>
<tr>
<td>VHH-S-RVG29</td>
<td>30</td>
</tr>
<tr>
<td>VHH-S-tet1</td>
<td>35.30</td>
</tr>
<tr>
<td>VHH-S-Anatoxin</td>
<td>47.50</td>
</tr>
<tr>
<td>VHH-S-RVMAT</td>
<td>47.75</td>
</tr>
<tr>
<td>VHH-RVG29</td>
<td>47.36</td>
</tr>
<tr>
<td>VHH-tet1</td>
<td>14.42</td>
</tr>
</tbody>
</table>

A second *in vitro* assay, the infection assay, determined the neutralizing capacity of cell-associated constructs. In this assay, neuronal cells were seeded as described above and treated with the VHH-avidin-peptide/protein construct. After an incubation of two hours, the cells were washed using three cycles of centrifugation, were resuspended to remove the unbound constructs, and were reseeded and finally exposed to the rabies virus. After an incubation of thirty minutes, virus medium was replaced by fresh medium and cells were incubated for 24 hours. At which time point, cells were fixed with methanol at –20 °C and stained to detect the viral nucleocapsid. To quantify the neutralizing capacity in this test, the number of infected cells per square millimetre was calculated. All RVG/tet1/anatoxin-streptavidin-linked VHH constructs efficiently blocked virus infection in the cells, resulting in a very limited cell infection of approximately two infected cells per square millimetre (Table 5.3). However, ‘naked’ VHH or the RVMAT linked constructs were also able to reduce the spread of infection, suggesting that they were also able to adhere to or enter cells and resist removal by subsequent cell washing steps. Thus in the infection assay, addition of a neurospecific peptide did not improve the antiviral capacity of the anti-rabies virus VHH.

**5.4.3 Cell-binding/uptake of constructs**

Prior to testing their BBB penetration or therapeutic capacities *in vivo*, all constructs were tested in an *in vitro* cell binding/uptake assay to visualize cell binding or uptake. Mouse neuroblastoma cells were seeded in 6-well plates, incubated for 48 hours, and then VHH-S-RVG/tet1/anatoxin constructs were added. 2.63 nmol of the construct was added per well. The
mixture was incubated for two hours. The cells were collected, centrifuged (2 minutes, 1200 rpm) and washed three times in PBS. After the third wash step, cells were re-suspended in 3 ml culture medium and re-seeded in a new 6-well plate.

Uptake or binding to the cell resulted in a cellular fluorescence signal (Figure 5.3). However, microscopic analysis was not able to differentiate whether the tested peptides were bound to the cellular membrane or whether they had actually entered the cells. Slight differences could be observed between different peptides. Whereas RVG29 and tet1 were observed to be concentrated at specific sites in the cytoplasm of the cell, the tetanus anatoxin binding was spread more evenly over the surface of the cells.

We did not fluorescently label the genetically fused construct and were thus not able to visualize cell binding/entry of these constructs in vitro.
Figure 5.3: Fluorescence microscopy images of the binding/uptake assays in neuroblastoma N2a cells. Binding of the VHH linked to the neurotropic peptides was visualized by using fluorescently labelled (FITC) streptavidin for the linkage. The nuclei were stained using DAPI blue. RVMAT linked to VHH was used as a negative control. Anatoxin-, tet1- and RVG-linked constructs all showed binding to the cells, but the binding pattern seemed to differ from one peptide to another. RVG and tet1 seemed to direct the binding to specific cytoplasmic sites in the cells, whereas VHH-S-Anatoxin was spread more evenly over the cells.
5.4.4 Brain distribution of VHH-avidin-RVG upon intranasal inoculation

To measure to which extent neurotropic constructs can enter the brain upon peripheral administration, we performed an experiment in which RVG-A-HRP or A-HRP was administered intranasally and the uptake in the brain was measured. Briefly, 0.6 nmol RVG-avidin-HRP or avidin-HRP was administered intranasally to mice under light anaesthesia. At different time points after administration, mice were euthanized, the blood was washed away from the brain by transcardial perfusion, and the brain and olfactory bulbs were tested for HRP enzymatic activity. Addition of the substrate (TMB) to the brain homogenates caused the formation of white floculae, most likely containing protein that denatured because of the drop in pH. As this influenced the photospectrometric read-out, we included a short centrifugation step to remove these floculae. This significantly improved background noise, but was unable to completely remove the background signal. A colorimetric reaction could be observed in the olfactory bulbs of mice treated with RVG-A-HRP, but also with A-HRP lacking a neurotropic peptide, up to seven days after intranasal administration (Table 5.4). However, it seemed that these proteins were unable to reach the rest of the brain, since no HRP activity was measured further up the brain. This indicates that intranasally administered proteins are able to unspecifically enter the olfactory bulbs early after intranasal administration.

Table 5.4: Comparison of the HRP activity in the olfactory bulbs and the rest of the brain at different time points after intranasal administration of 0.6 nmol RVG-A-HRP or A-HRP. Although HRP activity could be observed in the olfactory bulbs of all mice that received RVG-A-HRP until 7 days after administration, a similar HRP activity was observed when avidin-HRP was administered. No HRP activity was observed in the rest of the brain of any of the mice.

<table>
<thead>
<tr>
<th></th>
<th>Olfactory bulbs</th>
<th>Rest of the brain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h 48 h 72 h 96 h 168 h</td>
<td>24 h 48 h 72 h 96 h 168 h</td>
</tr>
<tr>
<td>HRP RVG-A-HRP</td>
<td>1.307 1.281 1.351 1.833 0.929</td>
<td>0.234 0.599 0.204 0.328 0.174</td>
</tr>
<tr>
<td>A-HRP</td>
<td>2.000 1.004 1.026 1.261 1.056</td>
<td>0.101 0.100 0.275 0.185 0.366</td>
</tr>
<tr>
<td>PBS</td>
<td>0.473 0.184</td>
<td>0.240 0.432</td>
</tr>
</tbody>
</table>

5.4.5 Treatment of infection in mice with neurotropic peptide-linked VHH

5.4.5.1 Pre-exposure treatment with VHH constructs

Mice were treated by administration of different VHH constructs in the nose at different time points prior to intranasal virus inoculation, allowing the construct to reach the brain before the virus arrives. The set-up of the experiments is presented in Table 5.5. In a first experiment, mice were
treated intranasally three days prior to virus challenge. This early treatment did not protect mice from lethal infection, nor did it prolong the median survival time. A similar result was obtained when the mice were treated with an equal dose of naked VHH (Figure 5.4). Median survival times within this experiment were respectively 8, 8, 9, 8 and 8 days for animals that received 0.9 % NaCl, VHH, VHH-A-RVG, VHH-A-tet1 or VHH-A-Anatoxin.

In a second experiment, mice were treated intranasally one day prior to virus inoculation via the same route. Similarly as for treatment three days before virus inoculation, survival times were not prolonged, nor were mice protected from lethal infection (Figure 5.5). Median survival times were respectively 8, 9, 8 and 8.5 days for animals that received 0.9 % NaCl, VHH, VHH-A-RVG, VHH-A-tet1 or VHH-A-Anatoxin.

Table 5.5: Set-up of the pre-exposure treatment experiments with VHH linked to neurotropic peptides/proteins.

<table>
<thead>
<tr>
<th>Group</th>
<th>Intervention at day…</th>
<th>-3</th>
<th>-1</th>
<th>0</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 IU VHH (n=7)</td>
<td>VHH IN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 IU VHH-A-RVG (n=7)</td>
<td>VHH IN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 IU VHH-A-tet1 (n=7)</td>
<td>VHH IN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 IU VHH-A-anatoxin</td>
<td>VHH IN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.9 % NaCl (n=7)</td>
<td>NaCl IN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 IU VHH (n=7)</td>
<td>VHH IN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 IU VHH-A-RVG (n=7)</td>
<td>VHH IN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 IU VHH-A-tet1(n=7)</td>
<td>VHH IN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 IU VHH-A-anatoxin</td>
<td>VHH IN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.9 % NaCl (n=7)</td>
<td>NaCl IN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.4.5.2 Post-exposure treatment with VHH constructs

Mice were inoculated with virus intranasally one day prior to intracerebral administration of the VHH construct. The set-up of the experiments is presented in Table 5.6. Despite the early administration of the construct, none of the VHH constructs were able to protect mice from lethal disease, nor could they significantly prolong survival of the animals. These results were similar to the results obtained with an equal dose of naked VHH (Figure 5.6; Figure 5.7). Median survival times within this experiment were respectively 8, 9, 8, 9 and 9 days for animals that received 0.9 % NaCl, VHH, VHH-A-RVG, VHH-A-tet1 or VHH-A-Anatoxin.
### Table 5.6: Set-up of post-exposure treatment experiments with VHH linked to neurotropic peptides/proteins.

<table>
<thead>
<tr>
<th>Group</th>
<th>Intervention at day...</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
</tr>
<tr>
<td>10 IU VHH (n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>10 IU VHH-A-RVG (n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>10 IU VHH-A-tet1(n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>10 IU VHH-A-anatoxin (n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>0.9 % NaCl (n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
</tr>
<tr>
<td>10 IU VHH (n=10)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>10 IU VHH- RVG (n=10)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>10 IU VHH- tet1(n=10)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>0.9 % NaCl (n=10)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td><strong>Experiment 3</strong></td>
<td></td>
</tr>
<tr>
<td>10 IU VHH (n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>10 IU VHH-A-RVG (n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>10 IU VHH-A-tet1(n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>10 IU VHH-A-anatoxin (n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>0.9 % NaCl (n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td><strong>Experiment 4</strong></td>
<td></td>
</tr>
<tr>
<td>10 IU VHH (n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>10 IU VHH- RVG (n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>10 IU VHH- tet1 (n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>0.9 % NaCl (n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td><strong>Experiment 5</strong></td>
<td></td>
</tr>
<tr>
<td>10 IU VHH (n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>10 IU VHH-A-RVG (n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>10 IU VHH-A-tet1(n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>10 IU VHH-A-anatoxin (n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>0.9 % NaCl (n=7)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td><strong>Experiment 6</strong></td>
<td></td>
</tr>
<tr>
<td>10 IU VHH- RVG (n=10)</td>
<td>Virus challenge</td>
</tr>
<tr>
<td>10 IU VHH- tet1 (n=7)</td>
<td>Virus challenge</td>
</tr>
</tbody>
</table>
The linear VHH constructs that were genetically fused to RVG or tet1, were administered to mice in an identical experimental setting, although the neutralizing dose for VHH-tet1 was lower (1.2 IU instead of 10 IU). As was seen for the avidin-linked constructs, none of the linear constructs was able to protect mice from lethal disease, nor could it significantly prolong survival of the animals (Figure 5.7). The results obtained were similar to those obtained with the avidin-biotin linked constructs (Figure 5.5). Median survival times within this experiment were 8 days for the animals that received 0.9 % NaCl and 9 days for the animals treated with naked VHH, VHH-RVG and VHH-tet1. It should however be noted that VHH-tet1 resulted in an equal median survival time, despite the fact that this construct was dosed about 10 times lower than the other constructs (0.9 IU versus 8 IU in the other groups and 10 IU used in the experiment with the avidin-linked constructs).

![Figure 5.6: Survival curves of mice treated intracerebrally with naked VHH or neurotropic VHH constructs 24 hours after intranasal virus inoculation. No significant differences could be observed between the groups treated with naked VHH or VHH constructs containing neurotropic peptides.](image)

![Figure 5.7: Survival curves of mice treated 24 hours after intranasal virus inoculation. Mice were treated intracerebrally with naked VHH or VHH that are genetically linked to RVG or tet1 1 day after intranasal virus inoculation. No significant differences could be observed between the groups treated with VHH fused to the neurotropic peptides or naked VHH.](image)
Upon intracerebral administration of the constructs, it is possible that the addition of a neurotropic peptide is redundant since the VHH is directly introduced into the brain. We therefore tested a second route of administration in which we administered 10 IU of each construct 24 hours after virus inoculation via the intranasal route (Table 5.6). The construct would thus ‘chase’ the virus using the same route. There was no delay in disease onset or reduction in mortality in mice treated with the VHH constructs compared to the control animals that were treated with naked VHH (Figure 5.8).

Similarly to the results of the avidin-linked constructs, the presence of a neurotropic peptide genetically linked to the VHH, was also unable to prevent lethal disease in the animals nor could it delay the onset of disease compared to animals treated with a control naked VHH (Figure 5.9). Median survival time was 8 days in all groups, and less variation in survival time was observed between the different groups than was seen with the avidin-linked constructs. The administered dose of VHH-tet1 was lower than for the other constructs (1.2 IU versus 10 IU for all the other groups) since this constructs could not be produced at a higher concentration.

Figure 5.8: Survival curves of mice treated intranasally with naked VHH or VHH constructs containing neurotropic peptides 24 hours after intranasal virus inoculation. No significant differences could be observed.
A third experimental setting involved the systemic administration of the VHH constructs, more specifically subcutaneously, 24 hours after intranasal virus inoculation. There was no delay in disease onset or reduction in mortality in mice treated with the VHH constructs compared to the control animals that were treated with naked VHH (Figure 5.10). The median survival times were respectively 8, 8, 9, 8 and 8 days for the mice treated with 0.9 % NaCl, VHH-A-RVG, VHH-A-tet1, VHH-A-anatoxin or naked VHH.

Administration of genetically fused constructs in the same experimental setting resulted in comparable results. The median survival times were respectively 9, 9 and 10 days for VHH, VHH-tet1 and VHH-RVG (Figure 5.11). We could not observe any significant differences between the groups, one mouse treated with VHH-RVG survived the infection.

Figure 5.9: Survival curves of mice treated intranasally with naked VHH or VHH constructs that were genetically linked to RVG or tet1 24 hours after intranasal virus inoculation. No significant differences could be observed between the groups treated with VHH fused to the neurotropic peptides, naked VHH or mock-treated mice.

Figure 5.10: Survival curves of mice treated subcutaneously 24 hours after intranasal virus inoculation. Mice were treated with naked VHH or avidin-linked VHH constructs. No significant differences could be observed between the groups treated with VHH fused to a neurotropic peptide, naked VHH or mock-treated mice.
Figure 5.11: Survival curves of mice treated subcutaneously 24 hours after intranasal virus inoculation. Mice were treated with naked VHH or VHH that are genetically linked to RVG or tet1. No significant differences could be observed between the groups treated with VHH fused to a neurotropic peptide or naked VHH.

5.5 Discussion

The objective of this study was to examine whether the addition of neurotropic peptides or proteins to the anti-rabies VHH would increase the potency of these VHH to treat rabies virus infection in vivo.

First, a selection of neurotropic proteins and peptides was made. RVG was chosen because it was shown to bind selectively to neurons and be transported through the BBB. In addition, since it is part of the rabies virus glycoprotein, it most likely follows the same entry mechanism as the virus. The tetanus toxin and tet1 both bind to neuronal cells. The tetanus toxin can travel within the neuronal network, but this remains uncertain for tet1. For safety reasons we used the detoxified tetanus anatoxin.

We developed two different strategies to link the selected peptides/proteins with anti-rabies VHH: a (strept)avidin-biotin linker system and genetically fusion of the peptide genes with the VHH using recombinant plasmid technology. The first strategy allows high versatility in the proteins and peptides which can be linked, and on top of that the availability of horse radish peroxidase (HRP) or fluorescein isothiocyanate (FITC) labelled (stept)avidin permitted the easy visualization of uptake or binding of these constructs to cells in our in vivo and in vitro assays. However, the disadvantage of this technique is that the stoichiometric composition of these constructs is dependent on probability. Genetic fusion of the constructs resulted in constructs with lower molecular weight and a fixed composition.
The functional characteristics (virus neutralizing capacity and capacity to bind to or enter neuronal cells) were tested using different virus-neutralisation assays with soluble extracellular VHH constructs (RFFIT) or cell-associated VHH constructs (infection assay). The results of our first in vitro assays were promising as all streptavidin-linked constructs were able to bind or enter into neuronal cells when they contained a neurotropic peptide/protein. Since we were unable to label the genetically fused construct, we could not visualize cell binding/entry for these constructs. However, confocal microscopy would be needed to determine the exact location of these constructs within the cell. Unfortunately, we did not have access to this equipment and were therefore unable to determine the exact location.

The neutralization assays, both with soluble (RFFIT) or bound constructs (infection assay), showed a good neutralization of virus in culture with all constructs, whether linked by genetic fusion or by avidin. The slight reduction in neutralization seen in the RFFIT can most likely be explained by limited steric hindrance by the avidin or the peptide on the VHH. Remarkably, we could find similar results between naked VHH and the constructs in the infection assay. This implies that VHH can also bind/enter cells and resist removal by several washing steps, despite the fact that this was not visualized in the cell binding/entry assay. It might be possible that linkage to streptavidin-FITC or biotinylation prevented the binding/entry of VHH. This made it difficult to prove the functional activity and the added value of the neurotropic peptides/proteins in vitro.

In addition we examined the possibility of (strept)avidin constructs to enter the brain upon intranasal administration. Upon intranasal administration of RVG-A-HRP, we found clear evidence of early (24 hours) entry of this construct into the brain, more specifically into the olfactory bulbs. Ultimately, this construct could be detected in the olfactory bulbs up to seven days after administration, but was never detected further in the brain. Still, it was difficult to assess the effect of the RVG peptide in this process since avidin-HRP, without neurotropic peptide/protein, was also able to enter the olfactory bulbs.

Despite these results, we still hypothesized that linkage with a neurotropic peptide would be advantageous for the VHH to reach the virus in the brain. We firstly tested this in a pre-exposure setting, in which we administered the avidin-linked construct intranasally before virus challenge. This would allow the construct to enter the brain before the virus does. Administration of the construct three days or 24 hours before virus inoculation did not result in any protective effect.

A similar result was obtained when we administered the construct in a post-exposure setting. Neither the avidin-linked or genetically fused constructs were able to prolong median survival time or rescue mice after intranasal virus inoculation. The route of administration, intracranially, intranasally or subcutaneously, did not impact the survival time and none of the constructs proved to be superior to naked VHH. However, these results should be interpreted with caution: for
the experiments with naked or half-life extended VHH we had access to highly concentrated VHH (up to 17 mg/ml), which allowed us to use higher doses of the VHH. Especially for the intracerebral inoculation this plays a major role since the volume that can be administered is very limited (20 µl/mouse). In this study, we did not have access to highly concentrated material. We were therefore unable to administer more than 7 µg VHH intracerebrally or intranasally for the avidin-linked constructs. In case of the genetically linked constructs (which were even less concentrated) we were able to administer 7 µg VHH-RVG, but only 2.5 µg VHH-tet1 intranasally, and as the volume for intracerebral administration is even lower, respectively only 4 µg and 2 µg were administered intracerebrally. As we showed in chapter 4, this dose is just below the critical dose (10 µg of naked VHH) needed to prolong survival of mice upon intracerebral treatment. Similarly, the doses that could be administered subcutaneously (84 µg of VHH) were significantly lower than the minimal doses effective upon intraperitoneal administration (10 mg of naked VHH).

It is therefore recommended to produce more concentrated material to repeat the experiments. This would require optimization of the production and possibly the use of a different expression system, most likely yeast instead of E. coli. This was not possible for the present work due to limitations in time and budget. While this work was in progress, it was reported that tet1 is unable to travel within the neuronal network [28]. This was in contrast with TeNT, which was shown to be able to travel within the network [29]. So far, no experimental data was available about whether RVG can travel from one neuron to another.

Another possibility explaining the limited effectiveness of these constructs in vivo, might be due to a different cellular destination of the constructs. As mentioned above, the technical limitations did not allow us to perform confocal microscopy in order to determine the location of the construct in the cells. It might therefore be interesting to perform some additional in vitro experiments prior to test higher doses of the construct in vivo. To determine the location of the construct relative to the virus in infected cells, a double incubation model could be developed. In this experimental setting the virus and the construct would be administered at different time points and stained with different fluorophores. Since in vivo we investigated whether the construct can “catch up” with the infection, the virus should be administered first. Ideally, this model would require the use of a fluorescently labelled rabies virus, rather than monoclonal antibodies directed against the rabies virus nucleocapsid as was used in the seroneutralization assays. In addition, this would allow live imaging and might allow the exact timing of co-localization. Although confirming the co-localization of the constructs would be important in all three cases, research has shown that both RVG29 and the rabies virus follow the same internalization route [30,31].

Since our mouse inoculation model leads to a very quick invasion of the brain, it is possible that the quantity of the used constructs were insufficient to confer protection. Based on our viral
kinetics, the olfactory bulbs, and maybe also a part of the midbrain, are already infected by the virus 24 hours after inoculation. At this stage, it is likely that higher doses of VHH are necessary to be able to neutralize the virus.

Thus far, we can conclude that the addition of neurotropic peptides/proteins to anti-rabies VHH, whether it be genetically fused or linked using avidin-biotin, does not have an added value in our model. It is possible that higher doses of the neurotropic constructs are needed to achieve better antiviral activity in the brain.


124


Post-exposure prophylaxis with half-life extended VHH and vaccine in mice

This chapter\(^1\) bundles the research results addressing the fifth and final objective of this thesis, namely to analyse whether the combined treatment of half-life extended anti-rabies (HLE) VHH and vaccine after virus exposure has an added value compared to single treatment with either compound. Firstly, we determined the protective effect of rabies vaccine in the intranasal animal model. Then, we examined the added value of combining the anti-rabies HLE Rab-E8/H7 VHH and vaccine for post-exposure prophylactic treatment.

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\(^1\) Adapted from: Terryn, S., Francart, A., Rommelaere, H., Stortelers, C., Van Gucht, S., 2016. Post-exposure treatment with anti-rabies VHH and vaccine significantly improves protection of mice from lethal rabies infection. PLOS Neg. Trop. Dis. 10(8)
6.1 Abstract

Post-exposure prophylaxis (PEP) against rabies infection consists of a combination of passive immunisation with plasma-derived human or equine immunoglobulins and active immunisation with vaccine delivered shortly after exposure. Since anti-rabies immunoglobulins are expensive and scarce, there is a need for cheaper alternatives that can be produced more consistently. Previously, we generated potent virus-neutralising VHH, also called Nanobodies®, against the rabies glycoprotein that are effectively preventing lethal disease in an in vivo mouse model. The VHH domain is the smallest antigen-binding functional fragment of camelid heavy chain-only antibodies that can be manufactured in microbial expression systems. In the current study we evaluated the efficacy of half-life extended anti-rabies VHH in combination with vaccine for PEP in an intranasal rabies infection model in mice.

The PEP combination therapy of systemic anti-rabies VHH and intramuscular vaccine significantly delayed the onset of disease compared to treatment with anti-rabies HLE Rab-E8/H7 alone, prolonged median survival time (35 versus 14 days) and decreased mortality (60 % versus 19 % survival rate), when treated 24 hours after rabies virus challenge. Vaccine alone was unable to rescue mice from lethal disease. As reported also for immunoglobulins, some interference of anti-rabies VHH with the antigenicity of the vaccine was observed, but this did not impede the synergistic effect. Post-exposure treatment with vaccine and human anti-rabies immunoglobulins was unable to protect mice from lethal challenge.

Anti-rabies VHH and vaccine act synergistically to protect mice after rabies virus exposure, which further validates the possible use of anti-rabies VHH for rabies PEP.

6.2 Introduction

Rabies virus ultimately causes an aggressive and lethal infection in the brain of humans and other mammals. Rabies virus is a model neurotropic RNA virus that belongs to the family Rhabdoviridae, Genus Lyssavirus [1,2]. The virus is transmitted through the saliva of an infected animal by biting or scratching. Once the virus enters peripheral nerves or neurons, it quickly replicates in the neuronal cytoplasm and progeny virus is transported through the neuronal network by crossing tight interneuronal synapses, eventually giving rise to encephalitis [3,4]. Each year, an estimated 59,000 people die from rabies and about 29 million receive post-exposure prophylaxis (PEP) after close contact with a suspected animal [5].

Passive antibody therapy with anti-rabies immunoglobulins (RIG) plays a major role in rabies post-exposure prophylaxis after high risk exposure [6]. Together with thorough wound cleansing, it is the first line of defence against the virus, and prophylaxis without RIG is associated with treatment failure [7,8]. Pioneering studies on the effects of anti-rabies serum date back to the late 1800s and early 1900s, and since 1954 the World Health Organisation (WHO) recommends the use of RIG in
combination with vaccination for rabies post-exposure prophylaxis [9]. Treatment with RIG and vaccine should be initiated as soon as possible after potential infection, with additional vaccine administrations in the following weeks to activate a full-blown and lasting immune response. Passive immunization with RIG serves to immediately neutralize the virus and close the gap between viral exposure and the vaccine-induced immune response [7]. In this regime, initial protection is offered by RIG, which is then gradually replaced by vaccine-induced antibodies mounted between day 0 and 7-14, providing continued protection to patients [10].

Rabies antibodies can be either from equine (ERIG) or human (HRIG) origin. Due to adverse effects, such as serum sickness, equine antibodies are now used under the form of pepsin-digested Fab fragments, but if available, HRIG is still preferred over ERIG [9]. The production of HRIG, however, requires sufficient numbers of immune donors and gives rise to the typical problems associated with biological products of human origin, such as the transmission of infectious agents [9]. The worldwide shortage and the high costs makes these products poorly available to developing countries, where rabies is endemic [7,9], the reason why the WHO recommends to develop alternatives [11].

VHH or Nanobodies® (a trade-name by Ablynx) are the smallest functional fragments (15 kDa) of heavy chain-only antibodies naturally occurring in Camelidae, and represent the antigen-binding variable domain. By nature VHH are hydrophilic and do not require hydrophobic interactions with a light chain, which allows high solubility, physicochemical stability and high-yield production in Escherichia coli, yeast or mammalian expression systems. The single domain nature and the small size of VHH also allows for easy formatting by genetic fusion into multimeric and multispecific constructs [12–14].

Previously, we generated a potent neutralizing anti-rabies VHH recognising two epitopes on the rabies glycoprotein, fused to an anti-albumin VHH to extend its serum half-life (HLE). The Rab-E8/H7-ALB11 was able to neutralize the virus at picomolar doses [15]. Post-exposure treatment with anti-rabies VHH at 24 hours after intranasal virus challenge could significantly delay disease onset in mice, and depending on the dose, could rescue part of the mice from lethal disease [15].

The main aim of this study was to examine whether the combined treatment with anti-rabies VHH and vaccine (Rabipur, Novartis) after exposure to rabies virus has added value compared to single treatment with either compound in the intranasal rabies virus challenge model, which is very well suited to study intervention strategies for prevention and prophylaxis [16]. Via the intranasal route the virus can directly access the brain via the olfactory epithelium, which results in a highly reproducible infection [17]. First disease signs appear at 7 days, which rapidly progress the following 2 days, requiring euthanasia at 8-9 days post inoculation (DPI). This model was recently also proposed as a valuable alternative to intracranial inoculation for rabies vaccine potency testing.
The typically short incubation period of this model (6.07 ± 0.59 days) is ideal to study the potentially beneficial effect of the combined passive (VHH) and active (vaccine) immunisation on disease outcome. Our results show that anti-rabies VHH and vaccine act synergistically to protect mice after rabies virus exposure, which further validates the possible use of anti-rabies VHH for rabies PEP.

6.3 Materials and Methods

6.3.1 VHH and antibodies

VHH directed against the rabies virus G protein were described previously [19]. Briefly, llamas were vaccinated using the inactivated rabies Human Diploid Cell Vaccine (HDCV, Sanofi, France) and RNA was extracted from peripheral blood lymphocytes. VHH genes were amplified from a cDNA library. Anti-rabies VHH were selected by panning phage libraries on plates coated with the native G protein. Multivalent VHH constructs were generated by the fusion of monovalent VHH into multimeric VHH constructs using flexible glycine-serine (GS) linkers [20]. In this study, we used the half-life extended VHH (HLE Rab-E8/H7-ALB11), containing two different VHH against the rabies virus spike protein and an anti-albumin VHH (ALB11) for half-life extension, and the non-HLE Rab-E8/H7 [15]. VHH were produced and kindly provided by Ablynx (Zwijnaarde, Belgium).

Human rabies immune globulins (HRIG) (Berirab®, CSL Behring GmbH, Germany) are gammaglobulins purified from plasma of vaccinated human donors.

6.3.2 Vaccine

Rabipur® (Purified Chicken Embryo Cell Vaccine, Novartis, Belgium) was reconstituted according to the manufacturer’s instructions and was administered via intraperitoneal or intramuscular injection. The vaccine contains at least 2.5 antigenic units (AU)/ml. It contains the inactivated Flury LEP strain produced on purified chick embryo cells.

6.3.3 Rabies virus

Challenge Virus Standard (CVS)-11 is a virulent classical rabies virus obtained from the American Type Culture Collection (ATCC reference VR959) and was grown in baby hamster kidney (BHK)-21 cells. For virus inoculation in mice, a dose of $10^{2.5}$ 50 % cell culture infectious doses (CCID$_{50}$) was used.

6.3.4 Mouse experiments and clinical follow-up

Six-to-eight weeks old female Swiss outbred mice (Charles River, France) were used. Mice were kept in filter top cages, water and feed provided ad libitum and exposed to a natural day/night light cycle. Intranasal (IN) inoculation procedures are described in detail by Rosseels et al. [16]. The
intranasal inoculation of rabies virus is an excellent technique to study antiviral treatment in the brain, since it leaves the brain mechanically intact, in contrast to intracranial inoculation, and yields a highly reproducible brain infection and disease outcome with little variation in the median survival time. This inoculation route has been used before for the evaluation of post-exposure prophylaxis of rabies in mice [21]. For intraperitoneal (IP) or intramuscular (IM) injections maximum volumes of respectively 1,000 and 100 µl were respected (50 µl per site in case of IM injections). Prior to intramuscular or intranasal administrations, mice were briefly anesthetized using isoflurane gas (IsoFlo, Abbott laboratories Ltd., United Kingdom), as described by Rosseels et al. (2011) [16].

Three retro-orbital bleedings were performed under isoflurane anaesthesia during the 28 day immunization period.

Mice were observed daily for signs of disease throughout the experiment until maximum 35 days post inoculation (DPI) (experimental end point). A score was attributed to weight loss (0 = absent, 1 ≥ 10 %), depression (0 = absent, 1 = lower (re)activity), hunched back (0 = absent, 1 = present), wasp waist (0 = absent, 1 = present),roughed hair coat (0 = absent, 1 = present), motoric incoordination (0 = absent, 1 = present), paresis (0 = absent, 1 = present) and paralysis of the hind legs (0 = absent, 1 = present).

Disease progression was represented by plotting the cumulative daily score in function of the days post inoculation (DPI). This score per mouse ranged from 0 (no disease) to 7 (severe nervous disease). In our experience, mice with a disease score of 6 or more die within 24 hours. Therefore, mice were euthanized by cervical dislocation when they reached a score of ≥ 6. Results were expressed as Kaplan-Meier survival curves.

Rabies virus infection in the brain was confirmed using real-time reverse transcriptase polymerase chain reaction (RT-qPCR) as described by Suin et al. [22], and by the fluorescent antigen test (FAT), performed according to the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Office International des Epizooties, 2008).

6.3.5 Determination of the viral load

The viral RNA load in the brain of mice was determined using RT-qPCR, as previously described [15,22]. Briefly, the brain was homogenized and RNA was extracted according to the manufacturer’s instructions (RNeasy kit, Qiagen, Hilden, Germany). Ribosomal 18S was used as a reference gene for standardization and delta cycle thresholds (ΔCt) values were calculated using the following formula: ΔCt = C_{t_ref} – C_{t}, with C_{t_ref} equal to 45, the number of cycles in this program.

6.3.6 Virus-neutralisation test

The virus-neutralizing titer of serum, antibody and VHH preparations was determined with the Rapid Fluorescent Focus Inhibition Test (RFFIT), according to the Manual of Diagnostic Tests and
Chapter 6


6.3.7 Statistical analysis

GraphPad Prism was used for statistical analyses of in vivo data. Differences in survival times were tested using the Log-Rank test with a Bonferroni post-test, differences in ΔCt values were tested using a Student’s t-test after normalization to the house-keeping gene. Differences in antibody titers were also tested using a Student’s t-test.

6.4 Results

6.4.1 Pre-exposure vaccination

To validate the protective effect of rabies vaccine (Rabipur, Novartis) in the intranasal rabies mouse model, mice were vaccinated with two intramuscular vaccine doses (0.25 AU/mouse), with a 3-day interval, following the schedule also used later on for PEP. This vaccination schedule is schematically represented in Table 6.1. Mice received a viral challenge 25 days after the last vaccine, allowing sufficient time for the development of an immune response.

Table 6.1: Set-up of the pre-exposure vaccination experiment and interventions in different treatment groups. Groups were treated with vaccine, HLE Rab-E8/H7 (VHH), or a combination of both.

<table>
<thead>
<tr>
<th>Group</th>
<th>Intervention at day…</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>28</td>
</tr>
<tr>
<td>Vaccine + VHH (n = 9)</td>
<td>Vaccine + anti-rabies VHH</td>
</tr>
<tr>
<td>VHH only (n = 8)</td>
<td>Vaccine</td>
</tr>
<tr>
<td>Saline (n = 10)</td>
<td>Anti-rabies VHH</td>
</tr>
</tbody>
</table>

The mounting of the humoral immune response in the blood after vaccination was monitored by assessing the rabies neutralization activity in vitro (RFFIT) in blood collected at different time points. In Figure 6.1, it is shown that mice that received the vaccine had detectable antibody titers eight days after the first dose (day -20), (mean 7.22 ± 3.28 IU/ml, range 3.73 - 12.62 IU/ml), which were well above the generally accepted protective threshold of 0.5 IU/ml. Antibody
titers continued to increase until 28 days later (day 0, mean 11.47 ± 4.77 IU/ml, range 6.01-14.81 IU/ml).

![Graph](image)

**Figure 6.1: Rabies neutralizing activity in the blood measured by RFFIT, following intramuscular (IM) vaccination at day alone or vaccination in combination with anti-rabies VHH (Rab-E8/H7-ALB11, 1.5 mg/mouse). Mice were vaccinated at day -28 and day -25, VHH was administered at day -28. Control groups consisted of mock treatment with saline (0.9% NaCl) or anti-rabies VHH only at day -28. Blood was collected at day -28 (prior to vaccination and VHH administration), day -25, day -20 and day 0 (= time of virus challenge). Both groups of mice that received anti-rabies VHH had high neutralizing titers at the early time points (day -25 and -20). Mice that received vaccine had neutralizing antibodies at day -20, which further increased to high levels at day 0. Mice treated with anti-rabies VHH only no longer had detectable VHH at day 0. Antibody titers in the vaccine + VHH group were significantly lower than in the vaccine only group at day 0 (** p< 0.005, *** p< 0.001). Error bars represent the standard deviation.**

To verify if the efficacy of the vaccine would be affected by the simultaneous administration of HLE Rab-E8/H7-ALB11, an interference phenomenon which is well known for anti-rabies immunoglobulins [23], a group of mice received besides the vaccine also a single dose of anti-rabies VHH in the same pre-exposure setting. In this regime, the first vaccination (day-28) was accompanied by anti-rabies VHH (Rab-E8/H7-ALB11) at a dose of 1.5 mg/mouse (corresponding to 60 mg/kg, 392,600 IU/kg), at the moment of the first vaccination (day -28). Vaccine (IM) and VHH (IP) were administered at separate sites. As reference groups mice were treated with anti-rabies VHH alone, or left untreated. In mice, the half-life of the anti-albumin VHH is approximately 1.5 days, hence the anti-rabies VHH will be removed from the circulation at the moment of viral challenge.

Figure 6.1 shows that the rabies neutralization titers of mice that were injected with anti-rabies VHH, whether or not in combination with vaccination, were high 3 days after VHH administration (day -25, mean 88.28 ± 58.05 IU/ml, range 0.61 - 149.18 IU/ml). As expected, anti-rabies VHH titers rapidly declined over time with the clearance of the VHH from the blood (day -20, mean 9.43 ± 6.04 IU/ml, range 0.16-15.57 IU/ml) and no detectable titers (< 0.5 IU/ml) on day 0. Mice that received both vaccine and anti-rabies VHH had a mean titer of 5.69 ± 3.03 IU/ml (range
1.73-9.37 IU/ml) at day -20, similar to mice that received vaccine alone, while at day 0, antibody titers were significantly (p<0.005) lower in the vaccine + VHH group (mean 5.15 ± 3.38 IU/ml, range 0.37-10.03 IU/ml), compared to the vaccine only group.

Figure 6.2 shows the results of a second independent experiment, in which a pre-incubated mix of either HLE Rab-E8/H7 or non-HLE Rab-E8/H7 and vaccine was administered intraperitoneally. The results confirmed that the simultaneous administration of vaccine and VHH, whether half-life extended or not, interfered with the immune response induced by the vaccine.

![Figure 6.2: Rabies neutralizing activity in the blood measured by RFFIT, following intraperitoneal administration (IP) of a pre-incubated mix of HLE VHH or non-HLE VHH and vaccine at day -28 and day -14. The control group consisted of mice receiving the vaccine without VHH. Blood was collected at day -28 (prior to vaccination and VHH administration), day -14 and day 0. Mice that received rabies vaccination had high antibody titers from day -14 onwards whereas mice that received the pre-incubated mix of HLE VHH + vaccine or non-HLE VHH + vaccine had significantly lower antibody titers on both days (*** p<0.0001, ** p<0.005, * p<0.01). Error bars represent the standard deviation.]

Mice were challenged by intranasal virus inoculation 4 weeks after the start of the vaccination (day 0). Figure 6.3 shows the survival curves of the vaccinated and control mice. Despite the fact that all mice had high neutralizing antibody titers at the time of challenge, only 50% was protected from disease and survived the challenge. In the remaining mice disease progression was delayed (median survival time 27 days versus 9 days in control group). Disease signs in vaccinated mice were different compared to control mice, which typically develop signs of depression, such as unresponsiveness to stimuli and isolation from the group. The vaccinated animals remained responsive to stimuli and aware of the environment, while developing ascending paresis, starting at the hind limbs, that gradually evolved into paralysis. Eventually, mice had to be euthanized because of severe paresis and paralysis.
The survival of mice that received the combination regime (Rab-E8/H7-ALB11 + vaccine) 4 weeks before viral challenge was substantially reduced compared to the mice that received only the vaccine (11% versus 50%). The median survival time of these mice was not significantly different from the control groups (10 days versus 9.5 days), despite the presence of relatively high neutralizing antibody titers at the moment of challenge. As expected, mice that received anti-rabies VHH were comparable to the control group. The presence of the anti-rabies VHH in the circulation hence seems to reduce the vaccine efficacy. This may indicate that in absence of the virus, the binding of the anti-rabies VHH to the vaccine may interfere with the induction of an effective humoral immune response.

### 6.4.2 Post-exposure prophylactic treatment with anti-rabies VHH and vaccine

In previous *in vivo* studies, post-exposure treatment with the anti-rabies VHH one day after virus challenge was shown to provide protection from disease and death in a dose-dependent manner [15]. The same set-up was used to examine the efficacy of the combination of vaccine with a single anti-rabies VHH dose after exposure to the virus, which is the main indication for the use of vaccine in humans. Two different experiments were conducted. In a first experiment mice were treated with IP administered anti-rabies VHH (Rab-E8/H7-ALB11, 1.5 mg = 7,852 IU/mouse) and IM administered vaccine (0.25 AU/mouse), twenty-four hours after challenge with a lethal rabies dose. A second vaccine dose was administered 3 days after the first. This treatment was than compared to
treatment with anti-rabies VHH at the same dose or the vaccine regimen alone. The anti-rabies VHH dose was the lowest effective dose in post-exposure treatment in previous studies [15]. Similar to the pre-exposure set-up, vaccinated mice received a second vaccination 3 days after the first dose.

In the second experiment, the same vaccination schedule was applied, but instead of anti-rabies VHH, mice were treated with human rabies immunoglobulins (HRIG, Berirab®, IP, 1 ml/mouse = 121.50 IU/mouse) at 24h after virus challenge. This is the highest volume and dose of the commercial HRIG product which could be administered to mice. Control mice were treated with HRIG alone. A schematic overview of both experiments can be found in Table 6.2.

The survival curves of the different treatment groups in the post-exposure prophylaxis setting are depicted in Figures 6.4 and 6.5. In the post-exposure setting, the combination of vaccination with anti-rabies VHH rescued 60% of mice (Figure 6.4), significantly better than the treatment with anti-rabies VHH alone which rescued only 19% of mice. The vaccine by itself in the post-exposure setting did not provide any protection, and disease was similar to the control group. The median survival time was significantly longer after the combined treatment (>35 days), compared to treatment with anti-rabies VHH (14 days, p<0.01) only, vaccine only (7 days, p<0.001) or the control group (8 days, p<0.001). Mice that were treated with the combination of vaccine and HRIG did not survive challenge, similar to mice treated with HRIG alone. The median survival time of mice treated with vaccine and HRIG was 9 days and treatment with HRIG alone resulted in a median survival time of 10 days.
Table 6.2: Set-up of the post-exposure treatment experiment and interventions in different treatment groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Intervention at day...</th>
<th>0</th>
<th>1</th>
<th>3</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Vaccine + VHH (n = 10)</strong></td>
<td>Virus challenge</td>
<td>Vaccine + anti-rabies VHH</td>
<td>Vaccine</td>
<td>End observation period - euthanasia</td>
<td></td>
</tr>
<tr>
<td><strong>Vaccine only (n = 10)</strong></td>
<td>Virus challenge</td>
<td>Vaccine</td>
<td>Vaccine</td>
<td>End observation period - euthanasia</td>
<td></td>
</tr>
<tr>
<td><strong>VHH only (n = 21)</strong></td>
<td>Virus challenge</td>
<td>Anti-rabies VHH</td>
<td>Saline</td>
<td>End observation period - euthanasia</td>
<td></td>
</tr>
<tr>
<td><strong>Saline (n = 7)</strong></td>
<td>Virus challenge</td>
<td>Saline</td>
<td>Saline</td>
<td>End observation period - euthanasia</td>
<td></td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Vaccine + HRIG (n = 10)</strong></td>
<td>Virus challenge</td>
<td>Vaccine + HRIG</td>
<td>Vaccine</td>
<td>End observation period - euthanasia</td>
<td></td>
</tr>
<tr>
<td><strong>HRIG only (n = 7)</strong></td>
<td>Virus challenge</td>
<td>HRIG</td>
<td>Saline</td>
<td>End observation period - euthanasia</td>
<td></td>
</tr>
<tr>
<td><strong>Saline (n = 10)</strong></td>
<td>Virus challenge</td>
<td>Saline</td>
<td>Saline</td>
<td>End observation period - euthanasia</td>
<td></td>
</tr>
</tbody>
</table>

Figure 6.4: Effect of post-exposure prophylactic treatment with vaccine and anti-rabies VHH on survival in rabies mouse model. Mice were intranasally inoculated with rabies virus followed by treatment with anti-rabies VHH (IP) 24 hours later, either alone or in conjunction with vaccine (IM). Vaccinated mice received a second vaccine dose 3 days later. Control groups consisted of mice that were not treated (virus only group), that received the vaccination regime only (vaccination group). Combined treatment with vaccine and anti-rabies VHH resulted in 60% survival, while treatment with anti-rabies VHH alone rescued 19% (p<0.01).
Figure 6.5: Effect of post-exposure prophylactic treatment with vaccine and human rabies immunoglobulins on survival in rabies mouse model. Mice were intranasally inoculated with rabies virus followed by treatment with human rabies immunoglobulins (HRIG) (IP) 24 hours later, either alone or in conjunction with vaccine (IM). Vaccinated mice received a second vaccine dose 3 days later. The control group consisted of mice that were not treated (virus only group). Combined treatment with vaccine and human rabies immunoglobulins did not differ significantly from treatment with human rabies immunoglobulins alone and was unable to rescue mice from lethal infection.

The viral RNA load in the brain of mice was also assessed (Figure 6.6). Mice that received the PEP with vaccine and anti-rabies VHH had significantly lower viral RNA loads than control mice or mice treated with anti-rabies VHH only (Figure 6.6).

Together these data show that in the post-exposure setting anti-rabies VHH acts synergistically with a standard vaccination regime to protect mice from disease after virus exposure.

Figure 6.6: Post-exposure treatment with vaccine and anti-rabies VHH: effect on the viral RNA load in the brain of mice. The viral load was determined at the peak of clinical symptoms in mice that developed disease (filled symbols) or at the end of the observation period (open symbols) in survivor (non-diseased) mice. The dashed line represents the limit of detection (= 5 ΔCt). Mice treated with vaccine + VHH had significantly lower viral RNA loads than naive mice (p<0.0001), mice treated with vaccine only (p<0.001) or mice treated with VHH only (p<0.05). Viral loads of diseased mice were also lower (25.85 ΔCt) in mice that were treated with vaccine + VHH compared to naïve mice (29.58 ± 1.29 ΔCt) or treated with VHH only (29.59 ±0.76 ΔCt). Survivor mice (vaccine + VHH, VHH alone) had comparably low viral loads (3.3 - 11.3 ΔCt).
6.5 Discussion

Post-exposure prophylaxis (PEP) for rabies consists of a combination of passive (human or equine immunoglobulins) and active immunisation (vaccine) soon after exposure. Anti-rabies immunoglobulins are expensive, scarce and often not available or affordable for people in developing countries, that are typically most at risk [24,25]. Also in Western countries, RIG are increasingly difficult to procure [26]. Cheaper and easier-to-produce alternatives are needed. Previously, we developed anti-rabies VHH (Nanobody®) capable of neutralizing virus at picomolar doses in vitro [15]. We also showed that post-exposure treatment with anti-rabies VHH only is capable of prolonging the incubation period of the disease in a dose dependent manner. In the current study, we evaluated whether post-exposure treatment with the combination of anti-rabies VHH (half-life extended Rab E8/H7-ALB11) and vaccine (Rabipur, Novartis) is better than single treatment with anti-rabies VHH or vaccine only. The combined treatment was tested using an intranasal challenge model of mice. Treatment was initiated at 24 hours after challenge.

In humans, rabies can have incubation periods as short as 4-6 days, especially if the virus is deposited in highly innervated facial tissues, as is often the case in children [27]. Failure of classic PEP is described for several cases, often with short incubation periods or when highly innervated tissues were infected, which allows quick entry of the virus in nerves [28–30]. In order for PEP to be effective, it is believed that the virus needs to be intercepted by passive or active immune effectors before invasion of the central nervous system [28]. In case of a short incubation period, with rapid invasion of the nervous system, PEP cannot intercept the virus in time to prevent brain infection.

Compared to anti-rabies VHH or vaccine alone, the combination therapy in a post-exposure setting significantly delayed the onset of disease, prolonged median survival time and decreased mortality. Sixty per cent of mice treated with anti-rabies VHH and vaccine survived the infection, in contrast to 0 % with vaccine only and 19 % with anti-rabies VHH only. This is in agreement with the observations from Servat et al., who also showed that PEP with vaccine only was unable to prevent lethal disease [31]. Post-exposure treatment with anti-rabies VHH only proved more effective than vaccine only. This partial protection is in line with studies previously described by our group [15]. We assume that the synergy between vaccine and VHH lies in the fact that anti-rabies VHH can immediately delay the spread of the virus and prolong the incubation period, which allows more (sufficient) time for the active immune response to mount and control the infection in part of the mice. Indeed, treatment with VHH prolongs the incubation period from six to ten days, and the earliest antibody and cellular immune response can be expected as soon as seven days after intramuscular vaccination with an inactivated rabies vaccine [32]. This hypothesis also explains the limited efficacy of the combined treatment with vaccine and HRIG. Indeed, in the current and a
previous study [15], we found that administration of HRIG to mice after lethal challenge merely prolongs the median survival time by one or two days. This limited prolongation of the incubation period is probably not long enough to mount an effective immune response, able to control the virus infection before it becomes lethal. Our results indicate that an active antibody response was induced in all survivor mice, corresponding to low residual levels of viral RNA (ΔCt ≤10) in the brain at the endpoint measurement (35 DPI).

Pre-exposure treatment with vaccine (IM) and VHH (IP) seemed to partially reduce the immunogenicity of the vaccine, a phenomenon that is also described for the combination of RIG and vaccine [23,33,34]. Mice that received anti-rabies VHH in conjunction with vaccine prior to virus challenge developed significantly lower antibody titers 4 weeks later and were significantly less well protected against virus challenge. Indeed, whereas mice receiving vaccine only had a 50 % survival rate and a delayed disease progression, only 11 % of the mice treated with vaccine and anti-rabies VHH survived infection and no delay could be observed. These results were confirmed in independent experiments in which a pre-incubated mix of rabies virus and VHH was administered simultaneously at the same site (Figure 6.6). Antibodies can interfere with active immunization via different mechanisms. Most of the described mechanisms are Fc dependent, like inhibition of the B-cell responses by binding to the Fc-receptor, cross-linking of the B-cell receptor and the complement system, or antigen removal by macrophages [35]. Only humoral, and not cellular, immune responses seem to be affected by the presence of specific antibodies [36]. Since the used anti-rabies VHH is not a full antibody and lacks the Fc domain, it is unlikely that these mechanisms are involved [37]. The half-life extended anti-rabies VHH can interact with the neonatal Fc receptor through the intermediate of albumin, but it remains an unlikely mechanism since the non-HLE anti-rabies VHH, lacking an albumin-binding VHH component showed similar reduction of the vaccine efficacy (Figure 6.2). Therefore a likely mechanism could be epitope masking. By binding to the surface glycoproteins of the inactivated vaccine virus, the anti-rabies VHH might shield recognition of the epitopes by the immune system [36]. The fact that the combination of anti-rabies VHH with vaccine still proved superior in PEP, argues for the relative importance of immediate passive immunisation in PEP, especially when the virus has easy access to nerves or neuronal cells.

Pre-exposure vaccination offered only partial protection upon intranasal virus challenge (50 % survivors). Half of the mice that were actively immunized with (inactivated) vaccine, both at 28 and 25 days before challenge, still developed lethal brain infection. This incomplete protection, even with high antigenic doses (2 x 0.25 AU/mouse), is also described by other researchers, using similar models [18]. Nevertheless, the applied vaccine schedule resulted in clear seroconversion of all mice, with virus-neutralizing serum titers well above the protective threshold of 0.5 IU/ml (range 6.01-18.04 IU/ml) at the moment of challenge. Moreover, the challenge occurred at four weeks after the
first vaccine administration, at the moment when the peak serological response can be expected [38,39]. The height of the neutralizing antibody titer in vaccinated mice did not correspond to the level of protection upon challenge. Some mice with titers up to 20 IU/ml still developed lethal disease.

The incomplete protection in the post-exposure setting may be explained by the aggressive nature of the used intranasal challenge model, in which virus is inoculated directly on a site that contains a high concentration of olfactory neuronal cells, providing a direct portal of entry to the central nervous system. In earlier studies we found spread of the virus in the olfactory bulbs of the brain already at the first day after inoculation [15]. Once inside the central nervous system, the virus is protected from several systemic immune effectors, which may limit the protection by the vaccine [40,41]. We therefore assume that the mice that survived the challenge after preventive vaccination or PEP with anti-rabies VHH and vaccine were able to develop a cellular immune response, capable of controlling the infection in the brain.

The intranasal challenge model is our preferred experimental model because of the high reproducibility, practicability, safety and animal wellbeing issues [16]. It may be that in an infection model with a longer incubation period and a more pronounced phase of peripheral virus replication in non-neuronal cells, preventive vaccination would be more effective, since vaccine-induced antibodies might be more effective to intercept virus spread between non-neuronal and neuronal cells. In our hands, intramuscular inoculation of rabies virus requires unnaturally high levels of virus in the inoculum (>10^{5-6} CCID_{50}) and yields variable inter-assay results, limiting its use for experimental comparison of intervention strategies [16].

Another remarkable finding was the different clinical picture observed depending on the vaccination status of the mouse prior to virus challenge. Naive mice typically showed signs of depression, such as isolation from the group, inactivity and unresponsiveness to stimulation. In contrast, pre-immunised mice remained alert and vivid, but developed ascending paresis, resulting in paralysis of all limbs, requiring euthanasia. Vaccinated mice developed disease after a longer incubation period (13.7 instead of 9 days) and had a longer morbidity period (3 instead of 1.5 days), which resulted in a longer median survival time (27 instead of 9 days), compared to naive mice. They also had lower viral loads in the brain at the peak of disease. The vaccine-induced immune response thus had a clear effect on pathogenesis and symptomatology. Iwasaki et al. also found that the host immune response has a clear impact on the development of, what they refer to as, either “encephalitic” or “paralytic” disease in mice. Rabies virus challenge in immunocompetent mice resulted in “paralytic disease”, with relatively low viral loads and a high extent of inflammation and damage in the brain. The same challenge in cyclophosphamide-treated mice resulted in the absence of an immune response and “encephalitic disease”, with severe general depression, only minor
paralysis, high viral loads, and less neuronal cell damage [42]. In our study, the pre-immunized mice developed a disease pattern similar to the immunocompetent mice of Iwasaki et al., whereas the naïve mice evolved comparably to the cyclophosphamide-treated mice. In human cases, the average survival time of paralytic rabies is twice as long, compared to the encephalitic (furious) form [42]. Patients with paralytic rabies typically remain fully conscious, while developing ascending motor weakness [43]. Also in dogs, paralytic rabies is associated with reduced viral load and more prominent inflammation [44]. Our observations further add to the evidence that paralytic rabies may be caused by an immuno(patho)logical response of the host to the virus infection.

In humans, passive immunisation with anti-rabies antibodies is expected to bridge the immunity gap between virus exposure and onset of the active antibody production induced by the vaccine. The half-life extension of the anti-rabies VHH is based on the addition an anti-albumin VHH component. In mice, addition of anti-ALB VHH extends the half-life to 0.5-1.9 days [15], while in humans it is extended up to 10-20 days [45]. It would therefore be feasible to formulate and dose anti-rabies VHH for humans to obtain protective levels (> 0.50 IU/ml) in the blood for 14 days, which would be sufficient for the active immune response to take over. Compared to (human) rabies immunoglobulins (150 IU/ml), VHH can be produced and formulated at very high potencies (>6,000 IU/ml). WHO recommends that rabies immunoglobulins are administered locally into the wound, however, due to the limited potency per ml of the rabies immunoglobulins, this is not possible for small wounds or injuries to nose, fingers or toes as it can cause compartment syndrome. VHH formulations containing high potencies per ml could overcome this problem and would be more suited for infiltration of the whole dose into small body parts.

These results provide evidence for the possible use of anti-rabies VHH together with vaccine for post-exposure prophylaxis of rabies. Early treatment with anti-rabies VHH can delay the incubation period of the disease, which allows more time for the vaccine-induced immunity to control the infection. The ease of production and high thermal stability of VHH are important advantages over the currently used anti-rabies immunoglobulins.

6.6 Acknowledgements

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Reference List


7 General Discussion

7.1 Need for alternatives for human rabies immunoglobulins

Rabies is one of the oldest recognized infectious diseases affecting humans [1]. This highly neurotropic virus causes an invariably fatal disease if left untreated. Typically, the virus is transmitted by animal bites as the saliva of infected animals contains high doses of virus. Once the virus is introduced into the (wounded) tissue, the virus infects neurons [2]. From there, the virus travels through the neuronal network, eventually reaching the brain [3]. Once the virus has reached the brain and symptoms of infection appear, treatment is not available [4]. However, before onset of the symptoms, the disease can be prevented by post-exposure prophylaxis administered soon after exposure. Post-exposure prophylaxis needs, besides wound care, a combination of active and passive immune treatment. The active component of this treatment consists of a vaccination schedule, whereas the passive component consists of immunoglobulins administered directly into the wound [5,6]. Passive immunisation has to span the period between the development of an active immune response and exposure. Because these immunoglobulins can neutralize the virus directly in the wound, before an active immune response is present, it is an indispensable part of post-exposure prophylaxis [7]. Indeed, absence of this component in post-exposure prophylaxis is often linked to failure of the treatment [8]. The currently used rabies immunoglobulins are purified from plasma of vaccinated human donors. Unfortunately, as human rabies immunoglobulins are expensive and scarce, they are often unavailable for people most at risk. The WHO encourages therefore the development of alternatives for passive immunity, making them more available to developing countries [9].

The research presented in this thesis had as a general aim the development and validation of a more affordable alternative for passive immunization in post-exposure prophylaxis, more
specifically we focussed in this thesis on the development and validation of several types of anti-rabies VHH for rabies virus prophylaxis in mouse models. To fulfil this aim, the thesis addressed a number of specific objectives.

Firstly, the intranasal infection model, used in this thesis, was validated by performing a retrospective analysis of the reproducibility of the clinical and virological outcome. Secondly, different monovalent, homo- or hetero-bivalent VHH were developed and tested in vitro and in vivo. This allowed us to determine the most potent construct and assess the potency of this anti-rabies VHH to delay infection and disease in vivo. Thirdly, the most potent VHH was further modified to increase the half-life and assess the impact of half-life extension on the prophylactic effect. Fourthly, the most potent bivalent VHH was linked to different types of neurotropic peptides/proteins in an attempt to target it better to the tissue of interest. Fifthly, we tested the half-life extended VHH in combination with vaccination for post exposure prophylaxis at 1 day after virus inoculation in mice.

7.2 Validation of the intranasal inoculation model (Chapter 3)

All animal experiments in this research were based on intranasal inoculation as a way to introduce the virus in the brain. This method was first described by Lafay et al., and was later adapted by our research group [10,11]. Since its optimization in 2008, the method has been used for the majority of experimental inoculations at our laboratory. This provided us with a large amount of data since 2008, allowing a thorough retrospective validation of the reproducibility of the technique over time between different operators, different strains or stocks of the rabies virus, etc. In addition, it permitted us to describe in detail the typical disease progression. This validation of the animal model is useful for the interpretation of experimental results with VHH and rabies post-exposure prophylaxis.

When comparing different inoculation techniques (intranasal, intracranial or intramuscular), we observed the lowest level of variation when mice were inoculated intracranially, closely followed by intranasal inoculation. Intramuscular inoculation showed the highest rate of variation in average survival times and overall mortality. These results are not surprising as intracranial inoculation puts the inoculum directly into contact with the tissue most sensitive to infection. However, this technique is a highly invasive and damages the delicate brain tissue. Also, the use of needles poses a potential risk to the operators. The tissue damage caused by the needle, the injection of a volume of 20 µl of inoculum, which is also packed with foreign molecules from the cell culture medium, induces a non-viral inflammatory response that can potentially influence the disease outcome [12]. In contrast, the intranasal inoculation is a non-invasive technique for the mouse, which poses virtually no risk to the operators, when wearing appropriate personal protective equipment. When the viral suspension is introduced in the nasal cavity, it comes into contact with the nerve terminals of the olfactory bulbs, via which the virus can infect the central nervous system [10]. We were able to show
the spread of the virus from the olfactory bulbs to the posterior parts of the brain. Upon intranasal inoculation, the virus quickly infects the olfactory bulbs (1-2 days after inoculation) and rapidly spreads to the rest of the brain (4 days after inoculation). A plateau in viral load is reached at about the same time as clinical disease symptoms become apparent. First disease signs appear 6 days after virus inoculation and are characterised by isolation of the group and apathy. As the disease progresses, mice move in an uncoordinated way or will no longer move spontaneously. Eventually, usually around 7 – 9 days after virus inoculation, mice will no longer respond to stimuli, at which point mice are euthanized. The CVS-11 rabies virus strain is well-adapted to mice and highly neurovirulent. The intranasal inoculation model brings the virus almost immediately into contact with neuronal tissue resulting in a short incubation period and a relatively aggressive type of infection. Therefore, only very potent prophylaxis approaches can be successful. The acute nature of the infection makes the model more representative for cases with a short incubation period. In humans severe bites to the hand, neck, face and head are generally associated with shorter incubation periods, which can in some cases be shorter than 7 days [13].

7.2.1 The use of VHH for the treatment of rabies virus in mice (Chapter 4.1)

We investigated the neutralizing potential of different VHH constructs (monovalent, homo- or hetero-bivalent) both in vitro as in vivo. VHH directed against the rabies virus glycoprotein were generated by vaccinating llamas using the inactivated rabies Human Diploid Cell Vaccine. After vaccination, RNA was extracted from peripheral blood lymphocytes, VHH genes were amplified from a cDNA library and anti-rabies VHH were selected by panning phage libraries. Monovalent VHH consist only of a single VHH, bivalent VHH are a combination of two (monovalent) VHH linked by a flexible linker. Homo-bivalent VHH consist of two identical VHH linked together and hetero-bivalent VHH consist of two different VHH. First studies showed that all VHH were able to neutralize the virus in vitro, but homo- and hetero-bivalent constructs proved superior. Indeed, multivalent formats of VHH increases the functional potency due to an avidity effect [14]. This was previously shown for an anti-respiratory syncytial virus bivalent VHH which demonstrated a 4,000-fold increase in neutralization compared to its monovalent counterparts [15]. The hetero-bivalent Rab-E8/H7 was eventually chosen as it proved superior over other VHH both in vitro as in vivo.

We then tested this VHH in different post-exposure settings. In a first set of experiments, we administered the VHH directly into the brain, as this is the main target organ of infection (no vaccination). In this setting, we determined the minimal dose required to offer protection when the VHH is administered soon after virus inoculation (24 hours), as well as the effect of delayed administration of the VHH (3 or 5 days after virus inoculation). We were able to determine that the minimal dose for protection could be set at 33 µg Rab-E8/H7 per mouse, when administered directly
in the brain at 24 hours after virus inoculation. At this dose a significant part of the animals were protected from disease. However, when Rab-E8/H7 was administered at later time points of infection (3 or 5 days after virus inoculation), the protective effect progressively diminished. This can be explained by the kinetic profile of the virus (Chapter 3). Indeed at 3 days post inoculation major parts of the brain, including the cerebrum and diencephalon, are already infected and viral loads in the brain are already relatively high, reaching a final plateau 2 days later. These results stress the importance of early treatment and emphasize the speed at which the virus travels through the brain.

In contrast to direct intracerebral treatments, in which relatively low doses of Rab-E8/H7 were able to prolong survival or protect mice from lethal infection, peripheral treatment with Rab-E8/H7 could only prolong the median survival time by one or two days, and was unable to protect mice from lethal infection, despite the high doses used (up to 10 mg per mouse, 300-fold higher than the intracranial dose). This reduced effect may be explained by the short half-life of the VHH in circulation and the fact that only a small fraction of Rab-E8/H7 will eventually reach the brain after systemic treatment. These results indicate that not only timing of treatment, but also the site of administration is important, and that a prolonged contact with sufficiently high doses of VHH may be needed to neutralize the virus in the brain.

7.2.2 The importance of half-life extension to increase treatment efficacy (Chapter 4.2)

Both the amplitude and duration of exposure to anti-rabies VHH are clearly important to successfully block or delay viral spread in the brain. The limited systemic half-life of VHH however may limit prolonged exposure of the brain. Both monovalent VHH (~15 kDa) as bivalent VHH (~31 kDa) are rapidly cleared by renal filtration, which reduces their half-life significantly. Half-life extension of the VHH may therefore increase the effectiveness of the VHH, especially after systemic administration.

Therefore, we increased the half-life of Rab-E8/H7 by the addition of a third anti-albumin VHH. This third VHH allows Rab-E8/H7 to reversibly bind to (human) serum albumin, a protein that is not cleared by renal filtration, resulting in an increased half-life. In humans the addition of a VHH directed to serum albumin increases the circulatory half-life to 10-20 days [16,17]. To determine the extent of the increased half-life in mice, we performed a pharmacokinetic study in which we compared the Rab-E8/H7 with or without the addition of the third VHH. From this study we could conclude that the addition of this VHH increased the half-life of the VHH from 1.16 h to 30.5 h. The limited increase in circulatory half-life observed in mice, is likely due to the fact that this VHH was originally directed to human, and not murine, serum albumin. The higher circulatory half-life, as compared to Rab-E8/H7, did however result in an increased exposure of the brain to the VHH.
This half-life extended (HLE) Rab-E8/H7-ALB11 significantly increased the median survival time of the animals upon peripheral administration at 1 day after virus inoculation. Whereas 10 mg Rab-E8/H7 increased the median survival time of the mice by one day without survival, at best, 1.5 mg Rab-E8/H7-ALB11 prolonged the median survival time by 6 days and resulted in a 43 % survival rate of the animals. The minimal systemic dose of HLE Rab-E8/H7-ALB11 required to increase the median survival time in mice by IP treatment at 1 day after virus inoculation was 0.5 mg/mouse.

Human rabies immunoglobulins have a typical half-life of 8 days in mice [18]. Remarkably, use of human rabies immunoglobulins in the same experimental setting (administration 24 hours after intranasal virus inoculation, 111 IU/mouse IP), was only able to increase the median survival time by one or two days, and failed to rescue mice from lethal infection.

Beside half-life extension by binding to serum albumin, we also examined the effect of another type of half-life extension. The addition of poly-ethylene-glycol (PEG) to Rab-E8/H7 increases the hydrophobic volume of the VHH and can prolong the half-life of the molecule. Although this method is expected to increase the half-life of the VHH to a similar extent, contrary to the addition of the anti-albumin VHH, PEGylation of the VHH did not have a superior therapeutic effect compared to half-life extended Rab-E8/H7-ALB11 \textit{in vivo}. This could be explained by a likely less effective brain penetration of the PEGylated VHH, due to certain properties of the PEG40 moiety.

The lower protection with PEGylated VHH and HRIG suggests that the increased efficacy of HLE Rab-E8/H7-ALB11 might not be solely due to the increased half-life of the molecule, but also by a superior capacity to pass the blood brain barrier.

7.2.3 The use of neurotropic peptides to target VHH to the brain (Chapter 5)

A major hurdle in the treatment of infections of the central nervous system is the presence of the blood-brain barrier [19]. The blood-brain barrier prevents potentially harmful substances from entering the brain, but also renders treatment of infections of the central nervous system notoriously difficult [20]. Neurotropic pathogens can take advantage of this and have developed ways to reach the brain while leaving the blood-brain barrier intact [21] and could therefore provide tool to develop strategies to better target therapeutics to the central nervous system.

We therefore chose to investigate the use of neurotropic peptides/proteins, derived from neurotropic pathogens, to better target/retain anti-rabies VHH to/in the brain and neurons. Based on a number of criteria like availability, possibility to cross the blood-brain barrier and binding to neurons, we performed a literature search to select three peptides/proteins: the RVG peptide, the (detoxified) tetanus toxin and tet1. The RVG peptide is a 29 amino acid peptide derived from the rabies virus glycoprotein and is the fragment responsible for binding to neuronal cells [22]. This 29 amino acid long peptide was shown to be able to transport siRNA into the central nervous system
from the circulation [23]. The tetanus toxin is a model neurotoxin that consists of two chains linked by a disulphide bridge. Whereas the light chain is responsible for its toxicity, the heavy chain serves as a transporter and is able to travel through the neuronal network [24,25]. Tet1 is a short, 13 amino acid peptide that was discovered using a random phage library and was shown to bind to the same receptor as the tetanus toxin. Since its discovery, it has been successfully used for the delivery of DNA to neuronal cells [26–28].

After the selection of the peptides/proteins, we developed two strategies to link these peptides/proteins to the hetero-bivalent Rab-E8/H7 VHH: the (strept)avidin-biotin linker system and genetic fusion using recombinant DNA technology.

VHH formulated with neurotropic peptides were able to bind to neuronal cells in vitro. Despite several washing steps, the construct remained tightly associated with the cells, whereas VHH linked to an unspecific peptide were unable to attach to or enter the cells. In addition, the neurotropic VHH constructs were still able to fully neutralize free rabies virus in the in vitro virus-neutralisation assay. Although neutralization was complete with the neurotropic VHH constructs, a reduction in neutralizing effect was observed (68.43 IU/nmol for the naked VHH versus 30-47.50 IU/nmol for the different neurotropic VHH constructs). Moreover, treatment of a freshly infected monolayer of cells prevented further (cell-to-cell) spread of the virus. It remains however difficult to determine whether this effect was due to the addition of a neurotropic peptide/protein since unformulated VHH and VHH linked to an unspecific peptide were also able to prevent the spread of the virus in the cell culture.

We were able to demonstrate that the constructs were taken up through the nose in the olfactory bulbs soon after intranasal administration using a streptavidin-horse radish peroxidase linkage and measurement of horse radish peroxidase activity in the brain tissue. As we only had low concentrations of these constructs at our disposal, we opted to only test the intranasal route of administration in tests. Although the constructs were able to reach the central nervous system through the nose, to our surprise, we found an almost equal uptake of streptavidin-horse radish peroxidase lacking the neurotropic component.

We could not observe an added value of the neurotropic VHH compared to ‘naked’ VHH to protect against rabies virus in vivo. In a head-to-head comparison of equal doses of neuropeptide-linked and control VHH, the protective effect was similar. Overall, HLE Rab-E8/H7-ALB11 still provided better protection than the neurotropic constructs. However, these results should be interpreted with caution as we were only able to test relatively low doses of neurotropic VHH in mice. The highest feasible doses administered in these experiments were 10 IU/mouse and 100 IU/mouse for intracerebral and intraperitoneal administrations respectively. In previous experiments with the naked or HLE VHH, much higher doses (231.5-46,300 IU/mouse depending on the route of
administration) were used in mice. Still, we compared to equal amounts of naked VHH and we could not observe improved protection. For sure, we would expect better protection if higher doses could have been used in mice, but it remains uncertain that linkage to the neurotropic peptide/protein would increase protective efficacy at higher doses compared to naked VHH.

The relatively low doses used in these experiments are explained by the fact that the VHH (naked or recombinant with RVG or tet1) were produced in E. coli in a small scale production unit at the Nanobody Service Facility of VIB, whereas for the other experiments VHH were produced in a large scale production facility in yeast by Ablynx. Due to limitations in budget and time we were unable to obtain more concentrated VHH stocks for the experiments with neurotropic peptides/proteins.

We are not sure why the neurotropic peptides did not improve antiviral efficacy. Possibly, different neurons or neuronal compartments are targeted by the neurotropic peptide and the virus, although we do not expect this for RVG. The efficacy of the chosen peptides to transport other molecules into or through neurons might be (too) limited in vivo. As described previously, rabies virus leads to quick invasion of the brain, leaving not much time for the used constructs to catch up with the virus and confer protection.

So far, only a limited number of successful applications have been published for these neurotropic peptides [23,29,30]. This might be due to the limited efficacy of these peptides/proteins to travel within the neuronal network. Most applications involve the transport and transfection of siRNA or DNA into neuronal cell bodies.

We can conclude that in our model and at the doses used, the addition of neurotropic peptides did not have an added value. At comparable (low) doses, addition of the neurotropic peptides/proteins did not prove superior to ‘naked’ VHH. Half-life extension using an anti-albumin VHH therefore remains the best strategy to improve efficacy of anti-rabies VHH in the mouse model.

7.2.4 VHH in combination with vaccine in post-exposure prophylaxis (Chapter 6)

An important aspect of any alternative for rabies immunoglobulins used in post-exposure prophylaxis, is the compatibility with rabies vaccination as both products would be administered simultaneously, albeit at different sites. It is thus important that the combinational treatment of active and passive immunisation proves superior to treatment with either component alone. We therefore used an experimental set-up mimicking post-exposure prophylaxis used in humans. Since the half-life extended Rab-E8/H7-ALB11 proved most effective in previous experiments, we used this VHH for the passive component of post-exposure prophylaxis. In this setting, mice were treated 24 hours after virus inoculation with VHH (1.5 mg/mouse administered intraperitoneally) and vaccine.
(administered intramuscularly), including a booster vaccination three days later. Treatment with vaccine or VHH alone yields respectively 0 and 15% survival, whereas combined treatment with vaccine and VHH significantly increased the survival rate to 60%. In addition, combined treatment also delayed disease onset and prolonged the median survival time in animals that still developed lethal infection. Most likely the synergy between the two components lies in the fact that VHH can immediately delay the spread of the virus, resulting in a prolonged incubation period and allowing more, or sufficient, time for the active immune response to mount. Indeed, treatment with HLE VHH prolongs the incubation period in mice from six to ten days, and the earliest antibody and cellular immune responses can be expected as soon as seven days after intramuscular rabies vaccination [31], which is exactly the purpose of passive immunity as it should span the period between exposure and the mounting of an active immune response. Serological analysis of blood samples showed that an active immune response was induced in all surviving mice.

A major concern when a treatment consists of a combined administration of antibodies and vaccine is interference with vaccine immunogenicity. This inhibitory effect has been observed with passive rabies immunization using immunoglobulins [32,33]. Although antibodies can interfere via different mechanisms, most of the described mechanisms are Fc dependent, like inhibition of the B-cell responses by binding to the Fc-receptor or antigen removal by macrophages [34]. We thus tested whether VHH interfered or not with the development of an immune response. Despite the absence of a Fc domain in VHH, the combined treatment seemed to partially reduce the immunogenicity of the vaccine. Mice treated with anti-rabies VHH and vaccine had significantly lower antibody titers and were less protected against virus challenge four weeks after vaccination. Theoretically, two mechanisms might be responsible for this effect: either via the indirect interaction of anti-albumin VHH and the neonatal Fc receptor or by epitope masking. The half-life extended VHH can interact with the neonatal Fc receptor through binding to albumin, but it is unlikely that this mechanism lies at the basis of the interference since non-half-life extended Rab-E8/H7, which does not present albumin-binding, shows a similar reduction of vaccine efficacy. This leads us to believe that the main mechanism is masking of epitopes on the vaccine virus by VHH, shielding it from recognition by the immune system.

In conclusion, like immunoglobulins, VHH are able to delay the onset of disease in mice and therefore VHH seem able to span the period between exposure and the mounting an active immune response. As the anti-albumin VHH used in these experiments was directed against human serum albumin, we have reason to believe that the half-life of this VHH in humans would be even longer and should be able to guarantee to induce serum neutralizing titers over 0.5 IU/ml for the first 10-14 days, which is the period necessary to induce an active immune response. VHH could therefore serve as valuable alternatives for the currently used (human) rabies immunoglobulins.
7.3 Rabies virus prophylaxis: future perspectives

We showed that VHH can serve as an alternative for immunoglobulins in post-exposure prophylaxis in mice, with the potential advantages that the costs associated with production are lower than those of HRIG, and that there is no longer a need for human donors and no biological risks, typically associated with blood-derived products.

Based on this work, we can recommend to move HLE Rab-E8/H7 forward for further tests including eventually in humans. Indeed, the results obtained in this study suggest that this VHH has high *in vitro* and *in vivo* neutralizing potency. *In vitro*, the 50% inhibitory concentration is 0.91 nM, which is in the typical range of VHH against other targets that previously proceeded to clinical trials in humans [14]. Half-life extended Rab-E8/H7 was also able to cross-neutralize different Lyssavirus species from phylogroup I [15]. Moreover, it is probable to expect that HLE Rab-E8/H7 will have even better pharmacokinetic characteristics in humans than in mice, since the anti-albumin VHH component was originally developed against human albumin and has better affinity for the latter than for murine albumin [16]. In order to further develop these VHH for use in humans, we believe that VHH should be tested first in an animal model which mimics as much as possible the human infection and treatment protocol. This model should therefore be susceptible for infection via the intramuscular route and result in a long incubation period of at least two weeks. It would be best to use one LD₅₀ dose, resulting in a 50% disease/no disease rate, which resembles better the outcome of natural exposure in humans. It would be best to apply the virus into an artificially inflicted cut wound, rather than using a needle to inject the virus intramuscularly. VHH should be infiltrated partly into the wound and partly administrated systemically, which resembles best the current treatment protocol in humans. In 1963, Dean *et al.* used guinea pigs for such a model to emphasize the importance of local wound treatment [7,35]. It remains to be determined which animal model would be best suited for this research, but we would opt for a hamster or guinea pig model as these animals, in contrast to for example canines, remain easy to handle, pose only a limited risk to the people handling the animals and are ethically better acceptable.

When testing these molecules in humans, it should be kept in mind that the major role of rabies immunoglobulins is to span the period between exposure and the mounting of an active immune response, which starts around 10 days after the first vaccine administration. With the expected half-life in humans, one dose of HLE Rab-E8-H7-ALB11 should be able to provide a protective serum titer of ≥0.5 IU/ml for 10 days in humans.

Additionally, the potential interference with the active immune response should also be investigated further in order to optimize the dose for humans. Rabies immunoglobulins are typically administered at a dose of 20 IU/kg. It has been shown that a higher dose (40 IU/kg) of rabies
immunoglobulins interferes with the vaccine-induced immune response. While, a lower dose (10 IU/kg) of rabies immunoglobulins does not induce sufficient levels of serum neutralization [36]. We would suggest to start testing with a minimal dose of 20 IU/kg, which is the standard dose of the current protocol with rabies immunoglobulins, potentially higher doses could also be explored as long as no interference with vaccine immunogenicity is observed. The vaccine response in combination with VHH administration, should be evaluated at different levels: the time of onset of first antibody production, the height of the antibody response at 4 weeks after first vaccination and the height of the antibody levels at later time points after vaccination (e.g. 1-5 years after vaccination), in order to have a complete picture of the interference. Determination of the boostability at later time points could also be considered. It would easily be feasible to use a dose of 20 IU/kg VHH in humans, which translates in a dose of 8.40 mg HLE Rab-E8/H7 for a person of 70 kg. This dose could easily be produced in a relatively small volume (~1 ml). Once the optimal dose of HLE Rab-E8/H7 for human treatment is determined, it can be tested in a clinical context. Since rabies virus infection leads to lethal disease, such a clinical study has to be designed with great caution. Evidently, it is risky to treat people with high risk exposure with an experimental product. We therefore propose to design such a study similarly as was done to test monoclonal antibody cocktails for rabies PEP in humans. In the latter trials, only “low-risk” exposures were treated with the experimental drug blinded with HRIG as control treatment. The distribution of patients into low or high risk categories is based on the location of the bite wound, but other risk factors might also be included into the analysis.

VHH might potentially also be used for the treatment of the clinical phase of rabies, for which so far no treatment option is available [3]. In this thesis, we showed that VHH were effective if sufficiently high doses were able to reach the brain, and that when high doses were administered directly into the brain, disease onset could be delayed and mice could be rescued from fatal disease [37]. However, this treatment only worked before onset of disease symptoms, most likely because disease is associated with high virus titers and it was not possible to administer sufficient amounts of VHH to the brain to neutralize all virus. Experimental work in laboratory animals, has shown that the presence of antibodies in the central nervous system, either produced by plasma cells in situ [38] or by opening the blood-brain barrier and allowing the entry of antibodies from the circulation [39], can successfully clear the infection. In this study, mice could be rescued when VHH were administered directly into the brain during the early stages of disease, but treatment at later stages did not prove effective. It should be taken into account that the VHH could only be administered once in the brain as intracerebral treatment is highly invasive and repetitive administration would be too traumatic for the brain. It might be that a prolonged in situ exposure of high doses of VHH might also be able to rescue mice at an advanced stage of infection and disease. A strategy to provide this type of
prolonged exposure with VHH in the brain might be the use of neurotropic virus vectors expressing VHH genes. Some adeno-associated virus vectors were shown to be able to specifically transfect neurons [39]. In the case of rabies virus infection, it is important that the production of the VHH would start as soon as possible after administration of the viral vector, but it is unclear how long it takes to have sufficiently high production levels in the brain after administration of this vector.

Only polyclonal immunoglobulins derived from plasma of horses or human donors are on the market and short term shortages have occurred in the past in several countries, even in Western countries, posing acute risk for people exposed to the virus during travel or to illegally imported pets or local bats [40]. In Belgium, about 250 patients are treated yearly by the Rabies Centre of the Scientific Institute of Public Health (WIV-ISP) following exposure to a suspect animal abroad or in Belgium (e.g. contact with bats). About 10% of these patients require also treatment with rabies immunoglobulins. In general, the rest of the patients do not qualify, because (i) they were already treated with immunoglobulins abroad, (ii) received the first vaccine 7 days or more before first presentation to the doctor, or (iii) were categorized as a low risk contact (no penetration of the skin or no contact with mucosae). Although the number of patients in Belgium requiring treatment with rabies immunoglobulins is limited (± 25/year), Belgium has encountered on several occasions problems in procuring sufficient amounts of commercial immunoglobulins in the past. The use of anti-rabies immunoglobulins is therefore restricted and rationed only for people at the highest risk. Development of alternatives, such as anti-rabies VHH or monoclonal antibodies, might provide a solution for the long term.

However, the clinical trials to develop such products are time-consuming and expensive and depend on the will of sponsors or big pharmaceutical companies to invest in such a program. Some trials in humans with cocktails of monoclonal antibodies have been initiated [41], but no results have been made public so far.

Following a literature search, we could only find one antibody cocktail that has entered clinical phase II/III [42]. As described above, the patients receiving the experimental treatment were those posing the lowest risk for the development of lethal disease. Although the trial has been finished, no data have been published so far. In the meantime, a short term solution to solve local shortages might also be that non-profit blood donation centres produce purified anti-rabies immunoglobulins from plasma of vaccinated donors. Based on the registers from our rabies serology laboratory, approximately 45% of healthy adults that are vaccinated against rabies for preventive reasons, develop antibody titers above 15 IU/ml, which is the minimal titer needed for successful purification of anti-rabies immunoglobulins from blood [43]. Purified immunoglobulins of about 9 immunized blood donors (>15 IU/ml) could cover treatment of 25 patients. To solve the problem of commercial shortages, a collaboration with the Red Cross, or another company that has the necessary
production units, could allow the installation of a stock at least sufficient to treat people in urgent need in Belgium. Potentially, this could be up scaled to production units for the treatment of exposed individuals in Europe.

On a global scale, a new approach for high scale production of anti-rabies biological is needed that can answer to the specific demands of regions most at risk. Considering the good results of anti-rabies VHH in the mouse PEP model, it would be very interesting to further investigate the potential of these VHH in other models and humans.
Reference List


8.1 Summary

Rabies virus causes an invariable fatal infectious disease in a large range of mammals, including humans. Chapter 1 summarizes the current literature on the rabies virus, including a brief historical overview and the currently existing pre- and post-exposure prophylaxis. Being present on nearly all continents and virtually able to infect all mammals, rabies virus poses a huge treat to humans. Lethal infection can be prevented by either vaccination prior to exposure or by a combined treatment with vaccine and anti-rabies immunoglobulins soon after exposure. Unfortunately, the latter is often unavailable to those most at risk, as the product is scarce and high costs are associated with the production. Therefore the general goal of this thesis was formulated in chapter 2, to develop and validate better alternatives for HRIG to be used for post-exposure prophylaxis of humans, which in the long term might increase the availability of effective post-exposure prophylaxis for people. We focussed on the development and validation of anti-rabies VHH, which is the antigen-binding domain of a heavy chain antibody of camelidae. More specifically the objectives were to (1) first validate the intranasal challenge model to test preventive or therapeutic intervention against rabies virus infection in mice, (2) compare different (monovalent, homo- and hetero-bivalent) VHH in vitro and in vivo, (3) assess the effect of half-life extension to improve the prophylactic effect of anti-rabies VHH, (4) examine whether addition of neurotropic peptides enhance the prophylactic activity and eventually (5) to examine whether the combined treatment of half-life extended VHH and vaccine has an added value compared to single treatment with either compound.

Chapter 3 shows the results addressing the first objective of this thesis; it provides a retrospective, statistical analysis of the intranasal inoculation technique used in this work and compares it to intracranial and intramuscular virus inoculations. This analysis shows not only that
intranasal virus inoculation is a very reproducible technique with a nearly 100 % mortality rate, but also describes the reasons why this technique is used throughout this thesis. This is a needle-free technique which requires only a low dose of virus resulting in a low risk for the operators and in contrast to intracranial inoculation, it does not damage the brain. Despite the low dose used, it shows very little variation in median survival times and mortality rates. This makes it a very useful model to test new treatment approaches which might show only small effects on survival rates.

**Chapter 4** bundles the research addressing the second and third objectives of this thesis; different VHH formats (monovalent, homo- and hetero-bivalent) were compared *in vitro* and *in vivo* and in addition it was investigated whether half-life extension enhanced the prophylactic effect of anti-rabies VHH. Firstly in **chapter 4.1**, the therapeutic potential of different anti-rabies VHH was tested. Due to their simple structure, VHH can easily be formatted into multimeric constructs, which allowed a comparison of the prophylactic potential of monovalent versus homo- and hetero-bivalent constructs. All constructs were able to neutralize the virus *in vitro*, but the results showed that the potency of the VHH constructs increased from monovalent over homo-bivalent to the hetero-bivalent constructs, with the latter having comparable or higher potency (0.14 nM IC$_{50}$) as compared to a rabies monoclonal antibody (0.17 nM IC$_{50}$). Co-administration studies in which low doses of anti-rabies VHH were incubated together with the virus prior to administration to susceptible organs, confirmed these results. The hetero-bivalent Rab-E8/H7 proved most potent in these tests and was therefore selected for further testing in this thesis.

The protective effect of this VHH was tested in pre- and post-exposure settings. Whereas a complete protection was observed in a pre-exposure setting, the outcome of post-exposure treatment with this VHH depended on the route of administration, dose and timing of treatment. Administration directly into the brain one day after virus inoculation could rescue part of the mice at a dose as low as 33 µg/mouse. Administration at later time points or systemic administration failed to protect animals from lethal infection, despite the use of significantly higher doses. Since VHH are very small molecules, their circulatory half-life is short (1.16 hours). This led to the question whether extension of the half-life VHH would increase the protection. In **chapter 4.2**, the effect half-life extension was investigated. Firstly, half-life was prolonged by the addition of a third VHH, directed to serum albumin, to the hetero-bivalent Rab-E8/H7. Systemic administration of this half-life extended VHH resulted in a significant increased survival rate compared to the hetero-bivalent VHH (71.4 % versus 0 % survival). This construct was also compared to PEGylated hetero-bivalent VHH and human rabies antibodies used for rabies post-exposure prophylaxis. Despite the comparable or longer circulatory half-life, neither one proved to be superior to the VHH coupled to an anti-albumin VHH. This indicates that addition of a third VHH directed against serum albumin is a better strategy than pegylation to increase the protective efficacy. Pharmacokinetic data showed that the hetero-
bivalent VHH was cleared from the brain within one day and the half-life extended VHH was
detectable up to ten days after administration. This corresponds with the delay in disease (4-10 days
depending on the dose) onset observed in mice.

Chapter 5 bundles research results addressing the fourth objective of this thesis which
examines whether linking VHH to neurotropic peptides increases the prophylactic efficacy of anti-
rabies VHH. After the selection of a number of neurotropic peptides or proteins, strategies were
developed to link these to the hetero-bivalent VHH. Three different peptides/proteins were included
in this study: RVG29, a 29 amino acid long peptide from the rabies virus glycoprotein responsible for
receptor binding, tet1, a 13 amino acid long peptide binding the same receptor as the tetanus toxin,
and the tetanus anatoxin, a detoxified tetanus toxin. The peptides/proteins were chosen because
they were available to the laboratory, can possibly cross the blood-brain barrier and are able to bind
to neurons. Peptides/proteins were linked using either avidin-biotin linkage or genetically fused to
create neurotropic constructs that were tested in vitro and in vivo. In vitro tests showed that all
neurotropic constructs were able to bind the neuronal cells and were able to neutralize the rabies
virus, albeit with a slight reduction in neutralizing potency, most likely explained by steric hinderance
by the avidin or the neurotropic peptide on the VHH. Unfortunately, these constructs were unable to
prove superior to unformulated VHH in vivo when administered in pre- or in post-exposure settings.
These results should be interpreted with caution as it was not possible to administer these
constructs in high doses. The neutralizing doses were in the range of 7-10 IU/animal, whereas
successful intracerebral treatment with VHH used doses in the range of 46.3-152.8 IU/animal.
Because of technical, budgetary and time limitations higher doses were not feasible in the project.

In chapter 6, we investigated the combination of HLE VHH and vaccine for post-exposure
prophylaxis, as specified in the fifth and final aim of this thesis. As described above, post-exposure
prophylaxis in humans consists of a combined treatment with vaccine and rabies immunoglobulins
soon after exposure to the virus. It was tested whether the administration of half-life extended VHH
in combination with a vaccination schedule was able to increase the survival rates of mice in a post-
exposure setting. The combined treatment of VHH and rabies vaccination, initiated soon after
exposure, increased the median survival and the survival rate of mice significantly compared to
single treatment with either VHH or vaccine. The treatment with VHH increased the incubation
period of the disease, allowing more time for an immune response to mount in response to the
vaccine. Mice that were treated with vaccine alone succumb to the infection before the vaccine
could induce a protective immune response. Despite the superiority of the combined treatment, it
could also be shown that VHH had a partial inhibitory effect on the immune response induced by the
vaccine. Still, this schedule could only offer partial protection This incomplete protection might be
explained by the aggressive nature of the intranasal rabies virus inoculation model.
Chapter 8

The general discussion is provided in chapter 7. The main conclusions of the different chapters are discussed as well as some future perspectives for the application of anti-rabies single domain antibodies.

The most important conclusions that could be drawn from this thesis are:

- Intranasal rabies virus inoculation is a non-invasive, highly reproducible model which results in a high mortality rate and reproducible incubation period, even over time and between different operators.

- Upon intranasal rabies virus inoculation the virus spreads quickly over the different parts of the brain, as soon as one to two days after virus inoculation, the olfactory bulbs of the inoculated mice become infected.

- Anti-rabies VHH are able to neutralize the rabies virus in vitro and in vivo when co-administered with the virus in sensitive organs like the muscle, brain and nose.

- The neutralizing potency of VHH increases strongly when VHH are combined in a homobivalent (single clone) or hetero-bivalent (different clones) format.

- Intracerebral treatment with anti-rabies VHH at doses as low as 33 µg can delay disease onset and partially rescue mice from lethal disease when administered early after virus inoculation, however treatment at later time points or systemic administration proved less effect.

- Half-life extension significantly increased the effectiveness of VHH when administered systemically, increasing the median survival time and survival rate of the treated mice. The effect was superior when the half-life was increased by addition of a third, anti-albumin VHH compared to VHH with an increased half-life by PEGylation.

- Engineering of VHH to increase the neurotropism, by addition of a third component that can cross the blood-brain barrier or travel through the neuronal network, did not prove more effective than unformulated VHH. Results should however be interpreted with caution as only relatively low doses were available to treat animals and treatment with higher doses might be required to see better results.

- The combinational therapy of half-life extended VHH and vaccination, as used in post-exposure prophylaxis, proved superior to treatment with either VHH or vaccination alone.
- Despite the absence of the Fc part in VHH, VHH still interfered to some extend with the development of an immune response upon vaccination, as demonstrated by lower neutralizing antibody responses observed in animals treated at the same time with vaccine and VHH compared to vaccine alone.

- These results provide evidence for the possible use of anti-rabies VHH as valuable candidates for the development of alternative post-exposure treatment drugs for rabies. The ease of production and high thermal stability of VHH are important advantages over the currently used anti-rabies immunoglobulins.
8.2 Samenvatting

Het rabiësvirus veroorzaakt een onbehandelbare, fatale infectie in een hele reeks zoogdieren, inclusief de mens. **Hoofdstuk 1** geeft een samenvatting van wat er momenteel in de literatuur beschreven is over het rabiësvirus, inclusief een historisch overzicht, alsook de huidige status van rabiësprevening vóór of na blootstelling. Omdat het virus op zowat elk continent voorkomt en theoretisch elk zoogdier kan infecteren, bestaat er een risico voor de bevolking. Een dodelijke afloop na besmetting met het virus kan voorkomen worden met behulp van preventieve vaccinatie vóór blootstelling of door een gecombineerde behandeling met vaccin en anti-rabiës immunoglobulinen vlak na de blootstelling. Spijtig genoeg zijn deze laatste vaak niet beschikbaar voor mensen die in risicogebieden leven omdat het product zo schaars is en er hoge kosten geassocieerd zijn aan de productie ervan. Het algemene doel van deze thesis wordt geformuleerd in **hoofdstuk 2** en betreft het ontwikkelen en valideren van betere alternatieven voor humane rabiës immunoglobulinen voor gebruik in post-exposure profylaxis bij mensen. Op langere termijn zou dit de wereldwijde beschikbaarheid van rabiës post-exposure prophylaxis kunnen verhogen. In dit werk werd specifiek gekeken naar de ontwikkeling en validatie van anti-rabies VHH, wat het antigeen-bindend domein van zware keten antilichamen uit kameelachtige dieren is. Specifieke objectieven van deze thesis waren; (1) het valideren van het intranasale inoculatiemodel voor het testen van preventieve of therapeutische interventies tegen infectie met het rabiës virus in muizen, (2) het vergelijken van de werkzaamheid van verschillende (monovalente, homo- en hetero-bivalente) anti-rabiës VHH in vitro en in vivo, (3) het onderzoeken van het effect van halfwaardetijdverlenging op het profylactische effect, (4) het onderzoeken of toevoegen van neurotroppe peptiden aan anti-rabiës VHH de profylactische activiteit verhogen en uiteindelijk (5) nagaan of het gecombineerd gebruik van VHH met een verlengde halfwaardetijd en vaccin beter is dan het gebruik van één van beide componenten alleen voor rabiës prophylaxis in muizen.

**Hoofdstuk 3** behandelt het eerste objectief van deze thesis; het omvat een retrospectieve, statistische analyse van de techniek waarbij muizen intranasaal worden geïnoculeerd met het rabiës virus, en vergelijkt deze met directe intracraniale en intramusculaire virusinoculaties. Deze vergelijking toont niet enkel aan dat het om een zeer reproduceerbare techniek gaat met een mortaliteitsgraad van bijna 100 %, maar laat ook toe om de redenen toe te lichten waarom deze techniek over heel de thesis werd gebruikt. Deze toedieningswijze omzeilt het gebruik van naalden en gebruikt slechts zeer lage virusdosissen, waardoor het risico voor de onderzoekers zeer gelimiteerd is, maar desalniettemin toont deze techniek weinig variatie in mediane overlevingstijden en mortaliteit. In tegenstelling tot intracraniale inoculatie, veroorzaakt het geen schade aan de hersenen. Het is dan ook een goede techniek om nieuwe behandelingen te testen en (kleine) verschillen in de overlevingstijd en kansen aan te tonen.
**Hoofdstuk 4** omvat het onderzoek in verband met het tweede en derde objectief van deze thesis. Het vergelijkt verschillende VHH formules (monovalent, homo- en hetero-bivalent) in vitro en in vivo, en beschrijft de impact van halfwaardetijdverlenging op het profylactisch effect van deze VHH. Eerst en vooral wordt in **hoofdstuk 4.1** het therapeutisch effect van verschillende anti-rabies VHH getest. De eenvoudige structuur van VHH laat toe om ze gemakkelijk om te vormen tot multimere constructen, wat toelaat het profylactisch potentieel van monovalente met dat van homo-bivalent of hetero-bivalent constructen te vergelijken. Alle formularies waren in staat het rabiësvirus te neutraliseren in vitro. De resultaten toonden ook aan dat het neutraliserend effect toename van monovalente, over homo-bivalent, naar hetero-bivalent constructen. Deze laatsten waren in staat om het rabiësvirus te neutraliseren op een gelijkaardig of hoger niveau dan een monoclonaal antilichaam (0.14 nM $IC_{50}$ versus 0.17 nM $IC_{50}$). Co-administratiestudies, waarin lage dosissen van VHH en virus werden vermengd vooraleer toegediend te worden in verschillende lichaamscompartimenten, bevestigden deze resultaten. Het hetero-bivalent Rab-E8/H7 VHH bleek uiteindelijk het meest potent en werd daarom ook geselecteerd voor verdere testen.

Het beschermende effect van dit VHH werd daarop getest door toediening aan muizen vóór of na blootstelling aan het virus. Hoewel er een volledig Bescherming waargenomen werd wanneer de muizen werden behandeld via intracraniale toediening vlak vóór blootstelling aan het virus, was het effect na blootstelling afhankelijk van de route, dosis en moment van toediening van het VHH. Wanneer het VHH onmiddellijk werd toegediend in de hersenen één dag na blootstelling kon een deel van de muizen worden gered. Als het VHH direct werd toegediend aan de hersenen gaf een dosis van 33 μg reeds een significante bescherming bij een deel van de muizen. Wanneer de toediening echter later gebeurde of via systemische toedieningen kon een dodelijke infectie niet worden vermeden, ondanks het gebruik van significant hogere dosissen. Het is echter wel zo dat VHH kleine moleculen (15kDa) zijn die zeer snel uit de circulatie worden gefilterd, waardoor ze een beperkte halfwaardetijd hebben (1.16 uur). Dit leidde tot de vraag of een verlengde halfwaardetijd een positief effect zou hebben op het beschermend effect van deze moleculen. Dit werd onderzocht in **hoofdstuk 4.2**. Eerst en vooral werd de halfwaardetijd verlengd door het toevoegen van een derde VHH, gericht tegen serumalbumine, aan het hetero-bivalent VHH Rab-E8/H7. Wanneer dit VHH systemisch werd toegediend, resulteerde dit in een significante verbetering van de overleving in vergelijking met het hetero-bivalent Rab-E8/H7. Dit construct werd ook vergeleken met gepegyleerde Rab-E8/H7 en commerciële humane antilichamen, gebruikt voor de profylaxis na blootstelling. Ondanks het feit dat deze een vergelijkbare of langere halfwaardetijd hebben in de circulatie, was er bij geen van beide een superieur effect waar te nemen in vergelijking met het Rab-E8/H7 VHH dat gelinkt was aan een derde VHH gericht tegen serumalbumine. Dit suggereert dat het toevoegen van een extra VHH, gericht tegen serumalbumine een betere strategie is dan pegylation.
om de beschermende werking van VHH te verhogen. Uiteindelijk, konden we op basis van farmacokinetiek aantonen dat het hetero-bivalente VHH na een dag niet meer detecteerbaar was in de hersenen terwijl het VHH met verlengde halfwaardetijd tot 10 dagen na toedienen nog steeds kon worden waargenomen. Deze verlenging komt overeen met een vertraging in het uitbreken van de ziektesymptomen (4-10 dagen afhankelijk van de gebruikte dosis), in de muizen.

In hoofdstuk 5 worden de resultaten gebundeld die het vierde objectief van deze thesis bestrijken; namelijk of het linken met neurotrope peptiden het profylactisch effect van anti-rabie VHH kan verhogen. Na een grondige selectie van een aantal neurotrope peptiden en proteïnen, werden twee strategieën ontwikkeld om deze te linken aan het hetero-bivalente VHH Rab-E8/H7. Drie verschillende peptiden/proteïnen werden in deze studie gebruikt: RVG29, een 29 aminozuren lang peptide afkomstig van het rabiesvirus glycoproteïne en verantwoordelijk voor binding aan de receptor, tet1, een 13 aminozuren lang peptide dat dezelfde receptor bindt als het tetanus toxine, en het tetanus anatoxine, een gedetoxifieerd tetanus toxine. De peptiden/proteïnes werden gekozen op basis van de beschikbaarheid voor het laboratorium, het feit dat ze mogelijk de bloedhersenbarrière kunnen kruisen en dat ze aan neuronen kunnen binden.

Peptiden/proteïnen werden ofwel gelinkt door gebruik te maken van het avidine-biotine systeem ofwel door ze genetisch te linken tot een recombinant construct. Uit de in vitro tests bleek dat alle neurotrope constructen aan cellen konden binden en dat ze alle in staat waren het virus te neutraliseren, al was het met een lichtjes lagere potentie in vergelijking met het ‘vrije’ VHH. Dit verschil zou kunnen verklaard worden door de mogelijke sterische hinder veroorzaakt door avidine of door het peptide op het VHH. Ondanks deze veelbelovende resultaten kon er geen superieure bescherming waargenomen worden in vivo wanneer deze werden toegediend voor of na blootstelling aan het virus. De resultaten moeten echter voorzichtig geïnterpreteerd worden aangezien het niet mogelijk was om de constructen in hoge dosissen toe te dienen. De neutraliserende dosissen toegediend lagen in de orde van 7-10 IE/dier, terwijl succesvolle behandeling met vrij VHH in de grootteorde van 46.3-152.8 IE/dier lag. Omwille van technische en technische redenen en de tijdsspan was het niet mogelijk om hogere dosissen te produceren binnen de termijn van dit project.

Hoofdstuk 6 behandelt het gebruik van VHH in combinatie met vaccin zoals beschreven in het vijfde, en laatste, objectief van deze thesis. Voor de behandeling van patiënten wordt gebruik gemaakt van een combinatie van vaccin en rabies immunoglobulines kort na een blootstelling. In dit laatste onderzoekshoofdstuk werd daarom getest of het gecombineerde gebruik van VHH met een verlengde halfwaardetijd en vaccinatie in staat zijn om de gemiddelde overleving in muizen te verlengen. Er kon aangetoond worden dat de gecombineerde behandeling, kort na virus inoculatie, de mediane overlevingstijd alsook het overlevingspercentage significant kon verlengen in
vergelijking met behandeling met VHH of vaccin alleen. De incubatieperiode was significant verlengd in de behandelde dieren, hoogst waarschijnlijk dankzij de toediening van het VHH, wat waarschijnlijk toeliet om een actieve immuunrespons te genereren tegen de infectie. Bij muizen die géén VHH kregen was de incubatieperiode te kort om een beschermende immuunrespons te genereren. Ondanks het superieure effect van de gecombineerde therapie, kon er worden aangetoond dat de gelijktijdige toediening van VHH en vaccin ook een inhiberend effect had op de immuunrespons. De gecombineerde behandeling bood bescherming aan 60% van de muizen, terwijl de rest uiteindelijk (na een verlengde incubatieperiode) stierf. De onvolledige bescherming kan verklaard worden door het agressieve karakter van ons model waarbij het virus onmiddellijk in de nabijheid van gevoelige cellen wordt gebracht.

Een algemene discussie wordt gegeven in hoofdstuk 7. De voornaamste conclusies uit de verschillende hoofdstukken worden besproken en uiteindelijk worden de toekomstperspectieven van anti-rabiës enkelvoudig domeinantstoffen (VHH) besproken.

De voornaamste conclusies die getrokken konden worden uit deze thesis zijn:

- Intranasale virusinoculatie is een niet-invasieve, zeer reproduceerbare techniek die resulteert in een hoge mortaliteit van bijna 100% en een reproduceerbare incubatieperiode, zowel in experimenten gespreid over de tijd als uitgevoerd door verschillende wetenschappers.

- Na intranasale toediening verspreid het virus zich snel doorheen de verschillende delen van de hersenen, reeds één tot twee dagen na virusinoculatie zijn de olfactorische bulbi van muizen geïnfecteerd.

- Anti-rabiës VHH zijn in staat om het virus compleet te neutraliseren in vitro en in vivo wanneer ze gelijktijdig met het virus worden toegeidiend in gevoelige organen zoals de neus, de spieren en de hersenen.

- Het neutraliserend potentieel van VHH stijgt sterk wanneer ze gecombineerd worden tot homo-bivalente (bestaande uit 2 identieke klonen) of hetero-bivalente (bestaande uit 2 verschillende klonen) constructen.

- In infectie-experimenten kan intracerebrale behandeling met anti-rabiës VHH ziekte uitstellen en een deel van de muizen redden van dodelijke infectie wanneer het vroegtijdig na virusblootstelling wordt toegeidiend. Wanneer de toediening gebeurt op
latere tijdstippen of via de systemische weg wordt toegepast is het duidelijk minder effectief.

- Verlenging van de halfwaardetijd van VHH geeft een significante verbetering van de effectiviteit, zeker voor systemische toediening, met een significante verlenging van de mediana overlevingstijd en de overlevingsgraad in muizen. Het effect was meest uitgesproken wanneer de halfwaardetijd werd verlengd door het toevoegen van een derde VHH gericht tegen serumalbumine en minder wanneer deze werd verlengd door PEGylatie.

- Het formuleren van VHH om het neurotropisme te verhogen, door het toevoegen van een derde peptide of proteïne waarvan aangetoond werd dat het de bloed-hersenbarrière kan doorkruisen of doorheen het neuronaal netwerk kan bewegen, bewees niet effectiever te zijn dan ‘vrije’ VHH. De resultaten moeten echter wel voorzichtig geïnterpreteerd worden aangezien enkel relatief lage dosissen beschikbaar waren voor de behandeling van de dieren en dat behandeling met hogere dosissen mogelijk nodig zijn om beter resultaten te bekomen.

- De gecombineerde therapie van VHH met een verlengde halfwaardetijd en vaccin, was superieur in vergelijking met de behandeling met VHH of vaccin alleen.

- Ondanks het gebrek aan Fc gedeelte in VHH was er toch een interferentie met de ontwikkeling van een immuunrespons na vaccinatie. Dit kon worden aangetoond door lagere titers neutraliserend antilichaam in het bloed van muizen die tegelijk behandeld werden met vaccin en VHH in vergelijking met muizen behandeld met vaccin alleen.

- Deze resultaten tonen aan dat anti-rabiës VHH mogelijk gebruikt kunnen worden als alternatief voor de bestaande behandelingen voor rabiës post-exposure profylaxis. Het gemak in productie en de stabiliteit van deze moleculen bij hogere temperaturen bieden een belangrijk voordeel in vergelijking met de huidig gebruikte immunoglobulines.
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Curriculum vitae

Personalia

Publications

Publications in international peer-reviewed journals.


Abstracts and oral presentations


Terryn, S., Francart, A., Rommelaere, H., Stortelers, C., Van Gucht, S. Post-exposure treatment with anti-rabies VHH and vaccine is superior to single treatment with VHH or vaccine to protect mice from rabies. BelVir 2015, December 18th, Brussels, Belgium. Abstract.