THE IMPORTANCE OF VIRUS NEUTRALIZATING ANTIBODIES AND BLOOD-BRAIN BARRIER PERMEABILITY ENHANCEMENT IN CLEARING RABIES VIRUS FROM THE CENTRAL NERVOUS SYSTEM

by

CHIEN-TSUN HUANG

(Under the Direction of Under the Direction of Zhen F. Fu)

ABSTRACT

Rabies has been known as a highly fatal zoonotic disease for centuries. It is widely accepted that there is no effective treatment, and rabies is almost always fatal once neurological symptoms develop. Although the loss of blood-brain barrier (BBB) integrity and infiltration of inflammatory cells have often been associated with pathological changes in the central nervous system (CNS) when infected by viruses, transiently increased BBB permeability during attenuated rabies virus (RABV) infection has been found to be helpful in clearing the virus from the CNS and preventing neurological sequelae. Furthermore, the clearance of attenuated RABV from the CNS was correlated with infiltration of B cells into the CNS, expressing high levels of κ-light chain mRNA. The main goal of this study is to provide a better understanding of factors influencing the pathogenicity of rabies viruses to develop a foundation for developing a possible therapy for clinical rabies. Our results demonstrated that virus neutralizating antibodies (VNAs) were not produced, and the BBB remained intact throughout wild-type RABV infections as previous studies reported. Based on these two major findings

during wild-type rabies infections, we developed a way to clear an established rabies infection from the CNS in a mouse model. This includes passive administration of VNAs and intracerebral injection of monocyte chemotactic protein 1 (MCP-1), a chemokine known to transiently enhance BBB permeability. The treatment was given to mice 5 days after infection with wild-type RABV. The results demonstrated that, even in the absence of B cells, administration of VNA in the periphery could lead to RABV clearance from the CNS and prevent the development of rabies once the BBB permeability is enhanced. In conclusion, VNA was found to be capable of clearing RABV from the CNS in both immunocompetent and immunocompromised mice, as long as the BBB permeability is enhanced.

INDEX WORDS: Rabies virus (RABV), Blood-Brain Barrier (BBB), Virus neutralizing antibody (VNA), monocyte chemotactic protein 1 (MCP-1)

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CHIEN-TSUN HUANG

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CHIEN-TSUN HUANG

Major Professor:	Zhen F. Fu
Committee:	Bridget Garner
	Kaori Sakamoto
	Robert Hogan
	Biao He

Electronic Version Approved:

Julie Coffield
Interim Dean of the Graduate School
The University of Georgia
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DEDICATION

Dedicated to my family members for their unconditional love and endless encouragement and support.

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TABLE OF CONTENTS

Page

LIST OF FIGURES (WITH LEGENDS)

Page

CHAPTER 1

INTRODUCTION

Rabies has been known as a deadly neurological disease for centuries (Jackson 2002) and remains a major threat to public health (Meslin, Fishbein et al. 1994, Martinez 2000). Although there are some rabies-free countries and islands, such as Japan, New Zealand, Portugal, Australia, and Austria, rabies is prevalent in all the continental regions of Asia, Africa, Europe, and America. It is estimated that rabies causes 50,000 deaths each year (Martinez 2000). Currently, post-exposure prophylaxis (PEP), comprised of vaccines and anti-rabies immunoglobulin, is very effective in preventing rabies only if it is initiated promptly after exposure (CDC 2010). It is widely accepted that there is no effective treatment, and rabies is almost always fatal once neurological symptoms develop (WHO 1992, Wilde 2007).

RABVs are believed to bypass the specialized features of the neurovasculature known as the blood-brain barrier (BBB) and enter the CNS via axonal transport directly from peripheral inoculation sites. Unlike humans, in which RABVs may take weeks to reach the CNS from the site of exposure and cause clinical illness, the spread of most RABVs to the CNS in mice is rapid, with virus generally being detectable in CNS tissues within 48 hours of infection (Hooper 2005). Several mouse models of rabies infections have been studied and significant differences between pathogenic and nonpathogenic

strains of RABVs were discovered, which provides more insights into the pathogenicity of RABV. First, induction of neuronal apoptosis by RABV was found to be associated with the expression of the G protein, but inversely correlated with pathogenicity (Sarmento, Li et al. 2005). G-mediated induction of apoptosis limits the spread of attenuated RABVs in the CNS of mice. In addition, death by apoptosis can significantly affect the efficiency of viral antigen capture by antigen-presenting cells and presentation to T cells, thus enhancing adaptive immune responses as well (Mazarakis, Azzouz et al. 2001). Second, attenuated RABVs, but not pathogenic RABVs, induced the expression of the genes involved in the innate immune and antiviral responses, especially those related to interferon-alpha/beta (IFN-α/ß) signaling pathways and inflammatory chemokines (Wang, Sarmento et al. 2005, Kuang, Lackay et al. 2009). Similar findings were also seen in humans who do not usually mount a strong peripheral immune response to natural wild-type RABV infection (Baltazard and Ghodssi 1954). Third, little or no CNS inflammation is observed in rabies patients and mice infected with pathogenic RABV, whereas extensive CNS inflammatory responses and necrosis were observed in mice infected with the attenuated RABV (Murphy 1977, Wang, Sarmento et al. 2005). The absence of significant immune cell infiltration into the CNS was also noted in humans who died from rabies. Fourth, BBB integrity is believed to remain intact during the course of pathogenic RABV infections (Roy and Hooper 2007) until the very end (Chai, He et al. 2014). In contrast, BBB permeability is enhanced during attenuated RABV infections (Wang, Sarmento et al. 2005, Kuang, Lackay et al. 2009). The integrity of the BBB might be an explanation for why immune effectors do not accumulate in the CNS tissues during infection of mice with pathogenic RABV strains, despite the development

of a peripheral virus-specific immune response. These findings suggested that a pathogenic RABV infection can be cleared after the virus has reached the CNS, providing that the appropriate immune effectors can also reach the infected tissues.

Recent studies from Wang, et al. 2011 have demonstrated that treatment with recombinant live-attenuated RABV expressing granulocyte-macrophage colony-stimulating factor (GM-CSF) (LBNSE-GM-CSF) via the intracerebral route 5 days after infection with wild-type viruses can lead to the clearance of not only the attenuated, but also the wild-type, pathogenic virus (Wang, Zhang et al. 2011). Direct intracerebral administration of recombinant RABV stimulated high levels of VNA and enhanced BBB permeability. On the other hand, intracerebral administration with UV-inactivated LBNSE-GM-CSF did not increase protection despite the fact that VNA were induced in the periphery (Wen, Wang et al. 2011). However, administration with monocyte chemoattractant protein-1 (MCP-1, also termed CCL2), known to enhance the BBB permeability (Stamatovic, Keep et al. 2003), significantly increased the protective efficacy of UV-inactivated LBNSE-GM-CSF. These observations confirmed that the lethal outcome of wild-type and pathogenic RABVs is likely due to a lack of virus clearance in the CNS as a consequence of the maintenance of BBB integrity and a weak immune response against RABV. However, direct intracerebral administration of live-attenuated RABV still possesses safety concerns for human use, and investigations of alternative methods with low safety concerns are needed. A better understanding of the factors that influence the pathogenicity of rabies viruses is needed in order to provide the foundation for developing a possible therapy for clinical rabies and to lower the mortality rates of rabies infection.

The present study provides us the opportunity to improve our understanding of the importance of VNA and BBB permeability in RABV clearance and further develop a treatment against an established rabies infection by answering the following questions:

1) Does wild-type RABV infection induce VNA production and BBB permeability change?
2) What mechanism does MCP-1 (a chemokine known to transiently enhance BBB permeability) utilize to enhance BBB permeability?
3) Are passively transferred VNAs able to cross the BBB and also be sufficient to clear the RABV from the CNS once the BBB permeability is enhanced?
4) Are antibody-producing B cells absolutely required to protect mice from lethal street RABV?

In the present study, to answer these questions, VNA were administered intravenously in conjunction with a chemokine, MCP-1 (known to enhance the BBB permeability), into mice after infection with wild-type virus. The results from this study will be beneficial for not only rabies, but also other CNS diseases for which the therapeutic agents must cross the BBB.

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CHAPTER 2

LITERATURE REVIEW

Part 1: The history of rabies

Rabies is one of the oldest diseases known in recorded human history, which can be dated back to the 23rd century B.C. The word "rabies" has variously been attributed to derivation from the Sanskirt *rabhas* ("to rage") or the Latin *rabere* ("to rave"). This word has been used to describe the diseases caused by the RABV since at least 1590 C.E. However, the condition of this disease affecting both human and animals was already documented in the Mesopotamian Codex of Eshnunna (Baer 1991, Baer 2009).

As early as in the 4th century B.C, rabies was stated by Aristotle as capable of infecting a very wide range of animals. Galen (2nd century AD) believed that only dogs were susceptible to rabies. Cardanus, a Roman writer, suggested the infectivity of the saliva of the rabid dog. The writer described the infectious material as "poison", which in Latin was "virus" (Baer 1991). Since the time of Celsus (50 B.C.E), the term "hydrophobia" was introduced and more widely used in the public as uncontrollable aversion to water observed in most rabies victims. The word is derived from the conjugation of the Greek *hydros* ("water") and *phobos* ("dread or fear") (Fleming 1872). Celsus also recommended a treatment by "cupping, burning, sucking or cauterizing the wound, or throwing patients unexpectedly into the pool" (Baer 1991, Baer 2009). Over

subsequent centuries, there was little progress in the knowledge of how to prevent and cure rabies. In the 1880s, microcopy had already been used to study pathogenic agents, and the bacillus of anthrax had been discovered (Smith 2005). However, viruses weren't identified until the invention of electron microscopy in early 1900's.

During 1881, while many scientists were still attempting to transmit rabies from man or animals to animals, Louis Pasteur reported success in demonstrating the neurotropic character of RABV by inducing disease via producing rabies by the injection of central nervous system material and spinal fluid into the brains of other animals. It also proved that the virus was present not only in the saliva of the rabid animal but also throughout its nervous system (Baer 1991, Baer 2009). While Pasteur was bothered by the experimental difficulties of the long incubation period, he later on shortened the incubation period to 7 days in which the virus and incubation period became fixed. He then developed a post-exposure rabies vaccine based the fixed virus, but was reluctant to test it on humans at that time (Baer 1991, Baer 2009).

However, during the Industrial Revolution, the fear of rabies reached peaked as endemic rabies spread rapidly among dogs and humans in London. It was also because of the public fear of rabies that led Pasteur to test his rabies vaccine on humans in 1885 (Baer 1991). Before the Industrial Revolution, rabies had been one of the most dreaded diseases throughout recorded history. However, it was never considered as severe as other transmissible diseases, such as syphilis or smallpox. The virus was never capable of spreading throughout an entire community due to its relatively long incubation period and the prominent clinical indications of infection, such as violent spasms or aggression, which allowed people to avoid close interactions with infected patients. Joseph Meister, a

9-year-old young boy bitten by a rabid dog, was Pasteur's first successful post-exposure rabies vaccine treatment case of humans (Wiktor 1985, Baer 1991). Later, as reported by Pasteur in 1886, only one out of 350 patients who had received the vaccine died (Baer 1991). Vaccination of animals against rabies also began around this period of time. After this success, the Pasteur Institute was built in France in 1888 to treat more rabies victims. By 1903, while most of the histopathologic signs of rabies had been recognized, Dr. Adelchi Negri reported the identification of what he believed to be the etiologic agent of rabies, the Negri body, which has been used as a diagnostic marker for RABV infection (Wunner 2013).

Today, there are still over 55,000 cases of rabies worldwide yearly (Meslin, Fishbein et al. 1994), and most are caused by dog bites (Cleaveland, Kaare et al. 2006). However, vaccination campaigns and quarantine/culling protocols for feral animals, especially dogs, has dramatically reduced the number of rabies cases (Cleaveland, Kaare et al. 2006).

Part 2: Rabies virus

Rabies virus (RABV) is an enveloped, negative-sense, single-stranded RNA virus, which belongs to the Lyssavirus genus of the Rhabdoviridae family. Phylogenetic analysis distinguished the genus *Lyssavirus* as seven genotypes (Bourhy, Kissi et al. 1993). These genotypes were further divided into two major phylogroups, which have distinctive immunogenic and pathogenic properties. Phylogroup I, more virulent than Phylogroup II, includes the worldwide genotype 1 (classic *Rabies virus*), the *European bat lyssavirus* (EBL) genotypes 5 (EBL1) and 6 (EBL2), the African genotype 4

(*Duvenhage virus*), and the *Australian bat lyssavirus* genotype 7. Phylogroup II includes the divergent African genotypes 2 (*Lagos bat virus*) and 3 (*Mokola virus*) (Badrane, Bahloul et al. 2001).

The family Rhabdoviridae and other families, such as Paramyxoviridae, Filoviridae, and Bornaviridae, constitute the order of Mononegavirales, which includes RNA viruses containing non-segmented, negative-stranded RNA genome (Rose 2000). The *Rhabdoviridae* family includes at least three genera of animal viruses, *Lyssavirus, Ephemerovirus, and Vesiculovirus*.

Rhabdoviruses have a typical bullet- or rod-shaped morphology, approximately 180 nm long and 75 nm wide, and a molecular weight of approximately $4.6x10^6$ (Madore and England 1977). The RNA genome of RABV encodes five viral proteins, which are highly conserved in order, namely nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and the viral RNA polymerase (L) (Finke and Conzelmann 2005). The length and structure of N, M, and L proteins are similar among all lyssavirus viruses but P and G proteins vary (Albertini, Ruigrok et al. 2011). The genetic information of the virus is present in the form of a helical ribonucleoprotein (RNP) complex, which contains the RNA genome, tightly encapsidated by the viral nucleoprotein (N), and the RNA polymerase complex which consists of the large protein (L) and its cofactor, the phosphoprotein (P). The RNP is surrounded by a lipid bilayer containing an inner layer of M protein and the transmembrane G proteins (Mebatsion, Weiland et al. 1999).

N protein is the most conserved among viral proteins of the lyssaviruses (Marston, McElhinney et al. 2007). Phosphorylation of N during viral RNA (vRNA) or complementary viral RNA (cRNA) is important in regulate vRNA transcription and

replication. Moreover, recent studies suggested that pathogenic determinants within N proteins affect the ability of RABV to evade host immune responses, which favors efficient propagation and spread of virus leading to lethal outcomes of infection (Masatani, Ito et al. 2011).

Both L and P proteins are involved in the transcription and replication of the genomic RNA (Schnell and Conzelmann 1995, Gupta, Blondel et al. 2000). The L protein is the RNA-dependent RNA polymerase. . In addition to polymerization of RNA, it is also responsible for mRNA capping, methylation, and polyadenylation (Schnell and Conzelmann 1995). The P protein is a non-catalytic cofactor, and a regulatory factor together by stabilizing the RNP complex, preventing its aggregation and maintaining a suitable form for specific encapsidation of viral RNA (Vidy, El Bougrini et al. 2007). The P protein is also largely responsible for antagonizing host IFN-induced antiviral response by interacting with STAT1 (Vidy, Chelbi-Alix et al. 2005) and promyelocytic leukemia protein (PML), (Blondel, Regad et al. 2002) and impairing interferon regulatory transcription factor-3 (IRF-3) phosphorylation (Brzozka, Finke et al. 2005). Diverse functions of P protein may be associated with its polymorphism of protein expression through phosphorylation modifications by two major kinases, RABV protein kinase and protein kinase C (Gupta, Blondel et al. 2000, Vidy, El Bougrini et al. 2007). Besides P protein, the P gene also encodes four other amino-terminally truncated products (P2, P3, P4, P5)(Chenik, Chebli et al. 1995). The cytoplasmic localizations of P and P2 are due to the N-terminal location of the nuclear export signal (NES), whereas the nuclear localizations of P3, P4 and P5 are due to the C-terminal location of the nuclear localization signal (NLS)(Vidy, El Bougrini et al. 2007).

The M protein plays an important role in virus assembly/budding by recruiting RNPs to the cell membrane, condensing into tightly coiled structures and budding enveloped virus particles (Mebatsion, Weiland et al. 1999, Finke, Mueller-Waldeck et al. 2003, Komarova, Real et al. 2007). In addition, it also regulates the balance of virus transcription and replication by inhibiting translation of capped polyadenylated mRNAs in virus-infected cells in vitro through a protein–protein interaction with the cellular translation machinery (Finke, Mueller-Waldeck et al. 2003, Komarova, Real et al. 2007).

The G protein is a trimeric type I membrane glycoprotein, which forms a spike extending from the viral membrane (Whitt, Buonocore et al. 1991). It is responsible for cellular recognition and low pH-induced fusion of the viral envelope with the endosomal membranes (Whitt, Buonocore et al. 1991, Gaudin, Ruigrok et al. 1993, Maillard and Gaudin 2002). It is also the major antigen target for production of virus-neutralizing (VNA) and protective antibodies (Cox, Dietzschold et al. 1977, Flamand, Raux et al. 1993). Two major antigenic sites (site II and site III), one minor site (site a), and several isolated epitopes have been identified (Benmansour, Leblois et al. 1991, Lafay, Benmansour et al. 1996, Maillard and Gaudin 2002). In wild-type RABVs, G proteins are generally expressed at relatively low levels compared to attenuated RABV, which likely enables the virus to escape immune recognition during infection and contributes to RABV pathogenesis by preventing neuronal apoptosis (Morimoto, Hooper et al. 1999, Sarmento, Li et al. 2005).

Part 3: Animal Reservoirs of the RABV

Rabies is a fatal disease that is considered a reemerging zoonosis throughout the world. Virus reservoirs are generally grouped into terrestrial species, such as Carnivora, and bat species (Rupprecht, Hanlon et al. 2002, Cleaveland, Kaare et al. 2006). Every year over 7 million people are exposed to RABV, and an estimated 55,000 people die from the infection. In comparison to other viruses, RABV infection kills more people than many diseases, such as yellow fever, dengue fever and Japanese encephalitis (Coleman, Fevre et al. 2004, Knobel, Cleaveland et al. 2005). Although RABV has been isolated from nearly all mammals, domestic dogs remain the source of infection for the vast majority (>95%) of cases of human rabies worldwide. This is especially true in developing countries, such as Africa and Asia, despite the availability of vaccines (Knobel, Cleaveland et al. 2005, Cleaveland, Kaare et al. 2006). Children are also represent more cases of rabies given their higher probability of being bitten by dogs when compared to adults (Pancharoen, Thisyakorn et al. 2001).

A major factor in the failure of rabies control in developing countries is the low level of political commitment arising from a lack of quantitative data on the true public health impact of the disease (Knobel, Cleaveland et al. 2005) and substantial economic burden of controlling it (Knobel, Cleaveland et al. 2005, Cleaveland, Kaare et al. 2006). In the United States, dog rabies has been largely brought under control through pet vaccination programs, and the number of human cases has declined dramatically during the past 60 years (Hampson, Dushoff et al. 2009). However, other domestic animals that are in contact with both humans and wildlife, such as cats, can transmit the virus from a reservoir species to humans and should be considered as potential vectors with exposures.

Cats have gradually replaced dogs as the most common reported rabid companion animals in the United States (Hanlon 2013).

The etiological agents of the lethal rabies infections, RABV, are found extensively in nature, each associated with diverse reservoir species in different geographic locations (Kissi, Tordo et al. 1995). In Europe, rabies emerged in red foxes (*Vulpes vulpes*) following a presumed spillover from domestic dogs. Within a few decades, the virus was circulating among foxes across much of Central and Western Europe, and the fox became an important reservoir of rabies in Europe (Ramsey 2008, Hooper, Roy et al. 2011, Freuling, Hampson et al. 2013). However, rabies in foxes has been largely controlled after the implementation of oral rabies vaccination (ORV) (Freuling, Hampson et al. 2013). In the United States, most of the human cases have been associated with unrecognized exposure to rabid bats, particularly two relatively rare bat species, silver-haired bats (*Lasionycteris noctivagans)* and Eastern pipistrelle *(Pipistrellus subflavus*) (Morimoto, Patel et al. 1996, Messenger, Smith et al. 2003). Although bat rabies is scattered widely and not limited to any specific areas, human rabies cases caused by rabies of these two species tend to cluster in the southeastern and northwestern United States (Messenger, Smith et al. 2003). In North America, besides bats, variants of RABV are also maintained by several other terrestrial species such as raccoon (*Procyon lotor),* striped skunk (*Mephitis mephitis*), coyote (*Canis latrans*; infected with the dog variant*)*, gray fox (*Urocyon cinereoargenteus),* Arctic fox (*Alopex lagopus),* red fox (*Vulpes vulpes*) (Woldehiwet 2002, Hanlon 2013). There is an association between geographic distribution of each antigenically and genetically distinct variant of RABVs and its reservoir species in the United States (Krebs, Mondul et al.

2001). Raccoon rabies is most commonly seen in the eastern United States and is attributed to the interstate translocation of rabies-infected raccoons to the mid-Atlantic region in the late 1970s (Baer 1975, Smith, Sumner et al. 1984, Coyne, Smith et al. 1989, Guerra, Curns et al. 2003). In comparison, skunk rabies is predominantly found in the north- and south-central states. Although all species of North American skunks are susceptible to rabies, the striped skunk is the most common species in areas of enzootic skunk rabies (Baer 1975). Therefore, skunk rabies often refers to the disease in striped skunks. The Arctic and grey foxes have distinct associated RABV variants and are especially important in polar and western American regions. Coyotes, on the other hand, are susceptible to a number of RABV variants, including raccoon rabies, gray fox rabies, skunk rabies, etc. Within the last two decades, several rabid coyotes were detected in southern Texas (Hanlon 2013). The South America, the main sources of rabies are vampire bats (Baer 1991). These bats are responsible for severe economic losses in cattle, causing paralytic rabies, and a limited number of human infections. However, dogs still remain the main vector of most human infections throughout Latin America (Acha and Malaga-Alba 1985). Although it has been assumed that almost any warm-blooded animal might be carrying rabies, no documented case of human rabies has ever been traced to small rodents (gerbils, chipmunks, guinea pigs, squirrels, rats, and mice) and lagomorphs (rabbits and hares) in the United States (Hankins and Rosekrans 2004). It has been proposed that they usually cannot survive an initial attack by a rabid animal. However, there are anecdotal reports of rabies transmitted by rats in Thailand (Kamoltham, Tepsumethanon et al. 2002) and by bandicoots in Sri Lanka.

Part 4: Life cycle of RABV infection

The RABV life cycle can be divided into three main phases. The early phase includes attachment to the host cell receptors, entry of the host cell by endocytosis, endosomal membrane fusion, virus particle uncoating, and helical RNP core release. The middle phase includes viral genome transcription, translation and replication within the host cell. The final phase includes virus assembly and budding, which releases the virus for subsequent infections (Finke and Conzelmann 2005, Wunner 2013). Unfortunately, the RABV behaves differently *in vivo* and *in vitro* in RABV susceptibility of cells, making the study of these events difficult, especially for the early phase, which has been studied in various cell culture systems (Wunner 2013).

RABV infection is initiated by virus attachment to a receptor molecule or cellular receptor unit (CRU) on the host cell surface (Reagan and Wunner 1985, Broughan and Wunner 1995, Wunner 2013).. No definitive receptor molecule for RABV attachment has found even after several plasma membrane molecules, such as lipids, gangliosides, and proteins, were studied (Conti, Hauttecoeur et al. 1988, Broughan and Wunner 1995). In addition to these molecules, three neuronal CRUs including nicotinic acetylcholine receptor (AchR) (Lewis, Fu et al. 2000), the neural cell adhesion molecule (NCAM) CD56 (Thoulouze, Lafage et al. 1998), and the low-affinity nerve-growth factor receptor (p75NTR) (Langevin and Tuffereau 2002), have been proposed to play the role of viral receptors. It has been suggested that RABV may utilize more than one receptor to initiate infection (Wunner 2013).

After binding to its cellular receptor(s), RABV penetrates into the cell through an endosomal transport pathway (Le Blanc, Luyet et al. 2005) or clathrin-coated pits, like

VSV (Tsiang, Derer et al. 1983). The transmembrane G protein mediates both receptor(s) recognition and membrane fusion. Fusion is triggered by the structural rearrangement of G protein induced under an acidic environment of the endosomal compartment (pH 6.3-6.5) (Gaudin, Ruigrok et al. 1993, Albertini, Baquero et al. 2012). Given the pH-dependent fusion activation, it has been assumed that there are at least three structurally distinct conformational states of G protein that possess different biochemical properties (Albertini, Baquero et al. 2012). Prior to fusion, the G protein is thought to be in a native state and binds to viral receptor(s) when the pH of viral surface is above 7 (Albertini, Baquero et al. 2012). Upon internalization, G is activated and exposes its hydrophobic domain to interact with the hydrophobic endosomal membrane (Albertini, Baquero et al. 2012, Wunner 2013). Once inside the low pH endosomal compartment, the G conformational shape changes. This exposes its fusion domain and allows it to interact with the target cell membrane. After fusion, the G protein adopts a fusion-inactive conformation, which is then highly sensitive to cellular proteases (Wunner 2013).

In the middle phase of RABV infection, viruses undergo an uncoating process by dissociating M protein from RNP which leads to the uncoiling of RNP (Wunner 2013). Transcription of viral genome RNA is then carried out on the negative-sense RNP by the viral RNA polymerase (the L–P polymerase complex) within the cytoplasm of infected cells (Albertini, Ruigrok et al. 2011). It is still unclear how the polymerase complex gains access to the viral RNA (vRNA). It is proposed that polymerase either recognizes a promoter at the 3'-end of the genome or resumes transcription at the next downstream internal mRNA start site on the viral genome (Albertini, Ruigrok et al. 2011, Wunner 2013). Subsequently, an positive-stranded leader RNA and five mRNAs coding for the N,

P, M, G, and L are synthesized during transcription. Following viral protein synthesis, viral transcription is switched to replication, which requires ongoing protein synthesis, especially N protein, which involves N-encapsidation-dependent synthesis of full-length positive strand cRNAs complementary to the complete negative strand vRNAs (Albertini, Ruigrok et al. 2011, Wunner 2013). Interestingly, the production of full-length genomic and antigenomic RNAs is disproportionate with up to a 50-fold excess of genomes over antigenomes, which possibly could be attributed to a stronger genomic promoter successfully competing with the anti-genomic promoter for polymerase (Finke and Conzelmann 1997). However, transcription of mRNAs remains the major mode of RNA synthesis throughout infection given that the viral mRNAs are always more abundant than vRNAs (Wunner 2013).

Accumulation of proteins can lead to the formation of cytoplasmic inclusion bodies, which have long been known as Negri bodies in neurons. Negri bodies are predominantly composed of viral N and P proteins, but contain viral RNAs (genome, antigenome, and every mRNA), which suggest that active viral transcription occurs within Negri bodies as well (Lahaye, Vidy et al. 2009).

The process of virus assembly begins with encapsidation of the viral progeny RNA and formation of RNP complex, vRNA-N-P, which requires sufficient production of viral N, P, and L proteins (Okumura and Harty 2011, Wunner 2013). M protein is the next viral protein to interact with the newly forming RNP structures in the cytoplasm. In addition to altering the structure of the RNP complex by condensing it into a tightly coiled form, M protein is also able to stimulate replication and inhibiting transcription through the association with newly-formed transcriptionally active RNP (Lyles and

McKenzie 1998, Finke, Mueller-Waldeck et al. 2003, Okumura and Harty 2011, Wunner 2013). Then, in preparation for budding, M protein accumulates on the cytoplasmic side of G-enriched microdomains on the plasma membrane as the RNPs condense into tightly coiled structures by interacting with M protein (Mebatsion, Weiland et al. 1999, Okumura and Harty 2011). To develop mature virions, M-RNP structures bud through the host cell membrane to obtain a lipid bilayer envelope (Wunner 2013). M-RNP can also bud through the cytoplasmic ER or Golgi apparatus to form virions and eventually be secreted from the host cell through the secretory pathway (Finke, Granzow et al. 2010, Wunner 2013).

In addition, like other viruses, RABV can also generate defective interfering (DI) particles. Although DI particles have shortened forms of viral genomes that have generally lost all essential viral genes for replication and encapsidation (Baer 1991), there has been indirect evidence that DI particles may be associated with attenuation of virus infection in some animal systems (Wiktor, Dietzschold et al. 1977, Bangham and Kirkwood 1990, Roux, Simon et al. 1991).

Part 5: Rabies pathogenesis and pathology

RABV is a highly neurotropic virus that causes an acute infection by invading the CNS through the peripheral nervous system. There are several sequential steps after the initial peripheral inoculation of RABV: 1) peripheral tissue replication, 2) spreading along peripheral nerves before reaching the spinal cord and brain, 4) dissemination within the CNS, and 5) centrifugal spread to various organs along nerves.

Although multiple routes of transmission have been reported for RABV, natural infection most frequently occurs via a bite. Previous experimental animal studies have proposed the idea that RABV remains at or near the site of inoculation for most of the incubation period (Baer and Cleary 1972). Muscle fibers may be a critical step for the virus to gain access to the peripheral nervous system (Jackson 2013). However, given their short incubation time, these experimental models provide little information about conditions that truly occur during the long incubation period of natural rabies infection in humans and animals (Jackson 2013). For human rabies cases without a recognized exposure history, mostly caused by silver-haired bat RABV (SHBRV), a superficial virus inoculation (rather than deeper tissue inoculation) is highly suspected since SHBRV was found associated with higher infectivity in cell types present in the dermis (Jackson 2013). However, it is still unclear how or from where the virus invades peripheral nerves after superficial exposures. In addition to bites, oral transmission, such as consumption of RABV-infected carcasses, might contribute to rabies in wildlife (Baer 1975), and contact of RABV with mucous membranes was found to be another possible route of transmission (Anderson, Williams et al. 1984). Iatrogenic human-to-human transmission of rabies through organ transplantation has also been well documented. At least eight cases of human rabies have resulted from RABV-infected cornea transplantation (Gode and Bhide 1988, Javadi, Fayaz et al. 1996, Jackson 2013). Rabies transmissions through transplantation of other organs, such as liver, kidney, lung, and iliac artery segment, have also been reported to cause death in humans (Burton, Burns et al. 2005, Srinivasan, Burton et al. 2005, Maier, Schwarting et al. 2010). In certain, unusual circumstances, such as laboratory accidents by releasing aerosolized RABV (Winkler, Fashinell et al.

1973) or aerosolized RABV in caves with large numbers of bats (Constantine 1962), transmission via aerosol can occur.

The RABV may enter the peripheral nervous system via the neuromuscular junctions. The RABV G protein is thought to be important in binding to neurospecific receptors. Three neurospecific receptors, nicotinic acetylcholine receptor (nAChR), neuronal cell adhesion molecule (NCAM), and p75 neurotrophin receptor (p75NTR), have been proposed to mediate RABV entering into nerve endings (Tuffereau, Benejean et al. 1998, Lafon 2005, Jackson 2013). Among these putative receptors, NCAM has the most wide-spread distribution in the nervous system, including presynaptic membranes and neuromuscular junction. RABV entry is facilitated through the internalization of RABV-NCAM receptor complexes by adsorptive endocytosis (Lafon 2005). Unlike NCAM, the CNS distribution of p75NTR and nAChR is not sufficiently ubiquitous. The nAChR cannot mediate virus uptake given its mainly postsynaptic location. However, it may improve the probability of uptake by concentrating viral particles in front of the neuromuscular junction (Lafon 2005). Although p75NTR is not present at the neuromuscular junction, its location at the dorsal horn of spinal cord suggests a possible role in trafficking of RABV by a sensory pathway (Lafon 2005). It was also speculated to participate in retrograde transport of RABV by forming the RABV- p75NTR complex that is transported into the cell following transcytosis (Lafon 2005).

Centripetal spread of RABV to the CNS occurs within motor and perhaps also sensory axons of peripheral nerves, although previous animal models disagreed with the importance of sensory pathway in virus transport (Jackson 2013). During this spread, RABV is believed to utilize fast axon retrograde transport. A transport rate of between 50

and 100 mm/day was reported in human dorsal root ganglia neurons in a cell culture system (Tsiang, Ceccaldi et al. 1991). Although RABV P protein was suggested to be important in axonal transport of RABV by interacting with dynein light chain 8 (LC8), studies showed that mutated dynein LC8 only led to minor effects on viral spread after peripheral inoculation of mice (Rasalingam, Rossiter et al. 2005). G protein was suggested by other studies to play a more important role than P protein in retrograde axonal transport (Mazarakis, Azzouz et al. 2001).

Once CNS neurons become infected, there is rapid dissemination of RABV along neuroanatomical pathways. As in the peripheral nervous system, RABV also spreads within the CNS by fast axonal transport. Studies showed that the deficiency of G protein limited the spread of RABVs in the brains (Etessami, Conzelmann et al. 2000). Therefore, rabies G protein is important and necessary for trans-synaptic spread of RABV from one neuron to another. Once the virus reaches the brain, it rapidly causes encephalitis and symptoms to appear.

RABV can be transported centrifugally to many peripheral tissues and organs, such as respiratory tract, cornea, liver, pancreas, cardiac and skeletal muscles, adrenal medulla, and renal parenchyma (Jackson 2013). Extremely high viral titers can also reach the acinar cells of the salivary glands through efferent secretory nerves. Rabid patients develop severe agitation, depression, hydrophobia, and paralysis followed by impaired consciousness and coma. Patients eventually die of circulatory insufficiency, cardiac arrest, and respiratory failure.

The neuropathological changes of the CNS in rabies victims is frequently unremarkable or relatively mild, with a variable degree of nonspecific changes similar to

those seen in other viral encephalitides, such as cerebral edema, meningeal vessel congestion, hemorrhage, mononuclear cell infiltration of the leptomeninges, perivascular cuffing, and microglial activation (Jackson 2013). Although some unique findings for rabies infections, such as Negri bodies and eosinophilic viral inclusions, can be found in many cases, the extent of rabies infection of the CNS is best highlighted by immunostaining for RABV antigen.

The minimal neuropathological changes of the CNS led to a hypothesis that rabies results from neuronal dysfunction rather than structural damage (Tsiang 1982). Studies suggest dysregulation of neurotransmitters and ion channels or altered host immune responses as the cause of clinical symptomatology, but the cause of invariable fatality remains uncertain (Suja, Mahadevan et al. 2011, Wunner 2013). A variety of studies of RABV infection in experimental animals and *in vitro* experiments have provided evidence that RABV may cause defects in neurotransmission, acetylcholine, serotonin, or γ-amino-*n*-butyric acid (GABA) levels (Wunner 2013). RABV may also alter electrophysiological properties of the neuron, functions of ion channels and nitric oxide production (Gourmelon, Briet et al. 1986, Gourmelon, Briet et al. 1991, Koprowski, Zheng et al. 1993, Iwata, Unno et al. 2000). However, the precise mechanism of neuron dysfunction and pathogenesis in RABV infection is still poorly understood and more studies are warranted.

Part 6: Factors affecting the pathogenicity of rabies virus

Unlike natural rabies infections, which only exhibiting minimal neuropathological changes, experimental infection with attenuated viruses, on the other hand, results in

extensive changes, such as inflammatory cell infiltration and apoptosis, in the CNS. Several animal models of rabies infections have been studied and significant differences between pathogenic and nonpathogenic strains of RABV were discovered, which provided more insights into rabies pathogenesis.

Apoptosis

In many viral infections, the destruction of infected cells by apoptosis can be either a mechanism for the release and dissemination of progeny virions or a defense strategy of multicellular host organisms for the destruction of infected cells and therefore preventing the spread of the virus (Allsopp and Fazakerley 2000, Baloul and Lafon 2003, Wunner 2013). Apoptosis can occur either through the mitochondrial (intrinsic) pathway within the infected cells or by the action of cytotoxic T lymphocytes, which induce the apoptosis of infected cells by activating Fas or triggering the release of cytokines and other cellular enzymes (Baloul and Lafon 2003). Viruses can facilitate their dissemination and promote infection by developing anti-apoptotic strategies, such as inhibition of the activation of caspases, encoding analogues of anti-apoptotic proteins, evading cytotoxic T lymphocytes attack, etc. (Baloul and Lafon 2003). Therefore, the role of apoptosis in the pathogenesis of naturally-occurring and experimentally-induced rabies infection has been investigated in considerable detail. Thoulouze et al. observed an inverse correlation between the induction of apoptosis and the capacity of a RABV strain to invade the brain, suggesting that inhibition of apoptosis could be a strategy employed by neurotropic virus to favor its progression through the nervous system (Thoulouze, Lafage et al. 2003). Similar findings were also reported in animal models or fatal human

rabies cases. Jackson et al. demonstrated the absence of neuronal apoptosis through TUNEL staining and activated caspase-3 immunostaining of neurons in the brains of fatal cases of human rabies encephalitis (Jackson, Randle et al. 2008). For animal models, wild-type virus SHBRV infection did not induce apoptosis in the spinal cord, and virus spread occurred to the brain. On the other hand, the laboratory attenuated CVS-B2C induced neuronal apoptosis in the spinal cord and was associated with the failure of virus spread to the brain and absence of neurological disease (Sarmento, Li et al. 2005). Apoptosis was further proved to be induced through both caspase-dependent and caspase-independent pathways. This hypothesis is supported by activation and up-regulation of caspases, including caspases 8 and 3, and apoptosis-inducing factor, respectively (Sarmento, Tseggai et al. 2006).

Induction of neuronal apoptosis by RABV was found to be associated with the expression of G protein, but inversely correlated with pathogenicity. These findings suggested that the laboratory attenuated, but not the wild-type RABV, induces apoptosis and the induction of apoptosis is mediated by viral glycoprotein (Sarmento, Li et al. 2005). However, the replacement of the proapoptotic G gene with a nonapoptotic G gene in CVS-NIV was sufficient to prevent destruction of the infected cells by apoptosis. Therefore, data suggest that commitment to apoptosis or survival depends primarily on determinants in the sequence of the G protein and is largely independent of the level of transcription or replication (Prehaud, Wolff et al. 2010). RABV neuroinvasiveness may be favored by the capacity of its G protein to promote survival signaling in the infected neuron. In addition, sequestration of Toll-like receptor-3 (TLR3) into Negri bodies has also been proposed as a possible strategy for RABV to protect the infected neuron against

apoptosis (Lafon 2011). The absence of neuronal apoptosis during pathogenic RABV infection significantly lowers the efficiency of viral antigen capture by antigen-presenting cells and presentation to T cells, thus limiting the induction of adaptive immune responses (Mazarakis, Azzouz et al. 2001).

Moreover, despite high levels viral antigens in the CNS indicated by immunocytochemistry and the lack of neuron apoptosis, migrating T cells underwent apoptosis during pathogenic RABV infections (Baloul and Lafon 2003, Baloul, Camelo et al. 2004). In contrast, T cell apoptosis was not observed during attenuated RABV infections (Galelli, Baloul et al. 2000). RABV facilitates the apoptosis of T cells by up-regulating the expression of molecules, such as FasL and B7-H1, in the nervous system. In mice lacking functional FasL or B7-H1, pathogenicity was drastically attenuated, indicating the critical role of this mechanism for RABV neuroinvasiveness.

Therefore, rabies neuroinvasiveness has been suggested as a result of three factors: 1) The absence of neuronal apoptosis facilitating intraneuronal survival and replication, 2) Apoptosis in inflammatory cells preventing elimination of the virus, and 3) Abrogation of host inflammatory response (Suja, Mahadevan et al. 2011).

The innate immunity

The innate immune response is the first line of defense against infectious agents before the adaptive immune response is established and involves the release of interferons, chemokines, cytokines, and the attraction of macrophages, NK cells, etc. Therefore, the ability of the virus to evade the innate immune response is crucial to the development of disease in a susceptible host.

The interferon system

The interferon (IFN) system serves as a defense mechanism, which activates both innate and adaptive immunity. Type I interferon (IFN-α/β) is produced in response to virus infection in most cell types, including neurons, and upon recognition of conserved exogenous pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) (Rieder and Conzelmann 2011). Unlike IFN-α/β, the expression of IFN-γ is mostly confined to activated immune cells, such as T cells and NK cells, although some neurons can also produce IFN-γ. Toll-like receptors (TLRs) and retinoic acid inducible gene (RIG)-like receptors (RLRs), namely, namely RIG-I (retinoic acid-inducible gene I) and Mda-5 (melanoma differentiation-associated gene 5), are important PRRs for the recognition of viral dsRNAs and ssRNAs. RABV is known to trigger RIG-I and Mda-5 mediated antiviral IFN response by detecting the 5'triphosphate base pairing of the viral genome (Brzozka, Finke et al. 2005, Kawai, Takahashi et al. 2005, Pichlmair, Schulz et al. 2006, Rieder and Conzelmann 2011). Although the MyD88-dependent TLR that is most likely to recognize rabies viral RNA is TLR7, which would recognize single-stranded RNA translocated into endosomes, TLR7-dependent activation of IFN expression by RABV was not observed in isolated human pDC and mouse $TLR7^{-/-}$ DC (Hornung, Schlender et al. 2004, Faul, Wanjalla et al. 2010). IFN-α/β and IFN-γ act through binding to the IFN-α/β receptor (IFNAR) and IFN-γ receptor (IFNGR), respectively, in an autocrine or paracrine fashion to activate JAK/STAT-mediated signal transduction pathways. IFNAR signaling involves tyrosine phosphorylation of STAT1 and STAT2 by TYK2 and JAK1, and formation of a heteromeric complex, known as IFN-stimulated gene factor 3 (ISGF3), which contains STAT1, STAT2, and IFN

regulatory factor 9 (IRF-9). IFNGR signaling involves tyrosine phosphorylation of STAT1 by JAK1 and JAK2, and formation of STAT1 homodimers, known as gamma-activated factor. ISGF3 and gamma-activated factor promote the expression of genes controlled by the interferon stimulated response elements (ISRE) and the gamma-activated sequences (GAS), respectively.

The ability of RABV to antagonize IFN production was indicated in studies in which attenuated RABV infection induced stronger up-regulation of various interferon-stimulated genes (ISGs) than wild-type RABV infection in mice (Wang, Sarmento et al. 2005). RABV P and N were then identified as the responsible IFN antagonist through counteracting transcriptional induction of IFN, IFN-induced STAT signaling, or the functions of antiviral proteins (Brzozka, Finke et al. 2005, Brzozka, Finke et al. 2006). Several studies suggested that P and N proteins facilitate inhibition of RLR-mediated IFN induction (Brzozka, Finke et al. 2005, Marschalek, Finke et al. 2009, Masatani, Ito et al. 2011). In cells infected with wild-type RABV (SAD L16), activation of IRF3 was hardly detectable. On the other hand, recombinant RABV expressing reduced amounts of P protein led to strong activation of IRF3 and IFN induction, and most importantly, attenuation in pathogenicity. P proteins lacking specific amino acids were severely compromised in counteracting phosphorylation of IRF3 and IRF7 by TBK1 or IKKi (Rieder, Brzozka et al. 2011). Therefore, phosphorylation of both IRF3 and IRF7 can be inhibited by P protein. In addition, P protein was found responsible for preventing IFN-α/β and IFN-γ-stimulated JAK-STAT signaling. P protein interacts with phosphorylated STAT1/STAT2, and retains activated STATs in the cytoplasm leading to the blockage of nuclear import and transcription of ISGs (Brzozka, Finke et al. 2006). For the N protein,

amino acids at positions 273 and 394 were found to be important for both evasion of host RIG-I-mediated antiviral response and pathogenicity (Masatani, Ito et al. 2011). Lastly, promyelocytic leukemia (PML) protein, also known as the tripartite motif protein 19 (TRIM19), may also play a role in IFN-induced antiviral activities against RABV (Blondel, Kheddache et al. 2010).

Chemokine expression

Chemokines are small (8–10 kDa) proteins, expressed by almost all types of nucleated cells and in charge of mediating leukocyte activation and/or chemotactic activities. Viral infections of the CNS can result in the expression of several chemokines and chemokine receptors by infiltrating inflammatory cells and also CNS resident cells, most importantly astrocytes and microglia. The expression of chemokines and their receptors can be associated with either neuroprotective or neuropathologic effects, depending largely on attracting T lymphocytes and macrophages (Dorries 2001, Lin, Tripathi et al. 2009, Zhao, Toriumi et al. 2009, Hosking and Lane 2010).

It was reported previously that induction of innate immunity, particularly chemokines, is strongly associated with RABV attenuation. Studies have shown that attenuated RABV, but not pathogenic RABV, induces the up-regulation of chemokines, such as MIP-1α (CCL3), RANTES, and IP-10, in mouse brains (Wang, Sarmento et al. 2005, Kuang, Lackay et al. 2009). The role of these chemokines was investigated in the mouse model infected with recombinant RABV encoding corresponding chemokine genes (Zhao, Toriumi et al. 2009, Zhao, Toriumi et al. 2010). The results showed that the expression of MIP-1α enhanced immunogenicity and reduced virus pathogenicity by

inducing innate immunity and recruiting dendritic cells and B cells. On the contrary, the expression of RANTES or IP-10 enhanced pathogenicity by causing neurological disease secondary to high and persistent expression of chemokines, excessive inflammatory cell infiltration, and marked enhancement of blood-brain barrier permeability. The differences in pathogenicity induced by these recombinant RABV were suspected to be due to the level and duration of the expression of chemokines instead of the virus replication rate. Therefore, transient, rather than persistent and high-level, expression of chemokines may be beneficial to the host during RABV infections.

Inflammatory response

Inflammation is a key factor to induce severe dysfunction and neuropathology with neuronal death during CNS infections (Brown and Neher 2010, Wunner 2013). However, the inflammatory response within the CNS triggered by lethal RABV infection in humans and animal models are known to be very limited in comparison with encephalitis elicited by other viruses (Fu, Weihe et al. 1993). Less pathogenic RABV strains tend to elicit more extensive CNS inflammatory responses and necrosis (Murphy 1977, Wang, Sarmento et al. 2005). Most studies show that inflammation does not promote viral infection, but instead, limits the dissemination of virions within the CNS. Treatment with minocycline, a tetracycline derivative with anti-inflammatory properties, led to reduced infiltration of T cells, but an increased mortality rate in experimental rabies infection (Jackson, Scott et al. 2007). Mouse models treated with steroid hormone also resulted in a higher mortality rate (Roy and Hooper 2007). Altogether, these findings indicate that the extent of inflammation is inversely correlated with rabies pathogenicity.

It is likely that the presence of CNS inflammation triggers a transient opening of the blood-brain barrier allowing immune effector infiltration for virus clearance and, in turn, decreasing the pathogenicity.

Limitation of the inflammation elicited by pathogenic RABV infection might be caused by several mechanisms: 1) reduction of inflammatory cell infiltration into the CNS, 2) maintenance of the integrity of the blood-brain barrier, 3) reduction of the release of inflammatory mediators, which can potentially compromise the neuronal function.

The adaptive immunity

An adaptive immune response is one which is developed against a specific microbe. This takes place in the lymphoid organs and relies on professional antigen-presenting cells, such as dendritic cells (DCs). The activated antigen-presenting cells, then, induce both humoral immunity and cell-mediated immunity.

A major factor of protection for RABV infection, demonstrated by the effectiveness of post-vaccination to virus challenge and numerous experimental studies, is the presence of virus- neutralizing antibody (VNA). It is generally accepted that humans do not usually mount a detectable antibody response to natural wild-type pathogenic RABV antigen until relatively late in the disease (Baltazard and Ghodssi 1954, Johnson, Cunningham et al. 2010). A study of human rabies cases in USA showed that although all patients did not have detectable RABV -neutralizing antibodies upon hospital presentation, seroconversion occurred within 10 days in some patients (Noah, Drenzek et al. 1998). Similar observations in some human cases in the UK in which

neutralizing antibodies were detectable weeks after hospital admission (Johnson, Cunningham et al. 2010). The concept of a possible delayed antibody response against rabies infection was utilized in a human survivor of rabies of bat origin in the USA. The patient was treated with coma induction as the peripheral antibody response continued to be established. High rabies antibody titers in both CSF and serum developed 8 days after treatment, which possibly contributed to the patient's survival (Willoughby, Tieves et al. 2005). However, attempts to repeat this treatment failed in most cases later on (Hemachudha, Sunsaneewitayakul et al. 2006).

The high mortality of natural rabies infection is probably due to the absence of protective antibody titers in both serum and CSF. Therefore, an earlier peripheral antibody induction or the ability of antibodies entering the CNS would likely limit virus propagation and enhance rabies survival. Passively transferred antibodies were demonstrated to be capable of inhibiting cell-to-cell virus spread and suggested that antibodies can contribute to virus from the CNS in rat models (Dietzschold, Kao et al. 1992).

G protein is known to be the major antigen stimulating production of virus-neutralizing and protective antibodies (Hooper, Roy et al. 2011). Laboratory attenuated RABV strains express high levels of G protein, which likely contribute to the generation of more effective antiviral immunity and therefore, lower pathogenicity (Morimoto, Hooper et al. 1999). On the contrary, Xue XH et al. demonstrated a possible inverse relationship between the expression of G protein and the induction of virus-neutralizing antibodies. The inactivated recombinant RABV expressing two copies of the glycoprotein elicited a higher level of neutralizing antibodies and provided better

protection in mice (Xue, Zheng et al. 2014). G protein is generally expressed at relatively low levels in cells infected with wild-type RABV, in turn, suggesting a relatively weaker peripheral immune response as observed in lethal human rabies cases (Cox, Dietzschold et al. 1977). However, no difference in adaptive immune responses, including neutralizing antibodies, was found in mice infected with either pathogenic (silver-haired bat RABV) or less pathogenic (CVS encoding a mutant G protein) RABV (Roy and Hooper 2007). The discrepancies in the results may be contributed by limitations in understanding natural rabies infections in rodent experimental models with different RABV strains.

T cell infiltration is considered crucial for controlling most CNS infections, such as West Nile virus infection, in which chemotactic CD8 T cells are important (Klein, Lin et al. 2005). Despite the fact that no neurons underwent apoptosis, T cell apoptosis was observed during fatal RABV infection in humans (Hemachudha, Wacharapluesadee et al. 2005, Tobiume, Sato et al. 2009). Similar findings were also found in mouse models infected with pathogenic RABV, but opposite results occurred in mice infected with attenuated RABV (Galelli, Baloul et al. 2000). Therefore, these results suggest a protective potential of T cells during RABV infection.

Blood-brain barrier (BBB)

BBB is a separation of the circulating blood from the brain extracellular fluid in the CNS. It is composed of endothelial cells, astrocyte end-feet, and pericytes (PCs) (Ballabh, Braun et al. 2004). However, BBB endothelial cells differ from endothelial cells in the rest of the body by the absence of fenestrations, sparse pinocytic vesicular

transport and more extensive tight junctions (TJs) (Liu, Wang et al. 2012). There are also adherens junctions (AJs), which stabilize cell–cell interactions in the junctional zone. Astrocytes maintain the TJs by tightly ensheathing the vessel by their end-feet, but they are not believed to have a barrier function in the mammalian brain. Besides maintaining endothelial TJs, pericytes are also thought to provide structural support and vasodynamic capacity to the microvasculature and regulate angiogenesis (Ramsauer, Krause et al. 2002). Under normal physiological conditions, the TJs limit BBB permeability which prevents transport of bacteria, large molecules, and most small molecules into the brain (van Sorge and Doran 2012). Small lipophilic substances, which are less than 400 Daltons, such as O2 and CO2, can diffuse freely across plasma membranes along their concentration gradient (Ballabh, Braun et al. 2004). Nutrients including glucose and amino acids enter the brain via transporters, whereas receptor-mediated endocytosis mediates the uptake of larger molecules including insulin, leptin, and iron transferrin (Zhang and Pardridge 2001, Ballabh, Braun et al. 2004). However, under certain conditions, such as inflammation, traumatic brain injury or hypoxic-ischemic shock, the BBB is compromised allowing for the passage of larger molecules (Abbott 2000).

Alteration of BBB permeability can occur in both bacterial and viral infections, such as Venezuelan equine encephalitis (VEE) virus, human immunodeficiency virus (HIV), Japanese encephalitis virus (JEV), and mouse adenovirus type 1 (MAV-1), Escherichia coli K1, etc. (Mathur, Khanna et al. 1992, Toborek, Lee et al. 2005, Gralinski, Ashley et al. 2009, Donoso Mantke, Karan et al. 2011, van Sorge and Doran 2012). Although the loss of BBB integrity and infiltration of inflammatory cells have often been associated with pathological changes in the CNS when infected by viruses (Phares, Kean

et al. 2006, Kim, Kang et al. 2009, Hosking and Lane 2010), transiently increased BBB permeability during attenuated RABV infection has been found to be helpful in clearing RABV from the CNS and preventing neurological sequelae (Phares, Fabis et al. 2007, Roy and Hooper 2007). The BBB permeability enhancement is often associated with the expression of chemokines/cytokines and the accumulation of immune cells. In a study of attenuated RABV infections, the expression of IP-10 was found best correlated with inflammatory cell infiltration into the CNS and BBB permeability enhancement among all up-regulated chemokines (Kuang, Lackay et al. 2009). These data indicate that attenuated RABV is capable of inducing expression of chemokines and infiltration of inflammatory cells into the CNS. On the contrary, the pathogenic strain, silver-haired bat RABV, failed to induce BBB breakdown and further prevented immune effectors to enter the CNS even though a strong peripheral virus-specific adaptive immune response was present in the host (Roy and Hooper 2007). During silver-haired bat RABV infections, treatment with a steroid hormone reduced the BBB permeability leading to increased mortality rates (Roy and Hooper 2007). Many studies have shown the loss of BBB integrity only occurs during attenuated RABV infections, but not pathogenic RABV infections (Roy and Hooper 2008, Kuang, Lackay et al. 2009, Zhao, Toriumi et al. 2009).

A major mechanism of BBB breakdown is through modulation of the tight junction (TJs) complex in the brain microvascular ECs (Spindler and Hsu 2012). The TJ complex is composed of transmembrane TJ proteins (occludin and claudins) and cytosolic TJ proteins (zonula occludens-1 [ZO-1]) that link transmembrane TJ proteins to the actin cytoskeleton. Virus infections have been found to induce BBB breakdown through direct disruption of the TJ complex or indirect disruption of the TJ complex

secondary to inflammatory cell infiltration and/ or up-regulation of chemokines (Toborek, Lee et al. 2005, Gralinski, Ashley et al. 2009, Murphy, Lalor et al. 2010). TJ protein (claudin-5, occludin, and zonula occludens-1) expression was found to be decreased during attenuated RABV infection, but not wild-type RABV infection (Chai, He et al. 2014). And the expression of chemokines/cytokines, particularly IFN-γ, in the brain is significantly higher than those infected with wild-type RABV (Chai, He et al. 2014). Therefore, this study suggests that the enhancement of BBB permeability and the reduction of TJ protein expression during attenuated RABV infection are indirectly caused by up-regulation of virus-induced inflammatory chemokines/cytokines.

Part 7: Rabies vaccines and preventative treatment

RABV has been one of the most fatal infectious agents throughout human history, and the majority of the human deaths were attributed to canine rabies exposure (Hampson, Dushoff et al. 2009). Rabies has been largely eliminated from domestic dog populations in Western Europe and North America, but continues to kill many thousands of people throughout Africa and Asia yearly. Therefore, implementation of both human and animal rabies vaccines is critical in rabies prevention and control. Rabies vaccines for humans and animal use both are produced *in vitro*, in which the growth of virus in cell culture allows large-scale antigen production for attenuated and inactivated vaccines. *In vitro* production also bypasses the need to infect live animals. Several RBAV strains, such as Challenge Virus Standard (CVS) and Pastuer virus (PV), have been used for the production of rabies vaccines (Reciilard 1996). All current human rabies vaccines are inactivated. Modified live and recombinant rabies vaccines are only used in animals.

Although administration of parenteral rabies vaccines is effective, it is largely limited to humans and domestic animals rather than free-ranging wildlife animals. Oral rabies vaccine administration started in the 1960s and has resulted in dramatic successes in the control of rabies in wildlife reservoirs (such as red foxes, coyotes, raccoons), but still limited to parts of Europe and North America (Cleaveland, Kaare et al. 2006). However, to this date, pre-exposure prophylaxis with rabies vaccination overall has been shown to dramatically reduce the number of cases in dogs, the incidence of human animal-bite injuries, and the number of human cases, primarily in children.

Postexposure prophylaxis (PEP)

In humans, rabies is almost invariably fatal once clinical symptoms develop. However, rabies deaths are preventable if PEP is implemented promptly and competently after an exposure (Shantavasinkul and Wilde 2011). Approximately 15 million people receive PEP every year (Shantavasinkul and Wilde 2011). PEP has proven highly effective in preventing infection and death. The rabies incubation period may be as short as a few days or as long as several years depending on the site of the bite, the size of the inoculum, and other unknown host factors. Therefore, in unvaccinated rabies-exposed patients, PEP must be started as soon as possible after local wound treatment. The WHO-recommended PEP protocol is a combination of local treatment of the wound, followed by active immunization with tissue culture-derived rabies vaccine and with or without passive immunization with rabies immunoglobulin (RIG) depending on the type of contact with the rabid animal. For type 1 (touching or feeding animals, licks on the skin), no treatments are needed. Type 2, including nibbling of uncovered skin, minor scratches

or abrasions without bleeding, and licks on broken skin, will require immediate vaccination. Type 3, the most severe type including single or multiple transdermal bites or scratches, contamination of mucous membranes with saliva from licks and exposure to bat bites or scratches, requires both vaccination and RIG. RIG and the first vaccination are given on the first day of treatment (designated day 0), and three additional rabies vaccinations are given on days 3, 7, and 14. It takes approximately 7-10 days before a protective level of endogenous rabies-neutralizing antibodies appears in the circulation following active immunization with rabies vaccines. Therefore, RIG is given to unvaccinated rabies-exposed patients to provide passive immunity to neutralize RABV until endogenous antibody production is fully established. There are two types of RIGs: human rabies immunoglobulin (HRIG) and equine rabies immunoglobulin (ERIG). HRIG remains the preferred RIG, but ERIG has been used frequently worldwide and is known to be effective as well. Anaphylaxis with ERIG is extremely rare, but serum sickness has been reported (Shantavasinkul and Wilde 2011). However, these adverse effects can be prevented by the administration of corticosteroids.

Pre-exposure rabies prophylaxis simplifies PEP by eliminating the need for immunoglobulin. It has been shown that rabies vaccines are capable of establishing long lasting immunity, and vaccine boosters can result in an accelerated antibody response. Therefore, patients who have previously received pre- or post-exposure rabies prophylaxis only need to receive two rabies vaccine boosters, on days 0 and 3 following an exposure. The protocol has been very effective in preventing rabies development. No human rabies deaths have been reported among patients who received booster vaccination after a prior pre-exposure rabies series of rabies vaccination. However, local

wound care still should be administered immediately after an exposure to minimize the risk.

Overall, PEP is highly effective, while most failures are due to delayed or improper treatments. However, controlling the canine and feline rabies vector in endemic areas of rabies and preexposure rabies administration of high-risk groups remains the most important strategy for rabies elimination.

Part 8: Therapy of rabies

Rabies PEP, including wound cleansing and active and passive immunization, is highly effective if given promptly after a rabies exposure. Nevertheless, to this date, there is still no effective therapy for those who develop rabies encephalomyelitis. There are many rabies case reports but only a few cases of treated patients have been published. Only 5 patients with acute illness have been reported to survive (Hattwick, Weis et al. 1972, Porras, Barboza et al. 1976, Control 1977, Alvarez, Fajardo et al. 1994, Madhusudana, Nagaraj et al. 2002, Hu, Willoughby Jr et al. 2007); however, only three patients had either no or mild neurologic outcome, while two died within 2-4 years as a result of complicated severe neurological sequelae. All but one of these survivors received rabies vaccine prior to the onset of their illness. The first and one full recovery from rabies occurred in 1970 (Hattwick, Weis et al. 1972). A 6-year-old boy from Ohio received a duck embryo vaccine 4 days after the exposure to a rabid bat. Extremely high VNA levels were observed in both serum and CSF, which have not been observed with vaccination. RABV was not isolated from brain, CSF, or saliva. Therefore, this outcome was suspected to be as a result of viral neutralization secondary to unusually high

antibody titers. Postvaccination encephalomyelitis was suspected in only patient given that the patient's clinical signs worsened after booster doses of the suckling mouse brain rabies vaccine, a vaccine of nervous tissue origin (Porras, Barboza et al. 1976). Another patient, a laboratory technician, survived but exhibited severe neurological sequelae after a possible exposure of aerosolized RABV while working with live RABV vaccine strains (Control 1977). This was the first reported rabies case in a pre-immunized individual and also the fourth well-documented case with transmission due to airborne RABV exposure. The only reported patient, who survived from rabies without any immunization treatment was reported in Wisconsin in 2004 (Willoughby, Tieves et al. 2005). One month after an unknown bat bite exposure, the 15-year-old girl started developing a series of neurological symptoms. VNAs were detected in both the serum and CSF upon hospitalization, and subsequently increased over time. She was then put into a drug-induced coma, induced by noncompetitive NMDA antagonist ketamine and midazolam. Other drugs, such as phenobarbital, ribavirin and amantadine, were also administered. After being discharged with neurologic deficits, she was reported to continue showing progressive neurologic improvement in 2007 (Hu, Willoughby Jr et al. 2007). The most recent case was a 15-year-old boy from Brazil, who was attached by a hematophagous bat and developed symptoms a month later (Saude 2008). Prior to the onset of symptoms, he received four doses of rabies vaccine and was then treated with therapeutically induced coma and other therapies ("Milwaukee protocol"). However, the patient survived with severe neurological sequelae. To this date, most of the rabies survivors exhibited significantly high VNA levels within the serum and/or CSF, which are likely the key to their survival.

The approach to the management of rabies normally should be palliative given that many aggressive approaches have proved to be unsuccessful. Human leukocyte interferon administration via intraventricular and intramuscular routes to patients with symptomatic rabies was not associated with any beneficial clinical effects (Merigan, Baer et al. 1984); however, it was not administered until the symptoms appeared. Antiviral therapy with intravenous ribavirin was proven unsuccessful (Kureishi, Xu et al. 1992). Furthermore, a combined trial with intravenous and intrathecal administration of either ribavirin or interferon-α also failed (Warrell, White et al. 1989, Jackson 2011). Although anti-RABV hyperimmune serum of human and equine origin, administered via intravenous and intrathecal routes, has prolonged the survival in some cases, no clear beneficial effects were observed (Hemachudha, Sunsaneewitayakul et al. 2003, Jackson 2011).

In unusual circumstances, patients in good health with relatively early disease and access to adequate resources and facilities can potentially be considered candidates for an aggressive therapy. Even therapy with single agents, such as ribavirin (Warrell, White et al. 1989) or interferon-α (Merigan, Baer et al. 1984), has reported to be ineffective, a combination of specific therapies may be more effective. Combinations of specific therapies have been used in other viral diseases. For example, ribavirin and interferon-α, provide a clinically synergistic effect in chronic hepatitis infection treatment (Lau, Tam et al. 2002). Proposed specific therapies for rabies include rabies vaccine, rabies immunoglobulin, monoclonal antibodies, ribavirin, interferon-α and ketamine (Jackson, Warrell et al. 2003).

Development of an immune response is associated with rabies encephalitis survival in animals (Jackson, Warrell et al. 2003). Theoretically, intramuscular administration of rabies vaccine should elicit humoral and cellular immune responses against human rabies infection. However, this attempt has failed and frequently proven to be unbeneficial. The failure has been attributed to the delayed immune response via the intramuscular route administration and the inactivated nature of human vaccines, which do not typically induce cytotoxic T cell response. A cytotoxic T cell response is observed in live attenuated and recombinant vaccines and believed to be important for viral clearance (Jackson, Warrell et al. 2003). Therefore, vaccines capable of inducing cytotoxic T cell response may be a potential treatment option for human clinical rabies in the future.

The aim of human rabies immunoglobulin administration is to promote RABV clearance. However, to this date, it has only proven to be beneficial while using as rabies postexposure prophylaxis before viral nervous system invasion. More specific rabies antibodies, RABV-neutralizing monoclonal antibodies, have been shown to clear RABV from the CNS before the onset of clinical signs (Dietzschold, Kao et al. 1992). This finding suggested that monoclonal antibodies may be more effective. Although a higher dose of immunoglobulin has also been suggested, it is still uncertain to what extent that immunoglobulin will enter the CNS given that blood-brain barrier is believed to remain intact during rabies infection (Roy and Hooper 2008). More studies regarding using antibodies as a therapeutic agent for clinical rabies are warranted.

Ribavirin is a board-spectrum antiviral agent that induces viral mutations and immunomodulatory responses, but its usage in clinical rabies remains unclear. Although

it has antiviral activity against RABV in vitro, its efficacy has not been determined (Bussereau, Picard et al. 1988). Moreover, there is also limited information about whether this agent can cross the blood-brain barrier, especially given that a rapid uptake into CSF was not observed after intravenous administration (Ferrara, Oishi et al. 1981).

Interferon-α (IFN- α) proteins are made naturally and released by host cells in response to the presence of pathogens, such as viruses or neoplastic cells. They can function as regulatory proteins and immunotherapy drugs for viral and neoplastic diseases. During viral infections, besides interfering with viral replication, IFNs also interact with innate immune cells to promote a transition from the innate to the adaptive immune response. Although the efficacy of this therapy has been demonstrated in animals, but no beneficial effect has been reported in human clinical rabies (Jackson, Warrell et al. 2003).

Ketamine is a dissociative anesthetic agent, which can cross the blood-brain barrier, as well as a noncompetitive antagonist of the NMDA receptor. Ketamine administered at a high dose has been demonstrated to inhibit RABV genome replication, leading to reduced RABV replication in vitro. In addition, RABV CNS distribution in animal models has suggested that the NMDA receptor is a potential receptor for RABV. All together, ketamine may be a potential therapeutic agent administered along with other antiviral agents in clinical rabies (Jackson, Warrell et al. 2003).

In conclusion, in situations in which a more aggressive approach is desirable, a combination therapy may be more effective than single agent therapy.

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CHAPTER 3

ENHANCEMENT OF BLOOD-BRAIN BARRIER PERMEABILITY IS REQUIRED FOR INTRAVENOUSLY ADMINISTERED VIRUS NEUTRALIZING ANTIBODIES TO CLEAR AN ESTABLISHED RABIES VIRUS INFECTION FROM THE BRAIN AND PREVENT THE DEVELOPMENT OF RABIES IN MICE[1]

[1]Chien-Tsun Huang, Zhenguang Li, Ying Huang, Guoqing Zhang, Ming Zhou, Qingqing Chai, Hua Wu, Zhen F. Fu. 2014. *Antiviral Research*. 110:132-141. Reprinted here with permission of publisher.

Abstract

Rabies virus (RABV) is a neurotropic virus that causes fatal disease in humans and animals. Currently, there is no cure for rabies once clinical signs appear. It is believed that once RABV enters the central nervous system (CNS), virus neutralizing antibodies (VNAs) in the periphery cannot pass through the Blood–brain Barrier (BBB) and into the CNS. Furthermore, it has been hypothesized that VNAs produced in the CNS by invading B cells, rather than those produced in the periphery and then transported into the CNS, are important in clearing RABV from the CNS. In the present study, mouse serum containing VNA was administered intravenously into mice after infection with wild-type RABV. Our studies demonstrate that exogenous administration of VNAs is crucial in the clearance of RABV from the brain and prevents the development of rabies in both immunocompetent and immunocompromised mice as long as the BBB permeability remains enhanced. This present study therefore provides a foundation for the possibility of developing VNA therapy for clinical rabies in humans.

Keywords: Rabies virus, MCP-1, BBB (Blood-brain Barrier), VNA (virus-neutralizing antibody), Occludin, Claudin-5

Introduction

Rabies virus (RABV) is a negative-sense and single-stranded RNA virus, which belongs to the Lyssavirus genus of the *Rhabdoviridae* family (Rupprecht 1996). Rhabdoviruses are enveloped with a typical bullet- or rod-shaped morphology and characterized by an extremely broad host spectrum ranging from plants to insects to mammals (Rupprecht 1996). Rabies has been known as a deadly neurological disease of both humans and animals for centuries (Jackson 2002) and remains a major threat to public health (Meslin, Fishbein et al. 1994, Martinez 2000, Knobel, Cleaveland et al. 2005, Jackson 2013). Each year, rabies causes more than 55,000 human deaths around the world (Meslin, Fishbein et al. 1994). Canine rabies is responsible for more than 99% of the human cases in Asia and Africa (Cleaveland, Kaare et al. 2006). In the United States, dog rabies has been largely brought under control through pet vaccination programs, and the number of human cases has declined dramatically during the past 60 years (Hampson, Dushoff et al. 2009). Most of the human cases in the USA have been associated with RABV found in bats, particularly the silver-haired bats with no obvious recognized exposure history (Morimoto, Patel et al. 1996, Messenger, Smith et al. 2003). Therefore, there is a need to develop therapeutics for clinical rabies although rabies can be prevented in humans after exposure (usually after an animal bite) by post-exposure prophylaxis (PEP), which is comprised of wound cleansing, vaccination, and administration of anti-rabies immunoglobulin. PEP is very effective if it is initiated promptly after exposure (CDC 2010). It is widely accepted that there is no effective treatment for rabies infection, which is almost always fatal once neurological symptoms develop (WHO 1992, Wilde 2007). Although human survivors have been reported

recently after treatment with the Milwaukee Protocol or a modification thereof (Willoughby, Tieves et al. 2005, MCW 2009), its effectiveness has been questioned (Hemachudha, Sunsaneewitayakul et al. 2006, McDermid, Saxinger et al. 2008, Jackson 2013).

Rabies clinical signs, especially neurologic signs, are believed to be indicative of virus replication resulting in neuronal dysfunction or injury in the central nervous system (CNS), where peripheral immune effectors have limited access (Yousaf, Qasim et al. 2012). Viral infection of the CNS poses unique challenges to the immune system with regards to controlling and eliminating the invading pathogens (Griffin 2003). The presence of a blood-brain barrier (BBB) provides a physical and physiological separation of the CNS from the periphery, and thus, cells and molecules cannot easily enter the CNS (Ballabh, Braun et al. 2004, Roy and Hooper 2008, Hosking and Lane 2010). Although enhancement of BBB permeability and infiltration of inflammatory cells have often been associated with pathological changes in the CNS when infected by viruses (Phares, Kean et al. 2006, Kim, Kang et al. 2009, Hosking and Lane 2010), transiently increased BBB permeability has been found to be helpful in clearing RABV from the CNS (Phares, Fabis et al. 2007, Roy and Hooper 2007). Induction of autoimmune CNS inflammation (experimental allergic encephalomyelitis) (Roy and Hooper 2007) or the administration of attenuated RABV (CVS-F3) (Phares, Kean et al. 2006), as well as recombinant RABV expressing three copies of the glycoprotein (G) (TriGAS) (Faber, Li et al. 2009) or immune stimulating molecules (for example, GM-CSF) (Wang, Zhang et al. 2011, Wen, Wang et al. 2011), all resulted in enhancement of BBB permeability, increased production of virus neutralizing antibodies (VNAs), clearance RABV from the CNS, and

prevention of rabies in the mouse model. Furthermore, clearance of attenuated RABV from the CNS correlates with infiltration of B cells into the CNS, expressing high levels of κ-light chain mRNA (Phares, Kean et al. 2006). Passively-transferred VNA via the intraperitoneal route was insufficient to mediate the CNS clearance of attenuated RABV in B-cell deficient mice (Hooper, Phares et al. 2009). These observations led to the hypothesis that it is the VNA produced in situ (CNS) by invading B cells, rather than those produced in the periphery and then crossed into the CNS, that are important in clearing RABV from the CNS (Hooper, Phares et al. 2009). Nevertheless, it has been reported that enhancing BBB permeability with the delivery of sufficient VNA to the brain may provide an effective treatment after CNS infection has been established (Liao, Yang et al. 2012). Recent studies have demonstrated monocyte chemoattractant protein-1(MCP-1, CCL2), a chemokine commonly expressed in the CNS during inflammation, when injected intracerebrally at a specific dose can lead to BBB permeability enhancement. This effect was believed to be due to alterations of TJ protein expression on endothelial cells (Stamatovic, Shakui et al. 2005). Therefore, in the present study, we used MCP-1, a member of the CC subfamily of chemokines, to enhance the BBB permeability and allow passively-transferred VNAs to reach the brain. Our results demonstrate that intravenous administration of VNA was found to be crucial in clearing RABV from the brain and preventing animals from developing rabies in both immunocompetent and B-cell deficient mice, as long as the BBB permeability is enhanced.

Materials and Methods

Viruses, cells, serum, and animals

Street RABV (Dog Rabies Virus from Mexico, DRV-Mexico) (Dietzschold, Morimoto et al. 2000, Zhang and Fu 2012) was propagated in suckling mouse brains. Mouse neuroblastoma cells (NA) were maintained in RPMI 1640 medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY). Recombinant murine monocyte chemotactic protein -1 (MCP-1) was purchased from PreproTech (Rocky Hill, NJ). RABV-antibody positive serum was prepared from the blood of ICR mice immunized with rRABV-GMCSF vaccines (Wen, Wang et al. 2011). RABV-negative serum was prepared from blood of naïve ICR mice. All sera were pooled and titrated for VNA. Four to six-week old female ICR mice were purchased from Harlan (Indianapolis, IN). Four to six-week old female C57BL/6J, B6.129S2-*Ighm*^{tm1Cgn}/J (B cell deficient) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were housed in temperature- and light-controlled quarters in the Animal Resources Facility, College of Veterinary Medicine, University of Georgia. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal procedures were approved by the Institutional Animal Care and Use Committee, University of Georgia (animal welfare assurance number: A3085-01). All efforts were made to minimize animal suffering.

Brain and serum collection

At indicated time points, whole blood was collected from mice, allowed to clot overnight, and spun down at 10,000 g for 10 min. Supernatant was isolated for serum antibody analysis. Then, mice were perfused with PBS at a rate of 2 ml/min for 10 min. Independent samples of mouse brains from each group were then homogenized and prepared as 10% (w/v) suspension in DMEM. Homogenized brain samples were centrifuged at 14,000 rpm for 20 min. Supernatant was harvested for virus titration, antibody detection and Western blot analysis.

Virus titrations

Virus titers were determined by direct fluorescent antibody assay (dFA) in NA cells. NA cells in a 96-well plate were inoculated with serial 10-fold dilutions of virus preparation and incubated at 34°C for 2 days. The culture supernatant was removed and the cells were fixed with 80% ice-cold acetone for 30 min. The cells were washed twice with PBS and then stained with FITC-conjugated anti-RABV N antibodies (Fujirebio, Malvern, PA). Antigen-positive foci were counted under a fluorescent microscope (Zeiss, Germany) and viral titers calculated as fluorescent focus units (FFU) per milliliter. All titrations were carried out in quadruplicate.

Quantitative real-time PCR (qRT-PCR)

A real-time (RT) SYBR green PCR assay was carried out in an Mx3000P apparatus (Stratagene, La Jolla, CA) to quantify the expression of viral genomic RNA (copy number) as well as mRNA of tight junction proteins (occludin and claudin-5) and

κ-light chain. Total RNA was extracted from brain homogenates using the Qiagen RNeasy kit (Qiagen, Redwood, CA) and treated with DNase (Qiagen). The reverse transcriptase and DNA polymerase were utilized from a one-step Brilliant II SYBR green qRT-PCR master mix kit (Stratagene). Each reaction was carried out in duplicate with approximately 100 ng of DNase-treated RNA and 5 nM each of primer pairs described previously (Faber, Pulmanausahakul et al. 2002, Phares, Kean et al. 2006, Braniste, Leveque et al. 2009, Armstrong, Wang et al. 2012). Amplification was carried out at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles in two steps: 95°C for 15 sec and 60°C for 1 min. For absolute quantification of viral genomic RNA, a standard curve was generated by using a serially-diluted RNA *in vitro* transcribed from a plasmid expressing RABV G, and the copy numbers of viral genomic RNA were normalized to 1 g of total RNA. For measurement of the mRNA for tight junction proteins and **κ**-light chain, the copy numbers were normalized to those of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Levels of gene expression in a test sample are presented as the fold increase over that detected in sham-infected controls.

Rapid fluorescent focus inhibition test (RFFIT)

VNA measurement was carried out by the RFFIT as previously described (Favoretto, Carrieri et al. 1993). 50 μl of serial five-fold dilutions of serum were prepared in Lab-Tek Chamber slides (Nalge Nunc International, Rochester, NY). 50% Fluorescing Foci dose (Fifty FFD_{50}) of challenge virus standard (CVS-11) was added to each chamber and incubated for 90 min at 37°C. NA cells (10^5 cells) were added into each chamber, and the slides were incubated at 37°C for 20 hrs. The cells were then fixed with ice-cold

80% acetone and stained with FITC-conjugated anti-RABV N antibodies for 1 hr at 37°C. Twenty fields in each chamber were assessed under a fluorescent microscope. The 50% endpoint titers were calculated according to the Reed-Meunch formula (Reed and Muench 1938). Then the values were compared with that of a reference serum (obtained from the National Institute for Biological Standards and Control, Herts, UK) and normalized to international units (IU/ml).

Total IgG ELISA assay

ELISA was carried out by using Mouse IgG Titer ELISA Kit (General Bioscience, Brisbane, CA) according to the manufacturer's instructions. The optical density was measured at 450 nm using a spectrophotometer (BioTek Instruments, VT). Determination of the total IgG concentration was performed by linear regression analysis using the software KC4 Signature Ver. 3.4 (Bio-Tek Instruments). A standard curve was prepared using known concentrations of mouse IgG provided in the ELISA Kit (General Bioscience). The IgG concentration of each sample and control was calculated from each corresponding reference standard curve using a 4-parameter logistic regression equation of the KC4 program. The results were expressed as μg/ml for both brain homogenates and serum samples.

Measurement of BBB permeability

BBB permeability was determined by measuring Sodium Fluorescein (NaF) uptake as previously described (Phares, Kean et al. 2006, Phares, Fabis et al. 2007, Kuang, Lackay et al. 2009). 100 μl of 100 mg/mL of NaF, used as a tracer, was injected

into the tail vein of each mouse. Peripheral blood was collected after 10 min and PBS-perfused brains were then harvested. Serum recovered was mixed with an equal volume of 10% trichloroacetic acid (TCA) and centrifuged for 10 min. The supernatant was collected after centrifugation and brought up to 150 µL by mixing with 5M NaOH and 7.5% TCA. Homogenized brain samples in cold 7.5% TCA were centrifuged for 10 min at 10,000 x g to remove debris. The supernatant was brought up to 150 µL by adding 5M NaOH. The fluorescence of serum and brain homogenate samples was measured using a spectrophotometer (BioTek Instruments) with excitation at 485 nm and emission at 530 nm. NaF taken up into brain tissues is expressed as the micrograms of fluorescence per mg of cerebrum or cerebellum divided by micrograms of fluorescence per µl of serum to normalize the uptake amounts of marker from peripheral blood at the time of brain tissue collection (Trout, Koenig et al. 1986, Phares, Fabis et al. 2007). Data are expressed as a fold change in the amount of tracer in tissues as compared to the values obtained for tissues from negative controls.

Western blot analysis

The brain homogenates were subjected to 8%-16% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Thermo Scientific, Rockford, IL). Separated proteins were electroblotted onto nitrocellulose membranes and incubated with primary antibodies overnight. After extensive washing with PBS, the blots were incubated with secondary antibodies. Proteins were detected by SuperSignal West Pico chemiluminescence (Thermo Scientific). Band signals corresponding to immunoreactive

proteins were captured and chemiluminescence intensities were analyzed using the ChemiDoc 4000 MP documentation System (Biorad, CA).

Statistical Analyses

Graphpad Prism 5 v. 5.01(1992-2009 Graphpad Software Inc., La Jolla, CA, USA) was used for statistical analysis. Survival curves were estimated through Kaplan-Meier method and statistical significance of survival rates between experimental groups was determined by log rank test. Student's t-test or one-way ANOVA was used to evaluate significant differences between experimental groups and control or experimental groups, respectively. Statistical significance was set at $P<0.05$.

Results

Intravenous administration of VNA can prevent immunocompetent mice from developing rabies if BBB permeability is enhanced.

Our previous studies revealed that intracerebral administration of rRABV-GMCSF or a combination of inactivated rRABV and MCP-1 can prevent mice from developing rabies by stimulating the production of VNA, enhancing the BBB permeability, and clearing RABV from the CNS (Wang, Zhang et al. 2011). To investigate if intravenous administration of VNA together with MCP-1 can protect mice from RABV infection, 4-6 week-old ICR mice were infected intramuscularly (biceps femoris muscle of left hindlimb) with 10 IMLD50 (50% mouse intramuscular lethal dose) of a street RABV (DRV-Mexico) as described (Wang, Zhang et al. 2011). At 3, 5, or 7 days post infection (p.i.), mice were treated intravenously from the tail vein with 150 µl

of RABV-negative serum or RABV-antibody positive serum (VNA titer: 8 IU). To enhance the BBB permeability, mice were also treated intracerebrally with 25 μg of recombinant murine MCP-1 (suspended in 40 μl of sterile ddH_2O), which is known to transiently enhance BBB permeability (Stamatovic, Shakui et al. 2005). Mice were observed daily for three weeks for the development of rabies, and the results are shown in Fig. 3.1-3.3. Treatment with VNA at day 3 p.i. significantly ($p<0.05$) enhanced the survival rate of the mice, but there was no significant difference in survival rates between mice that received MCP-1 or those without (Fig. 3.1). We suspected the treatment was given too early in the infection and that viruses were neutralized in the periphery before reaching the CNS. Treatment was subsequently delayed to day 5 p.i. (Fig. 3.2). Treatment with MCP-1 significantly increased ($p<0.05$) the survival rate from 0% to 60% in mice infected with DRV and treated with antibodies at day 5 p.i.. However, treatment with both MCP-1 and antibodies at a later time point, at Day 7 p.i., did not prevent the development of rabies infection in DRV-infected mice, and the survival rates between all four groups had no significant differences (Fig. 3.3). From these results, we concluded that treatment with MCP-1 and antibodies at 5 days p.i. will provide the best protection for mice against RABV infections.

Moreover, for the day 5 p.i. group, all infected mice treated with medium only died by day 14 p.i. Only 10% of the mice survived after treatment with the combination of MCP-1 and RABV-negative serum or with the positive serum alone without MCP-1. These results indicate that treatment with MCP-1 alone does not increase the survival rate after RABV infection in the absence of passively-transferred VNAs. Without MCP-1, VNA administered intravenously are unable to pass into the brain to clear RABV.

Combined treatment with RABV-antibody positive serum and MCP-1 increased the survival rate to 60% ($p<0.05$).

Intravenous administration of VNA is crucial in the clearance of RABV from the brain when the BBB permeability is enhanced.

To elucidate the mechanism behind the effectiveness of the combined treatment given at day 5 p.i., brains and sera from mice that survived (three weeks after treatment) from or succumbed (at the time of death) to rabies were harvested for VNA measurement, virus titration and detection of antibody **κ**-light chain by RT-PCR (Phares, Kean et al. 2006). The results are depicted in Fig. 2. High virus titers (>10^4 FFU/mL) were detected in the brains of mice succumbing to rabies and no virus was detected in the survivor brains (Fig. 3.4A). Similar results were also obtained when the viral genomic RNA was detected (Fig. 3.4B). High VNA titers were detected in the serum of surviving mice (Fig. 3.4C). Similar results were also observed in the brains of survived mice, particularly those that received MCP-1 treatment, which presumably enhanced the BBB permeability (Fig. 3.4D). The high VNA titers in the sera and brains also correlated with low viral loads in the brains (Fig 3.4A and 3.4B), suggesting that VNAs are crucial in clearing RABV from the brain.

The **κ**-light chain mRNA level in the brain was significantly higher in the surviving mice than in the dead ones (Fig. 3.4E). These results indicate that intravenous administration of VNA is crucial in the clearance of RABV from the brain when the BBB permeability is enhanced. However, detection of **κ**-light chain in the brain suggests that

plasma cells entering into the CNS could have produced VNA in situ to provide protection.

Passively transferred antibodies are able to reach the brain once BBB permeability is enhanced.

It has been shown that a single intracerebral dose of MCP-1 (25 ug) can induce FITC-albumin leakage to the mouse brain (Stamatovic, Shakui et al. 2005). To ensure that intravenously administered RABV VNAs are able to reach the brain after BBB permeability is enhanced by MCP-1, ICR mice were given RABV-antibody positive serum intravenously with or without MCP-1, and the antibody levels were analyzed 24 hrs later in both the brain and the serum. The results are depicted in Fig. 3. The level of VNA was found to be similar in the serum of mice with or without MCP-1 treatment (Fig. 3.5B), while the VNA levels in the brains of mice treated with RABV-antibody positive serum in conjunction with MCP-1 were significantly higher (~0.3 IU) than in mice treated with VNA-positive serum without MCP-1 (<0.1 IU) (Fig. 3.5A). Similar findings were also observed when total IgG levels were measured by ELISA (Fig. 3.5C & 3.5D). These results demonstrate that passively administered VNAs are able to pass through the BBB when BBB permeability is enhanced with MCP-1.

Enhancement of BBB permeability peaked at 36 hrs after administration of MCP-1 that correlates with the down-regulation of tight junction protein expression.

It has been shown that MCP-1 enhanced the BBB permeability 6 to 12 hrs after injection and the effect waned after 24 to 48 hrs (Stamatovic, Shakui et al. 2005). To

investigate if infection with wt RABV would change the dynamics of BBB permeability enhancement by MCP-1, mice were infected with DRV-Mexico and then treated with MCP-1 and RABV antibody-positive or negative serum at 5 dpi. NaF uptake was measured in mice at 12, 24, 36, and 48 hrs after treatment with MCP-1. As shown in Fig. 3.6, infection with DRV-Mexico alone did not change the BBB permeability when compared to the controls. The enhancement of BBB permeability was detected at 12 hr, reached a peak at 36 hr, and declined at 48 hr after MCP-1 injection, with the greater effect observed in the cerebrum than in the cerebellum (Fig. 3.6A & 3.6B). These studies indicate that infection with RABV does not change the dynamics of BBB permeability enhancement by MCP-1.

MCP-1 has been reported to attract leukocytes and to increase BBB permeability by redistributing tight junction proteins and reorganizing the actin cytoskeleton (Stamatovic, Keep et al. 2003). The expression of tight junction proteins (occludin and claudin-5) was measured by RT-PCR and Western blotting as described (Chai, He et al. 2014). An inverse relationship was found between the BBB permeability and the expression of tight junction proteins (Fig. 3.7A & 3.7B). When the NaF intake increased in the brains of mice after treatment with MCP-1 (12-36 hrs), the expression of tight junction proteins decreased (12-36 hrs). When the BBB permeability started to decline at 48 hrs, the expression of tight junction proteins increased. These observations were confirmed by semiquantitative Western blotting (Fig. 3.7C & 3.7D). These results suggest that MCP-1 enhances the BBB permeability possibly through down-regulation of tight junction proteins in the brain vasculature.

Treatment with VNA and MCP-1 is crucial for the clearance of RABV from the brain.

To determine if combined treatment with MCP-1 and RABV-antibody positive serum is crucial in clearing RABV from the brain, mice were infected with DRV-Mexico. At 5 dpi, mice were treated with MCP-1 with or without RABV-antibody positive serum. Animals were sacrificed at 12, 24, 36, 48 hrs after treatment. Animals were also sacrificed at the time when DRV-infected mice without any treatment developed hindlimb paralysis (reached the euthanasia criteria) at 10 dpi (indicated as D10) or survived at 15 dpi (indicated as D15) to ensure the clearance of RABV from the brain in an established infection. Brain and serum samples were collected from sacrificed mice for detection of VNAs and total IgG. Virus copy number and mRNA levels of κ-light chain were measured in the brain samples. The results are summarized in Fig. 3.8. All mice treated with MCP-1 without RABV antibody-positive serum reached euthanasia criteria before D15. Therefore, no samples were collected for this group (DRV+ MCP-1+N/ D15).

In mice treated with MCP-1 without RABV antibody-positive serum, no detectable levels of VNAs were found in the brain (Fig. 3.8A) or in the serum (Fig. 3.8B) during the observation period, suggesting that wt RABV (DRV-Mexico) is incapable of inducing VNAs. VNAs were detectable in the brains shortly (12 hr) after the combined treatment (MCP-1 and RABV antibody-positive serum). The level of VNAs in the brain reached a peak at 36 hr and then declined (Fig. 3.8A), corresponding to the enhancement of BBB permeability (Fig. 3.6). In the serum, VNA levels remained constant after administration (Fig. 3.8B). VNA levels in both the brain and serum continued to be significant at 10 and 15 dpi in mice treated with MCP-1 and RABV-antibody positive

serum while VNA remained undetectable in mice treated with MCP-1 but without RABV antibody-positive serum (Fig. 3.8A). These mice developed severe signs of rabies. On the other hand, no significant differences were observed in IgG levels in the brain and serum between DRV-infected mice treated with MCP-1 with or without RABV-antibody positive serum (Fig. 3.8C & 3.8D). IgG levels in the brains peaked immediately 12 hr after administration, which correlates with the timing of BBB permeability being enhanced by MCP-1 (Fig. 3.8C). Together, these results suggest that wt RABV fails to elicit VNA responses, and passively-transferred RABV antibody-positive serum, along with MCP-1, is needed for mice to maintain a protective VNA level in the brain.

As shown in Fig. 3.8E, virus copy numbers increased steadily in the brains of mice infected with DRV-Mexico and treated with MCP-1 plus RABV-negative serum. The highest virus copy number was observed on 10 dpi when infected mice developed severe clinical signs. In contrast, viral genomic RNA gradually decreased in the brains of mice infected with DRV-Mexico and treated with MCP-1 and RABV-antibody positive serum. Similar results were also observed for virus titers (data not shown). The decrease in virus copy numbers and titers corresponds to the increase of VNA in the brain. At 10 dpi, virus copy number in the brains of mice treated with RABV-negative serum was 1000 times more than in mice treated with RABV antibody-positive serum. Similar virus copy numbers were detected in the brains of mice infected with DVR-Mexico and treated with MCP-1 alone, and those without any treatment at 10 dpi. By 15 dpi (10 days after treatment), virus was no longer detectable in the surviving mice treated with MCP-1 and RABV antibody-positive serum. These findings indicated that MCP-1 alone is not

capable of clearing RABV from the brain. Instead, intravenous administration of VNA, in addition to MCP-1, is crucial in clearing RABV from the brain.

The copy numbers of κ-light chain as detected by RT-PCR were similar in the brains of mice treated with MCP-1 plus RABV antibody-positive or negative serum (Fig. 3.8F). The level was very low in the beginning stage of infection and reached high levels by 10 dpi. However, the copy numbers of κ-light chain were significantly higher in mice with MCP-1 treatment than without. These data may indicate that plasma cells induced by infection with DRV-Mexico can cross the BBB after treatment with MCP-1.

VNA in the absence of antibody-producing cells can prevent mice from developing rabies after BBB is compromised in B-deficient mice.

To exclude the possibility that VNA-producing plasma cells entering into the CNS are absolutely required to clear RABV from the CNS, the experiment as summarized in Fig. 1 was repeated in B-cell deficient mice. B6.129S2-*Ighm*^{tm1Cgn}/J (B-cell deficient) and C57BL/6J (background) mice were inoculated intramuscularly with10 IMLD50 DRV-Mexico. Half of the mice were left untreated and the other half treated intravenously with RABV antibody-positive serum in conjunction with MCP-1 at 5 dpi. Without treatment (MCP-1 or VNA), B-cell deficient and C57BL/6J mice all died by 12-14 dpi (Fig. 3.9). Eighty percent of the C57BL/6J mice treated with RABV-antibody positive serum and MCP-1 survived (Fig. 3.9), and high VNA levels and κ-light chain mRNA were detected in the brains of surviving mice (Fig. 3.10D & 3.10E). Only 25% of the B6.129S2-*Ighm*^{tm1Cgn}/J mice survived when treated at day 5 after infection (Fig. 3.9). Despite the fact that VNA titers were still high in the serum (Fig. 3.10C), VNA titers remained

extremely low in the brains (Fig. 3.10D). On the other hand, the survived ones had high levels of VNAs detected in the brains (Fig. 3.10D). Therefore, this relatively low survival rate may be due to the fact that circulating VNAs were not able to reach the brains given the transient effect of MCP-1 on BBB permeability, which declined 48 hrs after injection (Fig. 3.6). Thus, an additional dose of MCP-1 was given at day 7 after infection, which significantly increased ($p<0.05$) the survival rate from 25% (MCP-1 given only at 5 dpi) to 78% (MCP-1 given at both 5 and 7 dpi) in B6.129S2-*Ighm*^{tm1Cgn}/J mice (Fig. 3.9) and high VNA levels were detected in the brains (Fig. 3.10D). Yet, no **κ**-light chain was detected in the CNS of these mice (Fig. 3.10E). Virus was detected only in the brains of mice that succumbed to rabies, but not in those that survived (Fig. 3.10A & 3.10B). From these results, it is clear that antibody-producing plasma cells are not absolutely required to clear RABV if sufficient VNAs are allowed to enter the CNS.

Discussion

Our previous studies indicated that intracerebral administration of rRABV-GMCSF could stimulate the production of VNA, enhance BBB permeability, and clear RABV from the brain, therefore, preventing mice from developing rabies as late as 5 days after infection with a wt RABV. However, administration of inactivated rRABV-GMCSF did not improve the survival rate despite the fact that VNA was produced in the periphery. Yet, enhancing the BBB permeability by MCP-1 could improve the survivor rate of mice treated with inactivated rRABV-GMCSF (Wang, Zhang et al. 2011). In the present study, we demonstrated that exogenously delivered VNA is crucial in clearing an established infection by wt RABV in the brain and preventing the development of rabies

in immunocompetent and immunodeficient mice as long as the BBB permeability remains enhanced.

Despite natural infection of mice being rare, mouse models have been used in many rabies studies given their short incubation period. Unlike humans in which RABVs may take weeks to reach the CNS from the site of exposure and cause clinical illness, the spread of RABVs to the CNS in mice is rapid with virus generally being detectable in CNS tissues within 48 hours of infection (Hooper 2005). Clinical signs, particularly neurologic signs, are believed to indicate neuronal injury/dysfunction related to virus replication in CNS neurons. Hindlimb paralysis, a major neurological sign of rabies infection in mice, may not be the best endpoint for wt rabies infection in mice, especially given that infection of spinal neurons was believed to be the cause of hindlimb paralysis in infections with laboratory rabies strains (CVS-11). However, our previous studies demonstrated that the highest RABV titer was detected in the brains, at 9 dpi, right before RABV-infected mice develop hindlimb paralysis (Wang, Zhang et al. 2011). Therefore, it was used as the humane endpoint in this study to demonstrate the clearance of RABV from the CNS by passively transferred antibodies and also to minimize the suffering of the infected mice. The BBB is a physical and physiological barrier formed by the endothelial tight junctions, impeding the influx of most compounds from the peripheral circulation to enter the CNS (Ballabh, Braun et al. 2004, Abbott, Patabendige et al. 2010). Thus, the BBB plays an important role in maintaining homeostasis in the CNS (Neuwelt 2004), and enhancement of BBB permeability has often been associated with CNS diseases (Spindler and Hsu 2012). Some viruses, such as lymphocytic choriomeningitis virus and attenuated RABV, that infect the CNS can enhance BBB permeability resulting

in infiltration of inflammatory cells and pathological changes (Phares, Kean et al. 2006, Kim, Kang et al. 2009). In wt RABV infection, however, BBB permeability is not enhanced since RABV bypasses the BBB and enters the CNS via axonal transport directly from the site of peripheral inoculation (Roy and Hooper 2008, Hooper, Phares et al. 2009). Enhancement of BBB permeability is thus required for immune effectors to enter into the CNS to clear RABV and prevent the occurrence of rabies (Phares, Kean et al. 2006, Roy, Phares et al. 2007). To enhance the BBB permeability for RABV clearance from the CNS, lab-attenuated RABV (Phares, Kean et al. 2006) and recombinant RABV expressing three copies of G (Faber, Li et al. 2009) or GM-CSF (Wang, Zhang et al. 2011, Wen, Wang et al. 2011) have been directly injected into the brain and were found to stimulate VNA production and enhance the BBB permeability, resulting in the clearance of RABV from the CNS and prevention of the development of rabies in the mouse model after infection with wt RABV.

MCP-1 has been reported to transiently enhance the BBB permeability in mice without causing obvious side effects (Stamatovic, Shakui et al. 2005) and has been used to enhance the BBB permeability for RABV clearance in our previous studies (Wang, Zhang et al. 2011). In the present study, the role of MCP-1 in enhancing the BBB permeability was investigated, including the dynamics of BBB permeability change, the level of RABV VNA in the brain, and the down-regulation of tight junction proteins in the brain vasculature. A single intracerebral dose of MCP-1 enhanced BBB permeability significantly over the control mice. The enhancement reached a peak by 36 hrs, an observation similar to that previously reported (Stamatovic, Shakui et al. 2005). Administration of MCP-1 resulted in the reduction of TJ protein (occludin and claudin-5)

expression in our studies. It has been known that enhancement of BBB permeability correlates with the reduction of TJ protein expression in the brain vasculature (Kniesel and Wolburg 2000, Kirk, Plumb et al. 2003, Liao, Yang et al. 2012, Chai, He et al. 2014). Our results further support the functional role for the cytoplasmic anchorage of TJ proteins for brain endothelial barrier function. VNA levels in the brains were also measured to evaluate whether the compromised BBB allows antibodies to leak into the brain. The possible interference of circulating antibodies, particularly VNAs, in the brain vasculature was minimized by PBS perfusion before sample collection as previously described (Yu, Zhang et al. 2011). Although some residual antibodies which did not pass the BBB, still cannot be completely eliminated by perfusion, VNA in the brain was significantly higher in mice treated with the combination of VNA and MCP-1 than in mice treated with VNA alone, suggesting that a significant level of VNA was able to pass through the BBB with MCP-1 treatment. Therefore, enhancement of BBB permeability allowed intravenously administered VNA to pass through the BBB and into the brain, which inhibited RABV replication, leading to eventual clearance of RABV from the brain and prevention of the development of rabies. Without MCP-1, intravenously administered VNA cannot pass through the BBB to clear RABV in the brain and prevent the development of rabies. Enhancement of BBB permeability alone, however, cannot prevent the development of rabies in mice infected with wt RABV as has been demonstrated clearly in previous (Wang, Zhang et al. 2011) and this study. Although MCP-1 is capable of recruiting leukocytes into the CNS (Yadav, Saini et al. 2010), and intracerebral injection of MCP-1 may induce localized inflammation secondary to the mechanical stimuli, MCP-1(i.c.) alone or a combination of MCP-1(i.c.) and negative

serum (i.v.) is insufficient to clear wt RABV from the brain and prevent the development of rabies. These studies indicate that MCP-1 alone or mechanical stimuli of intracerebral injection does not lead to the clearance of RABV from the brain or increase survival.

VNA has been suggested to be produced in the brains of mice infected with attenuated RABV(CVS-F3), which may thus contribute to RABV clearance (Hooper, Phares et al. 2009). However, it has been known for a long time that natural infection with wt RABV does not induce a strong anti-viral immune responses (Baltazard and Ghodssi 1954). Hemachuda et al (1984) reported that less than 30% of human rabies patients developed VNA at the time of death. In this present study, no VNA was detected in mice infected with DRV-Mexico, and all mice died without any intervention. This finding further suggests that although non-VNA could be made in the brain, it does not contribute to survival during wt rabies infections. The mechanism by which wt RABV does not induce the production of VNA is not entirely clear at the moment and further studies are warranted.

It has been hypothesized that it is the VNA produced in situ (CNS) by invading B cells, not those produced in the periphery and then transported into the CNS, that are important in clearing RABV from the CNS (Hooper, Phares et al. 2009). Passively transferred VNA via intraperitoneal route failed to clear attenuated RABV from the brain in B-cell deficient mice. However, our studies summarized here demonstrate a different result in the wt RABV-infected mouse model, which is likely more relevant to clinical rabies in humans. The intravenously administered VNA is important in clearing wt RABV from the brain and preventing the development of rabies not only in immunocompetent mice, but also in B-cell deficient mice, as long as the BBB

permeability remains enhanced. Thus, production of VNA in situ (brain) by plasma cells is not absolutely required for wt RABV clearance from the brain. Similar results have also been reported for coronavirus-induced encephalomyelitis. Although recruitment of virus-specific antibody-secreting cells (ASC) mediated by CXCR3 has been found to be helpful in controlling coronavirus-induced encephalomyelitis (Marques, Kapil et al. 2011, Phares, Stohlman et al. 2013), viral recrudescence can be prevented in B-cell-deficient and Ig $M^{-/-}$ mice by intraperitoneal administration of JHMV-specific neutralizing antibodies (Lin, Hinton et al. 1999, Ramakrishna, Bergmann et al. 2003). Thus, these findings further demonstrate that production of VNA in situ (brain) by antibody-secreting cells (ASC) is not absolutely required for clearance of viruses from the brain.

It was found in our study that MCP-1 has to be administered twice (at 5 and 7 dpi) in B-cell deficient mice in order to achieve a similar rate of protection as found in immunocompetent mice after one injection. We attribute this to the transient nature of MCP-1 in enhancing the BBB permeability (Stamatovic, Shakui et al. 2005) and the low level of VNA present in the brain (~0.3 IU) despite the fact that VNA levels in the serum remain high (3-4 IU). Thus continued enhancement of BBB permeability is required for maintaining sufficient VNA levels in the brain to clear RABV. It was speculated that B cell deficient mice could mediate non-antibody-dependent antiviral mechanisms, and thus, play a role in the clearance of RABV from the brain. However, all mice died without being treated with RABV antibody-positive serum, and more mice survived when treated with MCP-1 twice than those treated only once, further indicating that VNA passing into the brain from the periphery played a decisive role in clearing RABV from the brain in the B-cell deficient mice.

In the present study, we were aware of some limitations, such as the possible role of non-antibody dependent antiviral mechanisms that cannot be completely excluded, and the treatment was applied before clinical signs appeared, even though virus was detectable at the time of treatment, possibly due to insufficient burden of neuronal injury/dysfunction. Further studies are warranted. However, our results demonstrated that VNA can be administered by intravenous route and is crucial for RABV clearance from the brain. The findings presented in this study not only provide a foundation for developing VNA therapy for human clinical rabies, but may also be a benefit for other CNS diseases where the therapeutic agents are required to cross the BBB.

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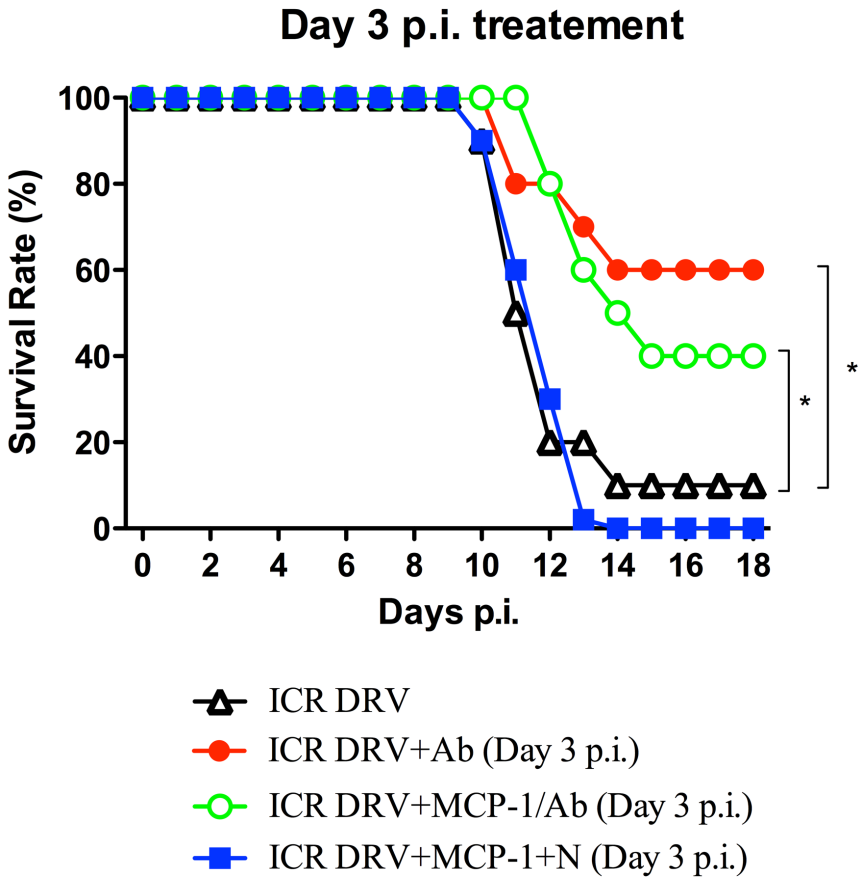

Figure 3.1. Protective efficacy of immune-competent mice treated with VNA with or without MCP-1 at 3 dpi after infection with DRV. ICR mice (groups of 10) at the age of 4-6 weeks were infected i.m. with 10 IMLD50 DRV-Mexico and then treated intravenously with RABV-negative serum (N) or RABV- antibody positive serum (Ab) with or without recombinant murine MCP-1 (25 μg/mouse, i.c.) at 3 dpi. Infected and treated mice were observed daily for 21 days and survivorship was recorded and analyzed. Asterisks indicate significant differences (* $p< 0.05$) between the indicated experimental groups.

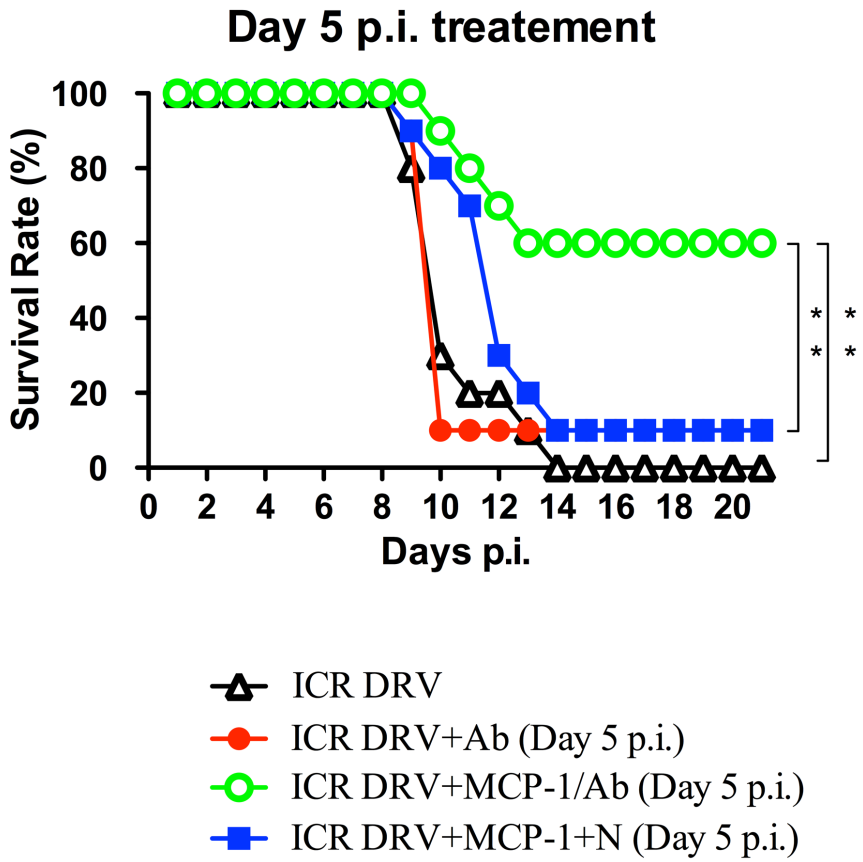

Figure 3.2. Protective efficacy of immune-competent mice treated with VNA with or without MCP-1 at 5 dpi after infection with DRV. ICR mice (groups of 10) at the age of 4-6 weeks were infected i.m. with 10 IMLD50 DRV-Mexico and then treated intravenously with RABV-negative serum (N) or RABV- antibody positive serum (Ab) with or without recombinant murine MCP-1 (25 µg/mouse, i.c.) at 5 dpi. Infected and treated mice were observed daily for 21 days and survivorship was recorded and analyzed. Asterisks indicate significant differences ** $p < 0.01$) between the indicated experimental groups.

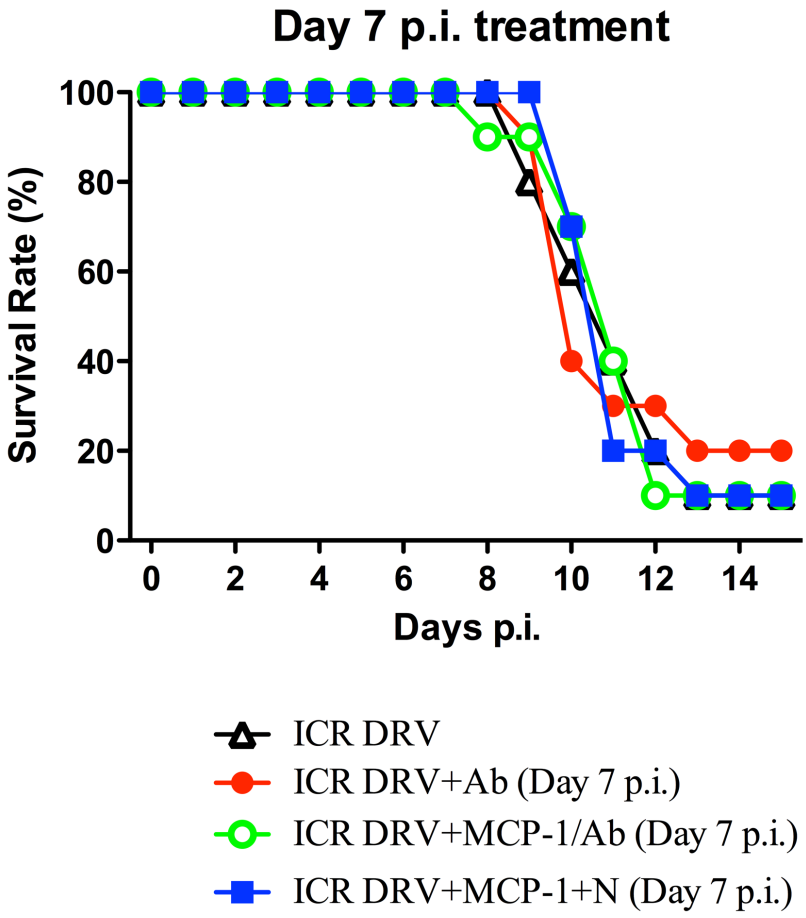

Figure 3.3. Protective efficacy of immune-competent mice treated with VNA at 7 dpi with or without MCP-1 after infection with DRV. ICR mice (groups of 10) at the age of 4-6 weeks were infected i.m. with 10 IMLD50 DRV-Mexico and then treated intravenously with RABV-negative serum (N) or RABV- antibody positive serum (Ab) with or without recombinant murine MCP-1 (25 μg/mouse, i.c.) at 7 dpi. Infected and treated mice were observed daily for 21 days and survivorship was recorded and analyzed.

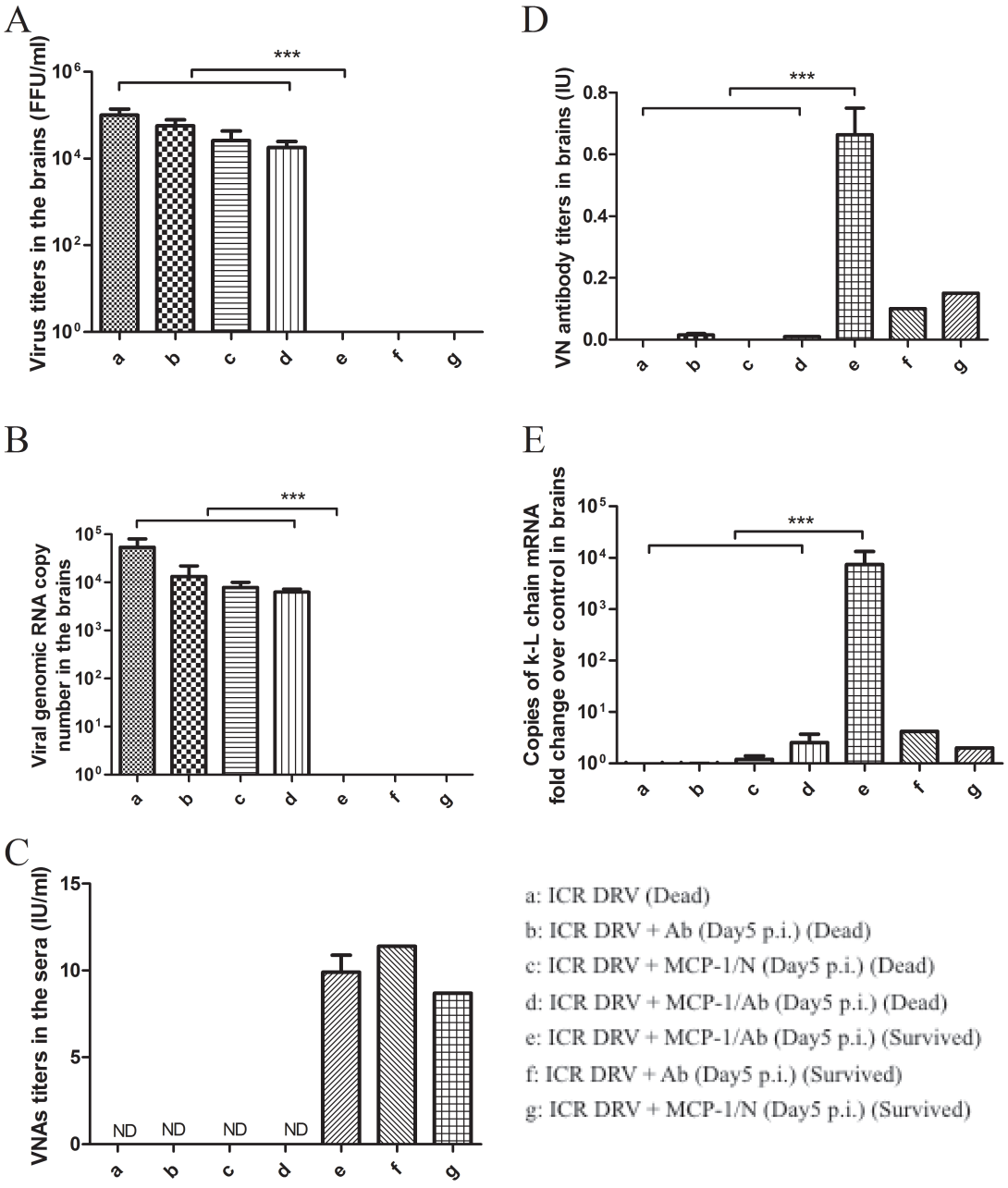

Figure 3.4. Virus titers, virus copy number, and VNA in brains, serum VNA, and the expression of κ-light chain mRNA in brains of immune-competent mice treated with VNA with or without MCP-1 after infection with DRV. ICR mice (groups of 10) at the age of 4-6 weeks were infected i.m. with 10 IMLD50 DRV-Mexico and then treated with RABV-antibody negative (N) or positive serum (Ab) with or without recombinant

murine MCP-1 at 5 dpi. Sera and brains were harvested once mice reached the criteria of euthanasia by developing paralysis in both hind legs (Dead) or survived through day 21 (Survived). Virus titers **(A)** and viral genomic RNA copy number **(B)** in brains, serum VNA **(C)** and VNA in brains **(D)** and the expression of κ-light chain mRNA in brains **(E)** were determined. Data from groups of n≥3 mice are presented as mean values ± SEM, except for groups f and g, in which only one C57BL/6 mouse survived. Asterisks indicate significant differences (*** $p < 0.001$) between the indicated experimental groups. ND: not done.

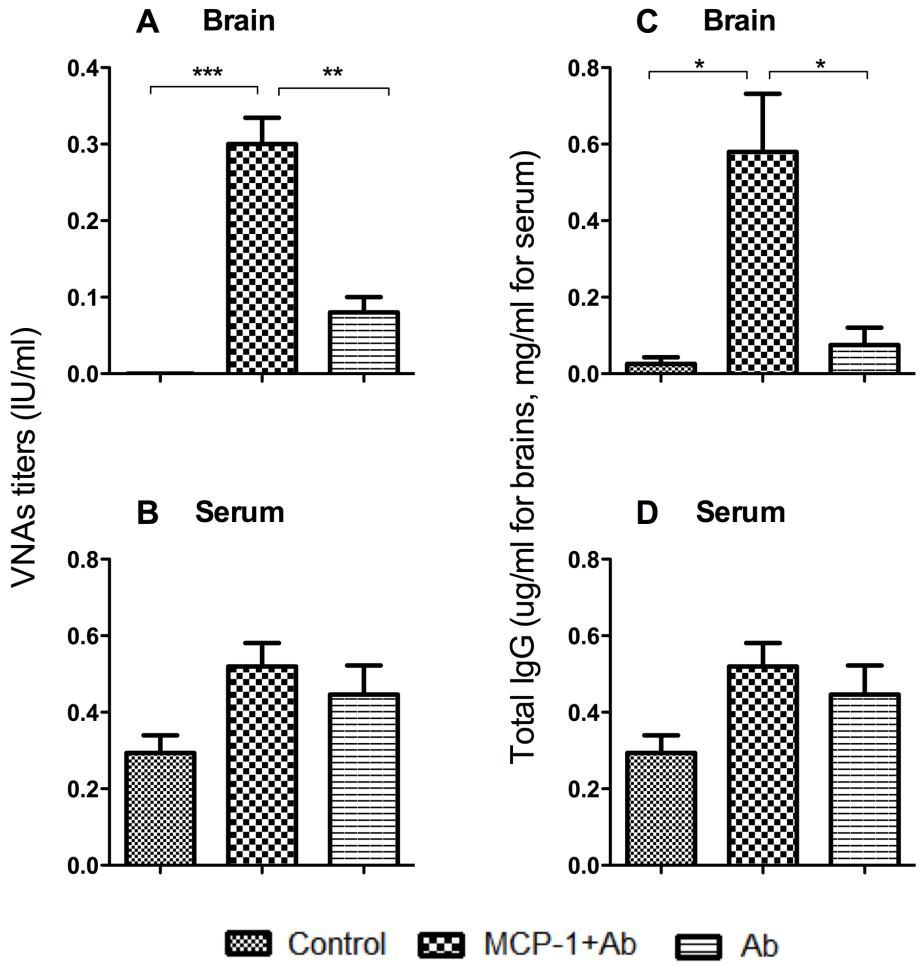

Figure 3.5. Serum VNA and total IgG in brains and sera of immune-competent mice treated with VNA and /or MCP-1. ICR mice (groups of 3) at the age of 4-6 weeks were treated with RABV antibody-positive serum (Ab) with or without recombinant murine MCP-1. Mice were euthanized 24 hr after the treatment, and both sera and brains were harvested. VNA in brains **(A),** serum VNA **(B)** , total IgG in brains **(C),** and serum total IgG **(D)** were determined. Data are presented as mean values ± SEM. Asterisks indicate significant differences (* $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$) between the indicated experimental groups.

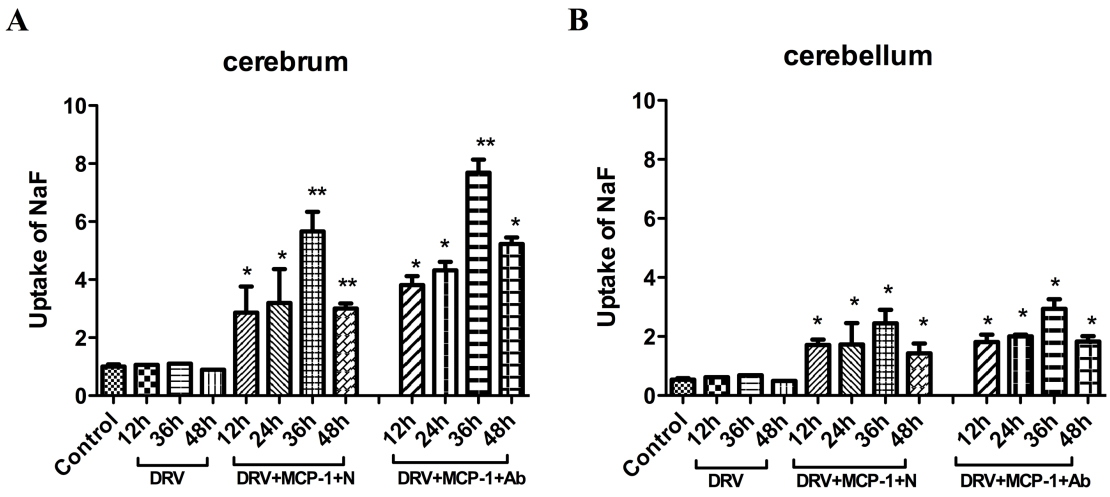

Figure 3.6. BBB permeability in cerebrum and cerebellum in brains of immune-competent mice treated with MCP-1 with or without VNA after infection with DRV. ICR mice (groups of 3) at the age of 4-6 weeks were infected i.m. with 10 IMLD50 DRV-Mexico and then treated with recombinant murine MCP-1 and RABV antibody-negative (N) or -positive serum (Ab) at 5 dpi. BBB permeability in cerebrum and cerebellum, (**A**) and (**B**), respectively, were determined at 12, 24, 36, and 48hr after the treatment. Data from groups are presented as mean values ± SEM. Asterisks indicate significant differences (* $p< 0.05$, ** $p< 0.01$) between the control and experimental groups.

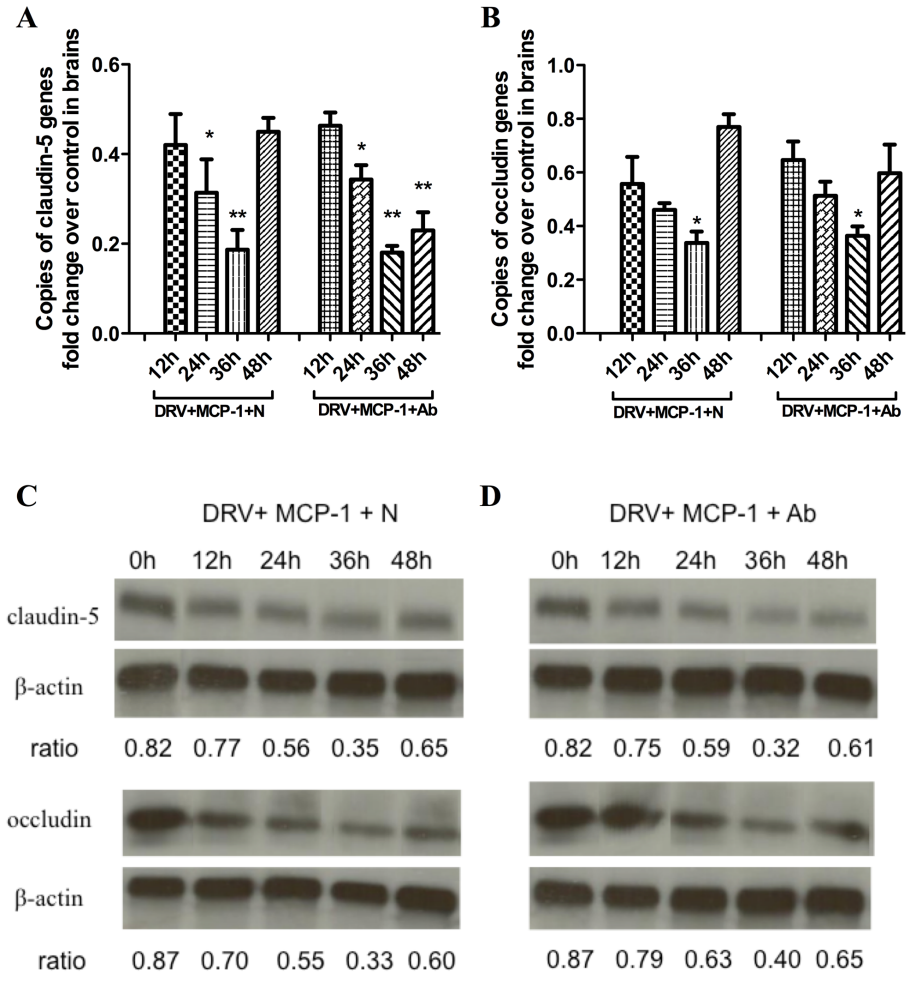

Figure 3.7. The expression of tight junction proteins (occludin and claudin-5) in brains of immune-competent mice treated with MCP-1 with or without VNA after infection with DRV. ICR mice (groups of 3) at the age of 4-6 weeks were infected i.m. with 10 IMLD50 DRV-Mexico and then treated with recombinant murine MCP-1 and RABV antibody-negative (N) or positive serum (Ab) at 5 dpi. mRNA and semiquantitative (Western blotting) expression of claudin-5 **(A, C)** and occludin **(B, D)** were determined at 12, 24, 36, and 48hr after the treatment. Data from groups are

presented as mean values ± SEM. Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$) between the control and experimental groups.

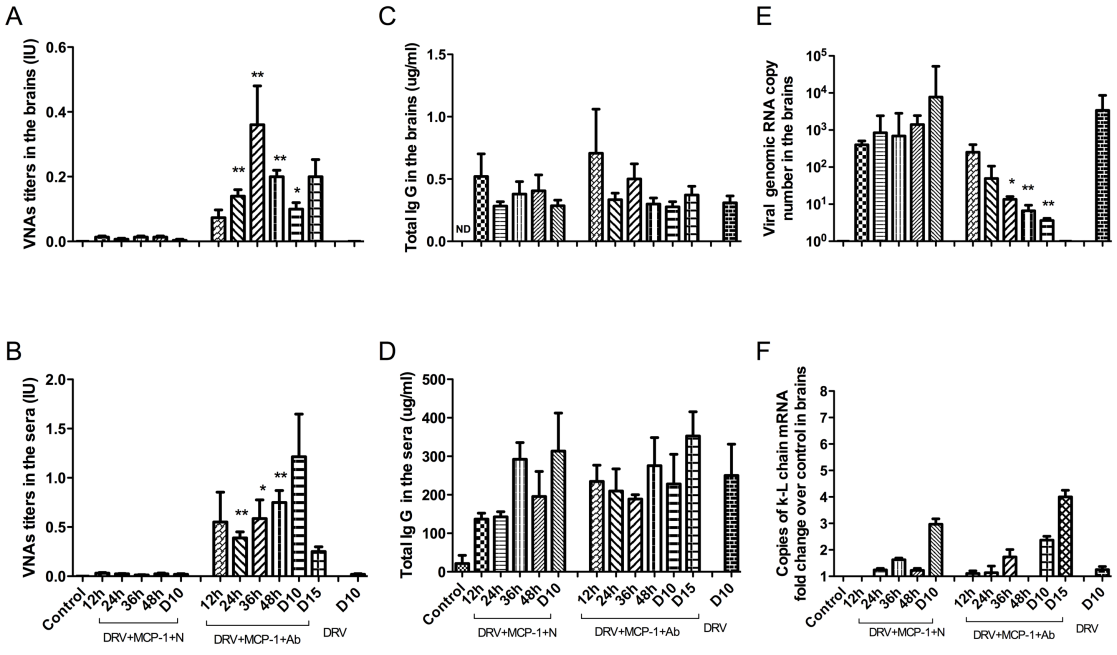

Figure 3.8. VNA and total IgG in brains and sera, viral copy number and the expression of κ-light chain mRNA in brains of immunocompetent mice treated with MCP-1 with or without VNA after infection with DRV. ICR mice (groups of 3) at the age of 4-6 weeks were infected i.m. with 10 IMLD50 DRV-Mexico and then treated with recombinant murine MCP-1(25 μg/mouse, i.c.) and RABV antibody-negative (N) or positive serum (Ab) at 5 dpi. Mice were euthanized at 12, 24, 36, or 48hr after the treatment) as well as at 10 dpi (indicated as D10), or survived at 15 dpi (indicated as D15). There is another group of one ICR mouse with only DRV infection and sacrificed at 10 dpi (DRV/ D10). Both sera and brains from all groups were harvested for determination of VNA in brains **(A)** and in sera **(B)** , total IgG in brains **(C)** and sera **(D)** , viral genomic RNA copy number in brains **(E),** and the expression of κ-light chain mRNA in brains **(F).** Data are presented as mean values ± SEM. Asterisks indicate

significant differences (* $p< 0.05$, ** $p< 0.01$) between the corresponding time points of MCP-1 with N or Ab groups.

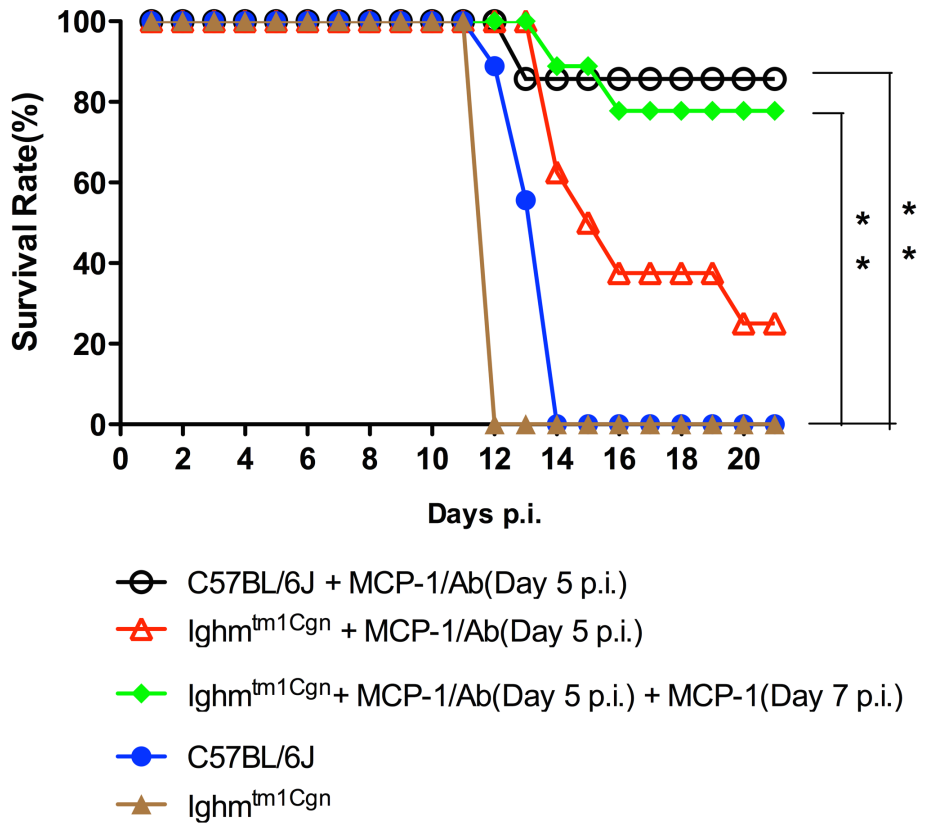

Figure 3.9. Protective efficacy in B-cell deficient mice treated with VNA and MCP-1 after infection with DRV. C57BL/6J and *Ighm*^{tm1Cgn} (B-cell deficient) mice (groups of 8) at the age of 4-6 weeks were infected i.m. with 10 IMLD50 DRV-Mexico and then treated with RABV- antibody positive serum (Ab) in conjunction with MCP-1 at 5 dpi or at both 5 and 7 dpi. Infected and treated mice were observed daily for 21 days, and survivorship was recorded and analyzed. Asterisks indicate significant differences (** $p<$ 0.01) between the indicated experimental groups.

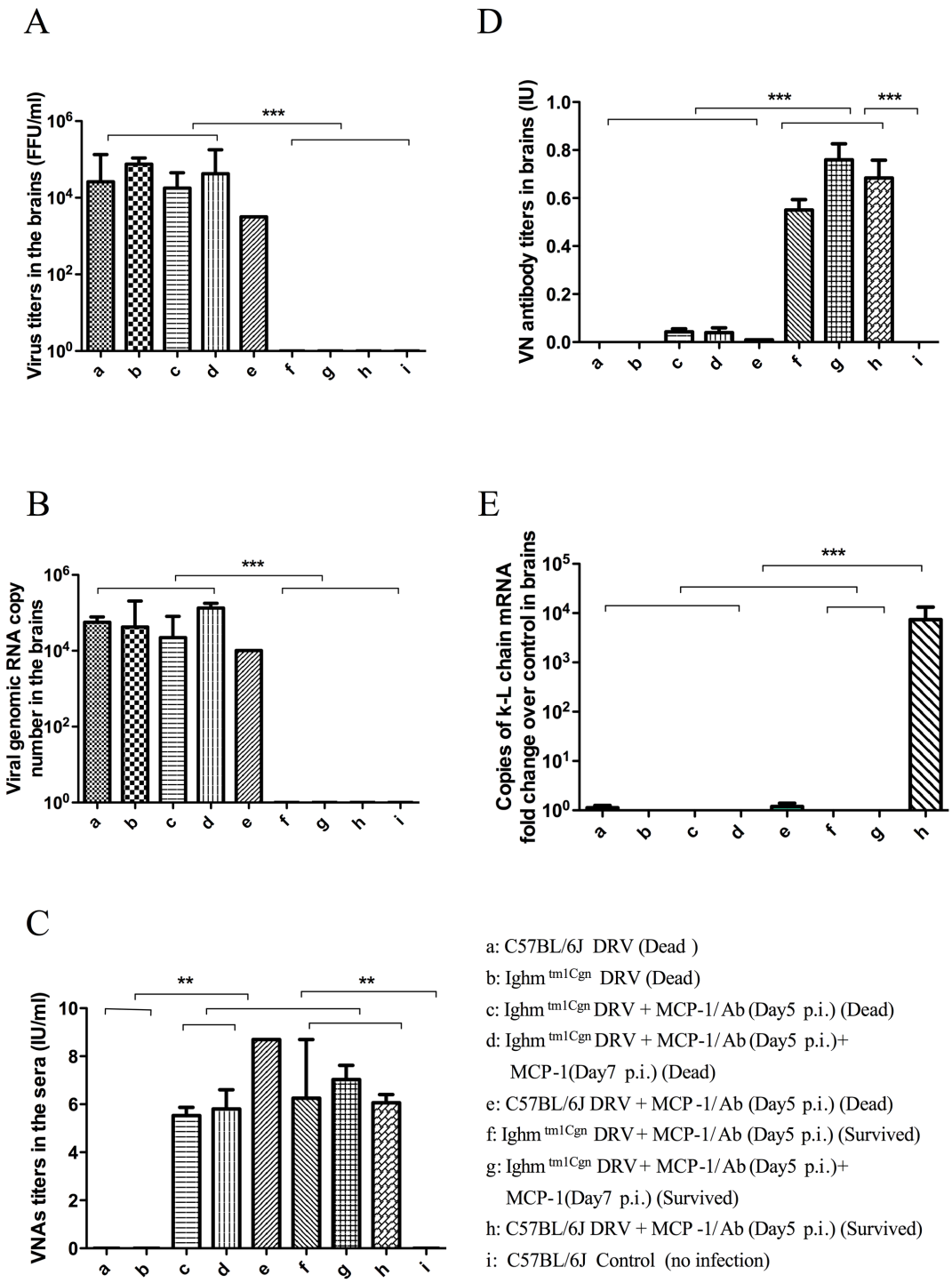

Figure 3.10. Virus titers and virus copy number in brains, serum VNA and the expression of k**-light chain mRNA in brains of B-cell deficient mice treated with**

RABV- antibody positive serum and MCP-1 after infection with DRV. C57BL/6J and *Ighm*^{tm1Cgn} (B-cell deficient) mice (groups of 8) at the age of 4-6 weeks were infected i.m. with 10 IMLD50 DRV and then treated with RABV- antibody positive serum (Ab) in conjunction with MCP-1 at 5 dpi or at both 5 and 7 dpi. Infected and treated mice were observed daily for 21 days. Both sera and brains were harvested once mice reached the criteria of euthanasia by developing paralysis in both hind legs (Dead) or survived through day 21 (Survived) for the determination of virus titers **(A)** and viral genomic RNA copy number **(B)** in brains, serum VNA **(C)**, VNA in brains **(D),** and κ-light chain mRNA in brains **(E)**. Data from groups of n≥3 mice are presented as mean values ± SEM, except for group e (only one C57BL/6J mouse dead). Asterisks indicate significant differences (** $p< 0.01$, *** $p < 0.001$) between the indicated experimental groups.

CHAPTER 4

CONCLUSION

The failure in enhancing BBB permeability and eliciting rabies-specific humoral immune responses have been believed to be two major determining factors for high pathogenicity of wild-type RABV (Roy and Hooper 2007, Kuang, Lackay et al. 2009, Johnson, Cunningham et al. 2010). In the present study, we again demonstrate the lack of BBB permeability change and VNA production during pathogenic wild-type RABV infection. Several studies have found certain approaches to be helpful in preventing lethal rabies infection by overcoming these two limitations observed in wild-type RABV infections, such as administration of attenuated RABV (CVS-F3) (Phares, Kean et al. 2006), recombinant RABV expressing three copies of the glycoprotein (G) (TriGAS) (Faber, Li et al. 2009), or immune-stimulating molecules (for example, GM-CSF) (Wang, Zhang et al. 2011, Wen, Wang et al. 2011). These all resulted in enhancement of BBB permeability, increased production of VNAs, and eventually, clearance of RABV from the CNS and prevention of rabies in the mouse model. In the present study, we hypothesized that passively transferred VNAs can reach the CNS in conjunction with MCP-1 injection. As we expected, passively transferred VNAs cannot only reach the CNS, but are crucial in preventing rabies lethal outcome by clearing the RABV. This

study demonstrates a very efficient way to clear the RABV present within the CNS where immune effectors have limited accesses, by treating only once.

Although traditional inactivated RABV vaccines have been used for pre- and post-exposure prophylaxis in humans with high safety and efficacy, they are not useful for delayed treatment and can even accelerate rabies (Rupprecht, Hanlon et al. 2002, Sampath, Parikh et al. 2005, CDC 2010). Other vaccines, such as the live-attenuated RABV vaccine and recombinant vaccine expressing RABV G, are limited to use only in wildlife animals (Brochier, Kieny et al. 1991, Schumacher, Coulon et al. 1993, Hanlon, Niezgoda et al. 1998). Recent studies suggested that live-attenuated vaccines expressing multiple copies of the G or immune-stimulating molecules not only have the potential to replace the traditional inactivated RABV vaccines, but can also clear RABV from the CNS if administered intracerebrally (Faber, Li et al. 2009, Wang, Zhang et al. 2011). However, intracerebral administration of live-attenuated or recombinant RABV still possesses safety concerns.

In the present study, we demonstrate that a combined treatment with VNAs and MCP-1 is highly safe and efficacious in the mouse model without a need for live RABV. Moreover, no adverse effects were observed clinically with either intravenous VNA or intracerebral MCP-1 injection. Our results also demonstrate that B cells are not absolutely required for this treatment to be effective for an established rabies infection in the mouse model. Therefore, it can potentially be given to not only immunocompetent but also immunocompromised rabies patients, and especially be useful for those who miss the optimal time period for PEP after rabies exposure. In conclusion, the findings

from this study will be beneficial for not only rabies, but also other CNS diseases for which the therapeutic agents must cross the BBB.

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